Effects of Fluoridated Water on Pineal Morphology in Male Rats

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2017

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Signature:

Aaron Mrvelj, Student	Date

Approvals:

Dr. Mark Womble, Thesis Advisor	Date
Dr. Jill Tall, Committee Member	Date
Dr. Jeffrey Coldren, Committee Member	Date
Dr. Salvatore A. Sanders, Dean of Graduate Studies	Date

Abstract

The pineal gland is a naturally calcifying endocrine organ which produces and releases the sleep-promoting hormone melatonin which also serves as an antioxidant. Fluoride is attracted to the calcium in the pineal gland and inhibits the synthesis and activity of melatonin, induces oxidative stress, and causes cellular changes to the neurons of the hippocampus. Morphological changes to the pineal gland have been demonstrated with increased age, exposure to light during a melatonin production period, or sleep deprivation. This study sought to examine the effects of fluoridated water on the morphology of the pineal gland. The effects of a fluoride-free flush were compared to fluoride treatment. Group 1, previously raised on fluoridated tap water served as a control that was sacrificed at the onset of the experiment. The remaining four groups were then subjected to a four-week fluoride-free diet with Group 2 being sacrificed at the end of this period. Group 3 was maintained on fluoride-free food and water while Groups 4 and 5 were switched to fluoridated water for the remaining four weeks. The fluoride-free flush resulted in an increase in the number of supporting cells and pinealocytes and a decrease in the nuclear diameter of pinealocytes, suggesting that the flush encouraged growth of the gland. Fluoride treatment had no effect on the number of supporting cells, but decreased the number of pinealocytes and their nuclear diameter, suggesting that fluoride is detrimental to the pineal gland.

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List of Abbreviations

Melatonin (MEL)	Also known as (aka)
Micrometer (μm)	Milliliter (mL)
Gram (g)	Hematoxylin and eosin (H&E)
Connective tissue capsule (CTC)	Retinohypothalamic tract (RHT)
Suprachiasmatic nucleus (SCN)	Paraventricular nucleus (PVN)
Action potential (AP)	United States (U.S.)
Period (per)	Cryptochrome (cry)
Clock (clk)	Cycle (cyc)
Clock-Cycle dimer (Clk-Cyc)	Period-Cryptochrome dimer (Per-Cry)
Superoxide dismutase (SOD)	Glutathione peroxidase (GPx)
Glutathione reductase (GR)	Catalase (CAT)
Reactive oxygen species (ROS)	Reactive nitrogen species (RNS)
Oxidative stress (OS)	Deoxyribonucleic acid (DNA)
Ribonucleic acid (RNA)	Computerized tomography (CT)
Hour (hr)	Centers for Disease Control and Prevention (CDC)
Pineal proteins (PP)	Sodium fluoride (NaF)
Lipid peroxidation (LPO)	Intraperitoneal (i.p.)
Alkaline phosphatase (ALP)	Parts per million (ppm)
Lethal dose (LD)	Environmental Protection Agency (EPA)
National Institute of Health (NIH)	Water (H_2O)
Celsius (C)	Liter (L)
Minute (min)	Magnification (X)
Analysis of variance (ANOVA)	Multivariate analysis of variance (MANOVA)

Intrinsically photosensitive retinal ganglion cells (ipRGC)

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June 26, 2016

Dr. Mark Womble
Department of Biological Sciences
UNIVERSITY

Re: IACUC Protocol # 04-16
Title: Fluoride's effect on sleep and pineal morphology in male rats.

Dear Dr. Womble:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration and determined it should be unconditionally approved for the period of June 24, 2016 through its expiration date of June 24, 2019.

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days ***prior*** to the protocol's yearly anniversary dates of June 24, 2017 and June 24, 2019. If you do not submit the forms as requested, this protocol will be immediately suspended. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Good luck with your research!

Michael A. Hripko
Associate Vice President for Research
Authorized Institutional Official

MAH:dka

cc: Dr. Walter Horne, Consulting Veterinarian, NEOMED
Dawn Amolsch, Animal Tech., Biological Sciences

Introduction

The pineal gland is a neuroendocrine organ responsible for producing and releasing the hormone melatonin (MEL). MEL is important for regulating sleep and also serves as an important direct and indirect antioxidant that is inhibited by fluoride. Naturally occurring calcium concretions (aka acervuli or brain sand) within the pineal are believed to attract fluoride, which has been found in abnormally high concentrations in the pineal of humans. Fluoride is added to public drinking water because it has been shown to be an effective topical treatment for dental health. However, the US national research council recommends more research on the systemic effects of fluoridated water which has been linked to crippling skeletal fluorosis and dental fluorosis in humans and inhibition of antioxidants, increased oxidative stress, increased risk of cancer, karyolysis, apoptosis, and decreased neuronal diameter in the hippocampus of rats. If water fluoridation is done in the interest of public health, more studies need to be conducted to assess the effects of chronic and acute fluoride exposure.

Since fluoride accumulates in the pineal gland and may alter cellular structure and function it may be more damaging to pineal functioning than previously thought. This study examines the morphological changes in the pineal gland associated with a fluoride-free "flush" and a fluoride treatment over an eight-week period. Animals provided fluoridated drinking water were compared to control animals on a fluoride-free diet to detect potential cellular changes in the pineal gland.

Pineal gland

The pineal gland is a midline pinecone-shaped organ located within the brain as part of the epithalamus. It is a neuroendocrine organ responsible for synchronizing

biological rhythms to environmental light/dark cues using the hormone melatonin (MEL). MEL is an important, evolutionarily conserved indolamine hormone and antioxidant, derived from the amino acid tryptophan, that is secreted by the pineal during darkness and acts to promote sleep (Pinel, 2010; Lan, 2001; Kaur *et al*., 2002; Purves, 2008; Widmaier *et al*., 2008; Bharti & Srivastava, 2009; Bharti & Srivastava, 2010; Hall & Guyton, 2016; Khavinson *et al*., 2012; Ramírez-Rodríguez *et al.*, 2014).

Normal pineal morphology

Pineal gland morphology of rats and humans has been studied using light and electron microscopy. Two types of human pinealocytes, light and dark, are distinguished by their staining characteristics using light microscopy, have been described (Redecker, 1993; Prosenc & Cervos-Navarro, 1994; Al-Hussain, 2006). Light cells stain more palely than dark cells (Allen *et al.*, 1982; Bastianelli & Pochet, 1993; Al-Hussain, 2006). In rats, the light and dark staining pinealocytes have similar enzyme content and are thus thought to differ only in their stages of differentiation or calcium aggregations (Huxley & Tapp, 1972). A third pinealocyte has been described in the human and rat pineal gland (Bastianelli & Pochet, 1993; Al-Hussain, 2006). However, data from rats based on enzymatic analysis suggests that these cells are macrophages or microglia (Karasek *et al.*, 1978) and thus they will not be discussed further. In rats and humans, investigators have reported pinealocytes forming rosettes and lobules of 8-10 cells with a fibrous background (Nathanson *et al.*, 1977; Boeckmann, 1980; Allen *et al.*, 1982; Becker & Vollrath, 1983; Bastianelli & Pochet, 1993; Jimenez-Heffernan *et al.*, 2015).

Light cells make up the majority of parenchymal cells in the human and rat pineal gland. Within the human pineal gland, light cells are round or oval, with short cell

processes, an average diameter of 9 μm, and a large 5.8 μm diameter round or oval nucleus (Huxley & Tapp, 1972; Al-Hussain, 2006). With electron microscopy, human pinealocyte are seen to exhibit nuclear infoldings, cytoplasmic vesicles, pigments, and rod-like ribbons, and cilia with a 9x2+0 microtubule pattern (Al-Hussain, 2006). Similarly, rat pinealocytes exhibit nuclear infoldings and lobation, and cytoplasmic vesicles, vacuoles, and lipid droplets (Karasek *et al.,* 1978; Allen *et al.*, 1982; Akinrinade *et al.*, 2015).

In rats and humans, dark pinealocytes occur much less frequently. In rats, dark cells are usually found in small clusters dispersed among the light cells (Allen *et al.*, 1982; Bastianelli & Pochet, 1993; Al-Hussain, 2006), while in the human pineal these cells occur singly at the periphery (Prosenc & Cervos-Navarro, 1994). Dark cells in humans are described as being round, oval, or elongated, with irregularly shaped nuclei and an average diameter of 9.4 μm. The nuclei average 6.4 μm in diameter, with condensed chromatin clustered against the inner surface of the nuclear envelope. The nuclear membrane demonstrated many infoldings resembling nuclear pellets, and there was extensive rough endoplasmic reticulum associated with the cytoplasmic face of the nuclear membrane (Al-Hussain, 2006). The morphological features of dark cells closely resembled light cells. Human dark pinealocytes also exhibit the same characteristics of short cell processes, nuclear infoldings, vesicles, pigments, rod-like ribbons, and cilia (Huxley & Tapp, 1972; Al-Hussain, 2006), while rat dark cells show intracellular vacuoles and lipid droplets with nuclear lobation (Karasek *et al.*, 1978; Allen *et al.*, 1982; Akinrinade *et al.*, 2015). Both light and dark cells produce and secrete the hormone melatonin.

Inside the pineal gland are intracellular and extracellular concrements of calcium called acervuli, or corpora arenacea, which is also known as brain sand (Nathanson *et al.*, 1977; Pinel, 2010; Lan *et al.*, 2001; Luke, 2001; Kaur *et al.*, 2002; U.S. National Research Council, 2006; Maronde & Stehle, 2007; Widmaier *et al.*, 2008; Bulc, 2010; Poloni *et al.*, 2011; Gerasimov, 2014). The acervuli of all mammals share the same structure, which differs from those of birds (Widmaier *et al.*, 2008). The origin of these structures is not well understood, but it is surmised that they are formed by the death and degeneration of pinealocytes or by calcium secreted by pinealocytes (Humbert & Pevet, 1995). There are individual variations and age-related differences in the number of acervuli in a pineal gland (Allen *et al.*, 1982; Becker & Vollrath, 1983; Humbert & Pevet, 1995). In rats, 98 acervuli were observed in 90 pineal glands using hematoxylin and eosin (H&E) staining (Becker & Vollrath, 1983). Researchers have found the majority of extracellular calcium concretions occurring in the subcapsular space, but they can also be found among the parenchymal cells (Allen *et al.*, 1982; Becker & Vollrath, 1983).

Calcium and phosphorus are the major components of acervuli, but trace amounts of sulfur, iron, silicon, chlorine, potassium, copper, and zinc were found in freeze dried specimens (Humbert & Pevet, 1995). Calcium was detected in higher concentrations in darkly staining pinealocytes using pyroantimonate to complex with calcium in electron-probe microscopy. Intracellular regions with the highest calcium concentrations, in increasing order of concentration, were lipopigments, mitochondria, and dense vacuoles. Acervuli calcium to phosphorus ratios, determined by x-ray microanalysis, differ within the gland with the highest ratios found in mitochondria, extracellular precipitate, and intracellular concretions. It was reported that acervuli are found in intra- and extracellular

spaces with differences in arrangement (Humbert & Pevet, 1995). Intracellular calcium "crystallite", containing hydroxyapatite crystals, is radially arranged while extracellular deposits display concentric spherical layers resembling a mulberry-shape (Humbert & Pevet, 1995; Bulc *et al.*, 2010; Kim *et al.*, 2012).

The growth of acervuli has been observed using synchrotron X-ray imaging with human subjects (Kim *et al.*, 2012). Using 3D reconstruction, this work revealed that peripheral acervuli form concentrically and remain small or aggregate together to form larger concretions. A single acervuli can develop into a large bumpy mulberry shape by concentric lamination or several acervuli can aggregate, with concentric laminae forming around the entire cluster. While the growth of acervuli is understood, it is believed that these structures begin when vacuoles containing high calcium ion concentrations grow until the cell disintegrates and the calcium concrements are released into the extracellular space (Bulc *et al.*, 2010).

Within the human and rat pineal gland, the secretory pinealocytes are accompanied by supporting glial cells and striated skeletal muscle fibers (Allen *et al.*, 1982; Becker & Vollrath, 1983; Al-Hussain, 2006). The rat pineal gland is surrounded by pia mater and a subpial connective tissue capsule (CTC) made of flattened cells a few layers in thickness (Krstic, 1979; Allen *et al.*, 1982). Fibroblasts are found among the collagen fibers in the CTC layer (Allen *et al.*, 1982). Deep to the pia mater and CTC lies a layer of glial cells that are superficial to the parenchyma of the gland (Allen *et al.*, 1982). Single or small bundles of striated muscle fibers with a transverse banding pattern are found in the parenchyma and CTC (Allen *et al.*, 1982; Becker & Vollrath, 1983). However, it is not said if the muscle fibers are continuous through the CTC into the

parenchyma. The function of striated skeletal muscle in the pineal gland is not understood, but appears to give rise to the pineal stalk, which attaches the gland to the diencephalon, at the cortical anterior region of the gland (Becker & Vollrath, 1983; Diehl, 1978).

Glial cells in the pineal of humans and rats are pyramidal, spindle-like, or triangular in shape, with short cell processes (Allen *et al.*, 1982; Bastianelli & Pochet, 1993; Al-Hussain, 2006). In rats, these cells vary in size and concentration in different regions of the pineal (Allen *et al.*, 1982; Bastianelli & Pochet, 1993). The ratio of pinealocytes to glia favors pinealocytes in humans (Allen *et al.*, 1982).

Vasculature, muscle fibers, and sympathetic nerve fibers extend through the capsule into the pineal gland (Allen *et al.*, 1982). The highly vascularized pineal gland lies outside of the blood-brain barrier (Allen *et al.*, 1982; Luke, 2001; Hall & Guyton, 2016; U.S. National Research Council, 2006). Blood vessels from the posterior cerebral arteries pass through the CTC and into the pineal gland where they become capillary networks that eventually empty into the internal cerebral vein (Allen *et al.*, 1982). Myelinated axons of postganglionic sympathetic neurons penetrate deep into the pineal gland, where they lose their myelin sheath (Allen *et al.*, 1982).

Melatonin

The synthesis and secretion of MEL is active during periods of darkness to promote sleep. Light levels are detected by intrinsically photosensitive retinal ganglion cells (ipRGC), which differ from rods and cones. The ipRGCs are located in the outer nuclear layer of the retina and project via the retinohypothalamic tract (RHT) directly to the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Purves, 2008;

Breedlove & Watson, 2013) (Fig. 1). During periods of detectable light, ipRGCs increase their action potential (AP) firing rate and in darkness the frequency of AP decreases. The ipRGCs that project via the RHT release glutamate in the presence of light, which binds to N-methyl-D-aspartate receptors on neurons in the SCN. The SCN neurons, in turn release the inhibitory neurotransmitter gamma-aminobutyric (GABA) acid at the paraventricular nucleus (PVN) of the hypothalamus. In the absence of light, the release of the inhibitory neurotransmitter GABA from the SCN is reduced, thus allowing the cells of the PVN to become active. The axons of cells in the PVN descend via the intermediolateral tract of the spinal cord to release acetylcholine and excite preganglionic sympathetic neurons in the lateral horn grey matter of the thoracic spinal cord. Axons from these preganglionic sympathetic neurons pass into the sympathetic trunk and ascend to the superior cervical ganglion, where they release acetylcholine to activate postganglionic sympathetic neurons (Lan *et al.*, 2001; Maronde & Stehle, 2007; Purves, 2008). These neurons in turn innervate the pineal gland and release norepinephrine to initiate the synthesis and release into the blood of the sleep-promoting hormone MEL (Reuss *et al.*, 1990; Redecker, 1993; U.S. National Research Council, 2006; Purves, 2008). The detection of light results in a cessation of MEL secretion.

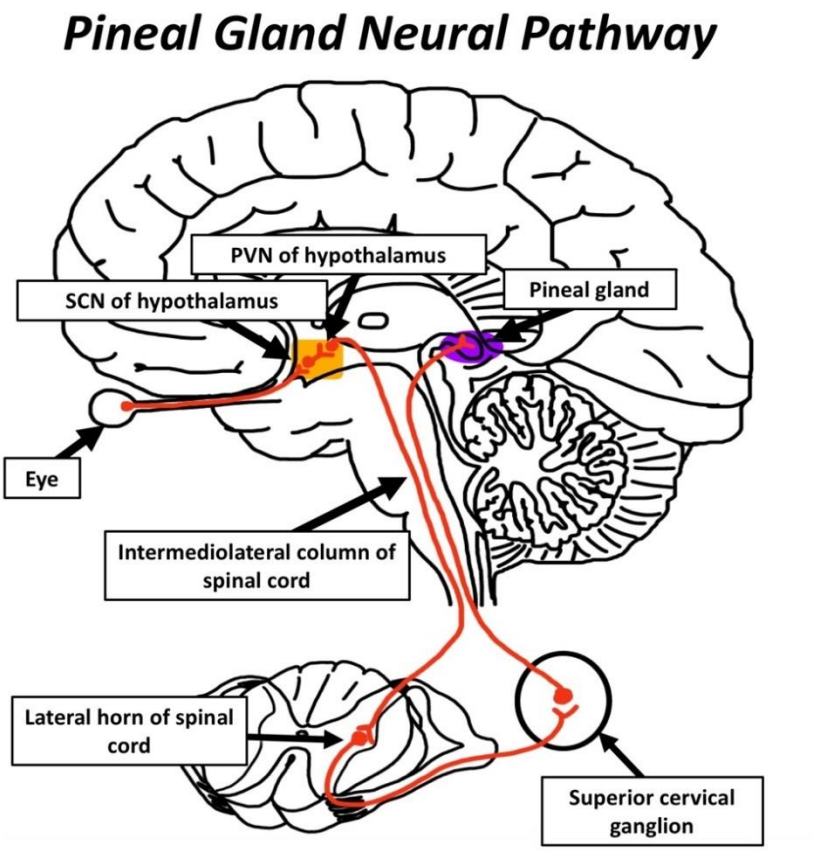

Figure 1: Neural pathway for signals traveling to the pineal gland. ipRGCs in the eye detect the intensity of light. In the absence of light ipRGCs activate the SCN via the RHT. Preganglionic sympathetic neurons send signals from the SCN via the intermediolateral cell column of the spinal cord to the lateral horns where postganglionic sympathetic axons ascend to the superior cervical ganglion. Axons from the neurons of the superior cervical ganglion ascend to the pineal gland where they release NE to stimulate MEL release.

During darkness, MEL released into the blood acts on the SCN to synchronize it with information about the time of day (U.S. National Research Council, 2006; Widmaier *et al.*, 2008). In the SCN, MEL binds to the melatonin receptors, MT_1 & MT_2. These are G-coupled protein receptors which inhibit expression of the clock genes *period (per)* and *cryptochrome (cry)* (Fig. 2). The genes *per* and *cry* code for the proteins Per and Cry which inhibit the Clock-Cycle (Clk-Cyc) dimer. The Clk-Cyc dimer is a transcription factor that promotes expression of *per* and *cry* genes. Per and Cry bind together to form the Per-Cry dimer which enters the nucleus of SCN neurons and inhibits the Clk-Cyc

dimer, thus slowing the transcription of Per and Cry. As the Per-Cry cycle subsequently declines, inhibition of Clk and Cyc transcription is reduced, resulting in the renewed production of Clk and Cyc. The cycle takes approximately 24 hours and acts to set our circadian rhythm on a molecular level (Breedlove & Watson, 2013). In this model, MEL inhibits the transcription of Per and Cry, which facilitate the production of Clk and Cyc, which in turn facilitate the transcription of Per and Cry.

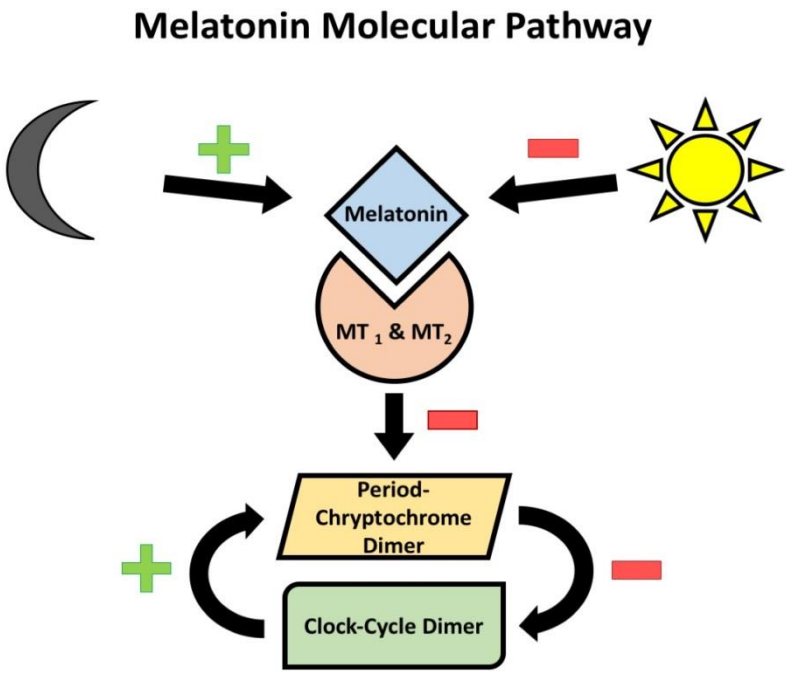

Figure 2: The MEL molecular pathway in the suprachiasmatic nucleus acts via the MT_1 & MT_2 receptors. Darkness increases melatonin production. Melatonin binds to MT_1 & MT_2 receptors to inhibit the production of the Period-Cryptochrome dimer. This in turn inhibits the production of the Clock-Cycle dimer, which increases production of the Period-Cryptochrome dimer. Light inhibits the production of melatonin which increases production the Period-Cryptochrome and Clock-Cycle dimers.

In addition to its role in circadian-synchronization, MEL serves as a powerful direct and indirect antioxidant (Lan *et al.*, 2001; Reiter *et al.*, 2001; Kaur *et al.*, 2002; Nation Research Council, 2006; Bharti & Srivastava, 2010; Pant & Rao, 2010; Bharti & Srivastava, 2011; Sahai & Sahai, 2013; Yan *et al.*, 2013; Bharti & Srivastava, 2014). Measures of blood plasma in rats have shown the MEL scavenges free radicals,

stimulates the activity of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT), and inhibits activity of radical generating enzymes (Bharti & Srivastava, 2010; Bharti & Srivastava, 2011; Bharti & Srivastava, 2014). Antioxidants mitigate cellular damage by donating electrons to radicals or reducing the production of free radicals.

Free radicals, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS), are byproducts resulting from cellular metabolism, radiation, or ingestion of toxins that cause oxidative stress (OS) when their concentrations reach toxic levels (Lykkesfeldt & Svendsen, 2007). At the cellular level, OS causes apoptosis, DNA and RNA damage, oxidation of amino acids, oxidation of cofactors used by enzymes, and lipid peroxidation (Zavodnik *et al.*, 2006; Lykkesfeldt & Svendsen, 2007; Hall & Guyton, 2016). This type of cellular damage has been implicated in several diseases, including Alzheimer's disease, cognitive decline, degeneration of the kidney, cardiovascular disease, stroke, heart attack, and liver damage (Reiter *et al.*, 2001; Widmaier *et al.*, 2008; Bharti *et al.*, 2014).

Altered Pineal Morphology

Previous research on altered pineal morphology was used to understand what changes might be seen during the current study. The morphology of the pineal gland can be affected by age or exposure to light during a dark phase. Age-associated morphological changes in the rat pineal consist of a thickening of the CTC, increased volume of acervuli, decreases in the pinealocyte to glial cell ratio, increased striated muscle fiber and adipocyte numbers, and within pinealocytes, increases in intracellular

lipid droplets, higher numbers of dense vesicles and clear vacuoles, condensed mitochondria, and mitochondria with longitudinally arranged cristae (Allen *et al.*, 1982).

In humans, the degree of pineal calcification, which has been found to increase with age, is also inversely related to MEL secretion. X-ray imaging and MEL assays found that an increased volume of acervuli was associated with decreased levels of MEL measured in the saliva (Liebrich *et al.*, 2013). These findings were corroborated with CT scans and assays (Mahlberg *et al.*, 2009), which also revealed a correlation between increased calcification and decreased MEL secretion in the urine. That study also found a correlation between increased age and increased degree of calcification, suggesting that calcification may lead to decreased MEL production. An increased glia to pinealocyte ratio resulting from both an increased number of glial cells per unit area and decreased numbers of pinealocytes is seen in aged versus young rats (Allen *et al.*, 1982 & Reuss *et al.*, 1990). In the study by Allen *et al.* (1982) older rats were seen to have alterations in organelle volumes, with mitochondria, endoplasmic reticulum, and the Golgi apparatus losing volume and lysosomes gaining volume.

In an experiment testing the effects of constant light exposure on the morphology of the pineal gland, a control group of rats on a normal 12:12-hr light-dark schedule was compared to two other groups exposed to either 45 days or 90 days of constant illumination (Gerasimov *et al.*, 2014). This experiment demonstrated a lower pinealocyte to glia ratio in the experimental groups, similar to aged rats. It was not mentioned if the altered ratio was due to an increase in glia or a decrease in pinealocytes. To examine the effect of light during sleep in rats, Karasek *et al.* (1988) compared the pineal morphology of a group of control rats living on a 14:10-hr light schedule for 7 days to a group

receiving a single 30-minute light pulse six hours after lights out. The animals were then immediately sacrificed. This experiment produced the same changes in organelle volumes as previously reported for aged rats (Allen *et al.*, 1982). Gerasimov *et al.* (2014) suggests that during (constant) light exposure the pinealocytes transition to a "rest" state, in which synthesis and secretion, facilitated by the above organelles, are halted resulting in decreased organelle size. However, rats deprived of sleep for five days were described as showing morphological changes within pinealocytes indicating increased synthesis and secretion activity (Lan *et al.*, 2001). Sleep deprived animals were found to have cellular structural alterations, including a disrupted cytoplasmic texture, dilated intracellular spaces, an increased cytoplasmic electron density, an increase in intracellular lipid droplets, large membrane bound vesicles, and dilated Golgi saccules, features that were not seen in pineal glands from control animals.

Fluoride

Fluoride, an anion, tends to concentrate in areas of the body containing the cations calcium, aluminum, or magnesium due to the attraction of their opposite charges. Organofluorines are fluoride-containing compounds which are lipid soluble, allowing them to passively traverse the mucosa of the gastrointestinal tract and penetrate the blood-brain barrier, where they can accumulate in the brain (U.S. National Research Council, 2006; Bharti & Srivastava, 2009; Czajka *et al.*, 2012; Lubkowska, 2012). Fluoride has been found in the pineal glands of humans and ducks using potentiometric measures (Luke, 2001; Kalisinska, 2014). Tissue samples of bone, muscle, and pineal gland were assayed using a fluoride ion specific electrode revealing abnormally high levels of fluoride in the human pineal gland (Luke, 2001). After extracting the fluoride,

the remaining pineal tissue was analyzed using atomic absorption spectroscopy to determine the calcium concentration. It was discovered that fluoride concentrations were correlated with the amount of calcium, suggesting that the fluoride anion was attracted to the positively charged calcium within the mitochondria, Golgi apparatus, cytoplasm, and nucleus of pinealocytes.

Interest in water fluoridation began in 1901 when Dr. Frederick McKay discovered reduced dental decay rates in areas of Colorado where residents had brown "mottled" teeth (Mullen, 2005). In 1931, the Aluminum Company of America correlated elevated levels of fluoride with reduced dental decay and browning of residents' teeth in Bauxite, Arkansas. This discovery inspired a six-year longitudinal study on the effects of water fluoridation in the United States (Centers for Disease Control and Prevention, 1999; Mullen, 2005). Six pilot studies were planned across the U.S. in which neighboring towns with similar rates of tooth decay, identified by survey, were selected in Michigan, New York, and Illinois. It was found that areas containing around 1 ppm of fluoride in the water had the benefits of reduced dental decay without the browning of teeth. Thus, the U.S. began adding fluoride to public drinking water to strengthen teeth and lessen tooth decay (Center for Disease Control, 2015).

By eliminating dental caries (Iheozor-Ejiofor *et al.*, 2015), water fluoridation is regarded as one of the 10 greatest public health achievements of the 20th century (Centers for Disease Control and Prevention, 1999). Iheozor-Ejiofor *et al.* (2015) argues that there is little reliable evidence showing that water fluoridation prevents dental caries, claiming that data evaluating the effectiveness of fluoride is outdated and does not take into account other medical advances made in the last 50 years. Studies assessing the

effects of fluoride on dental health found its effectiveness at preventing tooth decay dropping from 50% - 70% in the 1940s to 18% in the 1980s (Centers for Disease Control and Prevention, 1999). Instead, fluoridated toothpaste and regular dental checkups are postulated to be major factors contributing to the drop in tooth decay rates over this time period. This conclusion is based on a comparison of immature and mature decayed, missing, or filled teeth; the proportion of caries-free children; and changes in the proportion of caries-free children in populations exposed to fluoridated water versus non-fluoridated water. Thus, rates of tooth decay are similar between countries that fluoridate their water supply, at up to 4 ppm, compared to countries that do not fluoridate the drinking water (Czajka *et al.*, 2012). Furthermore, it has been shown that fluoride must be applied to the surface of adult teeth to be effective in preventing dental caries (U.S. National Research Council, 2006). In a review of the available data, it was concluded that systemic fluoridation via the water supply did not have an effect on the prevalence of dental caries (Czajka *et al.*, 2012). Therefore, it appears that fluoride only protects mature teeth when it is applied directly to the surface, and thus drinking fluoride-treated water does not bestow an anticaries effect. Despite this, the American Dental Association (2005) advocates for the use of fluoride in drinking water, saying that it benefits developing teeth by incorporating fluoride into the structure of the hydroxyapatite crystals of the tooth enamel, thus making the crystals harder. Interestingly, no fluoride was reported when examining hydroxyapatite crystals in the pineal gland. However, the presence of fluoride in drinking water also increases the risk of fluorosis.

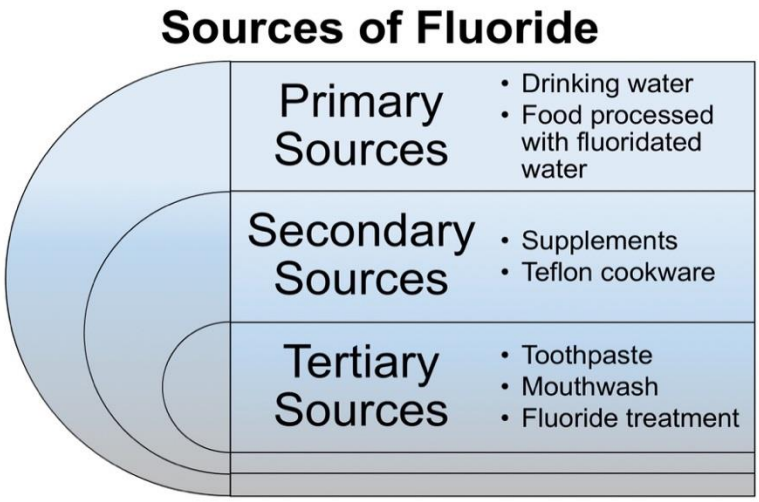

Figure 3: Common sources of fluoride exposure. Fluoride is also found in food made with fluoridated water, and in toothpaste, mouthwash, Teflon, and nutritional supplements. It is important to consider all sources of chronic fluoride exposure that may lead to fluorosis.

Effects of fluoride

Excess ingestion of fluoride leads to fluorosis. Dental fluorosis is identified by streaking of the teeth which may begin white (Browne *et al.*, 2005), but eventually becomes brown and pitted (Carton & Park, 2006). In bones, osteofluorosis hardens bones to the point of brittleness, thus making them more susceptible to fractures. Chronic exposure to fluoride levels as low as 2 ppm have been associated with stage II or stage III osteofluorosis, which are the highest stages and are deemed to be "crippling" (U.S. National Research Council, 2006). While fluoride may be an effective anticaries treatment and can harden hydroxyapatite crystalline structures, the material safety and data sheet for sodium fluoride classifies it as a mutagen (Science Lab, 2013) and research exploring the effects of fluoride on the hippocampus concluded that fluoride alters neural morphology (Akinrinade *et al.*, 2015). Fluoride is recognized to cause alterations to the nervous system and The United States National Research Council (2006) recommends more research to determine the risks associated with fluoride exposure.

In addition to MEL, the pineal gland produces a variety of proteins, peptides, and enzymes known collectively as pineal proteins (PP). Bharti and Srivastava (2009) and Bharti *et al.* (2014) used pineal proteins obtained from water buffalo pineal glands to examine blood plasma indicators of oxidative stress in rats. Pineal proteins were selected because the bulk of antioxidant research involving the pineal gland focuses solely on MEL. It is not known if PP are secreted by the pineal gland into the blood stream or if they act locally in the pineal gland.

Animals in experiments by Bharti and Srivastava (2009) and Bharti *et al.* (2014) were given 150 ppm sodium fluoride (NaF) via drinking water and either an intraperitoneal (i.p.) injection of PP, MEL, or saline. The results showed that the fluoride + saline group had decreased levels of several antioxidant enzymes, including CAT, SOD, GPx, and GR and a significantly higher measure of lipid peroxidation (LPO) than controls which indicates that fluoride induces oxidative stress and cell membrane oxidation. In contrast, groups treated with fluoride + MEL or fluoride + PP showed no significant difference in the levels of CAT, GPx, SOD, and GR compared to the control group. Thus these results suggest that MEL and PP can decrease the effect of oxidative stress induced by fluoride by acting as direct or indirect antioxidants. It is not known if fluoride acts to inhibit the synthesis of these antioxidant enzymes, is competitively inhibiting the active site of antioxidant enzymes, or if it promotes excessive production of free radicals.

Bharti and Srivastava (2010) also tested the effects of MEL and PP on additional biochemical indices of fluoride-induced oxidative stress in rats. Fluoride was administered either in drinking water at 150 ppm and blood serum concentrations of the

enzyme alkaline phosphatase (ALP) were measured. ALP is secreted by osteoblasts during bone formation and thus is a marker of osteogenic activity (Bharti and Srivastava, 2010). The animals in the fluoride treatment group had significantly increased levels of serum ALP compared to the no-fluoride control group. However, all animals except the fluoride + PP group had higher levels of ALP than the controls. These increased levels may indicate that the vehicle increased ALP levels or another confounding variable affected the measures and the increased levels of ALP was not due to fluoride. Interestingly, the fluoride + PP and fluoride + MEL treatment groups had significantly higher levels of ALP compared to the control and lower levels of ALP compared to the fluoride-only treatment group. Increased levels of serum ALP were also found in animals exposed to up to 10 ppm of sodium fluoride in their drinking water for thirty days (Akinrinade *et al.,* 2015). Both of these studies suggest that fluoride treatment may cause excessive activation of osteoblasts leading to abnormal bone formation and this excess activation can be reduced by MEL or PP.

Fluoride has been shown to alter the morphology of brain neurons. Histological examination of hippocampal neurons of rats treated with 2 ppm or 10 ppm of fluoride in drinking water for thirty days showed decreased cell diameter, with increased markers of cell death, increased cytoplasmic vacuoles, pyknosis, and karyolysis (Akinrinade *et al.,* 2015). Cathepsin D expression, used as an indicator of cell death, was increased in the the fluoride treatment groups, indicating increased rates of apoptosis and necrosis. These results were exacerbated when fluoride was combined with aluminum which facilitates the uptake of fluoride by cells. Levels of acetylcholine esterase in the hippocampus, used as a marker for the cellular mechanism of learning and memory formation, were

decreased. However, this difference was not significant unless fluoride was combined with aluminum. These results show that low levels of fluoride can alter neuronal morphology and possibly lead to neuronal death in the hippocampus.

Rats treated with high doses of fluoride (500 ppm) in their drinking water ate less food and subsequently had decreased body weight (Ekambaram & Paul, 2001). However, this dose of fluoride far exceeded the LD_{50} concentration of 52 mg/kg since 1 ppm = 1 mg/kg (Science Lab, 2013). My planned doses of 1.2 ppm and 20 ppm were chosen because 1.2 ppm the maximum amount of fluoride accepted by the EPA as being safe to add to drinking water, while 20 ppm was chosen to induce effects in the animal.

Hypothesis

The objective of this study was to assess the effect of fluoride on pineal morphology using three control groups and two fluoride treatment groups. Also examined were measures of health and animal well-being, including body weight and water and food consumption, and changes in pineal morphology associated with consuming fluoride-free food and water for eight weeks. Since fluoride has an affinity for the calcium found in high concentration in the pineal gland, and fluoride alters neuronal morphology, this experiment will examine how administering sodium fluoride in the drinking water of rats may affect the cellular structure of the pineal gland.

Methods

Protocol 04-16 was approved by the Northeast Ohio Medical University's Institutional Animal Care and Use Committee on August 31st 2016. Animal use adhered to the current Nation Institute of Health's Guide for the Care and Use of Laboratory

Animals, 8th edition. Animals were sacrificed using carbon dioxide asphyxiation and then decapitated.

Population

Twenty-five male Sprague-Dawley rats (Charles River, Ohio), initially weighing an average of 582.75 g, were housed for eight weeks in Youngstown State University's Biological Sciences' animal facility. The animals were twenty-six months-old and had been used in previous behavioral research projects unrelated to this study. Animals were housed in clear plastic cages (20' x 16" x 8.5") with metal cage tops. Each cage had bedding and contained three to four animals. Dry rat food with 0 ppm fluoride (Bioserv Rodent diet, AIN-76A; Prolab, 2015) was provided *ad libitum* throughout the experiment. The animal facility was on a reverse 12-hour light-dark schedule, with light periods being 10:30 am – 10:30 pm. The animals' water was changed daily and all accommodations not relating to water and food administration were provided by the animal care staff. All animals were monitored during water changes and weighed weekly to verify the well-being of the animals.

Pineal glands from all animals were harvested, but only seventeen glands were used to measure morphological features. The other animals were initially included to ensure that the appropriate number of pineal glands were obtained if any animals died or had to be removed from the experiment. One animal in Group 2 died during week four and one animal in Group 5 died during week two. One pineal gland in Group 2 was unrecoverable and the initial weights for animals in Group 1 were not recorded.

Animal wellness

All animals were observed daily and monitored for signs of distress. Fresh water was supplied daily and the amount drank was recorded as milliliters per animal per week. A measured amount of food was provided each week and the amount eaten was recorded as grams per animal per week. Body weight in grams was taken for each animal weekly.

Animal experimental groups

Animals were divided into five experimental groups, consisting of two to seven animals per group (Table 1; Fig. 4). Groups 1-3 served as control groups. To compare the effects of previous exposure to standard rat chow and tap water (0.9 ppm NaF) (Aqua America, 2016), the animals in Group 1 were sacrificed at the beginning of the experiment (day 0). All of he remaining animals were then placed on Milli-Q filtered water (Laboratory standard Type I ultrapure water; 18.2 MΩ*cm at 25 ℃) and fluoride-free food for four weeks to "flush" fluoride from their systems. The second control group, Group 2, was sacrificed at the end of this four-week flush (day 28). The last controls, Group 3, were maintained on fluoride-free food and water for an additional four weeks before being sacrificed at the end of week 8 (day 56).

The experimental groups, Groups 4 and 5, were used to compare the effects of exposure to fluoridated water. Animals in Groups 4 and 5 were initially placed on Milli-Q water and fluoride-free food on day 0. At the beginning of week 5 (day 29), Group 4 was changed to drinking water containing 1.2 ppm NaF and Group 5 was given drinking water with 20 ppm NaF. Both groups remained on fluoride-free food. Group 4 and 5 animals were sacrificed at the end of week 8 (day 56). Fluoridated water was prepared weekly using NaF powder (>99%, Sigma-Aldrich) dissolved into Milli-Q filtered water.

	Treatment Condition	Sacrificed	Number of Animals
Group 1	Tap H_2O (prior to start)	Onset of study (Day 0)	2
Group 2	Milli-Q H_2O (weeks 1-4)	Week 4 (day 28)	3
Group 3	Milli-Q H_2O (weeks 1-8)	Week 8 (day 56)	7
Group 4	Milli-Q H_2O (weeks 1-4) 1.2 ppm NaF H_2O (weeks 5-8)	Week 8 (day 56)	7
Group 5	Milli-Q H_2O (weeks 1-4) 20 ppm NaF H_2O (weeks 5-8)	Week 8 (day 56)	6

Table 1: Groups and treatment conditions.

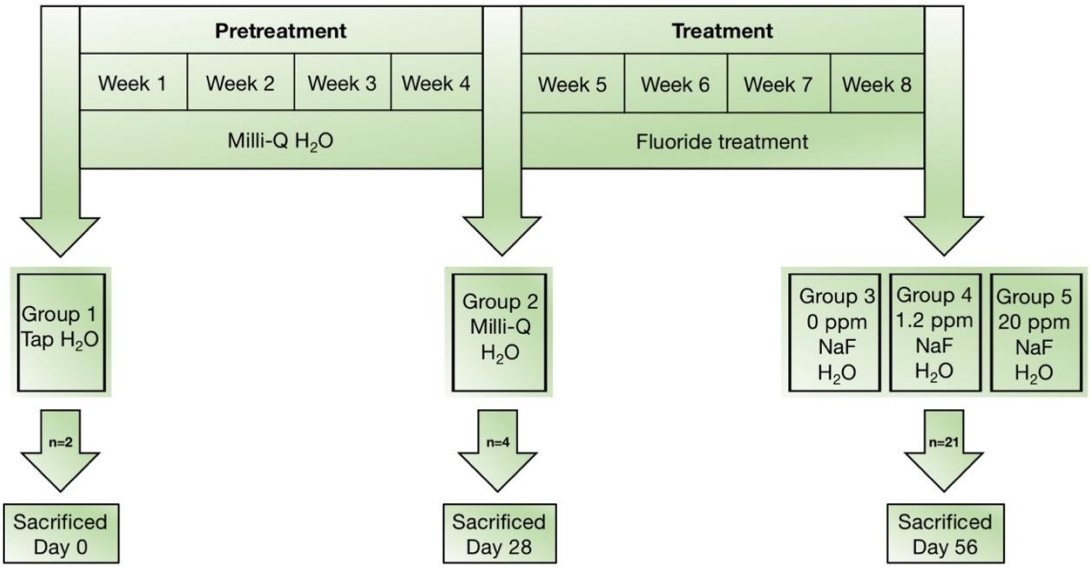

Figure 4: Graphical representation of the experimental design. Twenty-seven animals were divided into 5 treatment groups. At the onset of the experiment Group 1, which had been raised consuming fluoridated tap water and food, was sacrificed. Groups 2, 3, 4, and 5 began a four-week diet of fluoride-free food and water. Group 2 was sacrificed at the end of week four. For the next four weeks Group 3 received water with 0 ppm NaF, Group 4 received water with 1.2 ppm NaF, and Group 5 received water with 20 ppm NaF. Groups 3, 4, and 5 were sacrificed at the end of week eight.

Tissue processing and staining

Tissue processing and staining followed standard histological protocols (Humasen, 1972). Briefly, the pineal gland was removed, fixed in 10% buffered formalin overnight and stored indefinitely in 50% ethanol at 4° C. To prepare for paraffin embedding, the pineal gland was passed through a series of dehydrations in 70% and 95% ethanol (15 min each), and 100% ethanol (3x; 23 min each), then cleared using

xylene (2x 27 min each; 1x 23 min). The tissue was embedded in paraffin by submerging it in two changes of liquid paraffin at 56 ºC (12 hr.) then positioned within a tissue mold and allowed to cool and harden at room temperature. Pineal tissue blocks were sectioned using a microtome at a thickness of 7 µm and mounted on glass microscope slides. The slides had previously been "subbed" by dipping into a subbing solution (1 g gelatin and 0.05 g chromium sodium sulfate per 1 L of deionized water) and allowed to dry.

Pineal gland slides were stained with hematoxylin & eosin (H&E) (Table 2). Slides were initially passed through three changes of xylenes (10 dips each), then hydrated by passing through 100% ethanol (10 dips), and two changes of 95% ethanol (10 dips each) and into distilled water. Slides were then immersed in hematoxylin (10 min), passed through two tap water baths (10 dips each), moved into a bluing agent (5 dips) and rinsed with tap water (10 dips). They were then placed in 1% eosin Y (10 dips), two changes of 95% ethanol (1 dip; 4 dips) and dehydrated by passing through two changes of 100% ethanol (10 dips each), and three changes of xylene (10 dips each) and finally cover-slipped using Permount (Humasen, 1972).

Hematoxylin and Eosin Staining Procedure	1. Remove Paraffin
	2. Hydrate
	3. Hematoxylin 10 minutes
	4. Distilled H_2O 10 dips
	5. Distilled H_2O 10 dips
	6. Distilled H_2O 10 dips
	7. Bluing reagent 10 dips
	8. Distilled H_2O 10 dips
	9. Eosin (1%) 10 dips
	10. Ethanol (95%) 1 dip
	11. Ethanol (95%) 4 dips
	12. Dehydrate beginning at ethanol (100%)
	13. Cover-slip using Permount

Table 2: Hematoxylin and eosin staining procedure.

Morphological analysis

For each pineal gland three slides were chosen to be stained with H&E to characterize cellular morphology. Features used to distinguish between light and dark pinealocytes and supporting cells included the color and staining intensity of the cytoplasm and nucleus, the number of cell processes, and the size, shape, and chromatin density of the cell nucleus. Pinealocytes were differentiated from supporting cells by having a single cellular process, a round to rectangular cell body ranging from 7-11.2 μm in size, and a characteristically round to ovoid nucleus (Huxley and Tapp, 1972; Bastianelli & Pochet, 1993; Redecker, 1993; Al-Hussain, 2006). All non-pinealocyte nuclei which included glial cells, endothelial cells, and fibroblasts were counted as supporting cells. Light pinealocytes were more abundant in number, had lighter staining cytoplasm and nucleus that is more spherical, with less dense chromatin and a prominent nucleolus, compared to dark pinealocytes (Bastianelli & Pochet, 1993; Al-Hussain, 2006).

Cell counts and measurement were determined using a 5 x 5 reticle grid in the eyepiece of an Olympus CX22LED light microscope (Fig. 5). The reticle grid was calibrated with a micrometer revealing the side of a single square to be 170 μm in length at 40X magnification and 17.5 μm at 400X. Cell counts were made separately at the periphery of the pineal gland (defined as being within 87.5 μm from the surface of the gland) and in deeper portions of the gland.

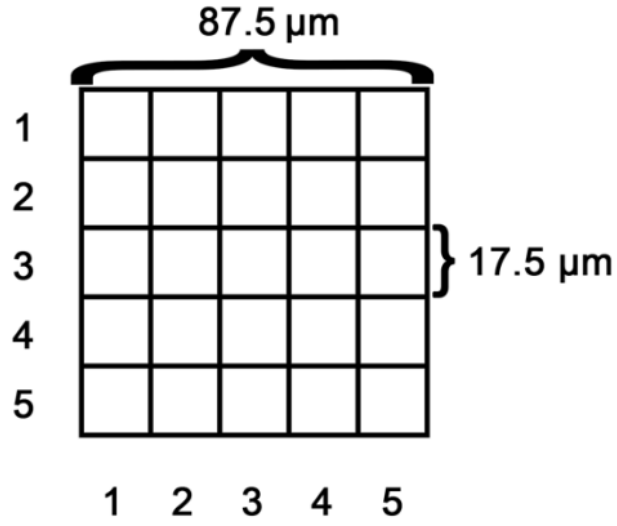

Figure 5: Grid reticle with relative dimensions at 400X magnification.

For cell counts and measurements, the microscope was initially set to 40X and the reticle was centered and aligned with a distinctive tissue landmark. Out of the twenty-five possible squares in the 5 x 5 grid, four squares were randomly selected. A random number generator (random.org) was used to generate ten sets of integers with each set consisting of numbers between one and five. The integer sets were used as coordinates corresponding to the reticle grid and the field of view was adjusted to center on the randomly selected square and zoomed to a magnification of 400X. At this magnification the full reticle grid measured 87.5 µm x 87.5 µm with an area of 7656 μm^2 which was defined as the unit area used for all tissue measurements and cell counts. All cells within the 5 x 5 grid (unit area) were counted. Any cell whose nucleus touched the outside border of the grid was excluded from the count. Each view was counted twice and averaged. If there was a discrepancy between the two cell counts greater than four, the area was counted a third time before averaging. Cells were characterized by their size, shape of nucleus, nuclear characteristics, color, and intensity of cytoplasmic staining

(light vs dark cells) as described above (Fig. 6). The nuclear diameter (in μm) of ten light pinealocytes and ten dark pinealocytes per slide were measured across the longest visible dimension.

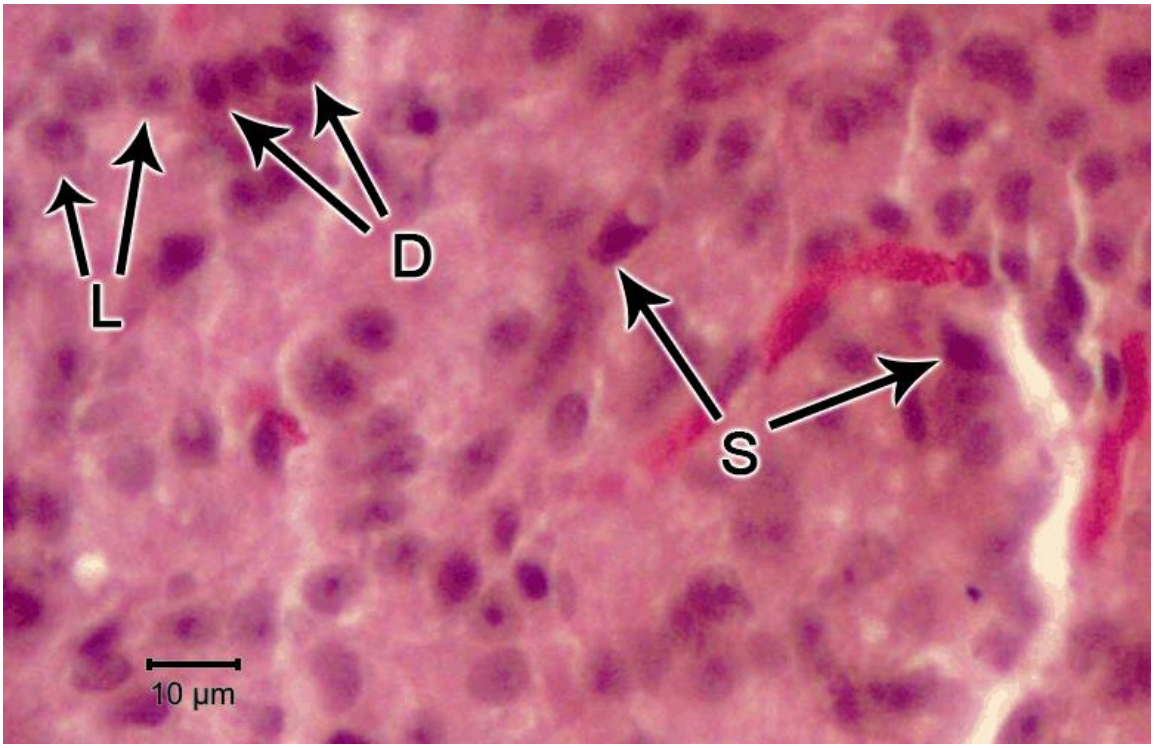

Figure 6: Photograph of light and dark pinealocytes and supporting cells using a light microscope at 400X magnification.

Statistical analyses

The following assumptions were made for analysis of variance data: the individuals in each group and were independently selected from a normally distributed population with a common variance and the effect of factors is additive. Assumptions for the multivariate analysis of variance were: observations were independently and randomly sampled from a population with equal covariance and dependent variables were normally distributed and lie on an interval scale. All data were reported as the mean ± standard deviation and data that violated homoscedasticity were log transformed. Tukey honest significant difference was used for all post hoc tests.

The weight of each animal was recorded weekly. Body weights were normalized and analyzed using a repeated-measures analysis of variance (ANOVA) (Table 3). Treatment conditions (Groups 3-5) were the between-subjects factor and time (day 0 and weeks 1-8) were the within-subjects factor.

The effect of a fluoride "flush", consisting of providing the animals with up to eight weeks of ultrapure water and fluoride-free food, on dependent measures of cell density (total number of cells per unit area, total number of pinealocytes per unit area, light pinealocytes per unit area, dark pinealocytes per unit area, ratio of light to dark pinealocytes, supporting cells per unit area, ratio of pinealocytes to supporting cells) were each analyzed using a one-way multivariate analysis of variance (MANOVA) with time (Groups 1-3) as the between-subjects variable (Table 3).

Measurements of cell density (total number of cells per unit area, total number of pinealocytes per unit area, light versus dark pinealocytes per unit area, ratio of light to dark pinealocytes, supporting cells per unit area, and ratio of pinealocytes to supporting cells) were analyzed using a two-way MANOVA to determine if there were differences based on treatment and location (Table 3). Furthermore, MANOVA was used to analyze differences in the ratio of light to dark pinealocytes based on their location. Treatment (Groups 1-5) and location (periphery versus deep) were the factors used with all cell density measures as the dependent variables. Levene's test for equality of error variance was significant thus the data was log transformed. Analysis for total pinealocytes, light pinealocytes, and supporting cells became non-significant and total cells, dark cells, ratio of light to dark pinealocytes, and ratio of pinealocytes to supporting cells became less

significant after transformation. The transformation of the data did not affect the outcome of analyses.

Measures of nuclear diameter were analyzed using a two-way ANOVA to determine if there were differences based on cell type (light versus dark pinealocytes) and treatment (Groups 1-5) (Table 3). Levene's test for equality of error variance was significant thus the data was log10 transformed. The transformation of the data did not affect the outcome of analyses.

	Between-subjects Factor(s)	***Within-Subjects Factor***	***Dependent Variable(s)***
Repeated-Measures ANOVA	Treatment (Group 3, Group 4, Group 5)	Time (day 0, week 1, week 2, week 3, week 4, week 5, week 6, week 7, week 8)	Percent change in body weight; Amount of food eaten; Amount of water drank
Two-way ANOVA	Treatment (Group 1, Group 2, Group 3, Group 4, Group 5); Type of pinealocyte (light, dark)		Diameter of nuclei
One-way MANOVA	Time (Group 1, Group 2, Group 3)		Cells; Pinealocytes; Light pinealocytes; Dark pinealocytes; Supporting cells; Light to dark pinealocyte ratio; Pinealocyte to supporting cell ratio
Two-way MANOVA	Treatment (Group 1, Group 2, Group 3, Group 4, Group 5); Location (periphery, deep)		Cells; Pinealocytes; Light pinealocytes; Dark pinealocytes; Supporting cells; Light to dark pinealocyte ratio; Pinealocyte to supporting cell ratio

Table 3: Statistical analyses (tests and variables).

Results

Animal wellness

The experiments reported here were designed to study the effects of fluoride treatment on pineal gland morphology. The well-being of animals was monitored throughout the experiment to verify that fluoride treatment did not result in decreased food or water consumption or weight loss. Previous research on the effects of fluoride indicated that animals administered 500 ppm of NaF in the drinking water consumed less food and had diminished body weight compared to control animals (Ekambaram & Paul, 2001). Body weight was therefore monitored throughout the experiment. To determine if fluoridated water had a detrimental effect on body weight, a repeated measures ANOVA was performed to analyze effect of time (day 0 and weeks 1-8) and the factor of treatment (Groups 3-5) on changes in body weight (Fig. 7). Results showed that body weight for these three groups increased significantly over time and there was no effect for treatment (Table 4). Pairwise comparisons revealed the animals (n=20) weighed significantly more during week 8 (days 50-56) (Normalized body weight: Group 3: 1.10±0.05, n=7; Group 4: 1.10±0.03, n=7; Group 5: 1.06±0.02, n=6; $p<0.001$) compared to their weight on day 0. There were no significant differences in weight gain between the groups. Therefore, 1.2 or 20 ppm NaF treatment did not have a harmful effect on the well-being of the rats.

	Value	F	df1	df2	Sig.	η^2	Power
Wilk's Λ	0.05	25.74	8	10	$p < 0.001$	0.954	1

Table 4: Results of repeated measures ANOVA of change in body weight over time.

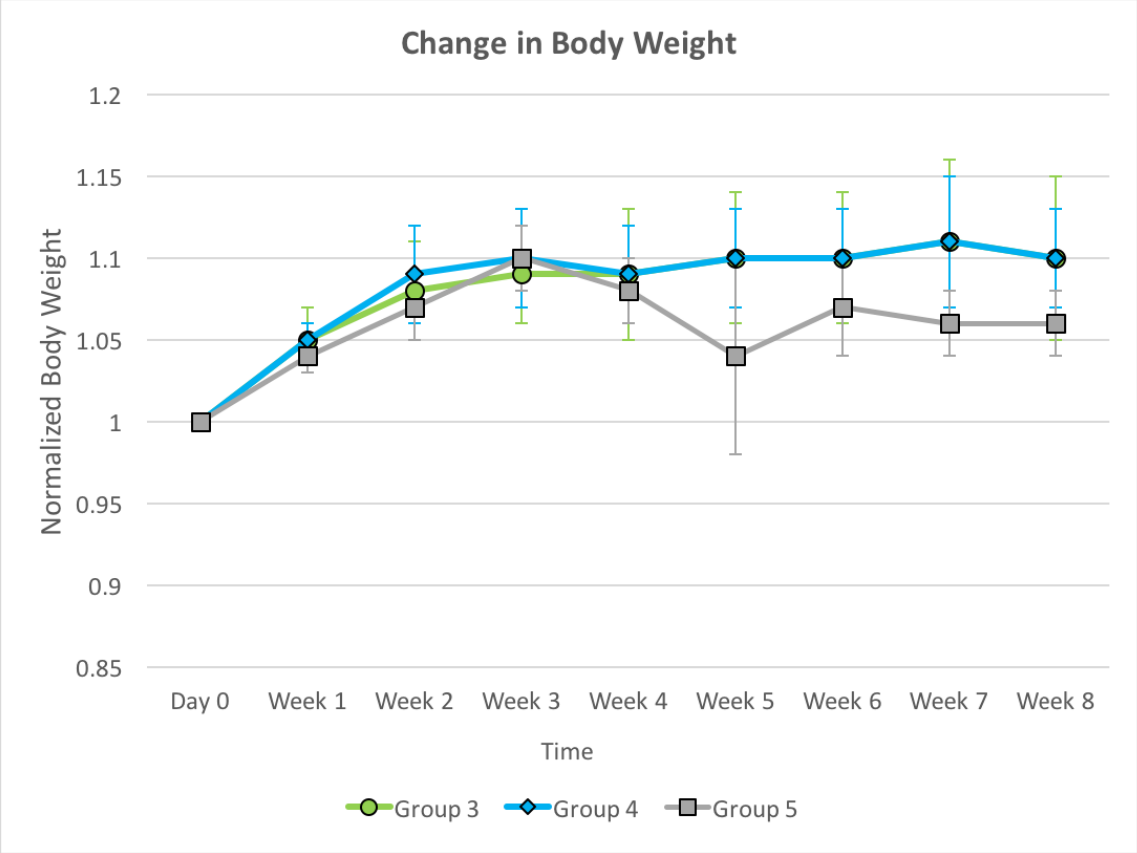

Figure 7: Change from initial body weight per animal for each group over time.

The amount of food consumed per animal was recorded weekly. Animals in all groups were provided with fluoride-free food throughout the eight-week experiment. To determine whether fluoride treated drinking water deceased food consumption, a repeated-measures two-way ANOVA was performed to analyze the effect of time (week 1-8) and treatment (Groups 3-5) on the amount of food consumed per animal per week (Fig. 8). Results indicate that there was no effect of time or treatment on amount of food eaten per animal per week.

Figure 8: Food eaten per animal per week.

The amount of water consumed by the animals was also recorded to verify that the addition of NaF to the drinking water did not adversely affect water consumption. All groups were given fluoride-free, Milli-Q filtered water for weeks 1-4. During weeks 5-8, Group 3 continued with fluoride-free water while Groups 4 and 5 were switched to drinking water containing fluoride (Group 4: 1.2 ppm NaF; Group 5: 20 ppm NaF). A repeated measures two-way ANOVA was performed to analyze the effect of time (weeks 1-8) and treatment (Groups 3-5) on the amount of water the animals consumed (Fig. 9). Results indicated that there was no effect of time or treatment on the amount of water consumed per animal per week. However, there was a non-significant drop in the amount drank by all groups on week 5 corresponding with the start of fluoride treatment and the death of an animal in Group 5.

These analyses show that up to 20 ppm of NaF in drinking water during weeks 4-8 did not affect body weight, the amount of food eaten, or water drank. Instead, the

animals in both the control and treatment groups ate steadily and body weight increased over time. The animals gained weight during weeks 1-4, after which body weigh stabilized during weeks 5-8. Food and water consumption remained stable throughout, and the addition of fluoride to the drinking water of Groups 4 and 5 did not affect consumption. Therefore, fluoride treatments did not have deleterious effects on animal well-being.

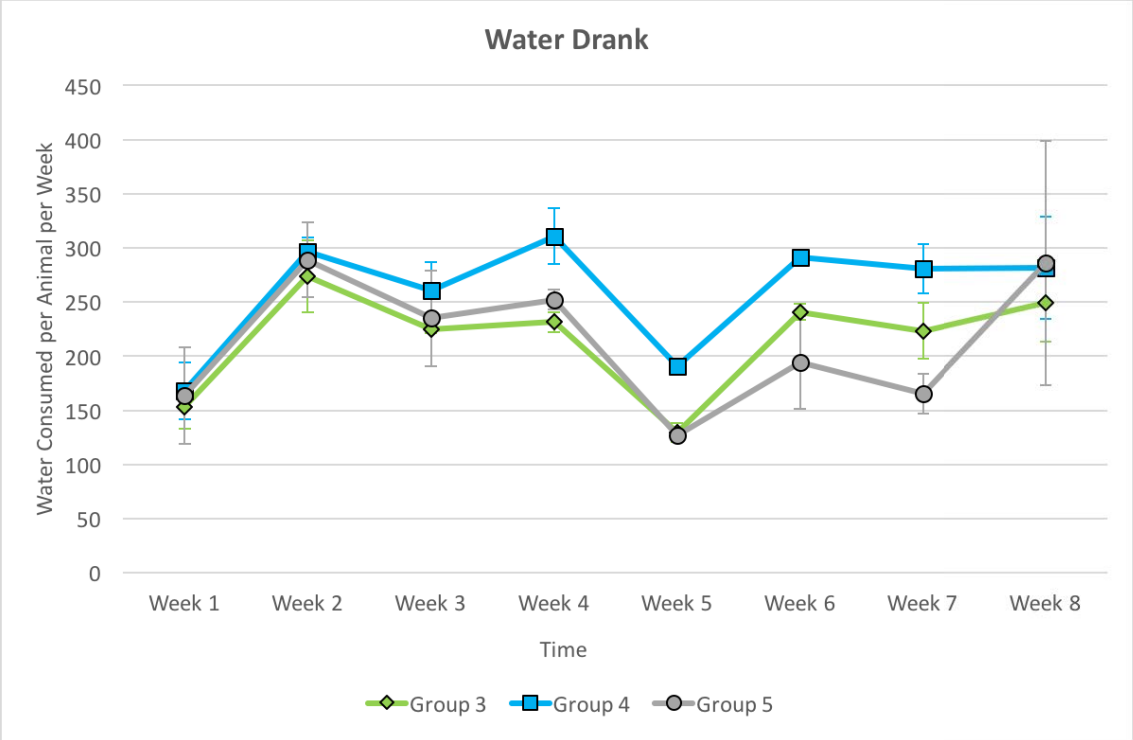

Figure 9: Water drank per animal per week.

Effects of fluoride-free flush

No background information or previous research was found that examined the effects of a fluoride flush on pineal gland morphology. Therefore, Groups 1-3 were used to asses the effects of fluoride-free food and water on the morphology of the pineal gland. Pineal glands obtained from animals previously raised with fluoridated tap water and sacrificed at day 0 (Group 1) served as a baseline control group. The morphology of

glands from animals given a fluoride flush for four weeks (Group 2) or eight weeks (Group 3) were then compared to this baseline. The repeated measures two-way ANOVA revealed significant differences in the number of cells, pinealocytes, light versus dark pinealocytes, and supporting cells per unit area, and in the ratios of light versus dark pinealocytes and pinealocytes to supporting cells (Tables 5).

Multivariate tests							
	Value	**F**	**df1**	**df2**	**Sig.**	**η2**	**Power**
Wilk's Λ location	0.97	.47	6	87	.83	0.03	0.18
Wilk's Λ treatment	0.53	5.35	12	174	p < 0.001	0.27	1
Wilk's Λ interaction	0.68	3.09	12	174	.001	0.18	0.99

Levene's test of equality of error variances				
	F	**df1**	**df2**	**Sig.**
Total cells	1.84	5	92	0.11
Pinealocytes	1.7	5	92	0.14
Light pinealocytes	2.33	5	92	0.049
Dark pinealocytes	0.91	5	92	0.48
Supporting cells	1.37	5	92	0.25
Light to dark pinealocyte ratio	2.64	5	92	0.03
Pinealocyte to supporting cell ratio	1.24	5	92	0.3

Table 5: Multivariate and Levene's test for homogeneity of variance results for two-way MANOVA for the fluoride-flush groups 1-3.

Counts of all cells within randomly chosen areas of each pineal gland were made to determine if a fluoride-free diet affected cell numbers. It was found that the total number of cells per unit area significantly increased with time during the fluoride flush (Fig. 10). Both Group 2 (40.78±8.88 cells/unit area, n=24, $p<0.001$) and Group 3 (41.13±8.38 cells/unit area, n=50, $p<0.001$) had significantly more cells compared to Group 1 (26.41±4.46 cells/unit area, n=24). There was no significant difference between Groups 2 and 3 in total cell number per unit area.

Total cells within the pineal gland include both pinealocytes and supporting cells. Pinealocytes were differentiated from supporting cells as described above, based on previously described morphological features (Huxley & Tapp, 1972; Al-Hussain, 2006). The increase in total cell numbers observed in Group 2 after four weeks of a fluoride-free flush was due to a significant increase in supporting cell numbers (Fig. 10), with Group 2 having 23.99±9.18 supporting cells/unit area (n=24), compared to Group 1 (12.21±3.40 supporting cells/unit area, n=24; $p<0.002$). Thus, four weeks of fluoride-free food and water is correlated with an increase in supporting cells. However, this increase was reversed beyond week 4, with no significant difference in supporting cell numbers between Group 1 and Group 3 (16.44±5.23 supporting cells/unit area, n=50). Thus a four-week fluoride flush appears to encourage the proliferation of supporting cells.

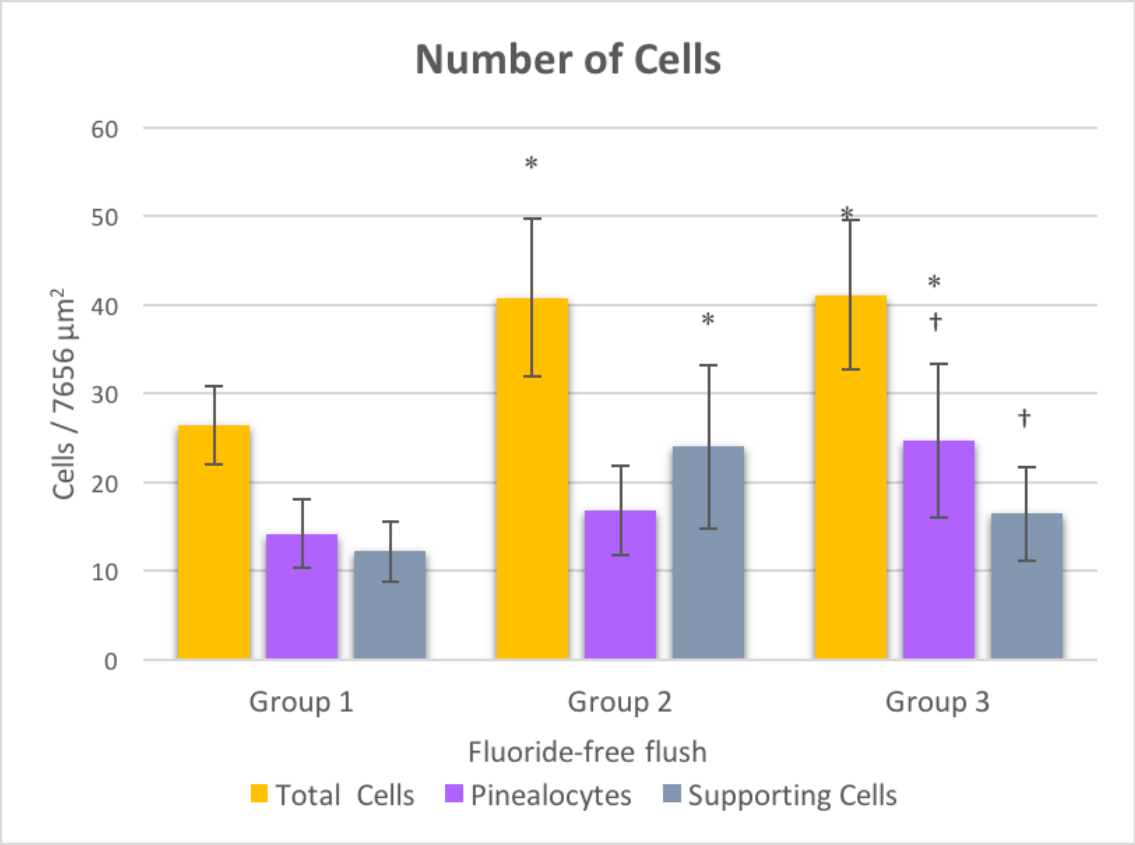

Figure 10: Change in the number of cells per unit area over time. * indicates a significant difference from Group 1 ($p < 0.05$). † indicates difference from Group 2 ($p < 0.001$).

In contrast, the increase in total cells observed in Group 3 was due to a significant rise in the number of pinealocytes per unit area, with Group 3 (24.69±8.60 pinealocytes/unit area, n=50, $p<0.001$) having significantly more pinealocytes per unit area compared to Group 1 (14.20±3.90 pinealocytes/unit area, n=24) or Group 2 (16.79±4.98 pinealocytes/unit area, n=24, $p<0.001$) (Fig. 10). There was no significant difference between Group 1 and Group 2 in the number of pinealocytes.

The Group 3 increase in total pinealocytes after eight weeks of fluoride-free flush resulted from increases in both light and dark pinealocytes (Fig. 11). Group 3 thus showed significant increases in both light cells (15.15±6.00 light cells/unit area, n=50) and dark cells (9.54±4.17 dark cells/unit area, n=50) compared to Group 1 (9.09±2.66 light cells/unit area, n=24, $p=0.006$ and 5.11±1.68 dark cells/unit area, n=24, $p<0.001$) or

Group 2 (10.25±3.14 light cells/unit area, n=24, p<0.001 and 6.54±2.65 dark cells/unit area, n=24, p=0.001). In addition, there were significantly more (p=0.026) dark pinealocytes in Group 2 compared to Group 1. Thus the results show that the eight-week fluoride flush resulted in significantly higher numbers of light and dark pinealocytes.

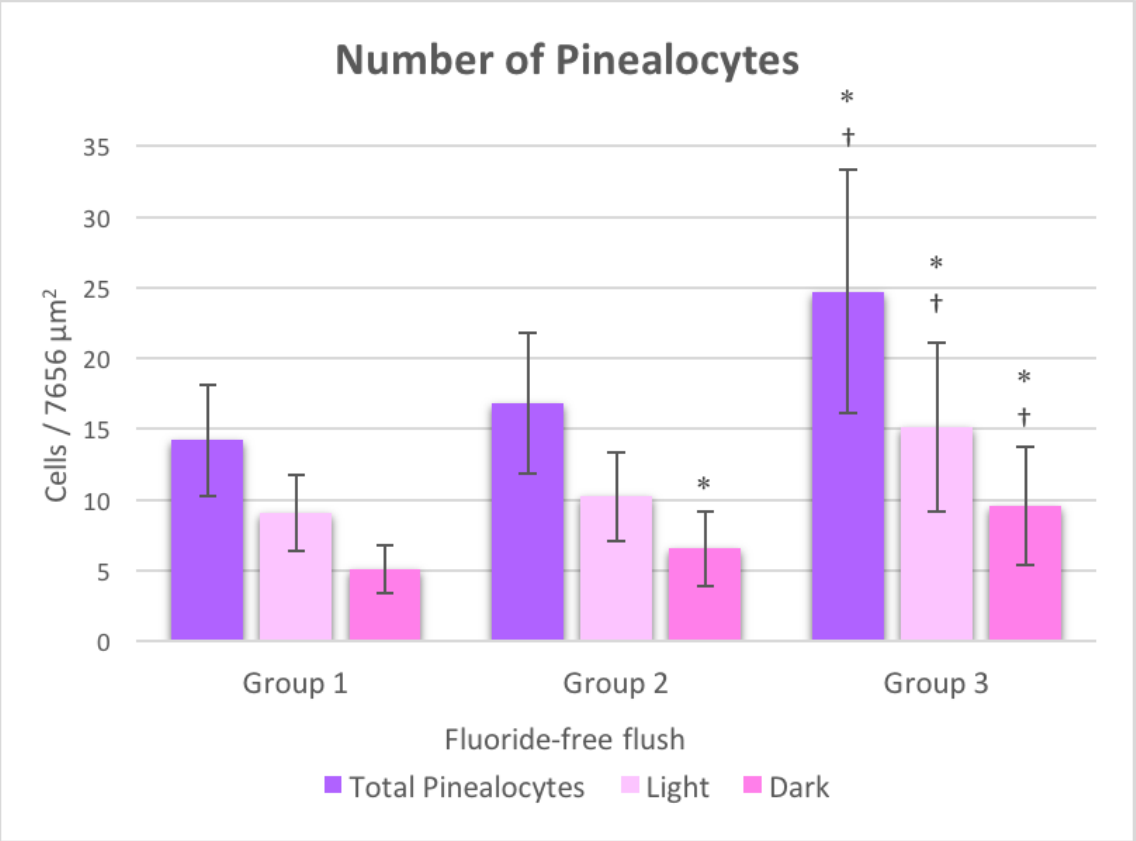

Figure 11: Change in the number of pinealocytes per unit area over time. * indicates significant difference from Group 1 ($p < 0.05$). † indicates difference from Group 2 ($p < 0.01$).

The ratio of pinealocytes to supporting cells was analyzed to determine if the fluoride-free flush affected pinealocytes and supporting cells differently. The ratio of pinealocytes to supporting cell initially showed a non-significant decline, but then increased (Fig. 12). The ratio of pinealocyte to supporting cell was not different between Group 1 (1.29±0.67 pinealocyte/supporting cell, n=24) and Group 2 (0.84±0.47 pinealocyte/supporting cell, n=24). However, Group 3 (1.75±1.03 pinealocyte/supporting

cell, n=50) did show a significant increase ($p<0.001$) in this ratio compared to Group 2, but not to Group 1. These results reflect the previous finding that Group 2 showed a selective increase in supporting cell numbers, thus lowering its pinealocyte/supporting cell ratio, while Group 3 had an increase in pinealocytes, thus raising its pinealocyte/supporting cell ratio. Therefore, the fluoride-free flush alters the ratio of pinealocyte to supporting cell due to an initial increase in supporting cell numbers at four weeks, followed by an increase in pinealocytes after eight weeks.

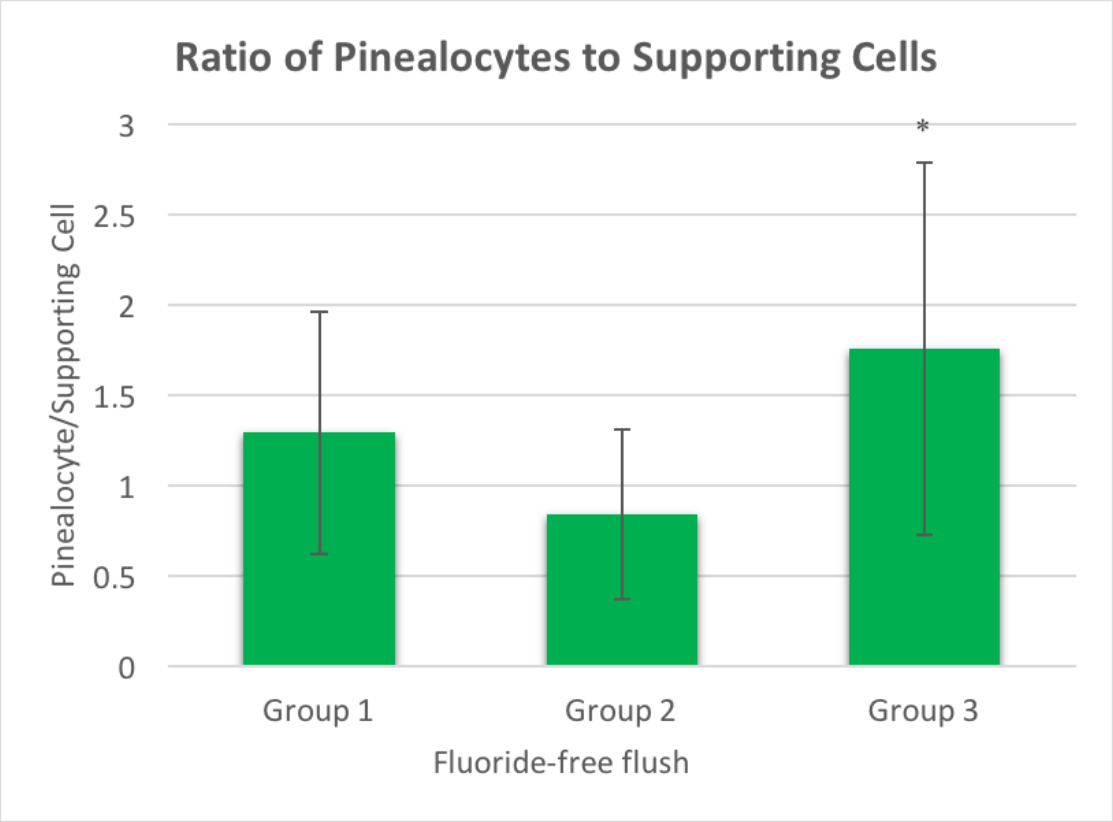

Figure 12: Ratio of pinealocytes to supporting cells over time. * indicates significant difference from Group 2 ($p < 0.001$).

Fluoride has been shown to shrink the nuclei and cell body of hippocampal neurons in rats (Akinrinade *et al.*, 2015). To determine the effects of a fluoride-flush, the diameter of pinealocyte nuclei were measured. It was found that fluoride-free food and water resulted in pinealocyte nuclear diameter being significantly smaller in both Group 2

(7.9±1 µm, n=40, p=0.005) and Group 3 (8.1±1.2 µm, n=80, p=0.018) compared to Group 1 (8.6±1 µm, n=40) (Fig. 13). Thus significantly smaller pinealocyte nuclei were seen after both 4 and 8 weeks of fluoride-flush. Since more pinealocytes were found in Group 3 (0 ppm NaF for 8 weeks) compared to Groups 1 (raised on 1.0 ppm NaF) and 2 (0 ppm NaF for 4 weeks), it is possible that the observed decreased in nuclear diameter is a sign of smaller pinealocytes due to mitosis, thus suggesting that an eight-week fluoride flush encourages pinealocyte cell division.

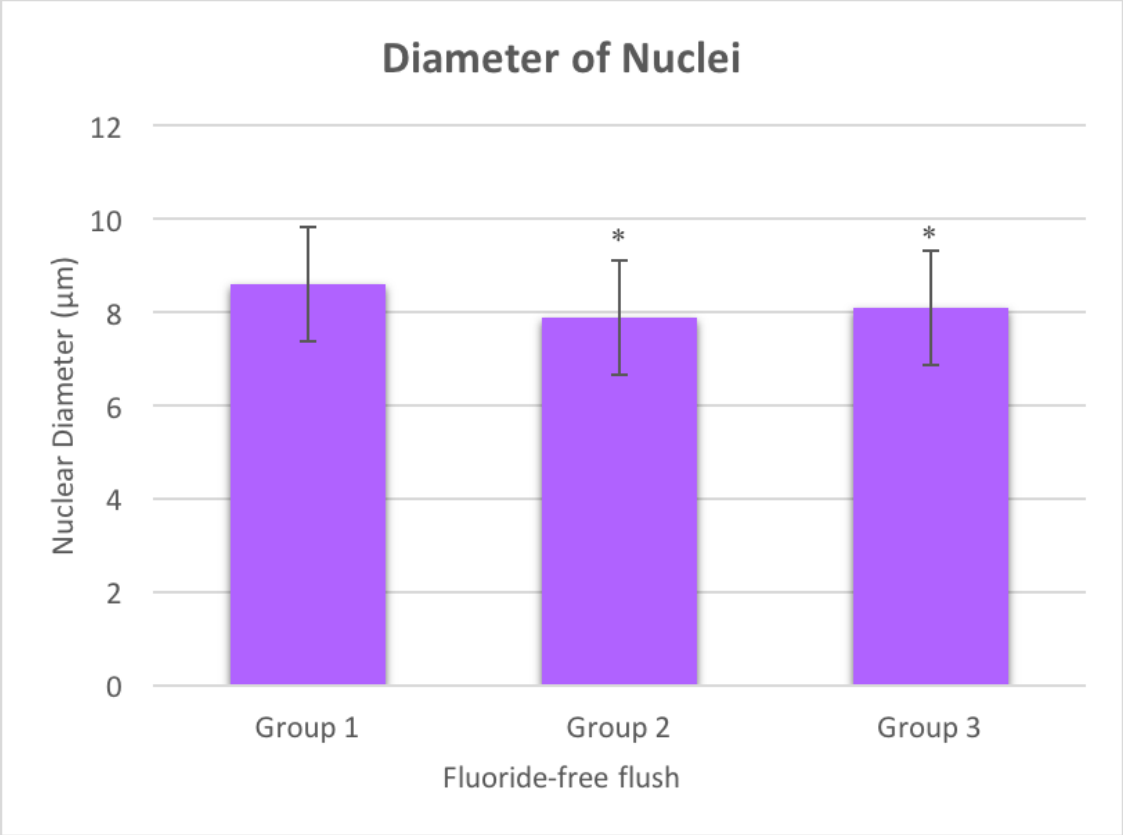

Figure 13: Diameter of pinealocyte nuclei. * indicates difference from Group 1 ($p < 0.05$).

Effects of fluoride treatment

Fluoride has been shown to accumulate in the pineal gland (Luke, 1997; 2001). Fluoride treatment also decreases body weight (Ekambaram and Paul, 2001), eating habits, neuronal survivability (Kaur *et al.,* 2009; Akinrinade *et al.,* 2015), and the size of

neuronal nuclei in rats (Akinrinade *et al.,* 2015). Rats exposed to increasingly hypoxic conditions for three months were also described as having qualitative differences in the number of light versus dark pinealocytes within the periphery versus deep pineal gland (Prosenc & Cervos-Navarro, 1994). To test the effects of fluoride treatment on pineal morphology, two groups of animals previously raised on fluoridated tap water were switched to fluoride-free food and water for four weeks (days 0-28), then changed to fluoridated drinking water for four weeks. Starting on day 29, Group 4 was given water containing 1.2 ppm NaF and Group 5 was given water with 20 ppm NaF. Control animals (Group 3) were maintained on fluoride-free food and water for the entire eight-week period. All animals were sacrificed on day 56 (week 8)

The results of the two-way MANOVA show a significant multivariate main effect for treatment condition (Groups 1-5), but no main effect for location (periphery versus deep) and a significant interaction between treatment and location (Table 6). Levene's test for homogeneity was significant for total cells, dark pinealocytes, ratio of light to dark pinealocytes, and ratio of pinealocytes to supporting cells after log transformation of data (Table 6). Based on the multivariate statistical significance univariate main effects were analyzed. Significant univariate main effects were found for numbers of cells, pinealocytes (both light and dark), supporting cells, and the ratio of pinealocytes to supporting cell (Table 7).

Multivariate Tests							
	Value	**F**	**df1**	**df2**	**Sig.**	**η2**	**Power**
Wilk's Λ Location	0.98	0.534	7	179	p = 0.81	0.02	0.23
Wilk's Λ Treatment	0.56	4.07	28	647	p < 0.001	0.14	1.00
Wilk's Λ Interaction	0.77	1.71	28	647	p = 0.01	0.06	0.98

Levene's test of equality of error variances				
	F	**df1**	**df2**	**Sig.**
Total cells	2.73	9	185	p = 0.005
Pinealocytes	1.18	9	185	p = 0.31
Light pinealocytes	1.11	9	185	p = 0.357
Dark pinealocytes	3.73	9	185	p = 0.0002
Supporting cells	1.38	9	185	p = 0.2
Light to dark pinealocyte ratio	5.44	9	185	p = 0.000001
Pinealocyte to supporting cell ratio	2.92	9	185	p = 0.003

Table 6: Multivariate and Levene's test of homogeneity of variance results of two-way MANOVA for fluoride treatment groups 1-5.

Between-subjects effects						
	Dependent Variable	**df**	**F**	**Sig.**	**η2**	**Power**
Location	Total cells	1	1.18	0.279	0.006	0.191
	Pinealocytes	1	0.68	0.41	0.004	0.13
	Light pinealocytes	1	0.65	0.422	0.003	0.126
	Dark pinealocytes	1	0.03	0.874	0	0.053
	Supporting cells	1	0.12	0.73	0.001	0.064
	Light to dark pinealocyte ratio	1	0.46	0.498	0.002	0.104
	Pinealocytes to supporting cell ratio	1	0.20	0.659	0.001	0.072
Treatment	Total cells	4	16.52	0	0.263	1
	Pinealocytes	4	14.74	0	0.242	1
	Light pinealocytes	4	9.68	0	0.173	1
	Dark pinealocytes	4	8.54	0	0.156	0.999
	Supporting cells	4	6.288	0	0.12	0.988
	Light to dark pinealocyte ratio	4	0.846	0.498	0.018	0.267
	Pinealocytes to supporting cell ratio	4	5.691	0	0.11	0.979
Interaction	Total cells	4	4.502	0.002	0.089	0.937
	Pinealocytes	4	1.025	0.396	0.022	0.32
	Light pinealocytes	4	0.788	0.534	0.017	0.25
	Dark pinealocytes	4	1.058	0.379	0.022	0.33
	Supporting cells	4	2.896	0.023	0.059	0.775
	Light to dark pinealocyte ratio	4	1.182	0.32	0.025	0.367
	Pinealocytes to supporting cell ratio	4	0.738	0.567	0.016	0.235

Table 7: Univariate results of two-way MANOVA for fluoride treatment Groups 1-5.

Total cell numbers per unit area were counted to determine if four weeks of fluoride treatment had an effect on the survivability of cells in the pineal gland. The results show (Fig. 14) that the number of cells per unit area was significantly decreased in both Group 4 (1.2 ppm NaF; 29.87±7.10 cells/unit area, n=49, $p<0.001$) and Group 5 (20 ppm NaF; 34.23±8.28 cells/unit area, n=50, $p<0.001$), when compared to Group 3 (0 ppm NaF; 41.13±8.38 cells/unit area, n=50). Interestingly, there were significantly more cells

in Group 5 compared to Group 4. Thus, the higher dose had less of an effect than did the lower dose on total cell numbers. The loss of cells in the gland with fluoride treatment is similar to previous findings showing that rats given either 2 ppm or 10 ppm NaF in drinking water exhibited molecular indicators of cell death in hippocampal neurons (Akinrinade *et al.* 2014).

Counts of pinealocytes and supporting cells were made to distinguish any differential effects of fluoride treatment. It was found that the number of pinealocytes significantly decreased with fluoride treatment (Fig. 14). Thus, compared to control animals (Group 3: 24.69±8.60 cells/unit area, n=50), fluoride-treated animals showed significant decreases in pinealocyte number (Group 4: 14.60±5.58 cells/unit area, n=49, $p<0.001$; Group 5: 16.93±6.61 cells/unit area, n=50, $p<0.001$). There was no significant difference in pinealocyte numbers between Group 4 and Group 5. In addition, no significant differences in supporting cell numbers were found between Group 3 (16.44±5.23 cells/unit area), Group 4 (15.54±6.39 cells/unit area), or Group 5 (17.30±7.24 cells/unit area) (Fig. 14).

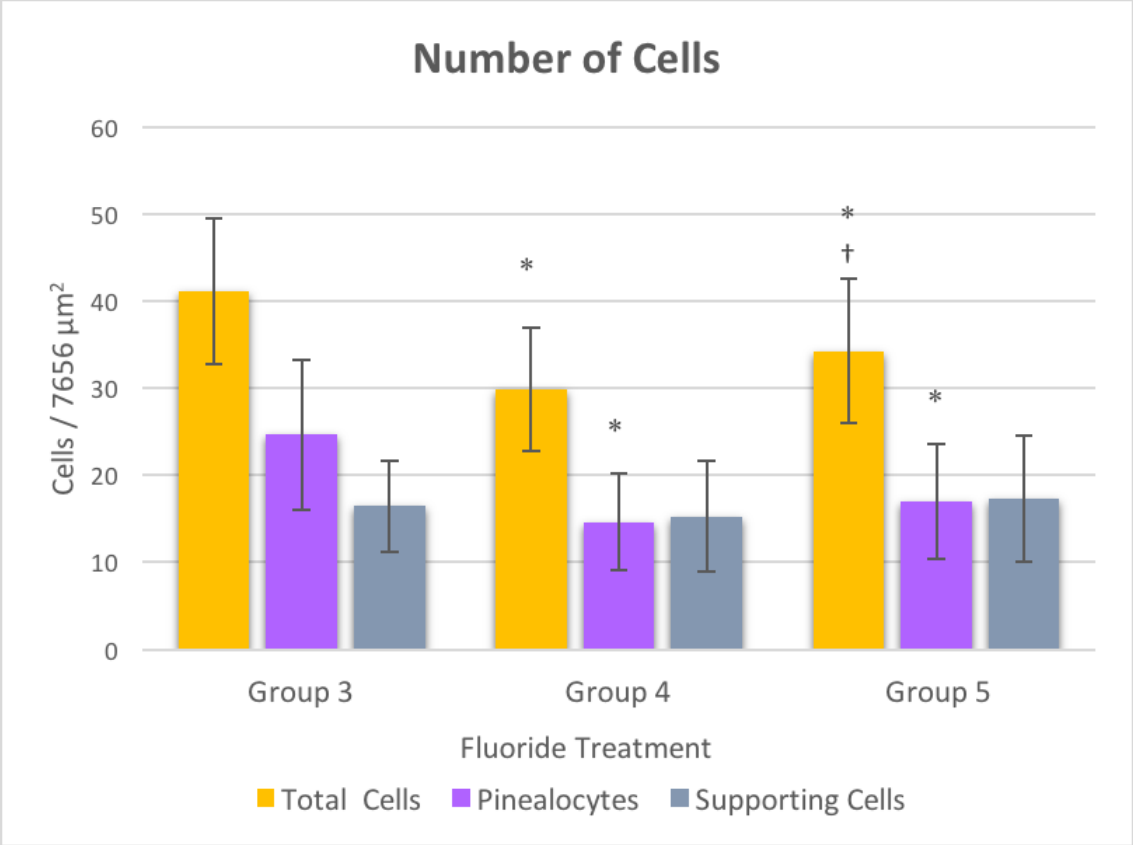

Figure 14: Number of cells per unit area for each group. * indicates a significant difference from Group 3 ($p < 0.001$). † indicates difference from Group 4 ($p<0.05$).

Pinealocytes are differentiated as either light or dark cell types (Huxley & Tapp, 1972; Karasek *et al.*, 1978; Allen *et al.,* 1982; Bastianelli & Pochet, 1993; Redecker, 1993; Prosenc & Cervos-Navarro, 1994; Al-Hussain, 2006). The number of light versus dark pinealocytes were thus also counted to determine if fluoride had similar effects on both cell types. It was found that fluoride treatment resulted in significant decreases in both light and dark pinealocytes (Fig. 15). Thus, Group 4 (1.2 ppm NaF; 9.59±3.74 cells/unit area, n=49, $p<0.001$) and Group 5 (20 ppm NaF; 10.06±3.76 cells/unit area, n=50, $p<0.001$) had fewer light pinealocytes compared to Group 3 (0 ppm NaF; 15.15±6.00 cells/unit area, n=50). Similarly, there were significantly fewer dark pinealocytes in both Group 4 (5.23±2.94 cells/unit area, n=49, $p<0.001$) and Group 5

(6.87±3.94 cells/unit area, n=50, $p<0.001$) compared to Group 3 (9.54±4.17 cells/unit area, n=50). For both light and dark pinealocytes, there were no significant differences in cell numbers between Group 4 and Group 5. Thus, fluoride treatment significantly decreased the numbers of both light and dark pinealocytes.

Despite these decreases, it was found that light cells were more abundant than dark cells in control glands (Group 3) as well as in the fluoride treated gland (Groups 4 and 5), which agrees with earlier reports (Huxley & Tapp, 1972; Allen *et al.,* 1982; Bastianelli & Pochet, 1993; Al-Hussain, 2006). Although no quantitative values for the ratio of light to dark pinealocytes was provided in these previous studies, the current work found no differences in the ratio of light versus dark pinealocytes between Group 3 (0 ppm NaF; 1.83±0.99 light/dark pinealocyte), Group 4 (1.2 ppm NaF; 2.25±1.49 light/dark pinealocyte), or Group 5 (20 ppm NaF; 2.56±4.08 light/dark pinealocyte) (Fig. 16). Thus, fluoride treatment affected both types of pinealocytes equally.

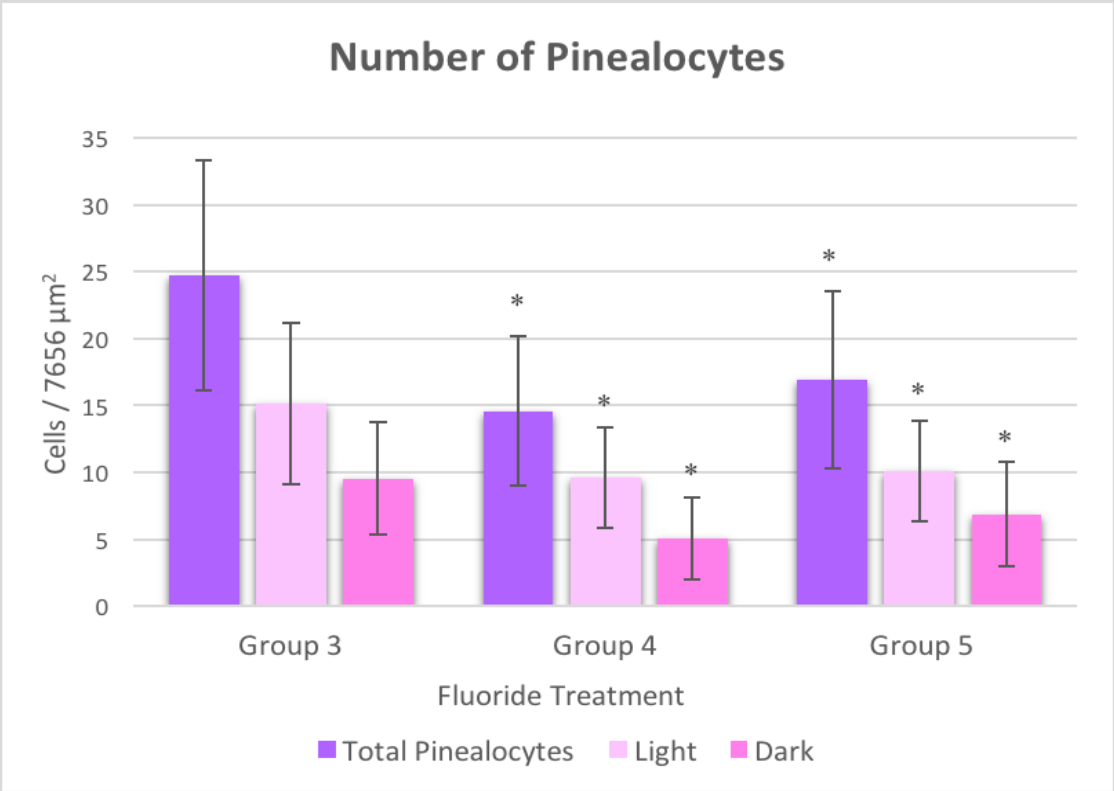

Figure 15: Number of pinealocytes per unit area for each group. * indicates a significant difference from Group 3 ($p < 0.001$).

The decrease in pinealocyte numbers following fluoride treatment, without any changes to supporting cell numbers, resulted in a significant decrease in the pinealocytes to supporting cell ratio (Fig. 17). Thus, this ratio dropped from 1.75±1.03 pinealocytes/supporting cell (n=50) in the control Group 3, to 1.22±0.93 pinealocytes/supporting cell (n=49, p=0.016) in Group 4 (1.2 ppm NaF) and 1.18±0.75 pinealocytes/supporting cell (n=50, p=0.001) in Group 5 (20 ppm NaF). This finding indicates that fluoride treatment preferentially affects pinealocytes but not supporting cells.

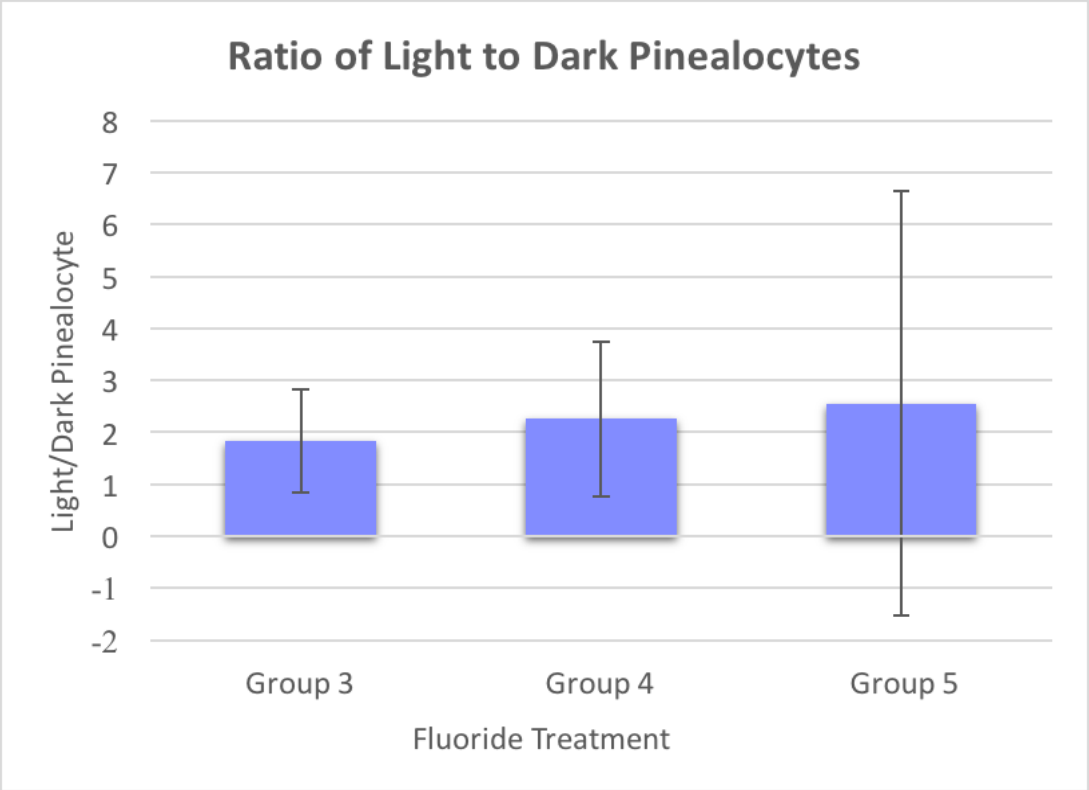

Figure 16: Ratio of light to dark pinealocyte.

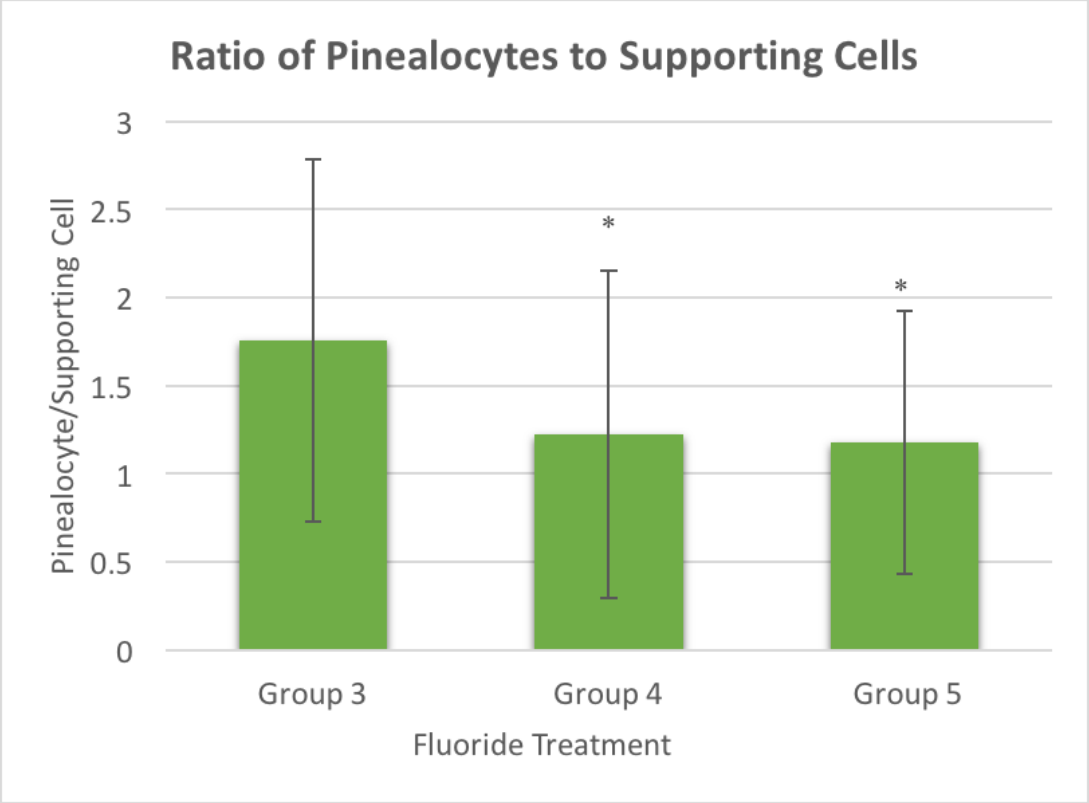

Figure 17: Ratio of pinealocytes to supporting cell by group. * indicates significant difference from Group 3 ($p < 0.05$).

A previous study reported finding more dark pinealocytes at the periphery of the pineal gland, but no information was provided regarding differences in light to dark cell ratios between the periphery versus deeper areas of the pineal gland (Prosenc & Cervos-Navarro, 1994). To look for differences in pinealocyte distributions based on location within the gland, cell counts were made at the periphery versus deeper areas. For this, the periphery was defined as being within 87.5 µm of the gland's surface. A two-way MANOVA found that there was a significant interaction ($F_{4,185} = 5.08$, $p = 0.001$) between location (periphery versus deep) and treatment (Group 3: 0 ppm NaF; Group 4: 1.2 ppm NaF; Group 5: 20 ppm NaF), but this interaction was not significant in subsequent univariate tests. Thus the numbers of pinealocytes (both light and dark) and supporting cells were not significantly different between peripheral and deep location within the pineal gland (Fig. 18).

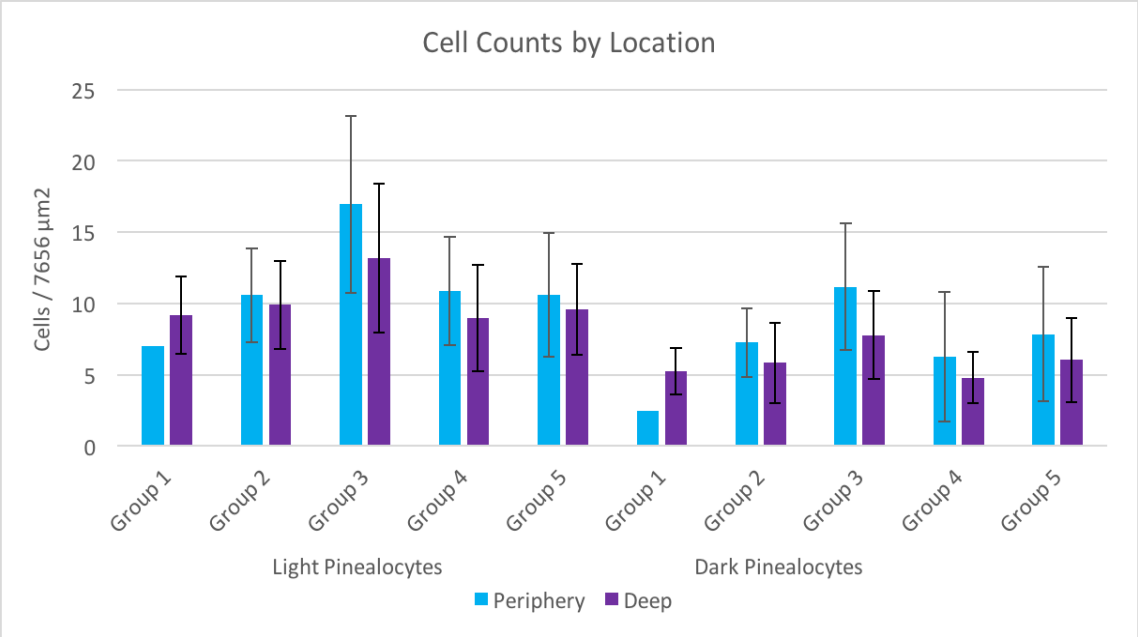

Figure 18: Number of light and dark cells in the periphery and deep pineal gland.

In rats, fluoride has been linked to reductions in the sizes of hippocampal neuron nuclei and cell bodies (Akinrinade *et al.*, 2015). Therefore, nuclear diameters were

measured to determine if fluoride treatment has a similar effect on the pinealocytes of rats. Results of the two-way ANOVA on pinealocyte nuclei diameter showed significant main effects for both the type of pinealocyte (light versus dark) and treatment condition (Group 3: 0 ppm NaF; Group 4: 1.2 ppm NaF; Group 5: 200 ppm NaF), but no significant interaction (Table 8).

Between-subjects effects					
	df	**F**	**Sig.**	**η**	**Power**
Type	1	29.45	p = 0.0000001	0.09	1
Treatment	4	4.59	0.001	0.06	0.94
Interaction	4	1.35	0.25	0.02	0.42
Levene's test of equality of error variances					
	F	**df1**	**df2**	**Sig.**	
	2.11	9	310	0.029	

Table 8: Results of two-way ANOVA for nuclear diameter.

Light pinealocytes had a smaller ($p<0.001$) nuclear diameter (7.9±1.1 μm; n=160) than dark pinealocytes (8.8±1.2 μm; n=160). It was found that only Group 4 (1.2 ppm NaF) showed a small, but significant increase in pinealocyte nuclear diameter (8.6±1.3 μm, n=80, p=0.003) when compared to the control Group 3 (8.1±1.2 μm, n=80). No differences were found between Group 5 with the highest fluoride treatment (20 ppm NaF) (8.4±1.4 μm, n=80) when compared to either Group 3 or Group 4 (Fig. 19).

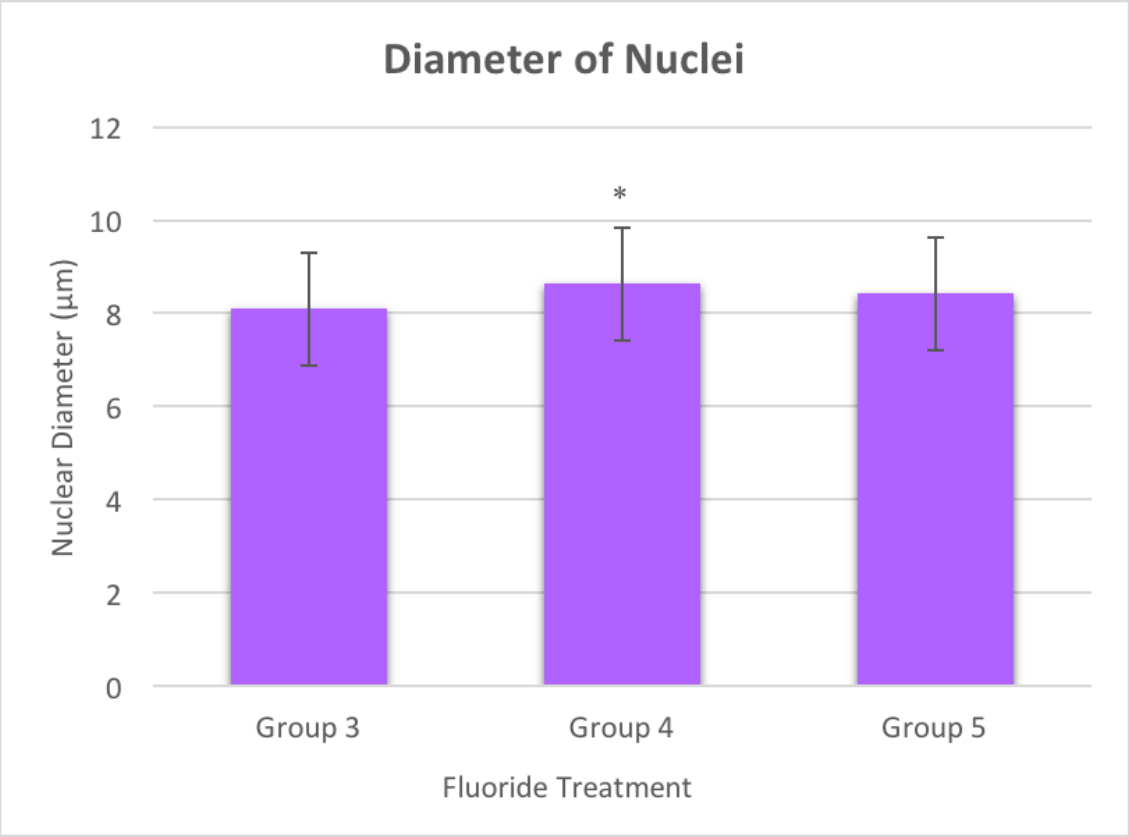

Figure 19: Diameter of pinealocyte nuclei for each group. * indicates a significant difference from Group 3 ($p < 0.01$).

Discussion

The present study examined the effects of fluoride on the cellular morphology of the rat pineal gland. Previous research found significantly higher concentrations of fluoride within the human pineal gland compared to muscle, bone, or brain tissue (Luke, 1997), suggesting that fluoride is preferentially accumulated within the pineal. Examination of the hippocampus from rats administered moderate levels of fluoride in their drinking water (2 or 10 ppm NaF) found decreased neuronal survivability and a reduction in the nuclear diameter of the remaining neurons (Akinrinade *et al.,* 2015). Changes to the morphological features of the rat pineal gland including a decrease in parenchymal cells (pinealocytes) and reduced pinealocyte to glia cell ratio, are seen with aging (Allen *et al.,* 1982; Reuss *et al.,* 1990) or disruptions to normal sleep patterns

(Karasek *et al.,* 1988; Gerasimov *et al.,* 2014). Therefore, it was hypothesized that the pineal gland of animals given fluoridated water may show a decrease in the number of pinealocytes and supporting cells, have an altered pinealocyte to supporting cell ratio, and the nuclear diameter of pinealocytes would be reduced. The present findings support the hypotheses that the number of pinealocytes and supporting cells would decrease and the pinealocyte to supporting cell ratio would be altered and disagree with the hypothesis of a reduction in pinealocyte nuclear diameter.

Prior to the start of the fluoride treatment portion of the experiment, a fluoride-free flush was done during which the animals were provided with fluoride-free food and water in an attempt to remove previously accumulated fluoride. Since no background information was found in the scientific literature which described the period of time necessary to remove fluoride from the pineal gland, a flush period of four weeks was used. It was found that switching the animals to fluoride-free food and changing their drinking water to Milli-Q filtered water for four weeks resulted in an increase in the total number of cells in the pineal gland when compared to controls raised on tap water (0.9 ppm NaF) and standard rat chow. The increase in the total cell number was due to an increase in the number of supporting cells.

Maintaining animals on the fluoride-flush for an additional four weeks (eight weeks total) did not result in any additional changes in total cell numbers. But, the increase in pineal gland cell numbers seen after eight weeks of flush was due to increases in the number of both light and dark pinealocytes rather than an increase in supporting cells. Thus there were more pinealocytes in glands from eight-week flush animals when compared to both four-week flush and tap water animals. The eight-week flush animals

also exhibited an increased pinealocyte to supporting cell ratio. Together, the flush findings indicate that switching the animals to Milli-Q filtered water and fluoride-free food is associated with an increase in supporting cells after four weeks, followed by an increase in pinealocytes after eight weeks. Although gland weights were not recorded, these findings suggest that the fluoride-free flush may have resulted in an increase in gland size, with the number of supporting cells increasing prior to an increase in the number of pinealocytes.

The amount of water and food consumed by the animals was recorded to assess the well-being of the rats and to determine the effect of fluoride treatment on food and water consumption. Previous findings indicated that rats given 500 ppm of NaF in drinking water for sixty days had reduced food consumption and weight gain compared to control animals (Ekambaram & Paul, 2001). However, in another study, rats given a twelve-week treatment of either 100 or 300 ppm NaF in drinking water did not show any differences in weight gain compared to control animals given tap water (Bataineh & Nusier, 2006). Therefore, the amount of food and water consumed and animal body weight were monitored in the present study to look for any effects of fluoride treatment. The analyses found that all animals, both fluoride-free controls and experimental animals (given water with 1.2 or 20 ppm NaF), gained weight and consumed food and water to the same degree throughout the experiment. Thus fluoride treatment was well tolerated and not harmful to the well-being of the rats.

In the current study, the highest dosage of NaF in the drinking water was 20 ppm for four weeks. Thus, the most likely explanation for the weight loss observed in the previous study is the dose of NaF used. The LD_{50} for NaF is 52 mg/kg (1ppm = 1

mg/kg_{H_2O} = 1 mg/L_{H_2O}) for rats (Science Lab, 2013) which exceeds the 500 ppm dose that resulted in weight loss (Ekambaram & Paul, 2001). The 500 ppm dose was chosen by these researchers to maximize NaF toxicity effects without significantly reducing animal survivability. In a different study, a dose of 300 ppm, which is also above the LD_{50} for NaF, was not associated with weight loss (Bataineh & Nusier, 2006). However, in these studies, the method s used to determine animal weight changes were no the same with weight being either compared to controls at the end of treatment (Ekambaram & Paul, 2001), or initial weight compared to final body weight (Bataineh & Nusier, 2006). Future research should use a repeated measures design to examine the effects of varying fluoride doses between 0 ppm and 500 ppm on weight gain and the consumption of food and water.

Brain, heart, liver, and kidney tissue of rats given 150 ppm NaF in drinking water showed increased levels of malondialdehyde and elevated activities of the antioxidant enzymes catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase, and reduced glutathione, which are all indicators of oxidative stress (Bharti & Srivastava, 2009; Bharti *et al.,* 2014). Similarly, the hippocampus of rats treated with either 2 or 10 ppm NaF in drinking water had increased levels of cathepsin D, which is a marker of cell death, compared to controls given distilled water (Akinrinade *et al.,* 2015). These findings suggest that fluoride can induce oxidative stress and may lead to cell death.

Therefore, cells within the pineal gland including pinealocytes (both light and dark) and supporting cells, were counted to determine if fluoride treatment was detrimental to the number of cells per unit area. It was found that rats given drinking water containing 1.2 or 20 ppm NaF had fewer total cells than did the fluoride-free

controls, but there were no differences in cell numbers between the two experimental groups. The reductions in total cell number following fluoride treatment was due to reductions in the numbers of both light and dark pinealocytes per unit area, with both of the fluoride treatment groups showing the same degree of light and dark pinealocyte loss. In contrast, there was no difference in the number of supporting cells per unit area in either of the experimental groups compared to controls. The loss of pinealocytes, with no change in supporting cell numbers, resulted in decreased pinealocyte to supporting cell ratios in both experimental groups. Similar decreases in the ratio of pinealocytes to supporting cells are seen in aged rats (Allen *et al.,* 1988) and rats exposed to constant illumination (Gerasimov *et al.,* 2014). The pinealocyte to glial cell ratio in animals exposed to constant illumination compared to control animals dropped from 17.01 to 15.57 pinealocyte/glial cell at 45 days and 16.95 to 13.93 pinealocyte/glial cell at 90 days. The results suggest that fluoride treatment resembles the effects of aging or constant exposure lighting, resulting in a selective loss of pinealocytes.

In humans, light pinealocyte nuclei and cell bodies are smaller in size than dark pinealocytes (Al-Hussain *et al.,* 2006). The present study found that in rats, light pinealocytes have significantly smaller nuclei than do dark pinealocytes. Compared to humans, rat pinealocyte nuclei are larger in size, indicating species differences between human and rat pinealocytes.

Research by Prosenc and Cervos-Navarro (1994) describe dark pinealocytes as occurring more frequently in the "periphery rather than the center" of the human pineal gland. However, these authors made no indication of how far the periphery extends into the pineal gland, and since there is no distinct cortical and medullary structure in the

pineal gland, their definition is subjective as was their description of the relative abundance of light and dark pinealocytes, with no cell counts or ratios reported. The present study counted light and dark pinealocytes at both the periphery, defined as 87.5 μm from the surface, and deeper areas of the pineal gland. No differences were found in pinealocyte numbers, light versus dark cells, or pinealocyte nuclear diameter based on their location within the gland.

The present study also examined the effects of fluoride treatment on the diameter of pinealocyte nuclei. It was found that animals treated with 1.2 ppm NaF had a larger pinealocyte nuclear diameter than did fluoride-free controls, while nuclei from animals treated with 20 ppm NaF were not different from controls. This may indicate that fluoride treatment has a biphasic effect on the size of pinealocyte nuclei, in which nuclei swell at lower doses of NaF and return to normal size or fragment at higher doses. Interestingly, the nuclear diameter of the 1.2 ppm treatment group was not significantly different from animals (Group 1) raised on tap water containing 0.9 ppm NaF, thus supporting the idea that low doses of NaF increases the diameter of pinealocyte nuclei. It is possible that the cells and/or nuclei are swelling or enlarging in the presence of 0.9 to 1.2 ppm NaF. In contrast, hippocampal neurons from rats given drinking water with 2 or 10 ppm NaF had shrunken cell bodies and nuclei (Akinrinade *et al.,* 2015). The differences in fluoride effects on nuclear size between pinealocytes and hippocampal neurons is therefore somewhat surprising considering the similar NaF treatment levels (1.2 and 2 ppm respectively). This suggests that fluoride has differential effects on pinealocytes and hippocampal neurons.

Conclusions

The current study found that switching animals to a fluoride-free diet and Milli-Q filtered water with 0 ppm NaF resulted in increases in the total number of cells, and in the numbers of supporting cells at four weeks and pinealocytes (both light and dark) at eight weeks, accompanied by a decrease in pinealocyte nuclear diameter. In contrast, fluoride treatment at levels as low as 1.2 ppm NaF, the maximum allowed by the EPA in U.S. drinking water, produced in the pineal gland a decrease in the total number of cells, with the numbers of both pinealocytes (light and dark) and supporting cells being decreased. There was also a small increase in size of pinealocyte nuclei with the lower level of fluoride treatment. This suggests that within the pineal gland a fluoride-free diet can reverse the effects of fluoride treatment.

Cells undergoing mitosis are generally smaller than mature cells and light pinealocytes are smaller and more abundant than dark pinealocytes suggesting that light and dark cells may be the same cell type being observed during different stages of cellular development. Furthermore, dark cells stain more darkly perhaps due to an increased number of ribosomes which are stained by hematoxylin. Therefore, it appears that dark pinealocytes may be actively synthesizing and storing MEL, while light pinealocytes are may represent pinealocytes that recently secreted their supply of MEL or they may be newly formed cells.

The morphological and cell number changes in the pineal seen here with fluoride treatment can be related to animal sleeping behavior observed in a previous experiment testing for the effects of fluoride treatment (Mrvelj & Shoup-Knox, 2013). In this study, rats provided drinking water containing 1.2 ppm NaF slept more than did control animals given fluoride-free distilled water, leading to the conclusion that fluoride

treatment disrupted sleep patterns. The results seem counterintuitive given the loss of pinealocytes following fluoride treatment observed in the present study which one expects would lead to lower levels of MEL and a drop in the amount of time spent sleeping. There were design problems in the previous study, but it appears that there is a relationship between fluoride treatment and sleep patterns. Since many homeostatic mechanisms are regulated by circadian rhythms and during sleep there may be other physiological implications that come with fluoride treatment.

Limitations

A limitation of the current study is that the presence of fluoride, melatonin, and acervuli were not quantified within the pineal gland. In the future, all of these should be quantified and then correlated with animal sleep patterns. Any observed change could then be correlated with the morphological changes and cell number counts reported in the current study.

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