DEVELOPMENTAL IMPACTS OF FLUORIDE AND ITS REMEDY ON *Xenopus laevis'* THYROID GLAND

BY

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

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Declaration by Supervisors

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DEDICATION

This thesis is dedicated to my family, friends and colleagues.

ABSTRACT

Fluoride is known to cause several non-skeletal health effects including neural, kidneys, endocrinal, thyroid gland, and disorders of the liver as well as interfering with metabolic processes when the doses are high. This study sought to evaluate developmental impacts of fluoride in the *Xenopus laevis* and the remedies using different treatments. The specific objectives of the study were to: evaluate acute toxicity of fluoride using *Xenopus laevis* embryos (FETAX test); reverse acute impacts of fluoride in *Xenopus laevis* embryos using T4 and KI; evaluate chronic toxicity of fluoride in the *Xenopus laevis* tadpoles and evaluate the effects of KI, T3, T4, and Methimazole on chronic toxicity of fluoride in *Xenopus laevis* tadpoles. This study used experimental research design. Breeding of the adults was done according to Frog Embryo Teratogenesis Assay Xenopus (FETAX) test. The fertilized eggs were divided into two groups for acute and chronic experiments. Eggs set aside for acute toxicity were used to set the experiment to test the effects of fluoride in the tadpoles and the ability of T4 (2 ppm) and Iodine (0.5 ppm) to reverse the effects. The setting of the experiment was set up the same way as the test for fluoride effects except; the LC_{50} calculated from the first part (452.8 ppm), was used as the standard test. Descriptive statistics, Abbott's adjustment, probit analysis (used to determine LC_{50} and $EC_{50)}$ and ANOVA was used to analyze the data. Teratogenic index was used to ascertain the degree of teratogenicity of the compounds. Development in chronic experiments was determined through the staging criteria of Nieuwkoop and Faber (1994). SVL was measured by using the cranial aspect of the vent as the caudal limit for the measurement. This was done using Motic® imagery software. Fertilized eggs were cultured for 17 days to attain stage 51. Tadpoles were fed with t- bites throughout the pre- exposure period (after NF stage 45/51) and throughout the experimental period. Iodine concentration was maintained at 0.5 ppm. Tadpoles exposed in AMA test were used to test for the thyroid gland impacts from the chemicals exposed to the *Xenopus laevis*. The study findings indicated that the tadpoles exposed to Fluoride exhibited abnormal behavior including non-response to touch even though they were still alive as they had visible heart palpitations. The level of significance of survival in the tadpoles was $p<.005$. Malformations across all the treatments were recorded. The tadpoles treated with F were insensitive to touch and remained completely immobile at 800 ppm F although were still alive because of visible heart palpitations. The study results revealed that the level of significance for the T4 treated tadpoles was $p>.005$, while those treated with Iodine $p<.005$. Comparing the response of the tadpoles between the Iodine treated and the T4 treated tadpoles showed that there was no significant difference between the responses as the p value was $p>.005$. Persistent exposure to fluoride multiplied the mortality, inhibited metamorphosis, and delayed development in tadpoles in both the acute and chronic experiments. T3 treated tadpoles recorded 100% mortality across all its combinations before end of experiment. All the treatments were able to reverse the impacts of fluoride exposure across all the concentrations although not fully. The study recommends the use of *Xenopus laevis* for similar studies as they are just as effective and for all relevant authorities to use this data to make all decisions.

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LIST OF ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
AMA	Amphibian Metamorphosis Assay
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CMI	Carbimazole
DIT	Diiodotyrosine
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
FD	Fluoride Deficiency
FETAX	Frog Embryo Teratogenesis Assay-Xenopus
HLL	Hind Limb Length
HPT	Hypothalamic-Pituitary-Thyroid
IE	Iodine Excess
ID	Iodine deficiency
IQ	Intelligence Quotient
KI	Iodine
MCIG	Minimal Concentration to Inhibit Growth
MIT	Monoiodotyrosine
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
PFCs	Perfluorochemicals
PLO	Pluronic Organogel
SCGE	Single Cell Gel Electrophoresis
SCHER	Scientific Committee on Health and Environmental Risks
SVL	Snout-Vent Length

T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroxine-binding globulin
TMJ	Temporomandibular Joint Disorder
TRH	Thyroid Releasing Hormone
TSH	Thyroid stimulating hormone
TTF	Thyroid Transcription Factor
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.0 Background

Fluorine is an abundant element on earth and is capable of combining with other elements to form a compound of Fluoride (Jha *et al.*, 2011). Due to its vast spread on the earth's crust, ground water has levels ranging from 1 mg/l to 25 mg/l (Perumal *et al.*, 2013). Additionally, Fluoride is also found in some fruits, vegetables and fish bone (Dratman *et al.*, 2020). It can be found in dust, wastes from industries, and when burning coal (Foda *et al.*, 2020). Therefore its presence in high quantities leads to an accumulation in the animal as well as in human bodies and can cause potential harm to their health (Domingo, 2012).

Fluoride insufficiency increases tooth decay but a prolonged contact to excess Fluoride leads to several health problems including mild teeth colorization to severe pitting, staining and enamel damage; and severe skeletal deformities and eventually loss of life may result in chronic cases. Other health effects include neural, liver disorders, thyroid, endocrine, and kidney in addition to the metabolic processes interference in high dosage (Ayoob *et al.*, 2006). Malnutrition, dietary goitrogens, Iodine deficiency and pollution has been shown to instigate non-toxic goiter (Bouaziz *et al.*, 2005; Trabelsi *et al.*, 2001).

Thyroxine (T4) and triiodothyronine (T3) hormones are produced in the thyroid gland which regulates some physiologic processes, including expected growth and development (Malin *et al.*, 2018; Mosonik, 2015). For the thyroid gland to function normally, it requires adequate consumption of iodine (100–200 mg/l) (Moturi *et al.*, 2002). Toxicity from Fluoride whether from water, food or air has caused serious public

health problems in several countries. Fluoride intake from water sources is significant irrespective of age (Onipe *et al.*, 2020). Although less than 1 ppm of fluoride intake from drinking water does not impact the performance of the thyroid or its structure (Ontumbi *et al.*, 2020). Endemic fluorotic areas have a higher intake of Fluoride. A heightened dietary fluoride ingestion has resulted in the enlargement of the thyroid, thyroid adenylate cyclase reduction, and a decrease in thyroxine (T4) and triiodothyronine (T3) in the blood (Trabelsi *et al.*, 2001).

Iodine is a vital element found in the soil and ocean waters, it aids the development of fetus and the synthesis of hormones. It is a key component in the production of thyroid hormones in the thyroid gland. Low levels of iodine result in hypothyroidism while high levels leads to hyperthyroidism (Waugh, 2019). Intake of high levels of fluoride leads to a reduction in the levels of iodine molecules in the thyroid thus hampering the production of the thyroid hormones (Foda & Shams, 2020). A halogen can replace another halogen that has a high atomic weight, as such, Fluoride (18.998 u) can easily displace Iodine (126.904 u) in the body because it is much lighter hence more reactive(Singh *et al.*, 2014).

Thyroxine (T4) is a prohormone that is produced in the thyroid gland and is inactive in nature. Thyroid stimulating hormone (TSH) regulates its production. The pituitary gland releases TSH. Deiodinases then mediate the conversion of T4 to Triiodothyronine (T3) which is the active hormone (Bayse *et al*, 2020). A low iodine level for T4 synthesis is common in the hilly regions distant from the sea (Dratman & Martin, 2020). Methimazole is a known antithyroid chemical that prevents thyroperoxidase which then inhibits the formation of thyroid hormones (Crane *et al.*, 2006).

Millions of people globally go through thyroid-associated problems. Thyroid cancer occurrence has risen from 2% to 5% in the last ten years. While in Africa thyroidal cancer prevalence has risen from 7.3 to 15 % in Africa; prevalence is increasing as considered by many authors (Kalk *et al.*, 1997; Sidibé, 2015). The thyroid gland is considered as the most fluoride sensitive tissues in the body. Fluoride causes interferences in the thyroid gland as well as giving rise to issues related to normal brain development and function (Panda *et al.*, 2015). There have been various studies published indicating several effects of fluoride in animals including *Xenopus laevis* with the most prominent malformations being reduced head to tail segment as well as an abnormality in the tadpoles' neuromuscular system proving that fluoride is an active teratogen that acts directly on growing embryos (Goh, & Neff, 2003).

In Africa, several nations have high fluoride concentrations beyond the limit of 1.5 mg/L set by WHO in groundwater used for ingesting (Mohan *et al.*, 2012; Thole, 2013). The East African rift-valley has excessive groundwater fluoride contents. Sudan, Tanzania, Uganda, and Malawi. This is also true to certain areas of west Africa, southern of Africa & northern Africa also have >1.5 mg/l F (Thole, 2013). A study by Wambu *et al*., in 2014 estimated that thirty six percent of children residing in Bondo-Rarieda are at a risk of getting fluorosis since the water they ingest from ground sources in this region have fluoride levels higher than the WHO recommended limit (Wambu *et al*., 2014). Kenya recommends 1.5ppm F in drinking water. Natural water sources in Kenya show concentrations above and below the optimum range, river waters have a low F level while groundwaters show much higher levels 19.369 mg/l- 80.798 mg/l in boreholes in the Njoro catchment area . The alkaline and saline lakes e.g. Elementaita (463 mg/1), Bogoria (738 mg/1), Lake Magadi (84 mg/l), and Lake Nakuru (344 mg/l) have abnormally high levels of fluoride (Pirahanchi *et al.*, 2020).

FETAX has a high degree of success when identifying mammalian teratogens on frog embryos. These studies indicate that sodium fluoride is capable of acting directly on the development of fetuses in mammals to cause malformations. Hence, this study sought to investigate the developmental impacts of fluoride and its remedy on *Xenopus Laevis'* Thyroid Gland.

1.2 Statement of the Problem

Fluoride can be found unequally in different amounts in the environment majorly from geogenic sources (Jha *et al.*, 2011). It is found in many areas in Kenya including the Kenyan highlands, western Kenya (Opazo *et al.*, 2020), and regions of Nyanza (Wambu *et al.*, 2014) where fluoride is washed down by the Rivers draining into Lake Victoria from the highland regions. Njoro river catchment has also recorded a high Fluoride level. Boreholes in the area also recorded higher amounts of Fluoride than what the WHO recommended levels (1.5 mg/l (1500 µg/l) (Singh *et al.*, 2014). Various conditions are endemic to regions that have elevated levels of fluoride in Kenya. Dental fluorosis (Nair *et al*., 1984), hyperthyroidism/ goiter and crippling skeletal deformations and in chronic cases death occur commonly. Fluoride also causes severe neurological, kidney, endocrine, thyroid, and liver disorders. Children are more susceptible to the toxicity of Fluoride in comparison to adults (Saeed *et al*., 2020) and have been known to show the following effects: low IQ levels on young children, physical and inadequate mental development and cretinism. Fluoride effects studies have mostly dwelled on dental and skeletal fluorosis at the expense of other effects which are endemic to high Fluoride areas. However, the extent to which fluoride affects iodine in a well-known system, *Xenopus laevis* that is affected by the metabolism of iodine has not been properly studied. This study therefore sought to study acute and

chronic effects of fluoride exposure and the reversal of these effects using thyroxine and iodine by use of assay, which involves *Xenopus laevis* embryos.

1.3 Justification of the Study

The water used by rural communities in Kenya is from natural sources that are untreated and there are no proper water quality monitoring measures put in place to be checking on the water. Kenya has a high prevalence of goiter despite mandated fortification of table salt with iodine. This shows that iodine fortification does not offer the solution in all cases. As a result of the indications of iodine prophylaxis being incapable of reversing all types of goiter, indicates the chance that not all goiters in Kenya are the directly linked to iodine deficiency. There have been reports of Fluoride toxicity in Kerio Valley the main region where fluorite is mined in Kenya. Hence; a need arises to carry out research to determine the possibility of an association between iodine, thyroxine (T4), triiodothyronine (T3), and methimazole and the acute and chronic effects of Fluoride.

Xenopus laevis offers a great model in this study since they are easy to keep and breed in the laboratory. They have also been well staged and characterized therefore, are easier to study for any changes that a substance may have on its development (Coady *et al.*, 2010b). There are less ethical considerations in place on use of *X. laevis* as compared to other choice of laboratory animals.

1.4 Objectives

1.4.1 Main Objective

To evaluate developmental impacts of fluoride in the *Xenopus laevis* using different treatments (KI, T3, T4, and Methimazole).

1.4.2 Specific Objectives

i. To evaluate the acute toxicity of fluoride using *Xenopus laevis* embryos (FETAX test)
ii. To reverse the acute impacts of fluoride in *Xenopus laevis* embryos using T4 and KI.
iii. To evaluate chronic toxicity of fluoride in the *Xenopus laevis* tadpoles
iv. To evaluate the effects of KI, T3, T4, and Methimazole on chronic toxicity of fluoride in *Xenopus laevis* tadpoles

1.5 Hypothesis

H_1 – fluoride causes acute effects when exposed to the *Xenopus laevis.*

H_1 – T4, KI reverses acute impacts of fluoride in *Xenopus laevis.*

H_1 – fluoride causes chronic toxicity in *Xenopus laevis* tadpoles.

H_1 – Iodine, T4, and T3 can control the thyroid hormone synthesis and reverse the histological effects of the thyroid gland caused by fluoride on *Xenopus laevis.*

1.6 Significance of the Study

Investigations of fluoride and the effects it has on human health have resulted in an increased interest worldwide and has led to many countries studying Fluoride extensively (Ahada *et al.*, 2019). This matter has received little attention in Kenya as there are only a few studies which have been conducted that mainly dealt with the occurrence of, and transportation of fluoride in several major groundwater sources in Kenya (Mbithi, 2017). The significance obtained from the study includes; creating relevant information for the evaluation of the impacts associated with Fluoride on wellbeing of humans. To comprehend the importance of exposure time to fluoride pollution as a longer exposure to fluoride causes several health effects to humans and animals. Authorities concerned with the environment will be able to use these findings

to form a basis for educating people on the risks that come up with exposure to fluoride and also come up with appropriate mitigating measures concerning the impacts of fluoride that occur naturally in the environment. Data from this study will form background information that can be used in other studies concerning the effects of fluoride contaminated water in several regions of the country that have the similar geology. It is anticipated that results arising from this study will assist relevant governmental and non-governmental organisations and scientific groups in making important decisions on water contaminated with fluoride.

1.7 Scope of the Study

This study focused on the developmental effects of fluoride and its remedy on *Xenopus Laevis,* Thyroid Gland. The study sought to: evaluate the developmental impacts of fluoride in *Xenopus laevis* ; evaluate acute toxicity of fluoride using *Xenopus laevis* embryos (FETAX test); reverse acute impacts of fluoride in *Xenopus laevis* embryos using T4 and KI; evaluate chronic toxicity of fluoride in the *Xenopus laevis* tadpoles and evaluate the effects of KI, T3, T4, and Methimazole on chronic toxicity of fluoride in *Xenopus laevis* tadpoles. The study was conducted through an experimental research design and covered a period between September 2018 and September 2019.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Concept of Fluoride; sources and effects

Fluoride is the simplest fluoride anion and a halogen element that occurs naturally. It may be commonly found in large quantities on the earth's crust, in water, soil vegetation, and animals. Fluoride salts are odorless and have a pronounced bitter taste. Fluoride is considered a weak base because it is partially soluble, yet it is corrosive in large quantities (Neuhold et *al.*, 1960).

Several minerals including fluorite contain fluoride ions, however, they are only found in tiny amounts in natural bodies of water. The quantity of fluoride present naturally in water sources varies significantly. People who consume high-fluoride water have better dental health and fewer tooth decay than those who drink low-fluoride water, according to large-scale studies. As a public measure, some countries added fluoride in their drinking water to address a deficiency. This is however still a contentious issue. Some argue that most individuals get enough fluoride from other sources, thus it's unneeded. Fluoridated toothpaste and mouthwashes, for example, are now frequently used. Furthermore, there have been claims that fluoridating drinking water can have negative health consequences and that the environmental dangers of the most prevalent fluoridating chemicals have not been adequately investigated.

Many sources of water contain fluoride, and in some countries, it is added to their drinking water. It's also found in dental care products. It's found in toothpaste gels, cement and tooth fillings, mouthwashes and some teeth floss brands (Carey, 2014). Fluoride supplementation is also advised in locations where the water doesn't contain fluoride. Drugs containing perfluorinated chemicals are some of the non-dental sources

of fluoride (PFCs), food & beverages prepared with water that has fluoride (Zohouri *et al.*, 2006), pesticides (Kaur *et al.*, 2019), waterproofed and some stain-resistant items with PFCs (Bennett *et al.*, 2015; Horowitz, 1995).

Fluoridation of public water, high fluoride concentrations in natural freshwater, fluoridated mouthwash or toothpaste, untested bottled water, improper uses of fluoride supplements, and specific foods can all cause excessive exposures to fluoride. Fluoride exposure may not always result from the chemical being added to water and dental products. Southern Asia, the eastern Mediterranean, and Africa, for example, have naturally high fluoride levels in their water for drinking (Malago *et al.*, 2017).

Addition of fluoride to water led to 35% less children reported with fewer decaying teeth, teeth extraction, or filling of baby teeth, according to a Cochrane review done in 2015 (OBE, 2015). There was a 15% rise in the number of children who did not have any decay in their infant's teeth and an increase by 14% if children with permanent teeth, indicating that fluoride can help prevent or reduce decay in children's teeth (Marinho, 2014). Fluoride can help to protect teeth from acid-induced demineralization (Jones *et al*., 2002). If the teeth are already damaged by acid, fluoride accumulation occurs in the demineralized regions and starts to strengthen the tooth enamel (Choi *et al*., 2010). The majority of public health agencies and medical organizations across the globe recommend fluoride to prevent children's and adults' teeth from decay (Baig *et al.*, 2004).

Too much fluoride can cause tooth discoloration and bone issues, among other things (Seraj *et al.,* 2012). 0.7 ppm is recommended for oral health. Whereby, over 4.0 parts per million (ppm) concentrations might be dangerous. Fluoride exposure at high levels when young during the formation of teeth may lead to minor dental fluorosis. Little

white streaks will be observed on the enamel of teeth. Although the general health of the teeth remains unaffected, heavy discoloration will be observed (Alvarez *et al*., 2009). Fluoride-containing mouthwash is not recommended for use by children under the age of six and must be monitored when cleaning their teeth to make sure that they dont ingest toothpaste (Khairnar *et al.*, 2015).

Skeletal fluorosis Excess fluoride exposure causes skeletal fluorosis, a disease that affects the bones. This causes discomfort and harm to joints and bones over a while (Littleton, 1999). This leads to an increased likelihood of fractures as the bones get harder and less flexible. The thickening of the bones and bone tissue accumulation can decrease the mobility of the joints (Wang *et al*., 2007).

Parathyroid issues: Excessive fluoride is capable of harming the parathyroid gland in rare situations. There may also arise hyperparathyroidism and unregulated release of parathyroid hormones. This leads to depleted calcium in bone structures and abnormally high concentrations of calcium in the blood. Low calcium content in bones makes them more vulnerable to fractures (Dey *et al.*, 2016).

Neurological issues: A study published in 2017 indicated that exposure to fluoride before birth lowers future cognitive results. The levels of fluoride in 299 pregnant women and their children ages 6 to 12 years were tested. They did an assessment of the children's cognitive capacity at four years old and a repeat of the same tests on the children at age six and twelve years. Poorer IQ scores were reported beyond a certain limit (Reddy *et al*., 2017; Sudheer, 2018). Fluoride was thus concluded to be neurotoxic and may harm the development of children (Shahid *et al.*, 2015).

Other health concerns from exposure to elevated levels of fluoride include; acne and other skin disorders (King, 2018); cardiovascular issues, such as arterial calcification,

increased high blood pressure, myocardial damage, cardiovascular insufficiency, heart failure, reproductive problems including lowered fertility and reports of early puberty in girls (Chinoy *et al.,* 1989), dysfunction of the thyroid, joints and bones issues e.g. osteoarthritis, cancer of the bones, and temporomandibular joint disorder (TMJ), and neural issues, such as ADHD. This may suggest that the risk-benefit ratio of fluoride be re-evaluated (Prystupa, 2011).

Fluoride poisoning can cause stomach discomfort, profuse saliva, nausea and vomiting, seizures, and muscular spasms when the exposure to fluoride is high over a short period time (Yolken *et al*., 1976). This is less likely to occur by drinking tap water. This is only likely to occur by unintentionally drinking water polluted from industrial fires or explosion. Of note is, most chemicals can be dangerous in big doses and are only beneficial in little doses (Gessner *et al*., 1994).

Fluoride Requirement in the Body: Fluoride levels in raw natural drinking water vary significantly among and within the nations. For example, in Ireland, the levels are between 0.01-5.8 ppm, in Finland, 0.1-3.0 ppm, and in Germany, between 0.1 & 1.1 ppm. In drinking water, fluoride quantities vary widely among various nations starting at 0.2 to 1.2mg/L (Schwarz *et al.*, 1972).

Fluoride is a harmful substance because it reacts with live-cell molecules, altering their shape and function. There are two forms of fluoride compounds: diffusible and non-diffusible, both of which are non-absorbable. NaF, HF, H_2SiF_6, and Na_2PO_3F are common diffusible preparations used in dental care products. CaF_2, MgF_2, and AlF_3 are among the ingredients in the non-diffusible preparation. Except for Na_2PO_3F, which an enzyme first digests before being absorbed and entering the circulation system, and if the preparation is more diffusible then its ionization is easier(MZ *et al.*, 2008).

With the exception of seafood and tea, the fluoride content in food and drink we eat on a daily basis seldom exceeds 1 ppm. Fluoride concentrations can range from 250 to 22,500 parts per million (ppm) in various forms of dental treatments performed at home. The use of fluoride may be classified into two categories: topical application, such as toothpaste and mouthwash, including fundamental preparations such as fluoridation of drinking water or the use of fluoridated tablets. A great number of clinical studies demonstrate the efficacy of fluoride in topical therapy by lowering caries occurrence. The fluoride used has been shown to reduce DMF by up to 50% (Marthaler, 2013) .

Sodium mono fluorophosphate formulation is commonly used in toothpaste (Na_2PO_3F). Fluoride forms a fluorapatite crystal, which strengthens enamel and protects it against acid, making it more insoluble. Fluorapatite crystal forms when the OH^- ion of the enamel is displaced by F^- ion, resulting in a more stable crystal structure. The hydrogen connection will be strengthened as a result of this. Fluoride can also enhance the crystal size, making it more resistant to acid. In terms of caries prevention, there are three main fluoride mechanisms: Fluoride, in conjunction with hydroxyapatite crystal, lowers enamel solubility in acid conditions during the pre-eruption stage.

Fluoride enhances remineralization and inhibits demineralization in the post-eruption phase. Fluoride inhibits bacterial glycolysis metabolic activity. The restrengthening of weakened enamel brought about by the acid effect in vitro provides confirmation of an occurrence of remineralization. When the enamel of the tooth has modest demineralization e.g. tiny caries lesion with white spots, this remineralization procedure is most successful (Zhang *et al.,* 2022). Overdosing on fluoride is quite unlikely. The lethal dosage of fluoride in people is predicted to be 32-64 mg per kilogram weight based on the incidence of fatal fluoride poisoning. Some

gastrointestinal issues will emerge if a dosage of 1 mg per kilogram weight (Han *et al.*, 2021).

2.2 Thyroid and iodine metabolism

The thyroid gland is a critical organ in the endocrine system. It's location is between the C_5-T_1 vertebrae of the *columna vertebralis*, beneath the larynx and the forefront of the trachea. The thyroid has 2 lobes (lobus dexter and lobus sinister) and is bound by the isthmus (Friedrich-Rust *et al.*, 2012). The glandular capsule has an external and internal folium which is enclosed in a fibrotic capsule: the thyroid. The thyroid is nourished by the external carotid artery & the inferior thyroid artery which branches off the subclavian arteries (Bursuk, 2012; Nguyen *et al.*, 2002)

The thyroid gland begins growing at 3 weeks of gestation. At 10 weeks gestation, the fetus organifies iodine which then initiates thyroid hormones synthesis. Thyroxine and TSH can be measurable in fetal blood. The synthesis of thyroglobulin in the thyroid of the fetus increases at the second trimester and elevated levels of T4 and TSH is also noted. The development of the hypothalamus by the fetus allows for the synthesis of thyroid-releasing hormone eventually increasing thyroid-stimulating hormones. Although TRH is transferrable from the mother to the fetus via the placenta, it is impossible to transfer TSH. The level of triiodothyronine (T3) increases towards the completion of trimester 2 and can be detected in the blood of the fetus although in only tiny amounts. The synthesis of T3 is further increased after birth. Transcription factors 1, 2 and paired homeobox-8 control the development of the thyroid gland. When the transcription factors work together, follicle cell growth & thyroid-specific proteins eg TSH receptor and thyroglobulin commence. When a mutation occurs in the transcription factors it is likely that hypothyroid babies are born as a result of thyroid agenesis or the thyroid gland secreting inadequate amounts of thyroid hormones

(Bhardwaj *et al.*, 2022). The principal useful component of the thyroid gland is the follicular cells which range between 100-300 µm. These follicles create a lumen that synthesizes a protein called thyroglobulin. The topmost part of the follicles comes into contact with the colloidal lumen while the bottom part of it with blood circulatory system through capillaries. This ensures that the thyroid hormones reach the target organs as intended. Calcitonin from parafollicular c cells affect calcium metabolism. T3 & T4 hormones released by the thyroid gland are responsible for normal bodily functions (Khan *et al.*, 2019).

Iodine metabolism: Iodine is a micronutrient that is essential in life. It is also fundamental in the synthesis of thyroid hormones. There are 3 forms of iodine in circulation; inorganic iodine (I^-), organic iodine before getting into the structure of the thyroid hormones, and as bound in the thyroid hormones. The molecular weights of the thyroid hormones T4 & T3 comprise mainly 65% and 59% respectively Iodine (Farebrother *et al.*, 2019). This is equivalent to 30% of whole-body iodine content. The other 70% iodine is distributed in other tissues of the body including the eyes, cervix, salivary glands, and mammary glands. This is of importance to the functions of the tissues (Sanyaolu *et al.*, 2021).

Thyroid hormone synthesis: When iodine is absorbed from the GIT system, it is instantly diffused in the extracellular fluid. T3 and T4 hormones are formed when iodine is added to tyrosine amino acids (Citterio *et al.*, 2019). T4 is synthesized in higher quantities in as much as T3 is the most efficient hormone. The synthesis of the thyroid hormone usually happens in 4 stages:

The first stage involves obtaining iodine through active transport to the thyroid follicular cells through the Na^+/I^- symporter pump. The commencement along with

continual transportation is controlled by TSH. Organification of iodine increases in the cells although it slows and stops after a certain point. This can then be concluded as a concentration-dependent mechanism. Perchlorate, pertechnetate, and thiocyanate inhibit iodine transport at this stage (Rolaki *et al.*, 2019). The second stage involves NADPH dependant thyroperoxidase enzymes oxidizing the iodine in together with H_2O_2. This takes place at the follicular lumen. Certain medications e.g. propylthiouracil and methimazole cause inhibition at this step (Benvenga *et al.*, 2018). The third stage involves oxidized iodine binding with the residues of thyroglobulin tyrosine. This is referred to as organification or the iodization of tyrosine. It is at this point that the synthesis of MIT or DIT is done. The above-mentioned types of hormones are inactive. The fourth stage involves coupling and compels for T4 and T3 synthesis from DIT and MIT. Other than being synthesized this way T3 hormone is made by metabolizing T4. Almost all the colloids in the thyroid follicle lumen are thyroglobulin. These contain 70% of thyroid protein that has 70 tyrosine amino acids. Its synthesis is dependent on TSH and it mainly takes place at the endoplasmic reticulum in the thyroid gland follicle cells. After synthesis, the thyroglobulin is transferred into apical cells and moved to the lumen of the follicles via exocytose, where it eventually takes part in the synthesis of thyroid hormones (Kim *et al.*, 2021).

Secretion of thyroid hormones: The thyroid hormones are bound to thyroglobulin and are kept in the lumen of the follicle cells. When TSH is secreted, apical villus is increased and the droplets of the colloids are trapped by the microtubules and transported through pinocytosis to the tip of the follicle cells. The lysosomes come into contact with the colloidal pinocytic vesicles which have thyroid hormones and thyroglobulin. The vesicles are then bound to lysosomes forming fagolysosomes. The fagolysososmes then move to the basal cell thereby hydrolyzing the thyroglobulin. As

a consequence of this tyrosine formed is excreted through diffusion facilitated by T4 and T3. Although it is of importance to note that not all the hormones that are detached from thyroglobulin go through the blood therefore, Iodothyronines such as MIT and DIT remain in the cell after which they are then reused. Some T3 is also formed from the deiodinization of T4. These occur through enzyme catalyzed reactions which occur in the thyroid follicular cells, to mean that a deiodinization process can also be referred to as dehalogenase. The deiodinization process results in approximately 50% iodine in thyroglobulin being returned and reused. Deficiency of iodine in people lacking this enzyme results in goiter. In such instances then the best remedy is giving them iodine treatment (Citterio *et al.*, 2019).

Thyroid hormone transportation: After the thyroid hormones are released for distribution, they all ultimately change into inactive form by reverse binding on the proteins synthesized in the liver. The thyroid hormones that bind to proteins inhibit hormone excretion in the urine where it is also deposited. This is to say that the hormone is active only as much as needed. Beyond this amount it becomes inactive. Thyroxin bound globulins are the main carrier proteins. T4 binds to this protein the most and causes the distribution of T4 into the fluids out the cells in abundance, although fewer T3 get attached. In as much as the total T3 and T4 is increased by TBG, free T4 and T3 are unaffected (A. Delitala *et al.*, 2022). Considering that T3 is capable of binding to fewer proteins, it remains active within the cells. Since carrier proteins have a high affinity to T4, T4 has an approximate half-life of 6 d, compared to T3 whose half-life is less than 1 day. Since T4 is bound to cytoplasmic proteins after it enters into cells they will affect it makes T3 more active (Al-Suhaimi, 2022).

Metabolism of the thyroid hormones: approximately 100 µg of thyroid hormones are produced in the thyroid gland with T4 being the majority. Nearly 40% of thyroxine is

turned to T3. This T3 with deiodinase enzymes are strong in periphery organs e.g. liver, kidney (Bursuk, 2012). For T3 to be formed, deiodination should take place in the 5' region of tyrosine. However, if this happens in the fifth atom in the inner circle, reverse triiodothyronine (rT3) which is inactive is formed. Deiodinase type I, type II iodothyronine deiodinase, and the inner circle deiodinase type III catalyzes deiodination. 5'-DI (type I) enzyme is mostly found in the kidneys, thyroid, and liver. Type II is found in the brain, placenta and hypophysis. Type III occurs in the brain, epidermis, and placenta. Type I and type II enzymes permit the transformation of inactive T4 to active T3. The only difference is that type I supplies T3 to plasma while theT3 that is formed type II enzyme remains in the tissue regulating the local concentration. The increases and decreases of thyroid hormones are managed by this enzyme e.g. in hypothyroidism inhibits the transformation of T4 into T3 is inhibited in tissues such as the brain. Hunger, acute stress, and certain drugs may cause changes to the transformation of T4 to T3.type III transforms T4 into the inactive reverse T3 (rT3) (Bursuk, 2012). As aforementioned, 40% of T4 transforms into T3. This accounts for 90% of the total T3. Therefore we can conclude that approximately ten percent of triiodothyronine is directly synthesized. Additionally, 40% of thyroxine is spent in forming reverse T3 (rT3). The 20% that remains is then excreted through urine or feces.

To control thyroid hormone synthesis and its secretion it needs to be maintained at certain amounts for the thyroid hormones to remain lively. As such, a crucial mechanism in the control of production and distribution of TH is the hypothalamus-pituitary-thyroid (HPT) axis. An auto-control mechanism depends on the iodine concentration(Feldt-Rasmussen *et al.*, 2021). Hypothalamus-pituitary-thyroid axis: This axis controls the synthesis of TH and its secretion. This process starts at the hypothalamus. The TRH is then released from hypothalamus into pituitary and TSH is

then secreted here. This is eventually moved by the blood which then signals the thyroid gland to synthesize TH leading to the start of secretion. If there is a lot of thyroid hormone synthesized the negative feedback system activates and the TSH and TRH become suppressed (Gavrila *et al.*, 2019). Thyrotropin-releasing hormone (TRH) is synthesized in the hypothalamus. TRH are then transported into the pituitary through the hypophyseal portal system which is important for secretion of TSH from thyrotrope cells. Specific receptors to TRH are present on the cell surfaces. TRH is also responsible for increased growth hormone secretions, follicle stimulating hormone & prolactin.

The thyrotropin-stimulating hormone (TSH) has a glycoprotein with α and β subunits. The α subunit is almost similar to hormones found in human chorionic gonadotropin, luteinizing hormone, also the follicle stimulating hormone. TSH activates Gs protein which then activates the adenylate cyclase enzyme. As this enzyme is activated, secondary messengers cAMP increase. This in combination with other enzymes leads to the development of thyroid follicle cells and thyroid hormone synthesis. Metabolism of TSH is done in the liver and kidneys. Its release is pulsatile, meaning secretion starts at night reaching its peak at midnight, and starts to decrease all through the day. The effects that arise from TSH are those occurring within minutes, those effects which occur in hours, and chronic effects. In spite of this, TSH doesn't affect the transformation of T4 to T3 in tissues in the periphery (Al-Suhaimi *et al.*, 2022).

The autoregulation in the thyroid: iodine concentration changes in the follicle cells in the thyroid causes an effect on the transportation of Iodine and auto-regulation is then formed. The synthesis of the thyroid hormone is prevented by increases in the levels of iodine in the follicle cells although there is an increase in synthesis as its amount reduces. The Wolf Chaikoff effect refers to when excess iodine stops the synthesis of the thyroid hormones. This is common to individuals who have

hyperthyroidism take anti thyroid drugs alongside iodine and they become euthyroid (Brix *et al.*, 2020).

The thyroid gland sensitivity increases because of TSH, even though TSH may not be stimulated by iodine deficiency. In addition to this the follicles in the thyroid gland become hypertrophic & hyperplastic, the gland increases in weight resulting in goiter arises. A rise in iodine leads to a reduction in TSH. In this case, the binding of iodine, synthesis of thyroid hormones, thyroglobulin secretion into the colloid, iodine entrapment, and cell hypertrophy decreases. The blood flow to the thyroid gland reduces.

Thyroid hormone effect occurrence: receptors are found in the cells in the thyroid gland. Some of the receptors can be found in the nucleus and most are found in T3. Since T4 is bound to carrier proteins extracellularly, the T4 is passed through the inside the cell which makes intracellular T4 amount low. In the intracellular region, there are hardly any free receptors that bind to proteins. More T3 is found intracellularly. This is because they bind less to the carrier proteins with the receptors showing a higher affinity towards T3 since they are free. Because of this, T3 has more potency in comparison to T4. This characteristic is due to the fact that T4 is transformed into T3 although T4 is high in amount, while in essence T3 is the efficient type (Delitala *et al.*, 2022).

Thyroid hormones pass through the cell membranes easily because they are soluble in lipids and as soon as possible T3 and binds to the receptors of the thyroid hormones in the nucleus of the cell. Although the receptors exist in the tissues, their effects differ. Thyroid receptors α are more effective in the brain, heart, kidneys, muscles, and gonads while Thyroid receptors β are more efficient in the liver and the pituitary. Enzymes are synthesized and they play an important role in forming thyroid hormone effects.

Thyroid hormones effects: Thyroid hormones have differing effects. They are categorized into effects on the cell, effects on growth, on the systems, and metabolism.

Thyroid hormones effects at the cell level: when protein synthesis increases, catabolism increases leading to an increase in basal metabolism. Metabolism in the cells increases by 60-70% when excess thyroid hormones are secreted (Bavarsad *et al.*, 2019). mRNA synthesis increases in the mitochondria when the thyroid hormones are released and protein production increases. Being that these proteins are respiratory chain proteins, the level of oxygen consumed also increases. Mitochondria numbers also increase as a result of the mitochondria activity being parallel to protein synthesis in the mitochondria (Mendez *et al.*, 2021) . Protein synthesis results in an increase the enzyme synthesis which affects the thyroid hormones. This leads to effects on the passage because of an increased production of transport enzymes in the membrane.

Effects of thyroid hormones on growth: the thyroid hormones are crucial for normal growth and development of muscles. Hypothyroidism in children is presented with a shorter stature because of early closure of epiphysis while hyperthyroid children are taller in comparison to their age-mates (Winter, 2021). The thyroid hormone is also of importance in the prenatal and postnatal brain development. There is a possibility that a fetus may not synthesize and secrete enough thyroid hormones during gestation which then suggests that developmental retardation may occur in prenatal and post natal stages. It is of importance to conduct tests on babies and those with inadequate levels are treated immediately for them to develop normally (Yamamoto *et al.*, 2020).

Thyroid hormones effects on metabolism: metabolic effects of thyroid hormones include effects on carbohydrates, protein and fats metabolism, vitamins, and an effect of body weight on the affected individuals (Yavuz *et al.*, 2019). . Effect of thyroid

metabolism on carbohydrates is both anabolic and catabolic. Increase in thyroid hormones results in an increase in enzyme synthesis which in turn increases carbohydrate metabolism. Thus glucose enters the cell easily, its absorption from the GIT system, glucogenesis & glycolysis, and insulin secretion is achieved.

The anabolic and catabolic effect on fat metabolism by the thyroid hormones results in a lipolysis effect on adipose tissues the amount of free fatty acid concentrations in plasma. Oxidation of fatty acids increases (Sinha *et al.*, 2018) . As a result, cholesterol levels and triglyceride amounts increase while reducing the level in their blood. This is mainly due to; thyroid hormones increasing resulting in an increase in the synthesis of receptors that are specific to 'bad' cholesterol and levels of cholesterol found in the liver, binding to lipoproteins in addition to a reduction in the triglyceride levels in the blood. It also fastens the conversion of triglyceride to cholesterol. Bile is produced in the liver using cholesterol which is then excreted from the intestines with feces. As a consequence of this, the adipose tissues, triglyceride, and cholesterol level in the blood decrease, while free fatty acids increases when there is an oversecretion of the thyroid hormones and vice versa for people with hyperthyroidism.

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A study done by Bursuk *et al*. (2012), showed by analyzing the bodily contents of control, hypothyroid and hyperthyroid individuals using the bioelectrical impedance analysis method, the percentage of fat decreases in hyperthyroid groups although they increase in hypothyroid individuals.

It was previously shown that anabolic effects increase the synthesis of proteins while the catabolic effect increases the destruction in instances where it is over secreted. Thyroid hormones are also responsible for the regulation of amino acid transportation as a need for the amino acids arises as the synthesis for proteins increases. Thyroid hormones facilitate the synthesis of proteins that are unique to the growth of cells. Thyroid hormones contribute to the expected growth in babies as it increases the synthesis of insulin-like factors in fetal hormones which then allows normal growth and development. Although hypothyroidism results in growth retardation, hormone replacement treatment can be done if a diagnosis is done early. Hyperthyroidism cases occur when the thyroid hormones are secreted over the required amount, resulting in muscle atrophies due to the protein catabolism (Sindhu, 2018).

Enzymes require vitamins as co-factors for them to have an effect. Vitamin C, riboflavin, folic acids, etc are common co-factors. A deficiency in these vitamins is expected in hyperthyroidism. Thyroid hormones are important for the transformation of carotene in food to Vitamin A. This does not take place in hypothyroid individuals because of inadequate thyroid hormones. It is important therefore to supplement vitamins for both hyperthyroid and hypothyroid individuals (Starchl *et al.*, 2021). Thyroid hormones also accelerate basal metabolism. As TH increases the increase consumption of oxygen, the synthesis of ATP rises the number and the activities of the mitochondria. This increases enzymes such as Na+ - K+ - ATPase, and Ca+ - ATPase. This results in a high level of temperature (Bursuk, 2012). With an increase in the basal

metabolic rates there is a reduction in body weight. Thyroid hormones are capable of reducing the amount of fat deposited. Hyperthyroidism cases report reduced weight, although their appetites increase. In hypothyroidism, however basal metabolism is reduced and an increase in weight occurs (Kalra *et al.*, 2021).

Thyroid hormones effects on systems: this mainly occurs through catecholamine. The hormones grows the number of β adrenergic receptor without necessarily causing effects to the catecholamine secretion. As a result of this, the pulse increases, flow rate increases, stroke volume increases, and widening of peripheral blood vessels occurs. Vasodilation of the blood vessels increases the skin's warmth including humidity. In a study by Bursuk *et al*.2012, the stroke volume, cardiac output, blood flow increases in hyperthyroid cases while reducing in hypothyroidism cases. Metabolism increases because of the high consumption of oxygen in cases where excess thyroid hormones are released. TH increases heart muscle contractions in instances where they are raised little by little. Over secretion decreases muscle strength and severe thyrotoxic individuals present a heart attack due to blockage of oxygen from getting into the heart muscles (Khan *et al.*, 2020). As thyroid hormones affect the skeletal system, it is important to check on the effects that they have on bones. For individuals with no thyroid hormone problems, their osteoblasts multiply rapidly, for hyperthyroidism individuals, the cortex of bones degenerates (A. P. Delitala *et al.*, 2020).

Thyroid hormones have an effect on how individuals respond to exposure to stimulants. When in excess, muscles get fatigued as this increases protein catabolism. One of the symptoms of hyperthyroidism is muscle tremors. This is due to increased activity in the neural synapses in the medulla spinalis regions which are in charge of muscle tones. These tremors, therefore, signify that thyroid hormones affect the central nervous

system. The excessive stimulant effect of the thyroid hormones causes sleeplessness and for hypothyroidism, the opposite is experienced.

Thyroid hormones have a major role in central nervous system development. TH are responsible for nerve myelination. When in deficiency during fetal development, neuronal development disorders occur in the brain, myelination slows, deep tendon reflexes are hindered, mental retardation, apathy, etc. in hyperthyroidism, anxiety, hyperirritability, and sleeplessness is observed in children (Farebrother *et al.*, 2019).

Research done by Bursuk *et al (*2012), on a comparison of blood parameters between hypothyroidic and hyperthyroidic groups showed an increase in blood viscosity in hypothyroidism groups because of higher blood count parameters as compared to hyperthyroidism individuals. Viscosity of blood in hypothyroidism individuals increased because of the high viscosity of plasma, this is in addition to blood lipids and fibrinogen being high.

Thyroid hormones speeds basal metabolism by controlling the actions of all the endocrine hormones. They improve the absorption of glucose in the GIT system. TH facilitates insulin increase by occasionally raising blood sugar. This is because growth hormones together with TH are crucial for normal somatic growth. It is due to the fact that TH increases growth factors & growth hormone synthesis and secretion. Thyroid hormones also control the production as well as the utilization of sex steroid hormones which in hypothyroidism leads to impotence, lack of libido, amenorrhea, menorrhagia, and polymenorrhea is seen as a result of sex steroid deficiency. This is mainly because TH affects prolactin secretion which is responsible for enabling mammals to produce milk.

Thyroid hormones increase cell metabolism which then increases the oxygen used which leads to high metabolism. As a result of this, blood flow increases, heart flow frequency, and volume because of vasodilation that is experienced. Hence, thyroid hormones are responsible for improved digestion, neural systems, muscle health, and the other endocrine organs (Elliott *et al.*, 2022). Hypothyroidism causes destruction of the thyroid gland and a deficiency of TSH secretion by the pituitary. Cretinism is found in children when there is a reduced or absence of thyroid hormones. Hypothyroidism amongst adults presents as fatigue, less sweat, cold and dry skin, swollen faces, slowing of movements and motor activity, and a delay in reflex motions (Omokore *et al.*, 2021). Thyroid hormones increase cell metabolism thereby increasing use of oxygen and the metabolic product increases. This increases flow of blood. This results in improved digestion, neural system, including the endocrine organs (Bigzad, 2022). Someone therefore born without thyroid hormones therefore develop mental and physical retardation (Kharbanda, 2020).

Susheela *et al*., (2005) did research on 7-18 year olds living in fluoride endemic areas that had enough iodine intakes. The drinking water in the region had an average of 4.77ppm F. The blood levels of the children had 3.96ppm F. The results from the study indicated that children exposed to excess fluoride undergo changes of thyroid hormone levels (Susheela *et al.*, 2005). Trabelsi *et al (2001)* did research on the effects of elevated levels of fluoride adult Winstar rats and were treated with iodine and sodium fluoride which was dissolved in their drinking water at fifteen days gestation and for fourteen days after they gave birth. The results from this study showed that the young rats that were treated had a 35% reduction in weight, 75% reduction in thyroxin blood level, 27% reduction of protein cerebellum, and 17% reduction of protein in the cerebellum (Trabelsi *et al.*, 2001). The rats that had been treated exhibited a reduction

in the external granular layer, the purkinje cells decreased and their apoptosis also increased.

A reduction in thyroxin level in the treated young rats led to the loss of weight. Fluoride also damages cell proliferation in the external glandular layer. This damage is made apparent by the neuron movement into the molecular and internal layers. Cell death in the treated rats also shows that death in granular cells was as a result of these cells not contacting the purkinje cells (Strunecka *et al.*, 2018). The effect of fluoride on thyroid gland growth and function in young pigs by Zhan *et al*.2006, for 50 days showed a reduced weight and T4 levels in the pigs, while the TSH increase. The T3 levels however remained the same. The thyroid gland accumulates iodine through Na/K-ATPase and Na/I symport. Fluoride interferes with the activities of this symport thus resulting in a reduction of iodine in the thyroid gland. In conclusion, this research showed that thyroid hormones play a key role in the regulation of growth, cell differentiation, and the metabolism of nearly all the tissues in the body. It also shows that the thyroid gland is sensitive to elevated fluoride levels especially, on its function and histology. High fluoride levels also cause slowed growth and are capable of changing the levels of thyroid hormones which is similar to those that are observed in iodine deficiency (Zhan *et al.*, 2006).

Zhao *et al*., (1998) did some tests using a total of 9 sets of water with differing amounts of iodine and fluoride on rats. The results of this study iodine in deficient amounts cause goiter. Fluoride in excess elevates the level of fluoride in bone and teeth. Adding iodine to fluoride affects the thyroid gland as well, as it showed that T3 secretion was inhibited. Fluoride can affect iodine uptake as the concentration changes and the duration of the treatments. In conclusion, iodine and fluoride affect each other in regards to goiter and fluorosis in the rats. Fluoride levels in the bones of rats with ID and FE appeared to be

high in comparison to iodine excess and fluoride excess treatment. Iodine excess treatment showed a decrease in the level of T3 in the blood serum. The histology of the rats in the fluoride excess treatment showed hyperplasia, reduced length of follicular cells, together with an increased diameter of the follicle cells. After 100 days the levels of T4 in ID + FE were high. This shows that fluoride effect on the thyroid gland decreases over time (Zhao *et al.,* 1998). Bouaziz *et al*. (2004) studied pregnant female rats treated with fluoride. Results indicate a reduction in T3 and T4, loss of weight, hypertrophy of the thyroid gland, and a significant reduction in the levels of iodine. In cases where the fluoride was done away with after birth, the young rats seemed to occur before death. This is exhibited as an increase in weight, an increase in the weight of the thyroid gland, and a reduction of TSH levels. To conclude this, fluoride causes hypothyroidism in female rats and their offspring (Bouaziz *et al.*, 2004).

Yaming *et al*. (2005) also did a study on Winstar albino rats and the results showed that a high intake of fluoride, low iodine level or a combination of the two causes a change in the DNA of the thyroid gland. The most important change observed being in those with high a amount of fluoride, and a low iodine level. The damages to the thyroid gland cells may break the DNA chains eventually leading to the thyroid gland dysfunction (Ge *et al.*, 2005). The thyroid gland is able to absorb, accumulate fluoride which in turn can cause changes in the structure of the cells in the thyroid follicles. Fluoride also inhibits the enzyme activities that play a role in the conversion of T4 to T3. Intake of fluoride together with iodine whether in sufficient amounts or insufficient may disturb the thyroid gland by increasing the TSH, reducing Na/K-ATPase, reducing enzymes, reducing the iodine binding protein and potentially damaging the DNA. These imbalances created by fluoride may in the long suppress thyroid hormone synthesis.

Although certain measures e.g. a diet with ascorbic acid, vitamin D, and vitamin E have the potential to overcome fluoride toxicity (Sharma *et al.*, 2022).

Methimazole has for long been used to treat hyperthyroidism in animals, most especially cats. At recommended levels, it is efficient in treating hyperthyroidism. Although formulated to be used on the skin of cats, oral administration of methimazole has proven to be more effective. It is recommended that 10mg per cat transdermal, should be administered for it to be effective.

2.3 *Xenopus Laevis* as a surrogate for thyroid function tests

Xenopus laevis is a good surrogate species in the study for thyroid gland disturbances as they go through metamorphosis that is dependent on thyroid hormones. *X.laevis* has an external early development free from the influences of the maternal state, this means that they can easily be experimented on, observed and any changes that may arise can be noted all through its developmental stages. Since *Xenopus laevis* go through metamorphosis that is only possible in presence of the thyroid hormones means that they are more suited to studies that go beyond postembryonic development. Maternal T3 & T4 in *X. laevis* eggs act as a source of the hormone to the tadpoles before they develop their own thyroid gland. It is also advantageous to use *Xenopus laevis* as they are able to breed freely as and when required in the laboratory when injected with HCG. They are able to lay many eggs at a time and they take a relatively short time between spawning and the time it takes to get to maturity. The embryonic development of *Xenopus laevis* has been extensively researched with established models for studying oocyte, egg, and the development of embryos (Masse, 2016).

2.4 Interaction of Fluoride and Iodine

Deficiency in iodine contributes to a reduced production of the thyroid hormone. ID may also intensify effects of chemicals that disrupt thyroid hormone production including fluoride. Iodine in sufficient amounts can counteract the effects that goitrogens e.g. fluoride, have on the thyroid gland. Synergistic effects of high fluoride and deficient iodine have also been found among both animals (Ge *et al*., 2005) and humans. There are few studies however that have acknowledged the use of iodine as a remediation measure for the effects of fluoride exposure to the thyroid gland. Studies in Canada show that 22% of the population aged 3-79 consumed low iodine (Organization, 2013).

Some reviews e.g. the one done by Waugh, 2019 pinpoint that fluoride prevents the sodium iodide symporter expression and functions which then leads to a reduced iodide absorption, its concentrating ability, and eventually iodine deficiency disorder (Waugh, 2019). Comprehensively, it is suggested that exposure to fluoride has a direct impact on the bioavailability of iodine in humans. Epidemiological studies indicate that iodine deficiency in combination with exposure to high fluoride levels results in high levels of development disorders and impaired cognitive function amongst children. It also suggests that fluoride and iodine have a synergistic interaction which is responsible for brain damage in individuals. Hypothyroidism due to low iodine intake/ absorption can occur at any age but of most significance is during gestation and childhood. This, therefore, means deficiency of iodine is still a major problem to public health officials and is one of the highest causes of brain damage that is preventable.

The scientific committee on food reported in 2002 that iodine absorption from food is reduced by fluoride whether from food or water. In Canada, recent studies show that urinary iodine concentrations were lower in fluoridated communities as compared to

non-fluorinated ones. Individuals with iodine deficiency have high urinary fluoride concentrations in comparison to groups of individuals with iodine sufficiency.

A study by Peckham *et al* (2014) revealed that drinking water containing high levels of fluoride can be a useful tool for predicting of the prevalence of hypothyroidism in an area. In west Midlands, an area that is fluoridated reported twice as much hypothyroidism as compared to the Manchester area which is non-fluoridated. Hypothyroidism is one of a major health problem which in addition to certain conditions such as iodine deficiency and fluoride exposure exacerbates its effects. This leads to a lot of questions with regards to the safety of adding fluoride to water provisions in the world (Peckham *et al.*, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design

Experimental research design was used in this study. The experimental design was important in this research in order to be able to study the possible effects of Fluoride on surrogate species i.e. *Xenopus laevis* and the possible interactions and remedial capacity of Iodine, thyroxine (T4) and triiodothyronine (T3) on these effects. These experiments were done for both short term (96h) and long term (21 days). Fluoride (NaF) was the source of test compound Fluoride ions used in the experiment. Iodine is used by the body to produce the thyroid hormones i.e. triiodothyronine (T3) and thyroxine (T4). T4 is the inactive prohormone while T3 is the active form of the hormone. Methimazole prevents the formation of thyroxine by interfering with peroxidase and iodine interaction with thyroglobulin. It was used in the experiment to ascertain whether the effect of Fluoride was encountered before the conversion of T4 or after the conversion of T4 to T3. Iodine, NaF, T3 were all analytical grade obtained from Sigma-Aldrich, T4 from aspen pharmacare and methimazole from avet pharmaceuticals.

3.2 Materials

The following equipment and supplies were used to conduct of both Frog Embryo Teratogenesis Assay Xenopus (FETAX) and Amphibian Metamorphosis Assay (AMA) test are: Eighteen small glass aquaria (10l), breeding tanks (20l), a heater fan, a thermometer, binocular dissection microscope, 4MP digital camera, Motic® digitizing software, petri dish (100*15 mm), analytical balance capable of measuring to 3 decimal places (mg), dissolved oxygen meter, pH meter, test chemical (NaF, Methimazole, KI, T3 and T4), male and female *Xenopus laevis*, labels, FETAX solution ,HCG (Human

Chorionic Gonadotrophic Hormone), de – chlorinated water, frog feed (z- bites and t – bites; these were prepared in the laboratory), chloroform, syringe and needles, incubator, plastic transfer pipettes, matchbox, scalpels, forceps, paraffin, formaldehyde, hematoxylin and eosin.

3.3 Experimentation Procedures

3.3.1 Test Species

Xenopus laevis was the best surrogate species for this experiment as their embryos are good models for the development of vertebrates as they are hardy and easy to maintain in the laboratory (Wheeler *et al.*, 2009). The amphibians' species were identified using dichotomous guidelines from (DeVito, 2003).

The frogs were left to acclimatize for six weeks in the holding tanks with dechlorinated water after collection from lake Victoria before they were injected with hCG for breeding. Z-bites were used to feed the frogs prior to the study and the temperature of the room was maintained at $25^{o}C \pm 2^{o}C$ during the experimental period below which death occurs. Water in the holding tanks was changed every 5 days.

3.3.2 Holding of Animals: Adult Care and Breeding

Adult breeding was guided by the standard guidelines of Frog Embryo Teratogenesis Assay Xenopus (FETAX) test. These are; in breeding, pairs of (3-5) of adult females and males are injected with human chorionic gonadotropin (hCG). Four pairs of the frogs were injected with 500IU for males and 1000IU for females of hCG (Osano *et al.*, 2002) dissolved in 0.9% saline solution on the dorsal lymph sac.

The breeding tank was filled halfway with FETAX solution (10 liters) and had a false bottom with mesh which allowed the egg mesh to fall into the bottom of the tank. The

tanks were then left in a room with a temperature of 23^0 C overnight after the frogs were injected in the evening at 6pm. The eggs deposited in the tank were then collected the following morning at 8 am. The adult frogs were then removed from the breeding tanks and the eggs collected. The fertilized eggs were identified by checking the formation of blastomeres.

3.3.3 Larval Care and Selection

Following the removal of the adults, eggs were checked for viability by use of a representative sub–set of the embryos from all breeding tanks. After selection the eggs were separated into two categories; one for use in the acute toxicity and the other to use in chronic toxicity.

3.4 Acute Toxicity

Eggs set aside for acute toxicity were used to test the effects of fluoride in the tadpoles and the ability of T4 and iodine to reverse the effects.

3.4.1 The FETAX Solution

Medium for growth also referred to Frog embryo Teratogenesis assay –Xenopus (FETAX) solution was prepared according to American Society for Testing and Material (ASTM 1998). This comprised 625 mg Sodium Chloride (NaCl), 96 mg Sodium hydrogen carbonate ($NaHCO_3$), 30 mg Pottasium chloride (KCl) 15 mg calcium chloride (CaCl), 60 mg calcium Sulphate ($CaSO_4.2H_2O$) and 5 mg Magnesium sulphate ($MgSO_4$), all analytical grade per liter of distilled water (Mouche *et al.*, 2011) all these chemicals were obtained from Sigma-Aldrich.

3.4.2 Fluoride Exposure

A stock solution of Sodium Fluoride was prepared to be used in the whole experiment. The eggs were counted into the petri dishes for exposure whereby each petri dish

contained 25 eggs that had been checked to be fertile. They were placed randomly in the incubator set at 25^0C. The experiment was set into duplicates while the concentration of fluoride (mol/mol) ranged at 50ppm, 100ppm, 200ppm, 400ppm and 800ppm and the control without Fluoride. Each pair of the test was treated as a block and the experiments done by complete random block design. After every 24h, dead tadpoles were removed while putting in fresh FETAX and the concentrations for the duration of the whole experiment ending at 96h. Parameters measured were mortality, development, and teratogenicity. Mortality was measured by counting the number of dead embryos every 24 h. Development was evaluated by measuring the total length of the tadpoles at the end of the experiment and teratogenicity was evaluated by observation and any malformation observed was recorded. Mortality was recorded every 24h while development and abnormalities were measure at the end of the experiment (96h).

3.4.3 Reversal of acute Fluoride Effects using T4 and KI

The experiment was set the same as the testing of effects of fluoride. The fertilized eggs were transferred into petri dishes and FETAX solution added. T4 (2 ppm) and Iodine (0.5ppm) was then added to the different concentrations of Fluoride. This was to test the capability of T4 and Iodine in reversing the effects of fluoride. Parameters tested were Mortality, Development and Teratogenicity.

3.5 Chronic Test: Amphibian Metamorphosis Assay (AMA)

The Amphibian Metamorphosis Assay (AMA) intends to empirically establish chemicals which inhibit the normal functioning of the hypothalamic-pituitary-thyroid (HPT) axis. This is a model that is specifically used to study the structures and functions of the Hypothalamus-Pituitary-Thyroid axis. This assay is essential as amphibian metamorphosis provides a well-known process that is solely dependent on the HPT axis

and is the only assay that focuses on the thyroid activity and how it affects animals that are undergoing morphological development. Tadpoles exposed in AMA test were used to test for the thyroid gland impacts from the chemicals exposed to the *Xenopus laevis*. The AMA test for thyroid glands effects states the onset of the experiment at day 17(stage 51). The tadpoles were acclimatized to the conditions before the exposure took place i.e. the12-12 h light and dark cycles, room temperature, feeding cycles and the culture medium to avoid distress which may result in deaths not associated with the experiment. To avoid crowding of the tadpoles, five tadpoles were used per liter of FETAX solution, which translated to 25 tadpoles, and 5 liters of FETAX per aquaria. There were 4 replicates for all test chemicals and control. The aquaria were placed randomly in the room so as not to favor any of the tests in terms of exposure to light, temperature and air circulation. The termination of the experiment was done at day 21. In the test, daily observations were made to record the mortality of the tadpoles including any other observable changes in their behavior. For the 21 days of the experiment, development stage, hind limb length measurement (HLL), snout-vent-length (SVL) and body weights of the tadpoles were performed at day seven and twenty one. The histology of the thyroid gland was performed at the end of the experiment, day twenty one.

3.5.1 Mortality and clinical signs

The daily recordings of the dead tadpoles and also any observable abnormal behavior and clinical signs exhibited by the tadpoles were noted.

3.5.2 Developmental stage

Day 7 and day 21 development of the tadpoles was established by using the staging criteria of Nieuwkoop and Faber (1994) which was used to determine whether the

development of the tadpoles was sped up, nonsynchronous, slowed, or unchanged at the point of comparison (day 7 and day 21).

3.5.3 Hindlimb length

The growth of the hindlimb is controlled by the thyroid hormones and is a great determinant of developmental stage. HLL was determined at day seven and day twenty one of the test. The length measurements were acquired digitally using Motic® imagery software.

3.5.4 Snout-vent-length and body weight

SVL was measured by using the cranial aspect of the vent as the caudal limit for analysis. This was done using Motic® imagery software. The tadpoles were then dabbed dry with paper towels getting it as dry as possible and the weight was established using a weighing scale.

3.5.5 Larval Culture and Feeding

Fertilized eggs were cultured for 17 days to attain stage 51. Tadpoles were fed t- bites throughout the pre- exposure period (after stage 51) and throughout the experimental period. Feeding regime was adjusted to meet the demand of the AMA. The newly hatched tadpoles were fed several rounds of food leading to at least once a day. The food quantity was measured to maintain a proper water quality and prevent the gill filters from clogging with food particles and detritus (30mg/animal/day shortly before the test initiation)

3.6 Exposure System

3.6.1 Water Quality

FETAX was used because it permits the normal growth and development of *X. laevis* tadpoles. The exposure to the chemicals was done via a static renewal system which required physically changing the water in the tanks. The major requirement for the static renewal to work optimally is that the renewal of the test water should be changed not more than 72 hours apart (OECD, 2004). When the water is left for longer, there is a risk of ammonia build up which would be toxic to the tadpoles. There is also a chance that left over foods might clog the gill filters and change the water turbidity.

3.6.2 Iodide Concentration in Test Water

Iodine concentration was maintained at 0.5ppm. This is because there must be an iodide in the test water for the larvae combination of water and dietary sources. However in the experiment without iodine, only deionized water was used and the feed was prepared without adding Iodine.

3.6.3 Test Chemicals

Stock solution of the test chemicals was prepared using Methimazole (0.005ppm), Thyroxine (T4) (11.652ppm), Triiodothyronine (T3) (8.135ppm), potassium iodide (0.5ppm) and Sodium Fluoride (452.8ppm). All these were prepared and stored separately for the entire duration of the experiment.

3.6.4 Exposure to the Test Organism

Tadpoles that attained stage 51 were used to test i.e. at day 17. The LOEC and NOEC data obtained from the acute data above was used in setting up the experiment. Fluoride, T4, T3 and Iodine were exposed individually and in combination. Parameters measured were development, mortality, teratogenicity, and thyroid gland development through

histopathology. Teratogenicity was measured by observing the abnormalities in the tadpoles and using the atlas of abnormalities, the specific kind of abnormality was identified. The experiment was terminated at day 21.

3.7 Remedial Capacity of T3 and T4 on the Thyroid Gland

Tadpoles exposed in AMA test were used to test for the thyroid gland impacts from the chemicals exposed to the *Xenopus Laevis*. The AMA test for thyroid glands effects states the onset of the experiment at day 17 (stage 51). The tadpoles were acclimatized to the conditions before the exposure took place i.e. the12-12hour light and dark cycles, room temperature, feeding cycles and the culture medium to avoid distress which may result in deaths not associated with the experiment. To avoid crowding of the tadpoles, 5 tadpoles were used per liter of FETAX which translated to 25 tadpoles and 5 liters of FETAX per aquaria. There were 4 replicates for each chemical and control. The aquaria were placed randomly in the room so as not to favor any of the tests in terms of exposure to light, temperature and air circulation. The termination of the experiment was done at day 21. The observation endpoints for the experiment were daily mortality, development stage, length of the hind limb, body length & wet weight, snout vent length and histology of the thyroid gland at the completion of the experiment.

3.8 Histology Process

The first step in a histologic examination was identifying sample tadpole from the aquaria. After removing the tadpole it was anaesthetized, the head was then cut off caudally to the eyes and fixed in formaldehyde for 48 hours. Dehydration was done through an ethanol series series and embedding done in paraffin. The face was then cut into sections of 8 µm and staining done with hematoxylin and eosin. An imaging of the sections was done under the same magnification and outlined so as to determine the glands under observation (Tietge *et al.*, 2005). The histological examination includes

changes as hyperplasia of thyroid follicles, hypertrophy of follicular cells, follicles size and the amount of colloid formation by use of the electron microscope. The digital photographs are then used to illustrate the changes (OECD, 2009).

3.9 Data analysis

The data was analyzed using descriptive statistics, Abbott's adjustment was used to adjust for mortality of tadpoles due to natural causes, ANOVA and probit analysis was used to establish the LC_{50} and EC_{50}. The teratogenic index was evaluated by dividing the LC_{50} by EC_{50} and is used in determining the degree of teratogenicity of compounds and the level of significance in the study was 0.05.

CHAPTER FOUR

RESULTS

4.1 Acute Toxicity of Fluoride using *Xenopus laevis* Embryos (FETAX test)

Mortalities were recorded in all concentrations of Fluoride including control set up which had FETAX solution alone. The mean percent mortality at 50ppm, 100ppm, 200ppm, 400ppm and 800ppm of F was 0.05, 0.35, 0.35, 0.55 and 0.67 respectively as shown in fig 4.1. The control had the lowest mean percent mortality at 5%. The tadpoles exposed to fluoride exhibited a concentration dependant immobility (>400ppm) they became moribund as they were unresponsive to touch but were still alive as they had visible heartbeats. The median lethal concentrations (LC_{50}) for fluoride on the *Xenopus laevis* larvae as determined by use of Probit analysis was 452.8ppm.

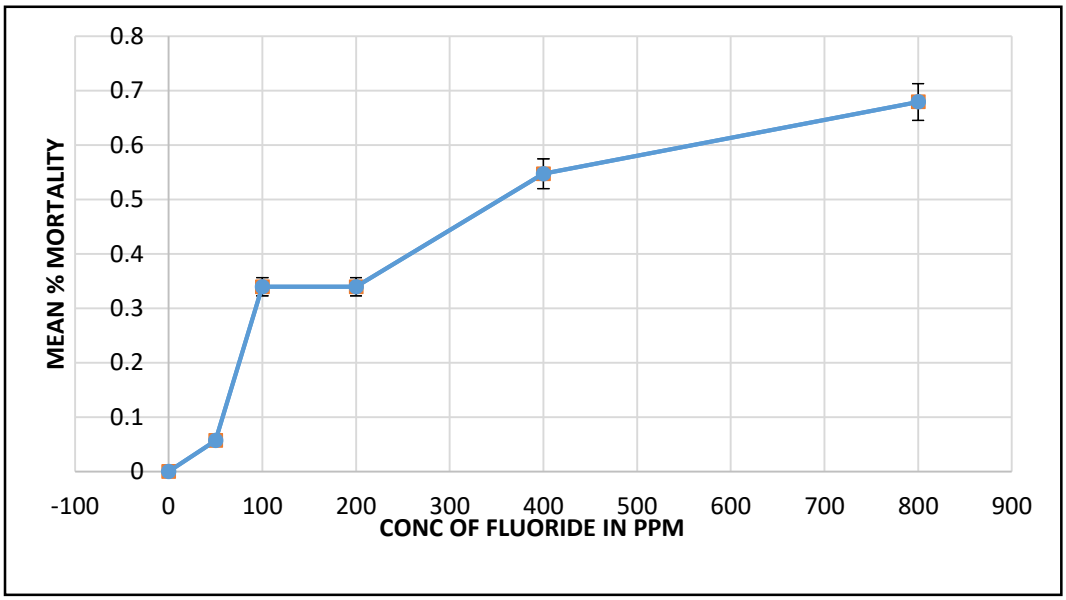

Figure 4.1 Mean % mortality of the tadpoles against the different concentration of Fluoride

4.1.1 Growth effects

Tadpoles in the control experiment had a mean length of 13.6 mm, as compared to the tadpoles treated with Fluoride, which was 13.4 mm at 50 ppm F, 12.9 mm at 100 ppm F, 12.6 mm at 200ppm F, 13.1 mm at 400 ppm F and 12.4 ppm at 800 ppm F. The tadpoles treated with fluoride showed a decrease in the whole-body length with increasing concentrations of fluoride with a p-value of $p<0.001$. Therefore, there is a significant difference in length between those with fluoride and the control. The reduction in length of the tadpoles was noted in both the deformed and normal tadpoles. The level of significance as per this test was $p<.005$.

4.1.2 Teratogenic effects

The 96hr EC_{50} of Fluoride as per this FETAX test was 452.8ppm. An atlas of abnormalities by Bantle (1991), was used to identify the deformities in the tadpoles(Bantle *et al.*, 1991). There were malformations observed in the tadpoles in control as well as those that were treated with Fluoride. The degree and severity of malformations however increased with an increase in concentration. Tail deformities were observed across all treatments including the control (8%). At F concentration of 50 ppm there was 4% of tadpoles with severe deformities and 16 % with tail deformities. In the 100ppm of F in the experiment there was 4% of tadpoles with severe deformities, 12% of tadpoles with stunted growth and 12% with tail deformities. At 200ppm of F there was 20% of tadpoles with stunted growth, 12% with tail deformities and 4 % with eye deformities. With 400ppm F there were 4% tadpoles with severe deformities, 8% with stunted growth, 16% with tail deformities and 8 % with eye defects. At 800ppm F there was 4% of tadpoles with cardiac edema, 24% with tail deformities and 16% with eye deformities.

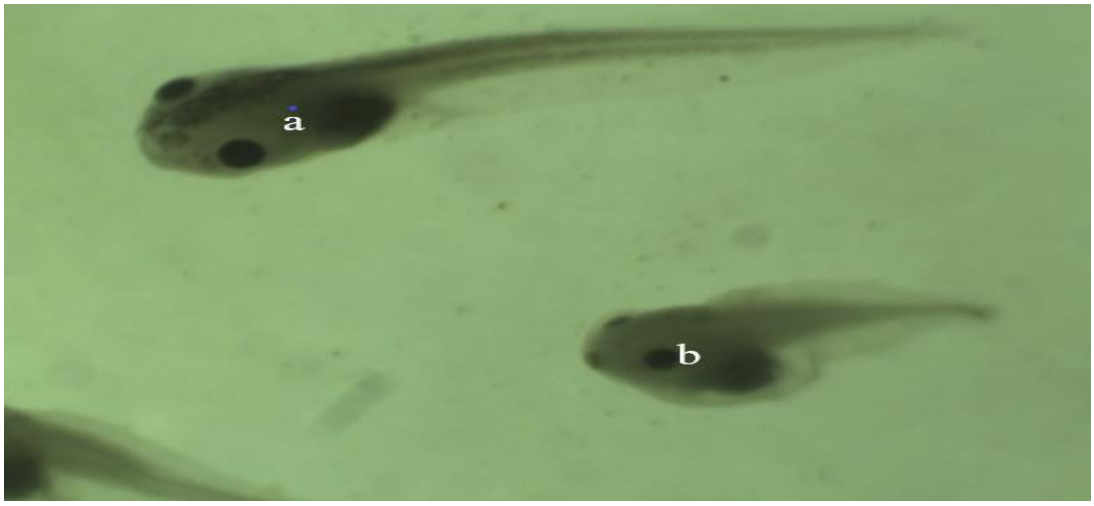

Figure 4.2: Tadpoles growth length between control (a) and Fluoride (b) (stunted growth)

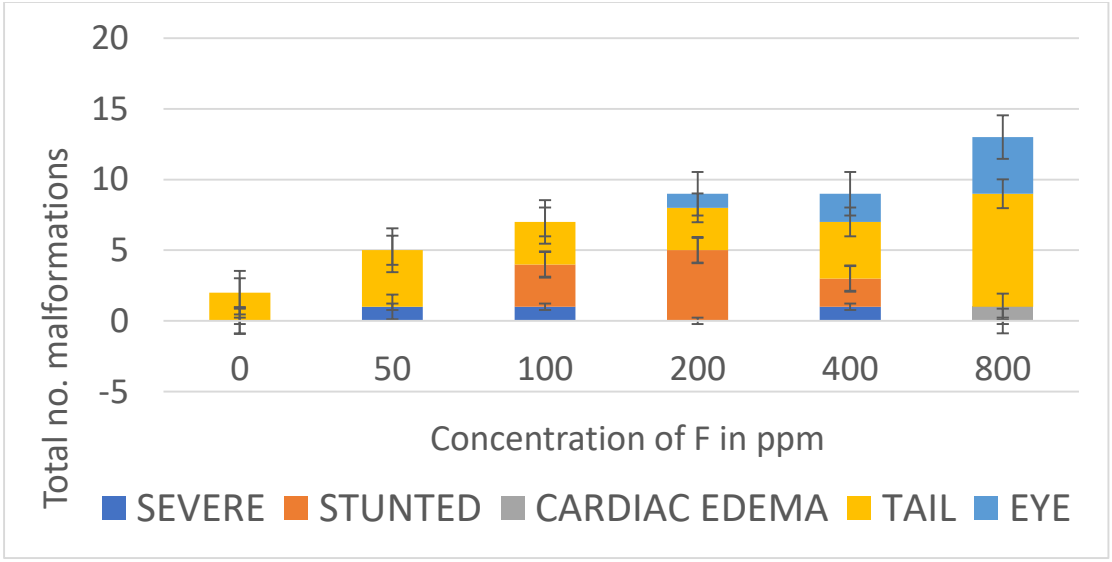

Figure 4.3: Representative graph of malformations recorded

The effective concentration 50 (EC_{50}) of fluoride as per this FETAX test was 452.8 ppm. The teratogenic index expressed as the ratio of LC_{50}/EC_{50} of F in this FETAX test protocol was 0.93.

4.2 Reversal of acute Impacts of Fluoride in *Xenopus Laevis* Embryos Using T4 and KI

4.2.1 Embryolethal effects

The mortality of the tadpoles with Fluoride treatment alone (0.68) was higher as compared to the tadpoles treated with Fluoride and Iodine (0.6), T4 alone (0.45), KI alone at (0.32) and those treated with fluoride and T4(0.35). An increase in the concentration of Fluoride increased the % death of the tadpoles in all the treatments $p>0.05$. At 800 ppm, the tadpoles were insensitive to touch and were immobile. The tadpoles treated with I and T4 did not show a difference in behavior between each treatment and the control tadpoles.

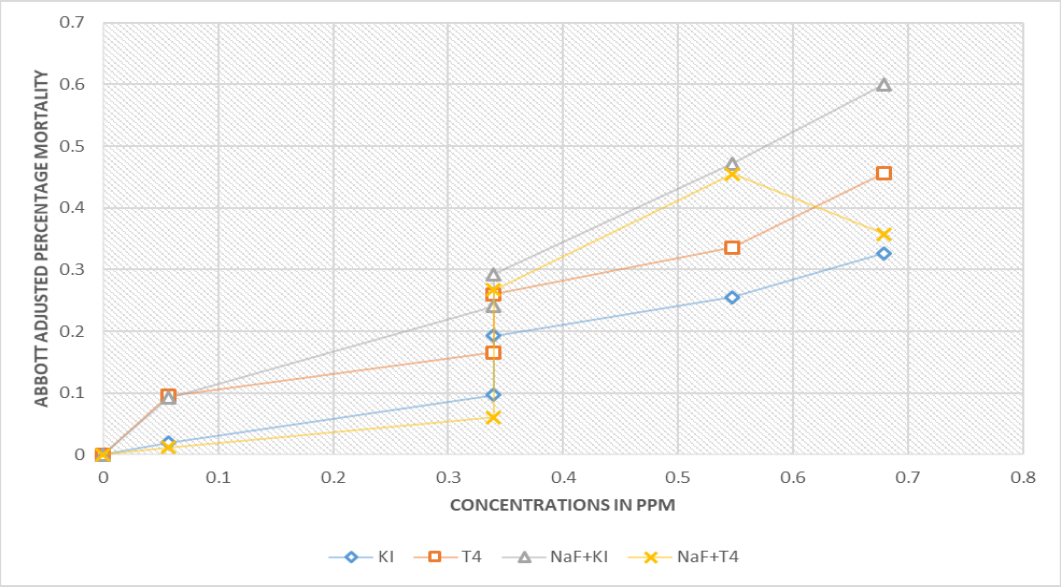

Figure 4.4: Mean Percent mortality of tadpoles treated with Fluoride alone, Fluoride and Iodine, and Fluoride and Thyroxine.

4.3 Growth effects

The mean size of the tadpoles in control at the end of 96 h was 13.6 mm. An increase in the mean length of the tadpoles was noted in all the tadpoles treated with T4 and I. Fig 4.5 shows that the mean length of the tadpoles treated with Fluoride alone was 12.88 mm; Iodine alone had a mean length of 12.58 mm while those with T4 alone had a mean length of 13.34 mm, F+T4 treated tadpoles had a mean growth of 13.16 mm and F+KI treated tadpoles had a mean length of 10.48mm. The p value for the treatments with F+KI and the F+T4 had p> .079.

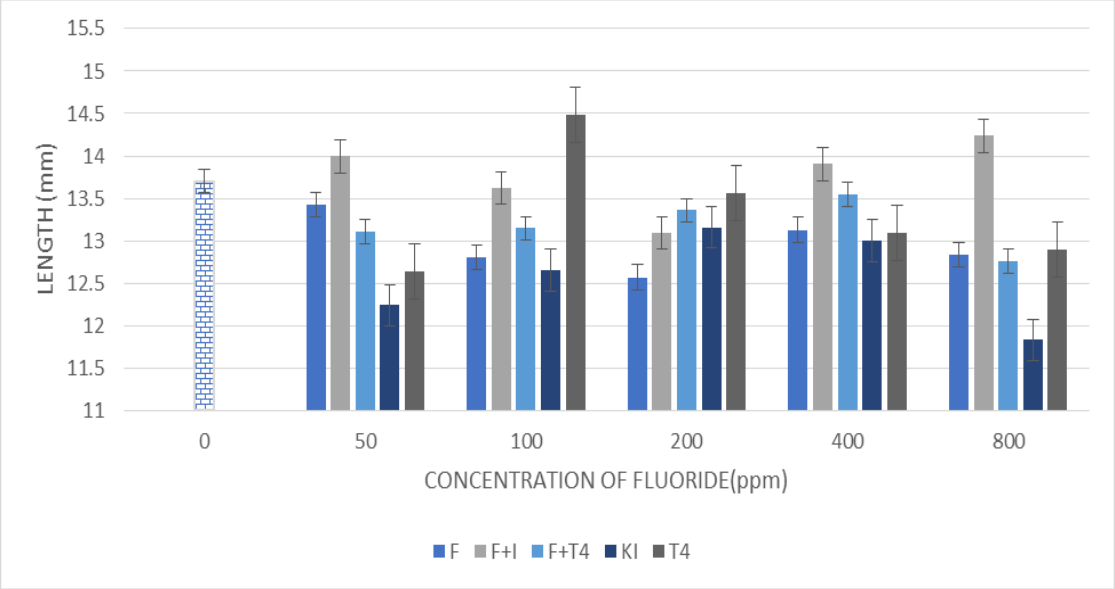

Figure 4.5: Comparison of Tadpoles Response to treatment Fluoride (F) with Fluoride & Iodine (F+I), those treated with Fluoride & Thyroxine (F+T4), Iodine alone (KI) and thyroxine alone (T4)

4.3.1 Teratogenicity

Tail deformities (fig 4.7) was recorded across all the treatments with the highest recorded at F (200ppm) and T4 at 20.4% of the tadpoles with this deformity. In the

Iodine treated tadpoles, 2.9% had cardiac edema, 3.1% had eye malformations, 15.6% tail malformations, 1.5% face malformations; 1.6% stunted growth (fig 4.9) and 1.3% severe malformations (fig 4.8). The T4 treated tadpoles had 17.8 %tail malformations, 4%stunted, 2%face malformations, 2% abdominal edema (fig 4.10) and 3.2% eye malformations.

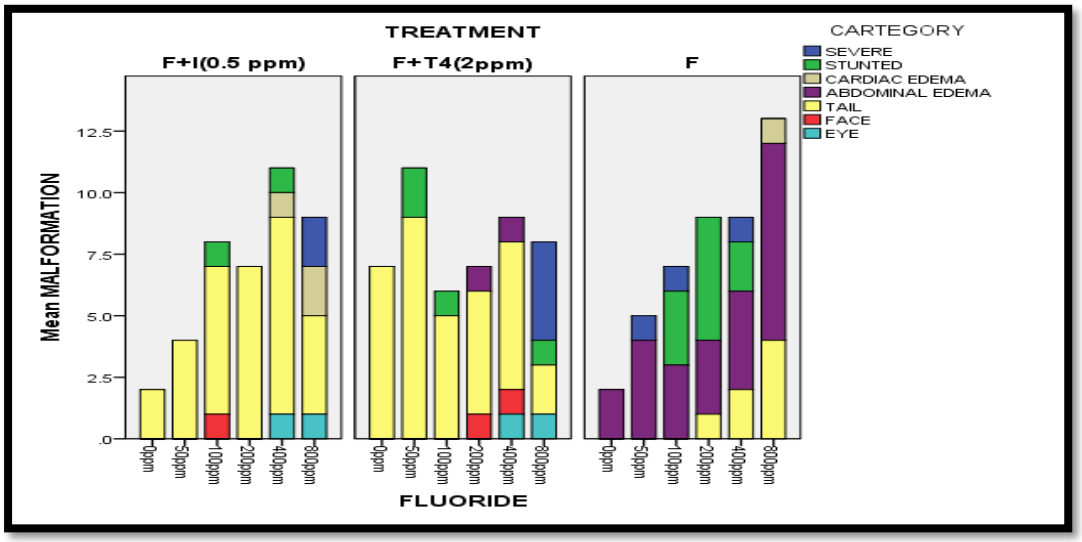

Figure 4.6: Malformations Recorded in F, F+I, F+T4 Treated Tadpoles

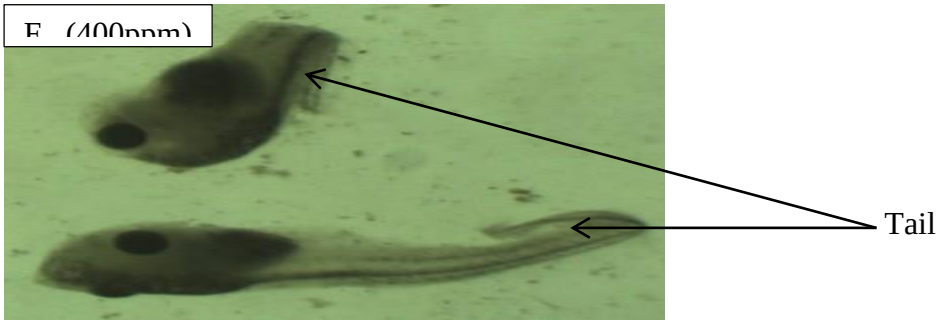

Figure 4.7: Tail Malformations in the Tadpoles

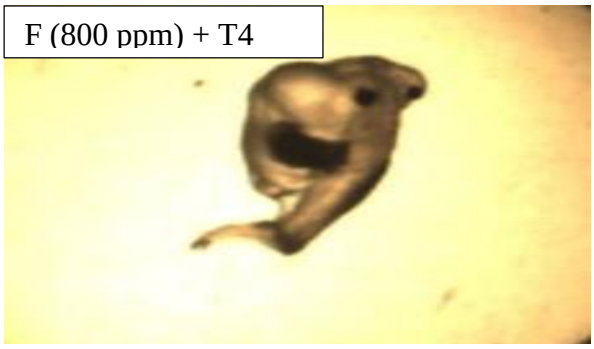

Figure 4.8: Severe Malformations

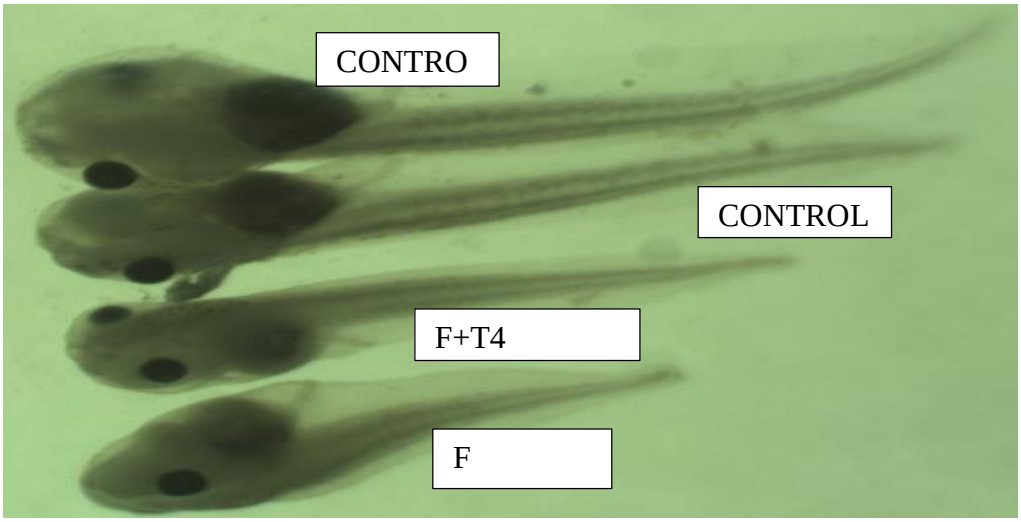

Figure 4.9: Stunted Growth in Tadpoles

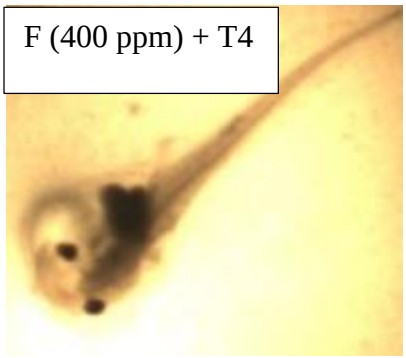

Figure 4.10: Abdominal Edema

4.4 To evaluate the effects of KI, T3, T4, and Methimazole on chronic toxicity of fluoride in *Xenopus laevis* tadpoles

The fourth and the last objective of the study were to reverse the chronic effects of Fluoride on *Xenopus laevis* using KI, T3 & T4.. Fig 4.12 shows that the control experiment had 32% mortality, with the highest mortality recorded in the study were those tadpoles that had been treated with T3, T3-I(T3 without Iodine), F+T3, F+T3+T4 and F+T3+T4+M had 100% mortality by day 15. All figures with T3 indicate triiodothyronine, T4 thyroxine, I iodine, M methimazole and F fluoride.

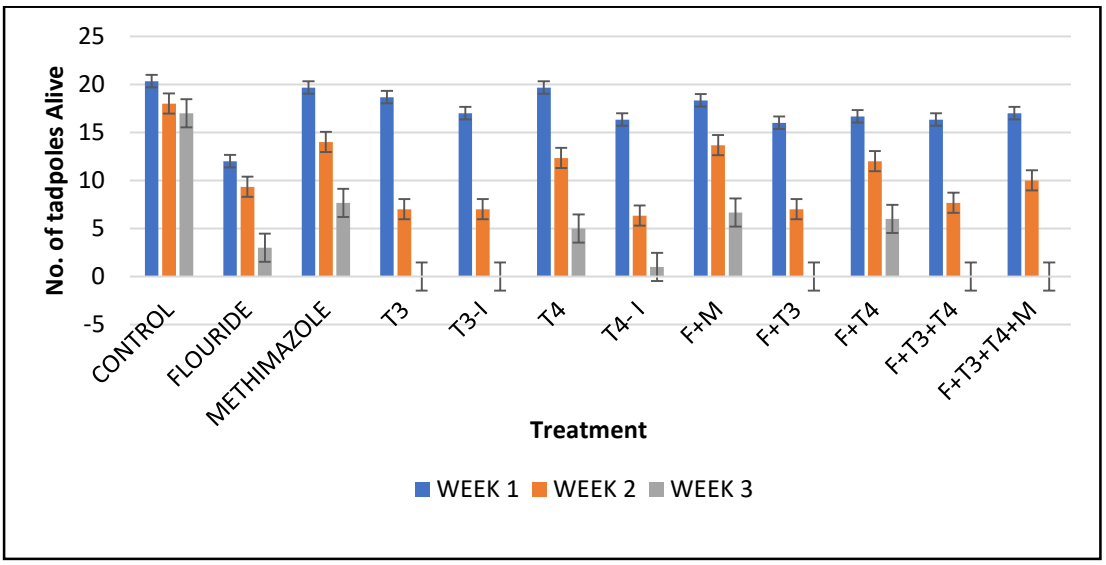

Figure 4.11Mean Mortality for Tadpoles under AMA Test

4.4.1 Teratogenicity

The atlas of abnormalities by Bantle (1991) was used to determine the deformities of the tadpoles. An increase in the percentage of tadpoles with deformities in comparison between day 7(0.5) and day 21(0.6) was also observed. Methimazole treated tadpoles had a 0.55 percent deformity on day 7 and 0.45 on day 21. The tadpoles with methimazole without iodine had 0.36 deformity and 0.45 deformities on day 21, T4 tadpoles had 0.35 deformities on day 7 and 0.4 on day 21, T4-I had 0.25 deformity on

day 7 and day 21, Fluoride and methimazole had 0.2 deformity on day 7 and 0.3 on day 21, F+T4 had 0.2 deformed tadpoles at day 7 and 0.3 deformities on day 21. Deformities on T3 treated tadpoles was only recorded on day 7, T3 with 0.1, T3-I with 0.15, F+T3 0.5, F+T3+M with 0.5 and (F+T3+T4) & (F+T3+T4+M) both had 0.5 deformities as described by fig 4.12 below.

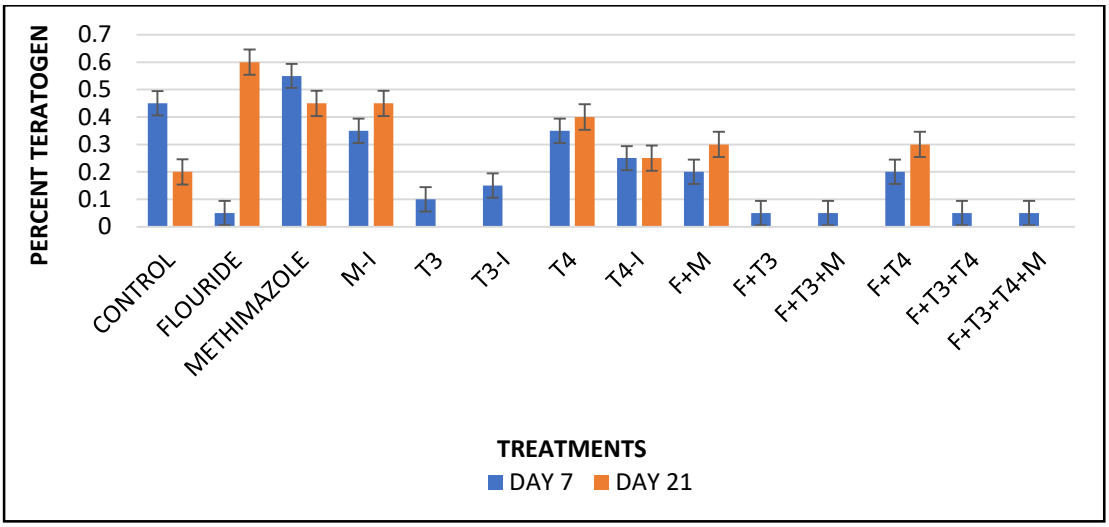

Figure 4.12 Percent teratogenic effects of tadpoles on Day 7 and Day 21

4.4.2 Hindlimb length

Hindlimb development was measured in terms of the sprouting of the hindlimb bud and not necessarily the length of the limbs. The tadpoles in control had the highest percentage of tadpoles developing a hindlimb with an increase from 80% as at day 7 to 92% at day 21, 70% of Methimazole treated tadpoles had hindlimbs at day21, T4 at 25%, F+M at 26% and F+T4 at 30% at day 21. On day 7, tadpoles treated with F=48%, M=67%, T3=30%, T3-I=37%, T4=62%, T4-I=58% F+M=15%, F+T3=7%, F+T4=37%, F+T3+T4=19% & F+T3+T4+M=6% as shown in table 4.1 below.

Table 4.1 Percent Hind Limb Development of live tadpoles

Treatment	Day 7 Examination	Day 21 Examination
Control	80%	92%
Fluoride	48%	0%
Methimazole	67%	70%
T3	30%	0%
T3-I	37%	0%
T4	62%	25%
T4- I	58%	0%
F+M	15%	26%
F+T3	7%	0%
F+T4	37%	30%
F+T3+T4	19%	0%
F+T3+T4+M	6%	0%

4.4.3 Snout-vent length (SVL)

The mean SVL of the tadpoles was highest at Control and Methimazole treated tadpoles at 13mm, followed by the tadpoles treated with T4 with 12mm, F, M-I and F+T3+M at 11mm, F+M and F+T3 at 10mm, F+T3+T4+M and T4-I at 9mm, T3-I at 7mm, F+T3+T4 and F+T4 at 5mm and T3 with the lowest at 3mm.

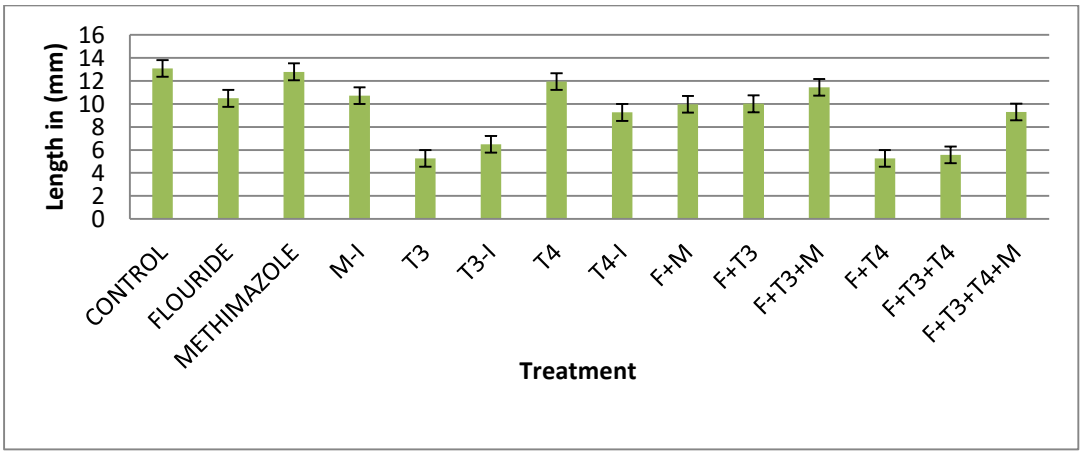

Figure 4.13 Snout Vent Lengths

4.4.4 Wet weight

The mean wet weight of the tadpoles was higher in the control, T4 and F+T4 treated tadpoles. There was however, a reduced mean weight of tadpoles across all treatments between week 1 and week 2 except for the tadpoles treated with T3 which had a slight increase in their weight.

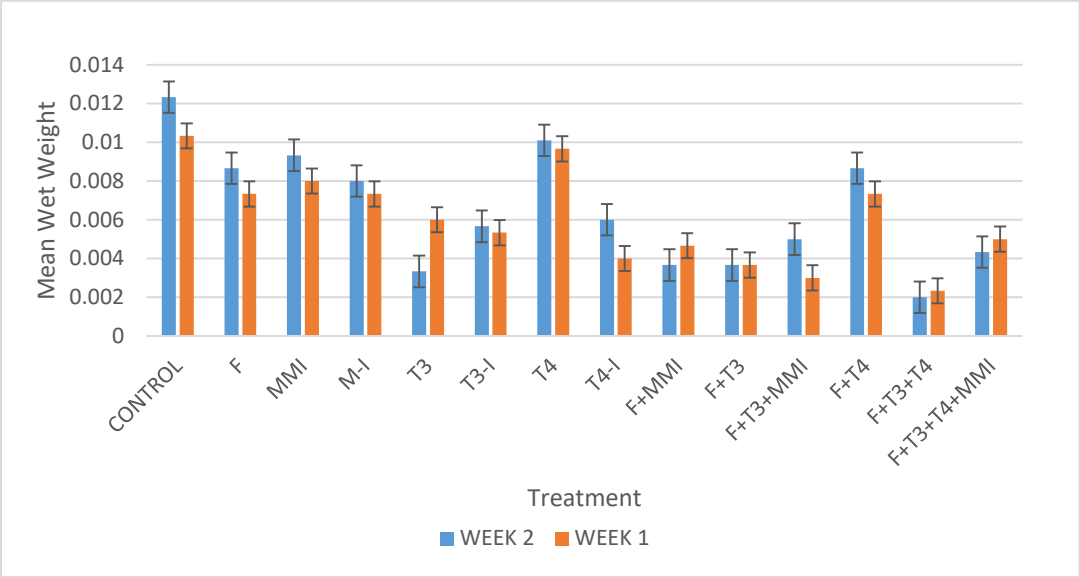

Figure 4.14 Mean wet weights

4.4.5 Thyroid Gland Histology

The histology done on the tadpoles showed only the tadpoles exposed to 11.65µg/l T4 had a developed thyroid gland. No other tadpoles showed a thyroid gland development including those with T4 in combination with other chemicals. The arrows in fig 4.15 shows where the thyroid gland in the sample was after a picture of the thyroid was taken under an electron microscope.

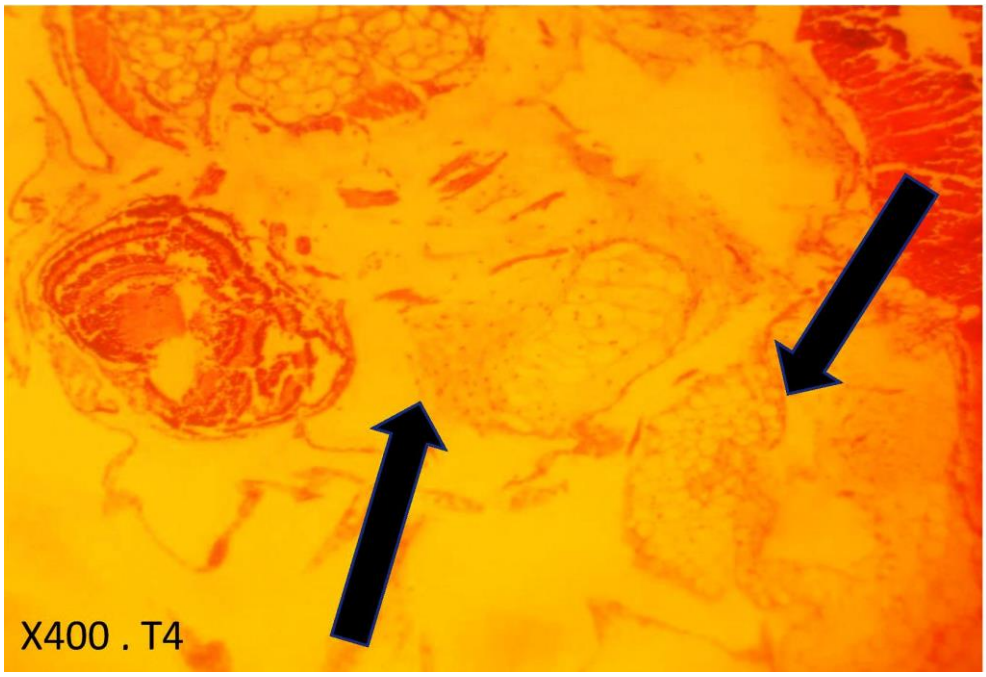

Figure 4.15 Thyroid Gland Histology

CHAPTER FIVE

DISCUSSION OF FINDINGS

5.1 Acute Toxicity of Fluoride using *Xenopus Laevis* Embryos (FETAX test)

This study findings indicated that the tadpoles exposed to Fluoride exhibited abnormal behavior including non-response to touch even though they were still alive as they had visible heart beats. This response was particularly high amongst the tadpoles at the concentration of Fluoride above 400ppm. The level of significance of survival in the tadpoles was $p<.005$. These findings were in agreement to Goh *et al.*, (2003), who showed that the most significant malformation caused by sodium fluoride are reduced head-tail lengths in addition to an impaired neuromuscular system in tadpoles (Goh *et al.*, 2003). The LC_{50}, EC_{50}, and minimal concentration to inhibit growth (MCIG) of sodium fluoride was within the range set for a teratogenic compound that has an effect on embryos. Given that FETAX has a high level of success in the identification of teratogens that affects mammals, the teratogenic action of sodium fluoride signify on the *Xenopus laevis* embryos signify that sodium fluoride can act directly on the fetuses developing leading to malformations.

The extreme mortality of the tadpoles between the first 24 hours of the study and at the termination of the experiment suggest that the jelly coating the eggs at the start of experimentation highly likely is responsible for its inabsorption into embryos given that sodium fluoride was administered aqueously as opposed to food administration (Osano *et al.*, 2002). As it is stripped off with time the jelly dissociates and allows for the embryos to absorb more Fluoride which in turn makes it toxic to the tadpoles. when T4 and I is added, the deaths of the tadpoles was significantly reduced. This was common for all the test concentrations (Figure 4.1; 4.5). A study of *Cyprinus carpio*

haematopterus agrees with this finding that increasing concentrations of Fluoride leads to increases in their mortality (Kaur *et al.*, 2019). Therefore, mortality is mostly dose dependent and the exposure time.

The reduced length of Fluoride treated tadpoles was noted as the concentrations of fluoride increased. This is in agreement with a study by (Goh *et al.*, 2003) which found that as the concentration of fluoride increased the length reduced. Spittle, 2018 also agrees that low iodine in addition to elevated levels of fluoride also leads to developmental disorder such as short stature as shown in Figure 4.2 (Spittle, 2018). Similar studies on anuran tadpoles in streams recording high amounts of fluoride also manifested reduced length of tadpoles in comparison to those found in streams with low levels of fluoride (Pollo *et al.*, 2019). Investigations into male zebrafish (*Danio rerio*) also exhibited reduced growth in high fluoride exposure areas (Jianjie *et al.*, 2016). Epidemiological studies have proven that iodine deficiency in combination with high fluoride exposure results in an elevated risk of disorders related to development including the level of intelligence in children (Waugh, 2019). I and F treated tadpoles showed a growth in length of the tadpoles in comparison to those that were treated with fluoride alone. This indicate that I treatment mitigated the development disorder of the tadpoles used in the experiment (Coady *et al.*, 2010a; Degitz *et al.*, 2005). Chen *et al*., (2016) showed that continual exposure to 50 mg NaF/L notably escalated mortality, prevented metamorphosis, and not on time development of *R. Chensinesis* and *R. Nigromaculata* tadpoles. Most notable malformation was tail flexure deformity in *R. chensinesis* tadpoles but not in *R. Nigromaculata* tadpoles, which resulted in bone remineralization and inadequate deposition of calcium in *R. Nigromaculata* tadpoles.

In the end, the study looked at suggestion that fluoride can additionally have an effect on skeletal ossification in frog species because of distinction in sensitivity or on the length of the exposure.

Malformations were noted in all the treatments. The tadpoles had lost response to touch especially as the concentrations of fluoride increased. This could be explained as fluoride inducing oxidative stress which can be manifested as sluggishness and lack of mobility as a result of a loss touch senses. This is because of the loss of sensation of touch of rats when they are exposed to high fluoride concentration which leads to an effect on medulla in the brain (Malin *et al.*, 2018). Studies in epidemiology have also connected exposure to high fluoride concentration to disorders of development and cognitive function in children (Waugh, 2019). Iodine and thyroxine treatment didn't fully do away with malformations, instead there was an increase in teratogenic effects recorded. In spite of this, teratogenic effects, severe deformities recorded reduced. Of importance is the eye deformities which increased in both I & T4 treated tadpoles. In early development of the tadpoles, deiodinases mainly function in the tissues of the eye and ears which would in normal cases would lower the amount of thyroid hormones resulting in thyroid receptors being unliganded. These receptors are of great importance in eye development (Fini *et al.*, 2012). This indicates that fluoride works by hindering thyroid hormone receptors of the tadpoles resulting in the malformations in the development of the eyes.

5.2 Reversal of acute Impacts of Fluoride in *Xenopus laevis* Embryos Using T4 and KI

The tadpoles exposed to F were not sensitive to touch and were immobile at 800ppm although were still alive because of visible heart palpitations. The tadpoles exposed with I and T4 didn't show a distinct change in their behavior between each of the treatment and the control. The analysis of variance showed $p>.005$ significance for the survival of F and T4 treatments and a $p>.005$ for tadpoles treated F with I.

Tadpoles treated with KI alone had a mean length of 12.58 mm and those treated with T4 alone had a mean length of 13.34 mm, F+T4 treated tadpoles had a mean growth of 13.16 mm and F+KI treated tadpoles had a mean length of 10.48mm. Even though there was an increase in length in the tadpoles treated with T4 and I as compared with those that had F+KI and F+T4. The slight decrease in length of tadpoles with Fluoride is in agreement with the earlier statement that Fluoride does cause retardation of the tadpoles. The two treatments showed an equal response of the tadpoles to the treatments.

5.3 Chronic Toxicity of Fluoride in the *Xenopus Laevis* Tadpoles

The results were in concurrence with results by Chai *et al*., (2017) which revealed that total length, snout-to-vent length (SVL), body mass and the development stage of tadpoles was repressed at 42.6mg/L F. Furthermore, metamorphosis was delayed and its size increased at the end of metamorphosis was evident after exposure to 19.8 mg/l F. On this concentration, bone mineralization of the larvae was restrained at the end of metamorphosis. Bone mineralization was however improved at 4.1 mg/l F. This study implies that in the presence of high F concentrations, death risks increase, metamorphosis is delayed and skeletal ossification is surpressed in *Xenopus laevis*. This was agreed by (Zhao, *et al* (2013) who demonstrated that the length and total weight

remained unaffected by exposure to fluoride across the concentrations of fluoride while metamorphic delays was only observed in 50 mg/l F (Zhao *et al.*, 2013). Metamorphosis of the tadpoles was inhibited with Fluoride exposure because it causes damages in the thyroid follicle cells in the thyroid gland which in turn leads to a reduction in the thyroid hormones that is necessary for the tadpole metamorphosis to be successful. Metamorphosis involves a lot of remodeling of organs and tissues in tadpoles, including skeletal system remodeling. Studies have shown that fluoride interferes with calcium ions deposits in the bones of tadpoles therefore causing a delay in the development of the skeleton leading to a prolonged mineralization and bone development hence an inhibition of metamorphosis (Chen *et al.*, 2016). Measurements in histomorphology showed an increase in colloid depletion in the thyroid gland while the diameter of the follicles was shortened at 50mg/l F. This study by Chen *et al.*, 2016 insinuate that fluoride may result in the damage of the follicular cells in the thyroid gland and these affect the thyroid system leading to delayed metamorphosis in addition to ossification of the bone tissues which prevent the deposition of calcium.

5.4 To evaluate the effects of KI, T3, T4, and Methimazole on chronic toxicity of fluoride in *Xenopus laevis* tadpoles

The death of all the tadpoles in the T3 treated tadpoles in this study may suggest that the T3 we used was much higher in concentrations than is normally required physiologically by the tadpoles was used or that the tadpoles do not require T3 at all and it is toxic at very low concentrations to it (Kowalik *et al.*, 2018). The wet weight of the tadpoles treated with T3 on day 7 was drastically reduced as compared to control tadpoles. This Yao *et al* (2017) attributes to T3 inducing a drastic metamorphosis and is a sensitive indicator that body weight is an able indicator of metamorphosis of the tadpoles (Yao *et al.*, 2017). *Xenopus laevis* tadpoles remain sensitive to substances

found in the environment since their habitats and its metamorphosis being controlled by the endocrinal system majorly the thyroid hormones. When metamorphosis is occurring, some hormonal factors are altered in addition to the changes that occur in the structure and function of the larvae. There are various ways that determine the thyroid hormone balance either directly or indirectly. Some agents that act directly causes changes in the synthesis of thyroxine and its secretion as a result of the effects it has on the peroxidases, iodine uptake in the thyroid, deiodinases and proteolysis. Concurrently, the indirect action as a result of the biochemical processes including sulfation etc. As a result of this, OECD and the EPA have each come up with their own guidelines which make use of African clawed frogs (*Xenopus laevis*) and frog metamorphosis for studies and tests of chemicals with potential to disrupt the endocrine system.

The poor functioning of the thyroid multiplied TSH at excessive fluoride became mentioned in children and adults in India who reside in areas that are endemic to high fluoride regions (Susheela *et al.*, 2005). They found that forty seven percent of youngsters living in a New Delhi region with a mean water fluoride degree of 4.37 mg f/l have shown clinical hypothyroidism on account of fluoride. Wang *et al*., (2020) recruited 571 children aged seven –thirteen years from endemic and non-endemic fluorosis areas in Tianjin, China. Results showed that each 1 mg/l increase fluoride in water resulted in a 0.13μiu/ml increase in levels TSH (Wang *et al.*, 2020). Same observation was made in young adults consuming water with 1 ppm of fluoride in northern Mexico have decreased T3 degrees (Ruiz-Payan, 2006). Each 1 mg/l increase in urinary fluoride becomes related to 0.09 μg/dl reduction in T4 (Ruiz-Payan, 2006). Kheradpisheh *et al.*, (2018) did a comparison of the amount of T3, T4, and TSH in human beings with hypothyroid and individuals without thyroid sickness, with fluoride

concentrations 0–0.29 and 0.3–0.5 mg/l in drinking water in Iran. This leads to a conclusion that fluoride affects the T3 and TSH even in low concentrations less than 0.5 mg/l (Kheradpisheh *et al.,* 2018)

A study on Talab Sarai region in Pakistan, an area with excessive fluoride content in the ingesting water (6.23 mg f/l) determined enamel fluorosis in 93% of one hundred thirty investigated kids with the age of 12 ± 3 years. Eighty percent of the youngsters displayed clean thyroid hormonal disorders, with 37% exhibiting excessive TSH and forty three percent with T3 and T4 disorders (Zulfiqar *et al.,* 2020). The analysis of hypothyroidism became almost two times as common in completely fluoridated regions in England, in comparison to non-fluoridated regions (Peckham *et al*., 2015). The population-based totally have a look at of the weighted sample of > 6M adults from age 18–seventy nine residing in Canada have increased amounts of urinary fluoride which also indicate improved danger of underperforming thyroid gland (Malin *et al*., 2018). Such studies prove that thyroid dysfunction occurs in each endemic fluorosis regions and in regions with CWF (Grandjean, 2019). In this study however, all the T3 treated tadpoles died before end of experiment. This finding is supported by the findings by Kowalik, who noted that an increase in the levels of T3 brought about thyrotoxicosis which was characterized by fastened heartbeats, irregular heart rhythms, muscle wasting and a reduction in bone mineralization and an altered CNS development (Kowalik *et al.*, 2018). The methimazole treated tadpoles reached stage 54 and were larger in size as compared to the control tadpoles since methimazole inhibits formation of T3. This supports the findings by (Coady *et al.*, 2010a) which showed that methimazole does not inhibit the thyrotoxicosis of the tadpoles at the onset of the experiment because the thyroid gland would then still be immature and not functional. An increase in the weight of the tadpoles' wet weight also means that there is a

disruption of the thyroid hormone in the tadpole development. This confirms that methimazole is an active thyroid peroxidase inhibitor. This was also reported by (Degitz *et al.*, 2005) who reported a similar finding that Methimazole inhibits the development of the tadpoles. Methimazole impede the development of the larvae and changes in the morphology of the thyroid gland in the form of reduced colloid, cellular hyperplasia, hypertrophy and glandular hypertrophy. The above result of this study is supported by the work of (Huq, 2008)which reported that methimazole can inhibit thyroxine synthesis by blocking TPO coupling of iodine to the tyrosine precursor contained in the thyroglobulin. Ammonium perchlorate disrupted iodide uptake by the follicular cells of the thyroid gland and inhibited forelimb emergence, hind limb development as well as tail resorption, is associated to significant hypertrophy in the epithelium of the thyroid follicles.

The hind limb length was also an important measure of development in the experiment. Even though the number of tadpoles with hind limb developing was higher as compared to any other treatment, the difference was in the length whereby the T4 treated tadpoles had a visibly longer hind limb as compared to the other treatments including the control. T4 treated tadpoles also had a reduced wet weight as compared to the ones in the control in both day 7 and day 21 of the experiment. This is attributed to the metamorphosis process which leads to a drastic loss in the weight of tadpoles, although not necessarily due to the ability of the tadpoles to produce and use endogenous thyroxine, may also be due to the stress levels the tadpoles face during the static renewal of the test water (Coady *et al.*, 2010a).

Histologically, the thyroid gland contains follicles. The colloid is wealthy in thyroglobulin, a protein owning many tyrosine residues to which iodine molecules connect to shape the thyroid hormones (Wang *et al.*, 2021). Studies in endemic areas

show that the thyroid gland responds to the fluoride burden. The TSH prompt the thyroid gland to produce thyroxine (T4) and triiodothyronine (T3). A high level of TSH suggests an inactive thyroid gland or hypothyroidism (Kleinau *et al.*, 2017). ALFX is capable of mimic TSH by linking its associated g protein. The thyroid gland histology was only possible for the tadpoles in T4 treatment. All the other treatments including the control tadpoles did not fully develop a thyroid gland at the end of this experiment. The thyroid gland appeared normal although the accelerated development of the thyroid gland as compared to the control means that T4 induced a faster development of *Xenopus laevis* thyroid gland.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Persistent exposure to fluoride multiplied the mortality, lead to inhibition of metamorphosis, and slowed development in tadpoles in both acute and chronic experiments. Exposure to fluoride increased the occurrence of deformities in tadpoles. Further, fluoride encourages bone mineralization in tadpoles and retards the calcium deposition in tadpoles. Accordingly, fluoride has specific consequences in tadpoles' development. Due to the submit-fertilization time to metamorphosis the accelerated ossification befell inside the species, with the longest time to metamorphosise, the extended skeletal ossification with fluoride may be related to an elevated response to fluoride or in a lengthened exposure period. Accordingly, the study concludes that fluoride can additionally affect development abnormally in distinct tadpoles as a result of variations in sensitivity or the period of the exposure.

Fluoride is a cumulative toxicant that can alter tissue accretion and resorption while also disrupting the equilibrium of bone mineral metabolism. Fluoride, inhibits the apposition charges of calcium ions at high concentrations, resulting in a longer mineralization period and, as a result, a delay in the growth of the skeleton. Furthermore, large doses of fluoride may result in the production of improperly mineralized bone of poor quality. Fluoride at high doses may easily cross the placental barrier and cause direct harm to the developing mammalian fetus, resulting in embryonic and fetal developmental defects in various species, including frogs.

The T4 and I in the acute experiments could reverse the effects of the Fluoride on the tadpoles although not entirely as was seen in the teratogenic effects. In the chronic

experiments, Methimazole was able to inhibit the development of the tadpoles and prevent the tadpoles from reaching the climax of metamorphosis. I, T4 treated tadpoles were able to reverse the impacts of Fluoride on the tadpoles. Histologically, T4 treated tadpoles showed an increased development in that they were the only tadpoles with a grown thyroid gland at the completion of the experiment. T3 treated tadpoles however would not reach the end of the experiment as all tadpoles had died before end of the experiment, even though earlier on experiment day 7 showed a higher rate of development as compared to the other tadpoles with the other treatments. This experiment would not yield much histologically due to only T4 treated tadpoles having developed a thyroid gland at the end of the experiment.

6.2 Recommendations

The study recommends the following;

1. Fluoride causes toxic effects to organisms and therefore there is need for relevant authorities' e.g. public health departments in counties to consider fluoride exposure to individuals and come up with ways to reduce these exposures.

2. *Xenopus laevis* is effective for use in laboratory experiments for study of toxicants and recommends further studies to improve on the other various endpoints of development e.g. histology.

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