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Reversal of Fluoride Induced Teratogenicity in *Xenopus laevis* using Iodine and Thyroxine

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Abstract

Fluoride is present naturally in the environment. However, human activities in the environment have increased the levels significantly. The effect of Fluoride exposure to Xenopus laevis embryo development was determined under Frog Embryo Teratogenesis Assay- Xenopus (FETAX). The endpoint of the study was mortality, whole body length and malformations which were recorded before and at 96 h. LC_{50} of Fluoride as per the FETAX test was 420.46 ppm and the EC_{50} was 452.802 ppm which indicated the TI of 0.93 therefore making Fluoride embryolethal. The study aimed to check on the ability of Thyroxine and Iodine to reverse the effects of F on the development of the tadpoles. There was a decrease in mortality but an increase in teratogenic impacts after treatment with Iodine and Thyroxine. The study found that the reversal of the Fluoride induced impacts on the tadpoles' treatments by Iodine and Thyroxine treatments did not show a significant difference (p value .079) in response of the tadpoles for each of the treatment.

Keywords: Fluoride, Thyroxine, Iodine, Teratogenic Index, Development, Reversal

INTRODUCTION

Fluoride in drinking water is generally high in some geographical areas for example, southern Asia (Bhattacharya et al., 2020), the eastern Mediterranean(Alhaj & Elhassan, 2020), and Africa (Onipe et al., 2020). Kenya is one of the countries in Africa that has high concentration of fluoride in drinking water. On the analysis of ground water in Nyanza regions (Bondo-Rarieda regions) have been found to have high levels of between 0.95-1.41 ppm in their spring water, pond water, open water and in stream water (Wambu et al., 2014). A study by (Mosonik, 2015) in Bomet, showed that all water sampled from boreholes in the area had a level of 4.51- 4.37 ppm and water pans with 2.46 -2.74ppm. In Njoro catchment area, analysis of ground water showed 2.4ppm in dams, 4.1ppm in wells, 5.5 in springs and 6.6 ppm in borehole water (Moturi et al., 2002). This shows that most parts in Kenya have fluoride higher than WHO standards of 1.5 ppm and 0.5-1.0 ppm for artificial fluoridation of water(Dratman & Martin, 2020) hence the need for de-fluoridation and treatment of fluoride impacts in the country. High Fluoride levels leads to an increase of; dental fluorosis(Alvarez et al., 2009), skeletal fluorosis, thyroid problems(Dey & Giri, 2016), neurological problems(Choi et al, 2012), heart failure, reproductive issues, acne and skin problems(Blanchard et al., 2020; Mishra, et al., 2020) to both humans and animals.

Iodine is an essential element found in the earth's soil and ocean waters, which helps in fetal development and hormone synthesis (Pearce, 2017). Iodine is the key component for production of thyroid hormone in the thyroid gland with low and high levels of

Iodine in the body resulting to hypothyroidism and hyperthyroidism respectively (Waugh, 2019). Excessive ingestion of Fluoride leads to lowering of the Iodine levels by replacing the Iodine molecules in the thyroid gland thereby an interference in the thyroid hormone production process (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. High concentrations of Fluoride together with low concentrations of Iodine result in a synergistic effect among animals and humans (Waugh, 2019).

Thyroxine (T4) is an aromatic amino acid that is important for the maintenance of life of an organism. It is a prohormone produced in the thyroid gland and is inactive in nature. Its production is regulated by thyroid stimulating hormone (TSH) which is released by the pituitary gland. Deiodinases then mediate the transformation of T4 to Triiodothyronine (T3) an active form of the hormone (Bays et al., 2020). Without T4, an organism has less chance of surviving and although they may survive for a while, they may be impaired with limited brain functions. Low provision of iodine ions for the synthesis of adequate amounts of T4 has been observed especially in areas with low iodine levels and especially in the mountainous regions far from the sea (Dratman & Martin, 2020). Fluoride causes inhibition of Na+, K+-ATPase activity an important regulator of the Sodium Iodide Symporter (NIS) functionality. NIS is important for effective absorption of Iodide in the intestines and the transportation and uptake of Iodine into the thyroid gland (Waugh, 2019).

Since the 1940’s all studies carried on Fluoride and embryonic development show different aberrations caused by action of Fluoride and its compounds more so aquatic life (Camargo, 2003). *Xenopus laevis* is a good model for this study as they are relatively large and robust and can be bred easily in simple salt solutions in the laboratory. They can be produced in large numbers by means of a simple hormone injection (hCg) and are easily manipulated. *X.laevis* provide an inductive signal which allow for one cell type to influence the development of another (Beck & Slack, 2001). *X. laevis’* development has also been well documented (Coady et al., 2010). There are also less ethical consideration rigors to be considered during the study. *Xenopus laevis* have therefore been used to conduct both acute and chronic exposures using FETAX and AMA test respectively (OECD, 2009).

Since FETAX has a high degree of success in identifying mammalian teratogens, the study employs this protocol to understand the embryotoxicity, teratogenicity and the remedy of fluoride effects using Iodine and Thyroxine.

Objective: To compare the ability of I and T4 to reverse the acute toxicity of Fluoride in *Xenopus laevis*.

MATERIALS AND METHODS

Chemicals and Kits

The materials include four breeding tanks (30cm×30cm×30cm) fiberglass, a portable heater fan , thermometer, dissecting microscope, digital camera with a four mega pixels resolution and micro function, Image digitizing software, Petri dish (100*15 mm), l-cysteine, NaOH, The pH meter, Test chemical (Fluoride (analytical grade), I(analytical grade), and T4 (levothyroxine)), FETAX solution ,HCG(Human Chorionic Gonadotrophic Hormone), de – chlorinated water, frog feed (z- bites and t – bites) , syringes and needles, labels, incubator, plastic transfer pipettes, matchbox, forceps and formaldehyde.

Test Animals *(Xenopus laevis)*

Male and female *Xenopus laevis* from Lake Victoria were collected from the field and taken to the Biotechnology Laboratory at University of Eldoret where the experimentation was being done.

Experimentation Procedures

Xenopus laevis were collected from Lake Victoria and left to acclimatize in dechlorinated water in the laboratory for 7 days before the onset of the experiment. Feeding was done using z-bites prior to the study and while the room was maintained at room temperature of 25ºC ± 2ºC during the experimental period as a temperature below 23^0C may cause deaths. The water in each holding tank was changed every five days. Adult breeding was conducted according to the standard guidelines of Frog Embryo Teratogenesis Assay Xenopus (FETAX) test. These standards are; in breeding, pairs of (3-5) of adult females and males are injected with human chorionic gonadotropin (hCG), 500IU for males and 1000IU for females dissolved in 0.9% saline solution on the dorsal lymph sac (Osano et al., 2002). Four adult pairs of *Xenopus laevis* were used in this experiment with each breeding pair in its own breeding tank.

The breeding tank was filled halfway with FETAX solution that had a false Perspex bottom with 1cm holes which allowed the egg 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. The eggs deposited in the tank were then collected the following morning. The adult frogs were then removed from the breeding tanks and the eggs collected. The fertilized eggs were identified by checking the formation of blastomers.

The medium for growth also referred to as the Frog embryo Teratogenesis assay – Xenopus (FETAX) solution was prepared according to American Society for Testing and Material (ASTM 1998) standards. This comprised of 625 mg Sodium Chloride (NaCl), 96 mg Sodium hydrogen carbonate (NaHCO3), 30 mg potassium 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. The 96-hour exposure system used was static renewal done once every 24 hours while removing the dead tadpoles.

A stock solution for Fluoride was prepared to be used during 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 then randomly placed in the incubator which was set at 25^0C. The experiment was set into triplicates where the concentration of fluoride ranged from 0 -800 ppm. The triplicate of each test was treated as a block and the experiments done by complete random block design. The medium was renewed, and dead tadpoles removed and recorded every 24 hours. The experiment was terminated at 96 hours. All the tadpoles alive at the end of the experiment were fixed in formaldehyde and stored. Parameters measured were mortality, development, and teratogenicity.

Reversal of Fluoride Effects using T4 and I

The experiment was set the same as the testing of effects of Fluoride. The Fluoride concentration was dosed into all petri dishes containing 25 eggs and FETAX solution then 2 ppm T4 (Coady et al., 2010) and 0.5 ppm Iodine (Guideline, 2008) was added appropriately at random. This was to test the capability of T4 and Iodine in reversing the effects of fluoride. Parameters tested were Mortality, Development and Teratogenicity.

Data Analysis

The data was analyzed using descriptive statistics, abbott's adjustment (to account for malformations in control), ANOVA and probit analysis was used to determine the LC_{50} and EC_{50}. The teratogenic index was determined by dividing the LC_{50} by EC_{50} and is used in determining the degree of teratogenicity of compounds (Osano et al., 2002).

RESULTS

Embryolethal Effects

Mortality of the tadpoles was recorded across all the concentrations of Fluoride with the highest recorded deaths (68.4%) at 800 ppm.

An increase in the concentration of Fluoride lead to an increase in mortality of the tadpoles. The survival rate of the tadpoles in the control was 95% with only 5% of the tadpoles dying. The 96 h LC_{50} of Fluoride as per the FETAX test was 420.46 ppm. At the highest concentration of Fluoride (800 ppm), the tadpoles were, although alive did not respond to touch.

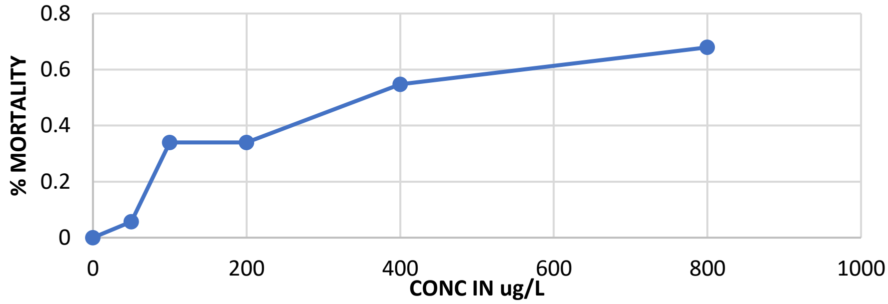

Figure 1: The Mean % Mortality of tadpoles in different concentrations of Fluoride.

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, 12.6 mm at 200 ppm, 13.1 mm at 400 ppm and 12.4 ppm at 800 ppm (illustration in fig 4 below). This showed a decrease in the whole-body length with increasing concentrations of Fluoride with a p-value of .000. 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.

Teratogenic Effects

The 96 h EC_{50} of Fluoride as per this FETAX test was 452.802 ppm. An atlas of deformities by Bantle (Bantle, 1991) was used to identify the deformities in the tadpoles. 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 100 ppm of F in the experiment there were 4% of tadpoles with severe deformities, 12% of tadpoles with stunted growth and 12% with tail deformities. At 200 ppm of F there were 20% of tadpoles with stunted growth, 12% with tail deformities and 4 % with eye deformities. With 400 ppm F there were 4% tadpoles with severe deformities, 8% with stunted growth, 16% with tail deformities and 8 % with eye defects. At 800 ppm F there was 4% of tadpoles with cardiac edema, 24% with tail deformities and 16% with eye deformities.

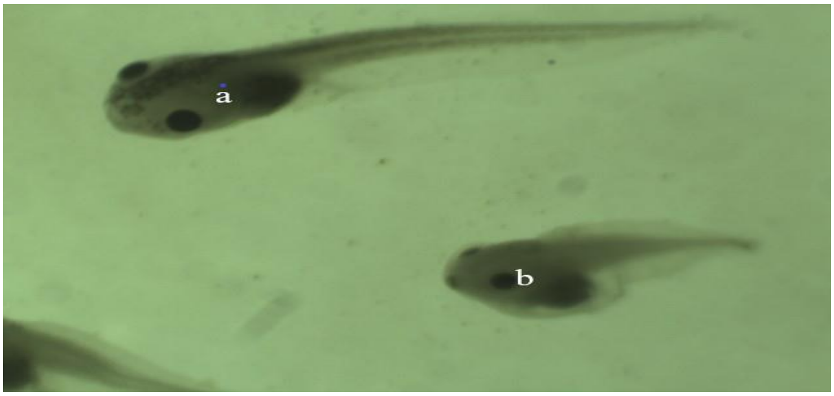

Figure 2: Tadpoles growth length between control (a) and Fluoride (b) (stunted growth).

The teratogenic index expressed as the ratio of LC_{50}/EC_{50} of F in this FETAX test protocol was 0.93.

ACUTE TOXICITY OF FLUORIDE REVERSAL WITH I & T4

Embryolethal Effects

The mortality of the tadpoles with Fluoride treatment alone (68.4%) was higher as compared to the tadpoles treated with Fluoride and Iodine (60%) , T4 alone (45%), KI alone at (32%) and those treated with fluoride and T4 (35%). There was also an increase in the percent mortality of the tadpoles across all the treatments with an increase in the concentrations of Fluoride. The analysis of variance showed P value of .558 significance for F and T4 treated tadpoles and a P value of .558 for tadpoles treated F with I. However, there is a significance of P value of .000 in the mortality of F only treated tadpoles. The tadpoles treated with F were insensitive to and remained completely immobile at 800 ppm. The tadpoles treated with I and T4 did not show a difference in behavior between each treatment and the control tadpoles.

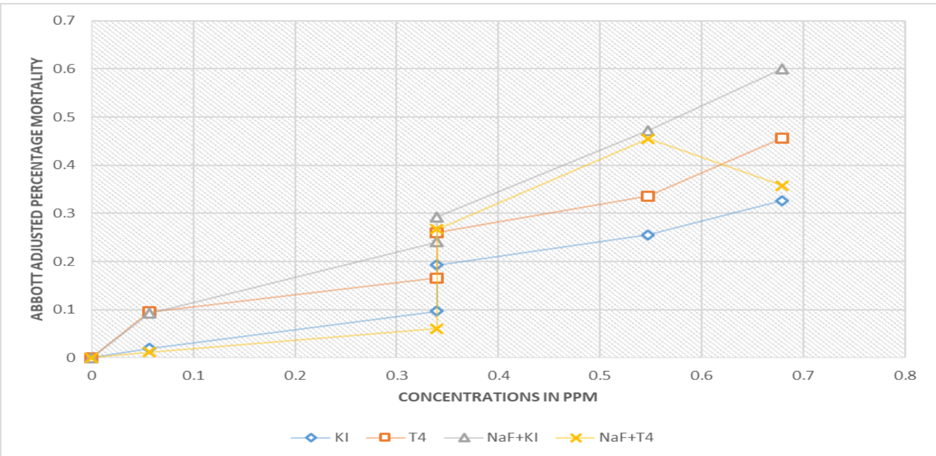

Figure 3: Abbott's adjusted graph of percent mortality of tadpoles treated with T4, KI, NaF+KI, and NaF+T4.

Growth Effects

The mean size of the tadpoles in control at the end of 96 hours was 13.6 mm. An increase in the mean length of the tadpoles was noted in all the tadpoles treated with I and T4. 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 both F+KI and the F+T4 had a significant value of .079.

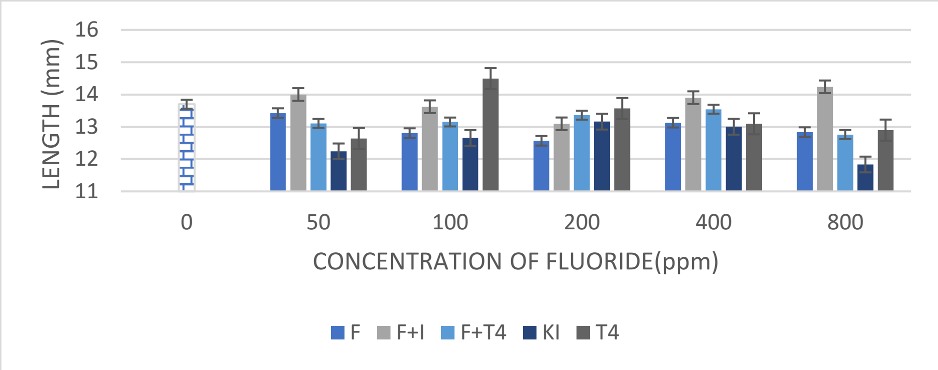

Figure 4: Graph of mean growth of the tadpoles treated with Fluoride & Iodine and those treated with Fluoride & Thyroxine.

Teratogenicity

Tail deformities was recorded across all the treatments with the highest recorded at F (200 ppm) and T4 at 20.4% of the tadpoles with this deformity. Abdominal edema was recorded in T4 and F treated tadpoles. Severe malformations were recorded across all the treatments, cardiac edema in I and F, eye deformities in I, T4 and face deformities in I and T4 treated tadpoles.

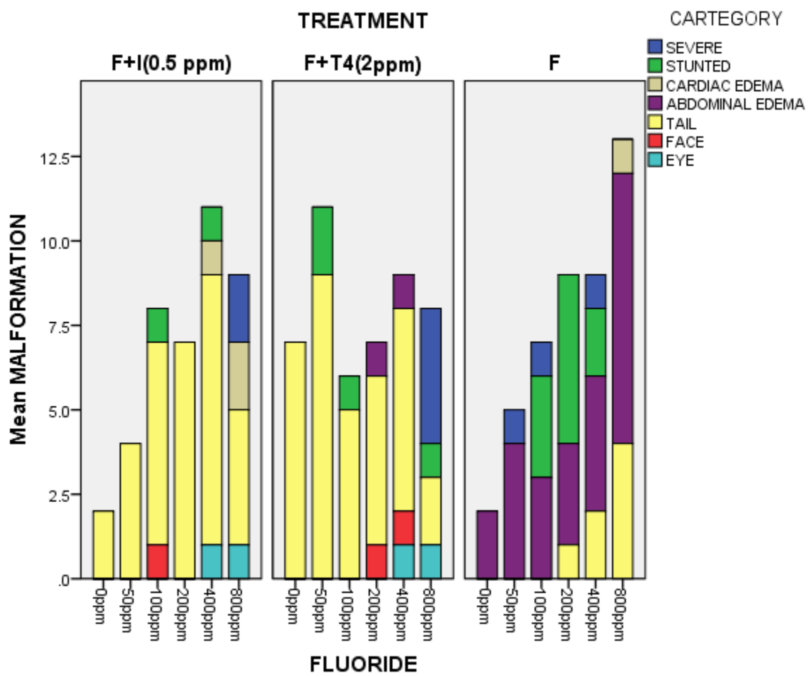

Figure 5: Malformations as recorded in tadpoles treated with Fluoride & Iodine and Fluoride & T4.

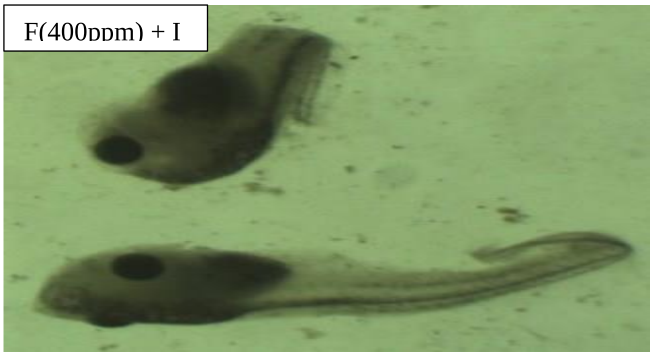

Figure 6: Tail Malformations.

Figure 7: Severe Malformations.

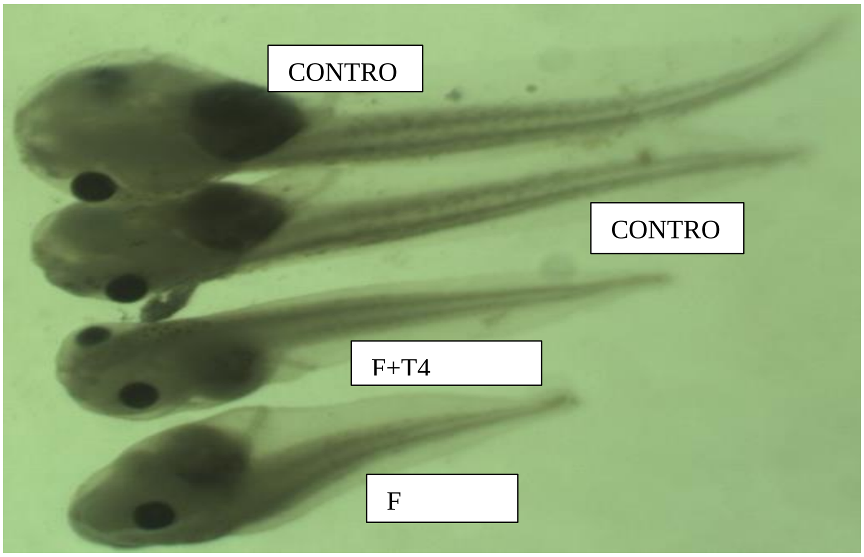

Figure 8: Stunted growth.

DISCUSSION

Fluoride is embryotoxic to *Xenopus laevis* tadpoles with the LC_{50} of 420.46 ppm and as the concentration of Fluoride increases there is an increase in mortality. The drastic increase in the mortality of the tadpoles between the first 24 hours of the study and the end of the experiment suggest that a remainder of the jelly coating the eggs at the onset of the experiment may contribute to its absorption into the embryos as Fluoride was administered through water and not food (Osano et al., 2002). An addition of T4 and Iodine in the test tadpoles showed a significant reduction in the mortality of the tadpoles across all the concentrations of Fluoride.

The reduction in length of Fluoride treated tadpoles recorded with an increase in concentration of Fluoride is consistent with other findings by (Goh & Neff, 2003) that the length of the tadpoles was dependent on the concentration of Fluoride. This also supports the findings by (Spittle, 2018) which supports the claim that low Iodine together with Fluoride led to development disorders including a short stature. A study of Anuran tadpoles in streams with high Fluoride also exhibited a reduction in length of the tadpoles as compared to those exposed to low Fluoride levels (Pollo et al., 2019). A study of male zebrafish (*Danio rerio*) also showed a reduction in their growth for those that were exposed to Fluoride(Jianjie et al., 2016). Synergistic effects have been reported in cases of high F and low I levels amongst both humans and animals. Epidemiological studies have found that the combination of I deficiency and Fluoride exposure culminates a higher risk of developmental disorders including intelligence in children(Waugh, 2019). The lengths of the tadpoles increased in the I and F treated tadpoles as compared to those with Fluoride alone suggesting that I treatment remedied the development of the tadpoles in this experiment. The T4 & F treated tadpoles also showed an increase in development (Coady et al., 2010; Degitz et al., 2005).

Malformations across all the treatments was recorded. The loss of response to touch by the tadpoles at a high concentration of Fluoride is attributed to oxidative stress which was characterized by sluggishness and immobility due to loss of sense of touch. This is attributed to mainly the defect in the neuromuscular system of frog tadpoles. A study by (Malin, et al., 2018) attributes the loss of sensation of touch of rats exposed to high rates of Fluoride to the effect that it has on the medulla part of the brain. Epidemiological studies have also linked high Fluoride exposure to developmental disorders in children including those of cognitive function in children(Waugh, 2019).Treatment of Fluoride tadpoles with Iodine and Thyroxine did not completely eliminate the malformations, in any case there was an increase in the number of tadpoles with teratogenic effects. Despite the increase in teratogenic effects there was a reduction in the number of tadpoles with severe deformities. The eye defects on the tadpoles were observed at higher concentrations on both the I and T4 treated tadpoles. During early development of *Xenopus laevis* deiodinases function in the eye and ear tissues which works by lowering the levels of the Thyroid hormone hence ensuring that the thyroid receptors are unliganded. The unliganded thyroid receptors are important in the development of the eye(Fini et al., 2012). This shows that Fluoride interferes with the thyroid hormone receptors of the tadpoles hence defects in the eye development. Which even under treatment but with high concentrations of Fluoride does not reverse the effects it has on the tadpoles.

This therefore suggests that the teratogenic effects once triggered by Fluoride cannot be fully reversed.

CONCLUSION AND RECOMMENDATIONS

In conclusion, Fluoride has significant toxic effects on *Xenopus laevis* even at low concentrations. The teratogenic index of Fluoride was found to be at 0.93 which affirms that it is embryolethal hence a coeffective teratogen. Both treatments with Iodine and Fluoride slightly increase the length and development of the tadpoles. Teratogenicity of Fluoride was increased with each of the treatment however the severe cases were reduced, eye deformities were also noted although did not reduce under treatment because of the effect fluoride has on the thyroid hormone signaling which plays an active role in certain tissues(eye) before the thyroid gland develops. The malformations produced by Fluoride exposure cannot be fully reversed by either Thyroxine or Iodine.

The study revealed that both I and T4 produced the same effect of reversal on the tadpoles.

The study recommends the re-examination of the safety standards of levels of Fluoride used in the manufacture of Fluoride based materials e.g. toothpaste. It also recommends further research into other remedial measures to avert the effects of Fluoride.

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