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To study the developmental and enzymatic effects of Cypermethrin on Zebrafish

Richa Shahwal, Arpita Srivastav and Dr. Arunima Sur*
Amity Institute of Biotechnology, Amity University Chhattisgarh
Corresponding author- arunimakarkun@gmail.com

Abstract

Cypermethrin is one of the most common toxic substances in freshwater systems used to control the pests of internal and external systems. In the present study, the toxicity of the development of Zebrafish embryos was carefully evaluated and investigated the effects of CYP exposure on apoptosis in zebrafish. 4-h post-fertilization (HPF) zebrafish embryos have been exposed to different concentrations of cypermethrin for up to 96 hours. Various morphological abnormalities such as curvature of the body, enema were observed at very high altitudes. The activities of Antioxidative enzymes, namely, superoxide dismutase and catalase of zebrafish larvae are performed in a concentrated manner. In addition, the toxic effects of cypermethrin investigated in fish with acridine orange (AO) staining, the result showed apoptotic cells mainly in the areas of the head, nervous system, body, heart, and tail. The results show that Cypermethrin induces oxidative stress and exhibits apoptosis. In this study, the developmental toxicity of cypermethrin research using zebrafish embryos helped to understand the various mechanisms of exposure to cypermethrin and suggested that Zebrafish serve as an appropriate model for the testing of toxic substances.

Keywords: Cypermethrin (CYP) Zebrafish Acridine orange Antioxidative biomarkers Acetylcholine esterase developmental toxicity.

Background to the Study

Indirect population growth leads to pollution and exposure to natural toxins. There is therefore a need to monitor the toxicity in order to determine the effects of exposure and to identify and understand the various mechanisms involved. A variety of toxic chemicals released from factories and fertilizers affect fish and wildlife. The reproductive health of fish, the duration of life, and the various characteristics and development of embryos and aquatic larvae are affected. Because of these toxic substances, the aquatic ecosystem exhibits harmful effects on fish populations. Humans are also exposed to a variety of natural drugs. When pesticides are used in agricultural fields they come into contact with the soil, and as they do not rot they store bio-accumulated in plants and animals which when used leads to health risks. However, research on human exposure is difficult due to the small number of children, longevity, large size, behavioral problems, and much more. Therefore, there is a need for in vivo models to study the effects of toxins. In this toxicity test, we use Cypermethrin as a common contaminant used to control and control pests in the agricultural and non-agricultural sectors as well. Cypermethrin is a pyrethroid pesticide, due to its unique insecticidal or antiseptic activity and has been declared a major pesticide in use (developed and developing countries). The sequence of cypermethrin is found in various rivers, streams, sediments, etc., and in some places, these were obtained at higher prices. Therefore, it is important to understand and study the effects of cypermethrin and its toxic mechanisms in order to maintain environmental safety and public health.

Various environmental problems have been caused by the aquatic environment through the influence and use of several pesticides. These studies are useful in determining various parameters such as developmental toxicity, oxidative stress, apoptosis, etc. Developmental toxicity can be tested by research into the embryo-larval phase of Zebrafish with water-based cypermethrin. Exposure of 120hpf is the best time for this test.

Today oxidative stress is an important topic in environmental issues. ROS are produced in all living things that use oxygen. Thus, toxic exposure creates an imbalance that leads to increased production of ROS, and since ROS is directly related to toxic substances, it leads to certain adverse effects caused by oxidative stress. As pesticides are the inducers of apoptosis. Cypermethrin has the capacity to induce or cause cell death that helps to eliminate cells that are unwanted. This can be done by Acridine orange staining which helps in detecting apoptotic signals

Objectives:

1. To study the effects of developmental toxicity in Zebrafish embryos exposed to Cypermethrin.
2. To study the enzyme activities when exposed to cypermethrin.
3. To study the apoptosis in the larvae of Zebrafish when exposed to cypermethrin.

Material and Methods:

1. **Maintenance:**

Zebrafish were kept in stagnant water in the tank and tossed with an aerator that helps maintain water quality by adding air to the water that the fish need continuously for a healthy and sustainable supply of oxygen. The water is changed every 24 hours to help remove waste and sewage. RO water is used for the system. The room temperature was generally maintained between 26-28.5 ºc and light conditions were 14: 10 hours (dark: light) and pH 6.8-7.5. Regular cleaning of the tanks is done to prevent the growth of algae or contaminants that may directly affect Zebrafish growth indirectly or indirectly. Tanks are rinsed with 70% alcohol before use.

2. **Feeding:**

Zebrafish were fed dry food twice a day and live foods (Brine shrimps) once a day. Brine shrimps (Artemia sp.) Eggs are purchased from local pet stores and hatched in a laboratory. As Artemia is present in the marine environment that is why salt was dissolved in RO water to maintain its properties and sodium bicarbonate was added to the area followed by continuous stirring for better melting. It was then stored in a continuous container of oxygen and light mixed with an aerator in an incubator bottle and left for 24 hours for proper ventilation. The next day the hatchlings were collected. These Brine shrimp were washed in clean water and fed to Zebrafish in each tank. Zebrafish should not be overused as its water nitrate level will increase which may interfere with performance, reproduction or death may also occur due to overeating.

3. **Breeding:**

The Zebrafish used for breeding was between 3 and 18 months old. Females are separated from males because of their large lower abdomen and males are thinner and darker in color. Usually, after feeding, breeding was done during the day. Females and males in a ratio of 2: 1 are transferred to the reproductive tank and kept under darkness overnight without interruption. The next morning the tank was kept under light for about half an hour. The embryos are placed on the ground and the fish are transferred to their tanks.

The eggs are then collected using a net and transferred to a container with a medium embryo.

4. **Raising of Larvae:**

Until the larvae hatch, fertilized eggs are stored in the incubator for 72 hours and the embryos' fluid was constantly changed and dead and separated from the fertilized ones daily from the container. By now, the larvae had been cut down and were ready for further research.

Experimental Design:

The 6hpf Live Zebrafish Embryos was taken for testing and divided into four different groups of 30 embryos each. All test groups were taken three times. The dosage for the four test groups is as follows: 1) 0 ug / l (Control) 2) 25 ug / l Cypermethrin 3) 100ug / l Cypermethrin 4) 200 Cypermethrin ug / l. There were no deaths during the test. Exposure was performed for 96 hours. The method used was changed daily to maintain water quality and the mortality of the treated embryos.

1. **Developmental toxicity of CYP induced Zebrafish embryos**

After 96h exposure to CYP, embryos were observed under the microscope for developmental abnormalities.

2. **Determining the apoptosis by Acridine orange staining**

Embryos after exposure to CYP 96 hpf were stained with acridine orange staining and kept in the dark for about 30 minutes. Then wash three times for 10 minutes with PBS. Tricane was used to abort embryos for 2 minutes. Spotted embryos were photographed and examined everywhere under a fluorescence microscope. The intensity of the larvae and the relative intensity was calculated to determine the degree of apoptosis or apoptotic signal of the larvae and morphological abnormalities.

3. **Acetylcholine esterase Activity**

10 larvae of each group were homogenized in phosphate buffer saline (PBS) (pH 8.0, 0.1M) in a homogenizer. 30 ul supernatant homogenate was added to the cuvette containing 195 ul of PBS. Then, 7.5 ul DTNB Reagent was added to the photocell. The absorption was measured at 412nm and when this had ceased to increase, the photometer split was opened so that the absorption was set to zero. On the substrate, 1.5 ul is added. Changes in absorbance were recorded and changes in absorbance per minute were noted. This was followed by Ellman's trial with some adjustments.

4. **Antioxidative enzyme parameters:**

4.1 Superoxide Dismutase (SOD)

This experiment was followed by Das et al. 10% of homogenate larvae were made in a buffer and centrifuged at 12000g for 20 minutes at 4 degrees Celsius and then supernatant. Protein estimation is done in the Lowry method. After that, 30ug protein for each group was taken and the reaction mixture was added and mixed well and placed in a preheated oven for 5 minutes. Now, 80 ul Riboflavin was added to all samples (in black and red light) and exposed to a 20-watt fluorescence lamp filtered in a foil box for 10-15 minutes. To stop the reaction, 1 ml of Griess reagent was added. Absorbance was taken at 543 nm against emptiness.

4.2 Catalase (CAT)

This enzyme activity is measured by Aebi et al. with minor changes. 0.5 ul of whole alcohol is added to 50 ul of the sample and stand for 30 minutes at 4 degrees Celsius. 45ul of the mixture is dispersed in a tube containing 5 ul of 10% triton-x-100. Now the sample was ready for use in catalase activity. A 10ul sample was added to 280 ul of PBS in the cuvette and the absorption was taken at 240 nm before the addition of H2O2 and then 10 ul of H2O2 was added to the cuvette and the absorption was recorded later for a period of 3 minutes.

4.3 Lipid Peroxidase (LPO)

Firstly 825ul of TBA reagent was added to the 50ul sample and then mixed and boiled at 95˚C in a water bath for 60 minutes. The color of the mixture was changed to pink and the sample was cooled, then centrifuged at 2000 g for 10 minutes, the supernatant was collected and the absorption was rated at 532 nm. This enzyme activity was determined by Okhawa et al.

Statistical Analysis

The values were expressed as mean±SD. Statistical analysis was performed by repeated measure one-way analysis of variance (ANOVA) using the SPSS software and the significant difference was set up at ($p<0.05$) at a 5% probability level.

Results:

1. **Effect of CYP on development of Zebrafish Embryos**

There have been various developmental abnormalities found in the Zebrafish Embryo induced to CYP. These defects include axis curvature, large yolk sac, and pericardial edema in zebrafish embryos as shown in Fig. 1. The curvature was observed even in low concentrations as low as 25ug/l but the body curvature in a higher dose, i.e., 200ug/l was very peculiar and observable. While in the control group, there were no morphological abnormalities.

2. **Effect of CYP on induction of apoptosis in Zebrafish embryos**

The larvae were stained with Acridine orange after being exposed to CYP for 96 hours. The apoptotic signal intensity was found to be significantly higher at a high dose of 200ug/l compared to the low dose and control group (Fig. 2a and 2b). The apoptotic signal is amplified in a dose-dependent manner mainly in the brain and spinal cord indicating that CYP plays a role in causing toxicity during the development of the Zebrafish embryo. There was not even a very low strength signal in the control group.

3. **Effect of CYP on acetylcholine esterase activity in Zebrafish Embryo**

Current data show that exposure to Zebrafish Embryos with CYP causes a significant decrease in the upper extremity group compared to the control group. Even at 100ug/l capacity there has been a significant reduction however there is a significant change in the low compared to the controls as shown in Fig. 3.

4. **Effect of CYP on Lipid Peroxidation**

This study showed that the content of the MDA level in the Zebrafish embryo is affected after exposure to a different concentration of 96h toxins as shown in the figure. The MDA level is significantly reduced in Fig.4

5. **SOD Activity in CYP induced Zebrafish Embryos**

During this study, the SOD activity of Zebrafish embryos treated with CYP showed a dependence on concentration control. The work was found to double up to a maximum of 200ug/l compared to the control group as shown in fig.5.

6. **Catalase Activity in CYP induced Zebrafish Embryo**

There has been a significant increase in the activity of catalase which is another antioxidant biomarker (Fig.6) .This activity is determined by the Aebi method. As compared to control, there is a focus on regulating the level of catalase that works against ROS production by acting as an antioxidant defense.

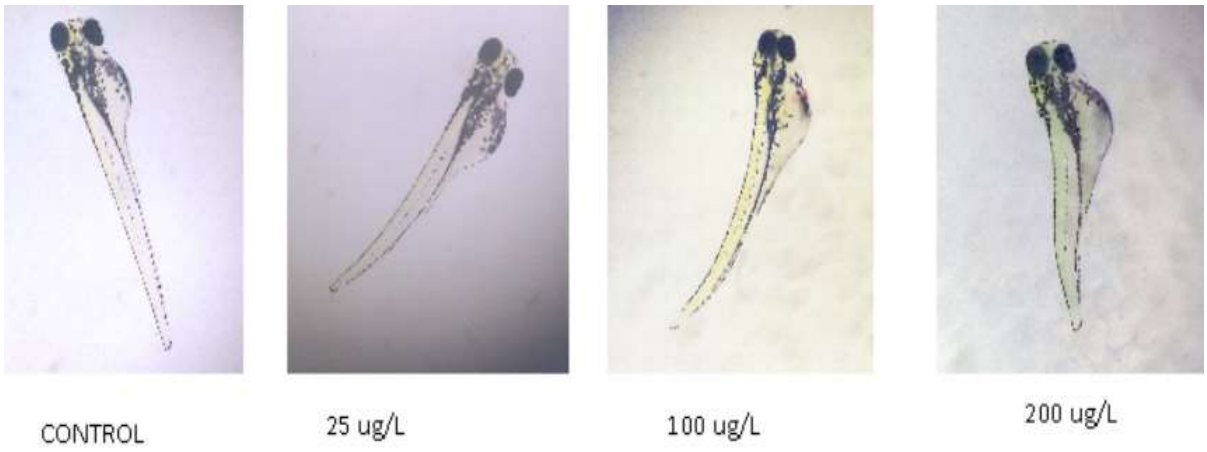

Fig. 1 Developmental abnormalities in Zebrafish embryos exposed to different concentrations of CYP

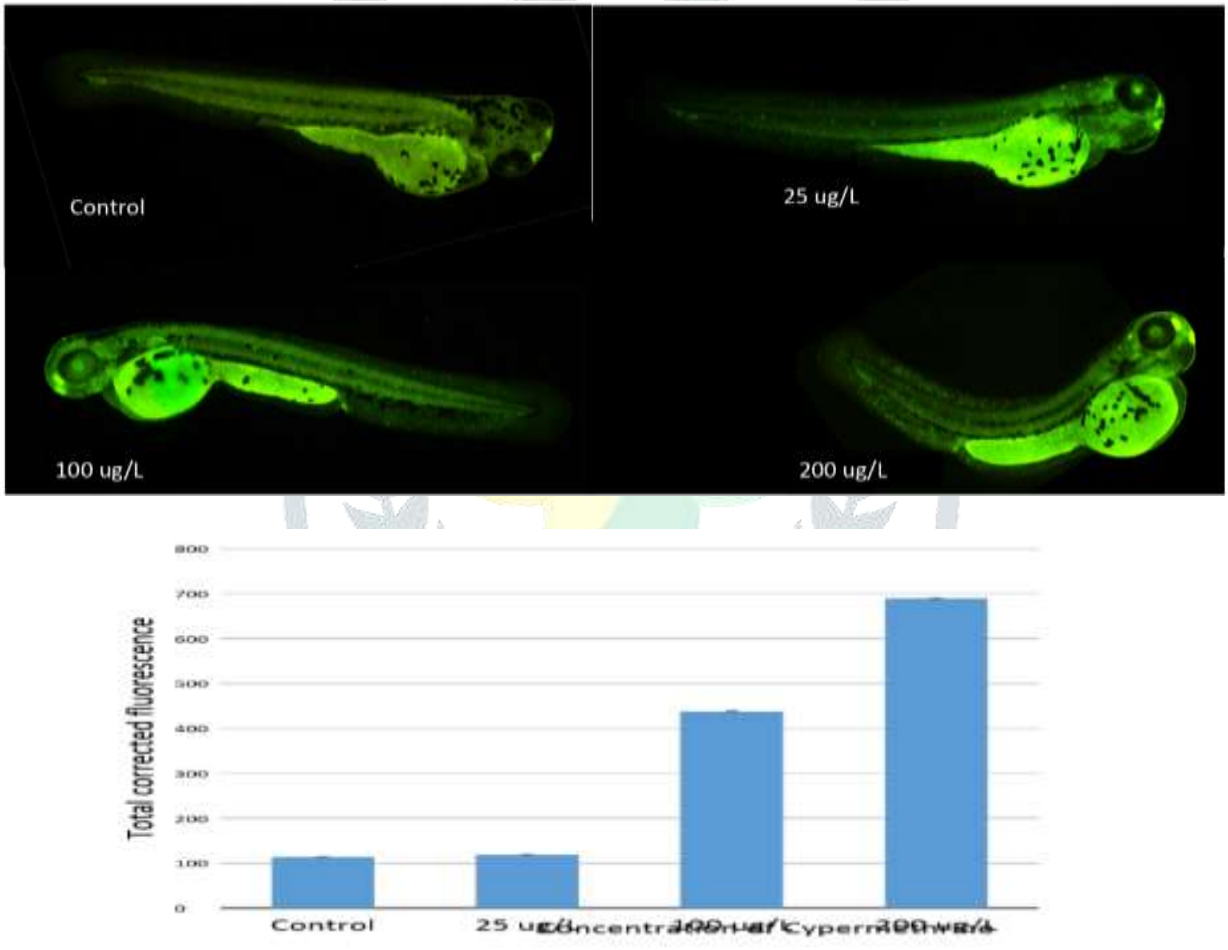

Fig.2 (a) Treated Zebrafish embryos stained with Acridine Orange and (fig.2 (b))the graph showing total corrected fluorescence

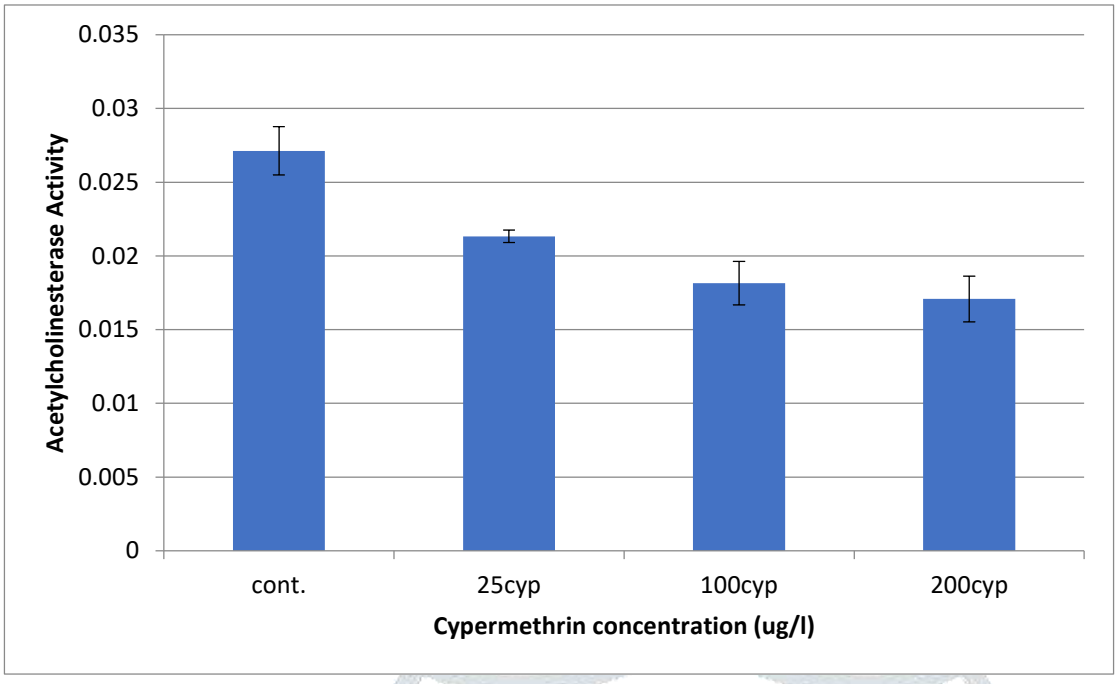

Fig.3 Acetylcholine esterase activity in Zebrafish embryo exposed to different concentrations of CYP

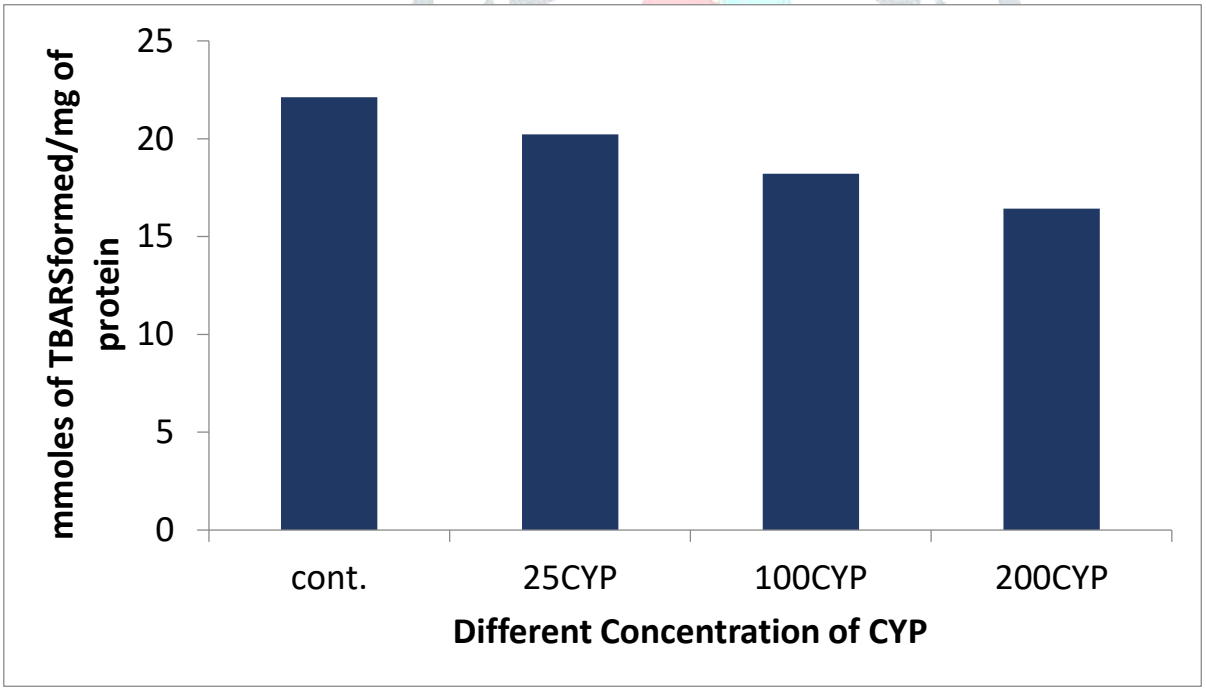

Fig.4 Lipid peroxidise activity in Zebrafish embryo exposed to different concentrations of CYP

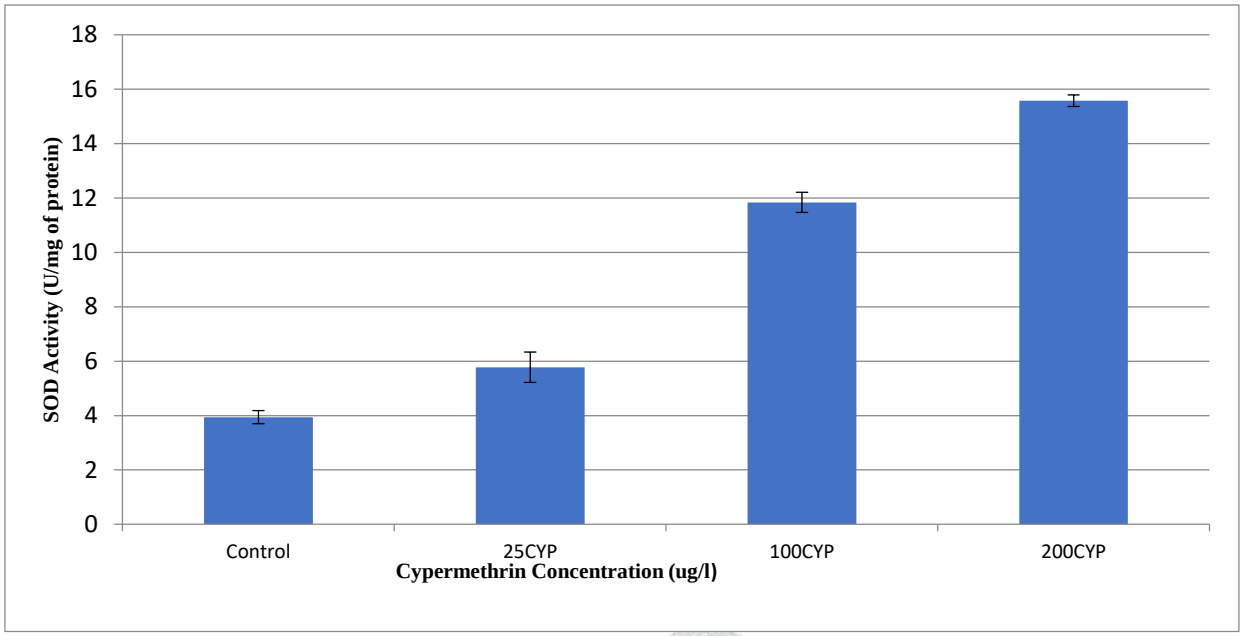

Fig. 5 SOD Activity (U/mg of protein) in Zebrafish embryo exposed to various concentrations of CYP for 96h

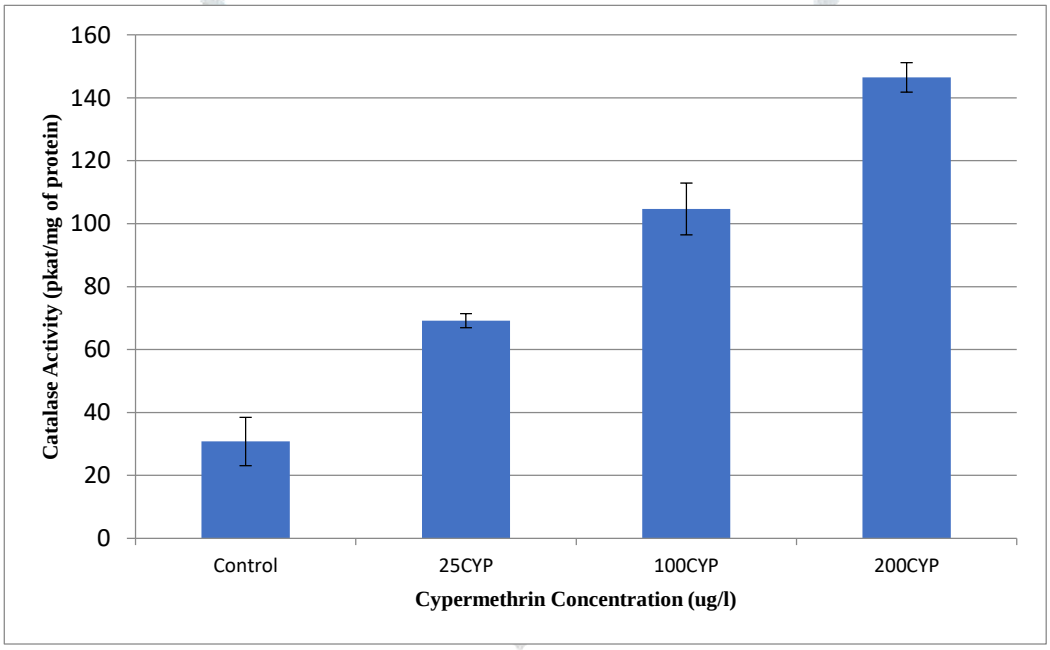

Fig.6 Catalase (pkat/mg of protein) Activity in Zebrafish embryo exposed to various concentrations of CYP for 96h.

Discussion:

As reported in various papers, cell apoptosis and toxicity have been caused by pesticides. Among the various toxins, Cypermethrin has the greatest potential for poisoning. The pesticide application includes an eco-toxicological risk assessment and pesticide recovery to maintain the natural health of biodiversity whether they are targeted or unintentional. Modern anthropogenic compounds have a responsibility to pollute the aquatic environment with energy. Of these compounds, pesticides are the most common because of their highly toxic properties. Pesticides show their toxic effect after exposure to a tense or temporary substance. The concentration

may vary depending on the need and this concentration may result in death and some harm to the person exhibiting different changes (Di Giulio & Shinton, 2008).

In this study, Cypermethrin caused a developmental disorder due to toxicity in Zebrafish embryos due to oxidative stress. After exposure to Cypermethrin, changes in body mass, pericardial edema, and a large yolk sac have been observed. The results also showed that SOD & CAT activities were highly regulated. All of these parameters indicate the developmental toxicity of Cypermethrin in the Zebrafish embryo. Oxidative ROS pressure is a major factor contributing to the abnormal development of fish during embryogenesis (Yabu et.al .2001; Yamashita, 2003).

The subsequent increase in lipid peroxidation due to Cypermethrin exposure is due to the incorporation of ROS which helps in the development of polysaturated fatty acids for oxidation and thus lipid peroxidation (Valavanidis et. Al, 2006). Treated fish have a higher lipid peroxidation rate as MDA content is increased compared to the control group.

Fish have a unique protective function against the harmful effect due to increased ROS or may not say that with the help of antioxidant enzymes, fish can protect against ROS.As our results are seen in increasing SOD & CAT levels. These additional functions help to eliminate the ROS produced by Cypermethrin. SOD converts superoxide into hydrogen peroxide and CAT converts hydrogen peroxide into hydrogen and water. Any ROS production of toxins when antioxidant enzymes counteract the harmful effects of oxidative stress. Therefore, in embryonic development the toxin of oxidative stress plays an important role in the production of Cypermethrin. The effects of oxidative stress caused by oxidative metabolism and the production of Cypermethrin have their own effect on the nervous system and lead to neurotoxicity. Oxidative damage caused by Cypermethrin treatment may be involved in changes seen in AchE activity in the brain. These changes in neurotransmission systems refer to neurotoxicological features of the toxicant. Hence it will be better to render important insights related to neurochemical and its molecular targets to elucidate Cypermethrin effect on the brain and the underlying mechanism.

In this study, the larvae were contaminated with acridine orange and the dye can interact with a nucleic acid that depicts apoptotic cells mainly seen in the eye, head, tail, and heart. In the case of AO staining, the developing nervous system is damaged after exposure to treatment due to the lipophilic nature of Cypermethrin which allows it to pass through the cell membrane and the blood-brain barrier without any means of transport (Mun et.al,

2005). The main purpose of AO staining is to detect apoptotic signals from engulfment due to metachromatic structures.

By studying the development of Zebrafish toxins can be linked to other edible fish based on what changes it causes when exposed to Cypermethrin as it comes from the same family namely cypiriniforms. Therefore, this can be compared to humans as humans and fish exhibit the same homology of 84%. Changes occurred in Zebrafish after treatment may be related to changes in people somewhere. The genes that cause mutations in fish also cause mutations in humans with high concentrations that we do not know can cause indirect DNA damage, side effects and various diseases. An in-depth analysis of the toxic effect of Cypermethrin in completely different environments can help to identify all the toxic effects of Zebrafish and its subsequent mechanism.

Conclusion:

In this study, Zebrafish embryos were used as a vivo model to study the effect of CYP on fish embryonic development and how to protect the antioxidant from oxidative stress due to this chemical. Therefore, after exposure to CYP for 96h, the parameters for observing oxidative stress and changes in development were analyzed. The result was found that the antioxidative activity in Zebrafish worms changed due to the increased level of oxidative stress caused by chemical exposure. These changes provide information and indicate the dangers of CYP in the aquatic environment. This current study will also eliminate the impact of CYP during fish growth stages. In addition, exposure to fish embryos by CYP has the potential to induce apoptosis as evidenced by our study that signal tension increases with a high dose indicating the basic mechanism of CYP toxicity in apoptosis. In addition, this result also suggests that Zebrafish could be a good model for studying the impact of environmental pollution on developing toxins.

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