

TOXICOLOGICAL STUDIES IN ZEBRAFISH AFTER EXPOSURE TO COPPER SULPHATE PENTAHYDRATE

Submitted in partial fulfilment of the requirements
for the award of

Master of Science
In
Medical Biotechnology and
Clinical Research
by

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INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)

Accredited with grade 'A' by NAAC

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DEPARTMENT OF MEDICAL BIOTECHNOLOGY AND CLINICAL RESEARCH

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **HAKSHAYA P(41101002)** and who carried out the project entitled **"TOXICOLOGICAL STUDIES IN ZEBRAFISH AFTER EXPOSURE TO COPPER SULPHATE PENTAHYDRATE"** under our supervision from December 2022 to May 2023.

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DECLARATION

I, **Ms. HAKSHAYA P (41101002)** hereby declare that the project report entitled **"TOXICOLOGICAL STUDIES IN ZEBRAFISH AFTER EXPOSURE TO COPPER SULPHATE PENTAHYDRATE"** was successfully done by me under the guidance of **Dr.A.DAYANANDAN** is submitted in partial fulfilment of the requirements for the award of Master of Science Degree in Medical Biotechnology and Clinical Research.

Date: 05/05/2023

Place: CHENNAI



Signature of the candidate

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ABSTRACT

The active sites of many proteins contain the necessary ion copper. Free radicals are hazardous to cells and other creatures and are created when this metal is present in excess. The effects of metals contained in food or the environment, particularly copper, have been widely studied using fish. Different metals have been found to cause different adaptive responses in adult fish. But up until now, little was known about the reactions that copper in the water causes in fish during their early life stages. Here, dissolved copper sulphate at two distinct concentrations (80 and 120 ppm) was administered to zebrafish fish for a period of 96 hours, and acute toxicity tests were created to determine the effects. The changes were then assessed using methods such as protein estimation, antioxidant assays, and agarose gel electrophoresis.

The zebrafish (*Danio rerio*) is used as a model to perform in vitro experiments and developmental toxicity studies. Zebrafish may be used to determine the toxicity of samples in early screening assays, often in a high-throughput manner. The zebrafish embryotoxicity model is at the leading edge of toxicology research due to the short time required for analyses, transparency of embryos, short life cycle, high fertility, and genetic data similarity.

Liver, the largest gland is a vital organ. It is the metabolic “engine-room of the body”. Almost all the drugs, foods and water constituents are metabolized and detoxified in the liver, and as such it is often exposed to maladies resulting in a number of clinical syndromes. The mobilization of these heavy metals to the aquatic ecosystem alters the physicochemical property of water which is hazardous for aquatic organisms. Heavy metals mainly enter the fish body through gills, body surface and digestive tract during ingestion of metal accumulated food materials.

Key words : Copper, zebrafish , protein estimation, antioxidant assays and agarose gel electrophoresis.

S.NO	TITLE	PAGE NUMBER
1	List of Abbreviations	3
2	List of Figures	3
3	List of Tables	4
4	List of Graphs	5
5	Introduction	6
6	Review of Literature	13
7	Aim and Objective	16
8	Materials and Methods	17
9	Results and Discussion	28
10	Summary and Conclusion	39
11	References	41

LIST OF ABBREVIATIONS:

IAA – Isoamyl alcohol

SDS – Sodium Dodecyl Sulphate

NaCl – Sodium Chloride

NaOH – Sodium Hydroxide

IUPAC – International Union of Pure and Applied Chemistry

U.S. EPA – United States Environmental Protection Agency

LD – Lethal Dose

EDTA – Ethylene Diamine Tetra Acetic Acid

LIST OF FIGURES

FIGURE NO	TITLE OF THE FIGURE
1.6	Zebrafish – <i>Danio rerio</i>
4.2.5	Dissected zebrafish showing gills, liver, intestine
5.1	Zebrafish before and after treatment
5.4	Agarose gel electrophoresis
5.6	Histological section of the normal and treated and head of zebrafish

LIST OF TABLES:

TABLE NO	TITLE OF THE TABLE
1.1	Classes of chemicals evaluated for toxicity in zebrafish
1.2	Chemical properties between Basic copper and Copper sulphate
5.1	Body weight
5.2	Liver weight
5.3	Protein Estimation
5.4	Changes in CAT enzyme between control and treated animals
5.5	Changes in SOD enzyme between control and treated animals
5.6	Changes in Vitamin E between control and treated animals
5.7	Changes in GST enzyme between control and treated animals
5.8	Changes in GSH enzyme between control and treated animals

LIST OF GRAPHS:

GRAPH NO	TITLE OF THE GRAPH
5.1	Graphical representation of body weight between control ,80 ppm and 120 ppm groups.
5.2	Graphical representation of liver weight between control ,80 ppm and 120 ppm groups.
5.3	Graphical representation of protein estimation between control ,80 ppm and 120 ppm groups.
5.4	Graphical representation of changes in CAT enzyme between control, 80 ppm and 120 ppm groups.
5.5	Graphical representation of changes in SOD enzyme between control, 80 ppm and 120 ppm groups.
5.6	Graphical representation of changes in Vitamin E between control, 80 ppm and 120 ppm groups.
5.7	Graphical representation of changes in GST enzyme between control, 80 ppm and 120 ppm groups.
5.8	Graphical representation of changes in GSH enzyme between control, 80 ppm and 120 ppm groups.

CHAPTER - 1

INTRODUCTION

1.1 ZEBRAFISH: A SUITABLE VERTEBRATE MODEL FOR TOXICITY ASSESSMENT

Despite the fact that studies on laboratory rodents provide reliable information for extrapolating toxicant effects to human health, these are more expensive, time consuming, and legally constrained. Studies with different animal species may be representative for "higher," more sophisticated creatures because genes, receptors, and molecular processes are highly conserved across animal phyla. The fruit fly and nematode worm are examples of model invertebrate creatures, whereas the medaka, zebrafish, frog, chick, mouse, and rat are examples of model vertebrate organisms . There are notable parallels in the morphology of all vertebrate embryos, which is particularly indicative of how highly conserved gene programming and development are in the early life stages of all vertebrates. Additionally, similar to how chemicals from the mother's body are transferred through the placenta to the embryo and foetus of mammals (Jacobson et al., 1984), Fish, amphibians, and bird eggs receive chemicals from the female before they are laid. The embryo is then exposed to chemicals through the egg.

1.2 ADVANTAGES OF THE ZEBRAFISH

Zebrafish have many benefits for usage as a toxicological model species as well as for other disciplines (Spitsbergen and Kent, 2003; Teraoka et al., 2003a). This is demonstrated by the rise in recent articles that have utilised this organism. Less than 100 zebrafish-related publications were submitted yearly in the early 1990s. This increased to \$1,000 at the turn of the century and currently amounts to an annual average of about \$3,500.

Zebrafish are a better choice for a toxicological model than other vertebrate species because of their size, ease of care, and early morphogenesis. Zebrafish adults are much shorter than other fish species, such as trout, at just one to 1.5 inches long . This significantly decreases housing space and upkeep expenses, and there are

now a number of businesses that specialise in zebrafish aquariums that can house thousands of fish. The ideal breeding and maintenance settings for zebrafish have also been well established due to its long history as a species used in laboratories (Westerfield, 1995).

1.3 ASSESSMENTS FOR TOXICOLOGICAL ENDPOINTS

The growth of zebrafish has been extensively studied (Kimmel, 1989; Kimmel et al., 1995). The main morphological changes up to and including pharyngulation can be observed without obstruction since zebrafish eggs are transparent from fertilisation until the tissues start to get thick and colouring begins (at around 30-72 h post fertilisation (hpf)). Pigmentation can be prevented *in vivo* by treatment with 0.003% phenylthiourea or removed by bleaching after fixation, thereby extending this period of unobstructed assessment. Therefore, using little magnification, adverse effects of chemical exposure on development of the brain, notochord, heart, and jaw, trunk segmentation, and measurements of size can be assessed quantitatively. Also, unlike rodents, embryological development can be continually followed in live individuals rather than harvested embryos and fetuses. In addition, zebrafish embryos that are malformed, missing organs, or displaying organ dysfunction, can usually survive substantially past the time in which those organs start to function in healthy individuals. For example, mutant zebrafish such as *silent heart*, *still heart*, and *slow mo* (Chen et al., 1996), and toxicant-exposed embryos with heart abnormalities (Antkiewicz et al., in press; Incardona et al., 2004;) survive well beyond 24 hpf when the heart normally begins to beat (Kimmel et al., 1995). This is in contrast to rodent embryos with malformed hearts that tend to die *in utero*.

1.4 ACUTE TOXICITY AND ADVERSE EFFECTS TO THE JUVENILE AND ADULT ZEBRAFISH

Acute toxicity studies using zebrafish are limited. Examples of toxicants investigated include lead and uranium (Labrot et al., 1999), malathion (Kumar and Ansari, 1986), metronidazole (Lanzky and Halling-Sorensen, 1997), anilines (Zok et al., 1991), and colchicines (Roche et al., 1994). Apparently the main reason few studies have utilized juvenile and adult zebrafish is because the overarching value of zebrafish lies in its genetics and developmental biology. Several studies have exposed embryos to

graded concentrations of a chemical and assessed endpoints of toxicity as juveniles. For example, hatching, morphological abnormalities and survival were assessed for up to 35 days post fertilization (dpf) in zebrafish exposed to lindane, deltamethrin, and atrazine (Gorge and Nagel, 1990). Effects on sex differentiation were also assessed for 17 α -ethinylestradiol (Andersen *et al.*, 2003). Likewise 17 β -ethinylestradiol altered sex differentiation in the juvenile, caused modified sexual characteristics in the adult male, and inhibited egg production (Brion *et al.*, 2004).

Table 1.1: Classes of Chemicals Evaluated for Toxicity in Zebrafish

Chemical classes	Type of toxicity study	Reference
Metals		
Copper and zinc	Susceptibility to infection by listeria after exposure to metals	Rougier <i>et al.</i> , 1996
Copper, nickel, mercury, cobalt, lead	Toxicity: dose response, effects on hatching and survival	Dave and Xiu, 1991
Methylmercury	Functional impairment and delayed mortality syndrome	Samson <i>et al.</i> , 2001
Aluminium, cadmium, iron	Toxicity: dose response, effects on hatching and survival	Dave, 1985
Lead and uranium	Acute toxicity and toxicokinetics	Labrot <i>et al.</i> , 1999
Cadmium	Ectopic apoptosis induction	Chan and Cheng, 2003
Cadmium	Abnormal somite patterning and defects in axonogenesis	Hen Chow and Cheng, 2003
Tributyltin	Reproductive toxicity	McAllister and Kime, 2003

1.5 COPPER SULPHATE PENTAHYDRATE :

1.5.1 Chemical Class and Type:

- Copper sulfate is an algaeicide, bactericide, and fungicide. When it is mixed with calcium hydroxide it is known as Bordeaux mixture. The International Union of Pure and Applied Chemistry (IUPAC) name for this active ingredient is copper (2+) sulfate or copper (II) sulfate. Other names include copper (2+) tetraoxidosulfate or copper (II) tetraoxidosulfate.
- Formulations include basic copper sulfate, copper sulfate monohydrate, copper sulfate pentahydrate, and copper sulfate anhydrous. Their Chemical Abstracts Service (CAS) registry numbers are 1344-73-6, 1332-14-5, 7758-99-8, and 7758-98-7, respectively. Pesticides containing copper sulfate monohydrate and/or copper sulfate anhydrous have been cancelled by the United States Environmental Protection Agency (U.S. EPA).
- Copper sulfate has been used in the United States since the 1700s, and it was first registered for use in the United States in 1956. The U.S. EPA completed the reregistration of copper sulfate in 2009.
- Copper sulfate is an inorganic salt that is highly soluble in water. The copper ion is the component of copper sulfate with toxicological implications.
- Copper is an essential mineral, and the recommended dietary allowance of copper for human adults has been set at 900 µg/day.
- Copper is also a ubiquitous element. It can be found in the environment and in foods and water.

1.5.2 Physical / Chemical Properties:

Copper sulfate pentahydrate and basic copper sulfate are the only copper sulfate forms contained in currently registered pesticide products. Chemical properties for these two forms are summarized in the table below.

Table 1.2: Chemical properties between copper and copper sulphate

Active Ingredient	CASRN	Formula	Copper (%Cu)	Form	Vapour pressure (mmHg at 25 °C)	Molecular weight (g/mol)	Specific gravity / density	Solubility (water)
Copper sulfate pentahydrate	7758-99-8	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	25.4	Blue crystals, granules or powder	Non-volatile	249.65	2.286 SG (15.6 °C / 4 °C)	148g/kg (0 °C), 736g/kg (100 °C)
Basic copper sulphate	1344-73-6	$3\text{Cu}(\text{OH})_2 \cdot \text{CuSO}_4$	54.2	Light blue / green fine powder	Not found	468.29	0.800-0.900 SG	Insoluble (soluble in acids)

1.5.3 Acute Toxicity:

Oral

- The acute oral LD₅₀ in rats is 450 to 790 mg/kg. The U.S. EPA considered copper sulfate pentahydrate to be moderately toxic by ingestion.
- Humans may be exposed to copper in drinking water. Volunteers drank purified water with copper at concentrations ranging from 0-12 mg/L. They reported nausea starting at 4 mg/L and vomiting at 6 mg/L. Solutions containing copper and orange flavor raised the NOEL to 6 mg/L for nausea and 12 mg/L for vomiting. Hence, flavored beverages contaminated with copper may lead to higher exposures.
- Scientists measured total copper ion in people's blood serum after they ingested copper sulfate. Mean blood copper levels of 287 µg/L Cu were correlated with mild toxicosis and levels of 798 µg/L Cu to severe toxicosis.

Dermal

- Copper sulfate is not a skin irritant. It was classified by the U.S. EPA as very low in toxicity for dermal irritation. The dermal LD₅₀ was greater than 2000 mg/kg in rats.
- Copper sulfate caused severe eye irritation from day 1 in rabbits that were exposed up to 21 days, and it was ranked as highly toxic by the U.S. EPA for primary eye irritation.
- No data were found regarding the potential for copper sulfate to cause dermal sensitization in any species.(Reregistration Eligibility Decision (RED) for Copper)

1.6 TAXONOMY

The zebrafish is a derived member of the genus *Danio*. It has a sister group relationship with *Danio kyathit* (Hutton, A. T. Nomenclature of Inorganic Chemistry, IUPAC Recommendations 2005)



Fig 1.6: Zebrafish - *Danio rerio*. Image taken from <https://www.google.com/url>

1.6.1 Scientific Classification

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Cypriniformes

Family : Cyprinidae

Genus : Danio

Species : D.rerio

1.6.2 Distribution

The zebrafish is native to the streams of South-eastern Himalayan region (Hartshorn, R. M.; Hutton, A. T. Nomenclature of Inorganic Chemistry, IUPAC Recommendations 2005) . The zebrafish arose in Ganges region in Eastern india and is also native to Pakistan, Bangladesh, Nepal and Myanmar. It commonly inhabits streams, canals, ditches, ponds and slow moving to stagnant water bodies, including rice fields.

Zebrafish have been introduced in Japan, United States as well as in Australia, where they are kept in aquariums (Froese, R. and D. Pauly. Editors Danio rerio. FishBase. Retrieved on 2007-04-07.). They have also been sighted in Colombia, presumably having escaped from an aquarium.

1.6.3 Description

The fish is named for its five uniform, pigmented, horizontal blue stripes on the side of the body, all of which extend to the end of the caudal fin. Males are torpedo shaped and have gold stripes between the blue stripes , females have a larger, whitish belly and have silver stripes instead of gold. The zebrafish grows to 3.8 cm, lives for around 5 years, and produces 300-500 eggs per spawning.

1.6.4 Varities

Recently, transgenic zebrafish have become commercially available that express green fluorescent protein, red fluorescent protein, and yellow fluorescent protein. These fishes are tradenamed GloFish. Other varities include golden, sandy, longfin and leopard.

The Leopard danio, previously known as Danio franken is a spotted colour morph of the zebrafish Danio rerio caused by a pigment mutation(Watanabe M, Iwashita M, Ishii M, Kurachi Y, Kawakami A, Kondo S, Okada N. 2006) .

CHAPTER – 2

REVIEW OF LITERATURE

Copper sulfate (CuSO_4) as industrial effluent is intentionally or unintentionally released into water bodies and accumulates in the fish. Because of its numerous applications, CuSO_4 can be hazardous to non-target creatures, producing direct alterations in fish habitats. Acacetin is a flavonoid present in all vascular plants that are extensively dispersed in plant pigments and responsible for many natural hues. However, the impact of acacetin on mitigating the toxic effect of CuSO_4 in the in-vivo conditions is not known. The toxicity of acacetin was determined by measuring the survival, deformities and heart rate after treatment with various concentrations to larvae. The protective effect of acacetin was also observed in CuSO_4 exposed zebrafish larvae by reducing malformation, mortality rate and oxidative stress. Meanwhile, the acacetin-protected larvae from CuSO_4 effects through the molecular mechanism by suppressing pro-inflammatory genes (COX-2, TNF- α and IL-1) and upregulating antioxidant genes (GPx, GST and GR). Overall, our findings suggest that acacetin can act as a protective barrier against CuSO_4 -induced inflammation in an in-vivo zebrafish larval model.(Mahima Singh,2022)

β -Sitosterol, which is used extensively in pharmaceuticals, nutraceuticals, and cosmetics, has high nutritional value along with immunomodulatory and anti-inflammatory properties. In this study, we investigated the antioxidant and anti-inflammatory effects of β -sitosterol in zebrafish and explored the associated molecular mechanisms. In an in vivo antioxidant experiment, zebrafish (*Danio rerio*) larvae were treated with different concentrations of β -sitosterol and then exposed to a nonlethal concentration of CuSO_4 to induce oxidative stress. Treatment with β -sitosterol at 70 or 100 $\mu\text{g/mL}$ significantly reduced CuSO_4 -induced oxidative stress in the zebrafish, demonstrating the strong antioxidant activity of β -sitosterol. Treatment with β -sitosterol protected zebrafish larvae against oxidative damage from CuSO_4 by upregulating the expressions of *sod* and *gpx4b*. In a zebrafish model of inflammation, pretreatment with β -sitosterol before CuSO_4 exposure inhibited neutrophil recruitment and damage to lateral line neuromasts, indicating a potent anti-inflammatory effect derived from reductions in the expressions of *il-8* and *myd88*. The results demonstrate the antioxidative and anti-inflammatory activities of

β -sitosterol and suggest that β -sitosterol may be useful for the treatment of various inflammatory diseases (PengZhang,2023)

Metals are constantly released in aquatic systems from natural and anthropogenic sources such as industrial activities, domestic sewage discharges, mining, farming, electronic waste, accidents, navigation traffic as well as climatic change event like floods (Babu *et al.*, 1990; Sandu *et al.*, 2008; Zhang *et al.*, 2016).

Metal pollution can arise most commonly from the mining, smelting and nuclear fuel production. Electroplating activity is the primary source of chromium and cadmium contamination in aquatic systems. Through precipitation of their compounds or by ion exchange in soils, heavy metal pollutants can localize and lay dormant. Unlike organic pollutants, heavy metals do not decay and thus pose a different kind of challenge for remediation.

Plants and microorganisms are tentatively used to remove some heavy metals from the contaminated site. Plants which exhibit hyper accumulation can be used to remove heavy metals from soils by concentrating them in their biomass. Some plants are used in the treatment of mining tailings where the vegetation is eventually incinerated to recover the heavy metals. Heavy metals like lead, mercury, iron, cadmium, aluminium and magnesium are present in water bodies reached sediment and enter the food chain through plants and other aquatic organisms cause heavy metal poisoning in case their level in the water is high.

Electroplating is a plating process that uses electrical current to reduce cations of a desired material from a solution and coat a conductive object with a thin layer of the material, such as a metal. Electroplating is primarily used for depositing a layer of material to a surface that otherwise lacks some properties such as abrasion and wear resistance, corrosion protection, lubricity and aesthetic qualities. Another application of electroplating is to build up thickness on undersized parts. Water pollution is caused by discharge of domestic or urban sewage, agricultural waste and industrial wastes into water bodies that makes the water unsuitable for drinking and other purposes. This renders the water useless for humans and also endangers aquatic life.

Metal uptake by aquatic organisms is a two-phased process involving initial rapid adsorption or binding to the surface, followed by slower transport into the cell interior (Crist *et al.*, 1988). In epithelial tissues the last step is the rate-limiting factor in transepithelial movement of metals (Foulkes, 1988). Metal transport inside the cell is carried out either by diffusion of the metal ion across the cell membrane or by carrier protein driven active transport (Brezonik *et al.*, 1991).

Heavy metals have become a major environmental pollutant today. In higher concentrations they may be lethal to aquatic organisms (Schroder, 1973; Rubin, 1974). The effect of heavy metals on behaviour, reproduction, blood chemistry, survival, growth of fish and other aquatic organisms have been reviewed by Katz (1975). A number of authors have reported the effect of heavy metals on aquatic organisms particularly fishes (Geetha *et al.*, 1996; Olsson *et al.*, 1998; Van den Belt *et al.*, 2000).

The ethanol extract of *C. cyrtophyllum* displayed prominent antioxidant effects through radical scavenging activity in vitro and decreased production of ROS in a CuSO₄-induced zebrafish inflammation model. In addition, in vivo results in zebrafish suggest that the ethanol extract from *C. cyrtophyllum* also inhibits oxidative stress via the upregulation of sod and the downregulation of hsp70 and gadd45bb. EE also inhibited inflammation via the downregulation of the inflammatory genes cox-2, pla2, c3a, il-1, il-8, mpo, and il-10. These in vivo results help to elucidate the protective mechanism against CuSO₄ toxicity of the ethanol extract of leaves of *C. cyrtophyllum*. Taken together, these findings provide a pharmacological basis for the use of *C. cyrtophyllum* leaves in the treatment of inflammatory disorders. (Thu Hang, 2020)

CHAPTER -3

AIM AND SCOPE

3.1 AIM :

To evaluate the changes in the body of zebrafish which is treated with copper sulphate pentahydrate

3.2 OBJECTIVE :

- To measure the changes in the body weight, organ weight and total protein in control and copper sulphate treated animals.
- To analyse the fragmentation of DNA using agarose gel electrophoresis.
- To understand the histopathological conditions of the head of the zebrafish by Hematoxylin and Eucine staining.
- To analyse the antioxidant enzyme activities in the liver for control and copper sulphate treated animals.

CHAPTER – 4

MATERIALS AND METHODOLOGY

4.1 MATERIALS :

- Fish tank – 3 (4 fishes in each tank).

SPECIMEN :

- 5 months old zebrafishes –male and female

CHEMICALS:

- Copper sulphate
- Tricaine methane sulfonate solution
- 2X Sample Buffer
- β – mercaptoethanol
- Tris HCl
- NaCl
- Isoamyl alcohol
- EDTA
- SDS
- Phenol
- Milli-Q water
- Tris Base
- Boric acid
- Agarose
- Ethidium bromide

INSTRUMENTS:

- Vortex
- Centrifuge
- Micropipette
- Dry bath
- 96 well plate
- pH meter

- Shaker
- Nanodrop
- Homogenizer

4.2 METHODOLOGY:

4.2.1 MAINTENANCE OF FISH

The fishes (5 months) brought to the laboratory were acclimatized in large fish tanks before they were used for the experiment.

They were maintained for 25 days in reverse osmosis (RO) water and were fed daily with fish feed.

4.2.2 CHEMICAL PREPARATION :

Chemical ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) is taken from the infectious disease biology lab in Sathyabama IRC. It contains 25.45% of copper and 36.07% of water.

4.2.3 PREPARATION OF COPPER SULPHATE SOLUTION:

- Stock solution (I) – 2000 µg of copper sulphate powder dissolved in 1000 µl of distilled water.
- Stock solution (II)- 500 µl of Stock solution (I) is dissolved in 500 µl of water.
- The elevated dose sequence of the chemical was 80 and 120 ppm from stock (II) and dissolved in 1 L of water in each tank respectively. 12 zebrafishes were taken and placed as 4 members in 80 and 120 ppm tanks. They are in observance for 96hrs with daily feed after adding the toxicity solution.

4.2.4 TRICAINE METHANE SULFONATE :

Tricaine methane sulfonate is used as an anaesthesia before dissecting the fish

PREPARATION:

- Tricaine powder – 400mg
- Double distilled water – 97.9ml
- 1M Tris – 2.1ml

400 mg of tricaine powder is dissolved in 97.9ml double distilled water and 1M Tris

4.2.5 DISSECTION OF ZEBRAFISH LIVER AND HEAD:

After fish had been treated with Tricaine solution , the liver and head has been dissected from the body after treated in copper sulphate solution for 96 hours by fresh surgical needle from three fishes – Control, 80ppm and 120ppm.

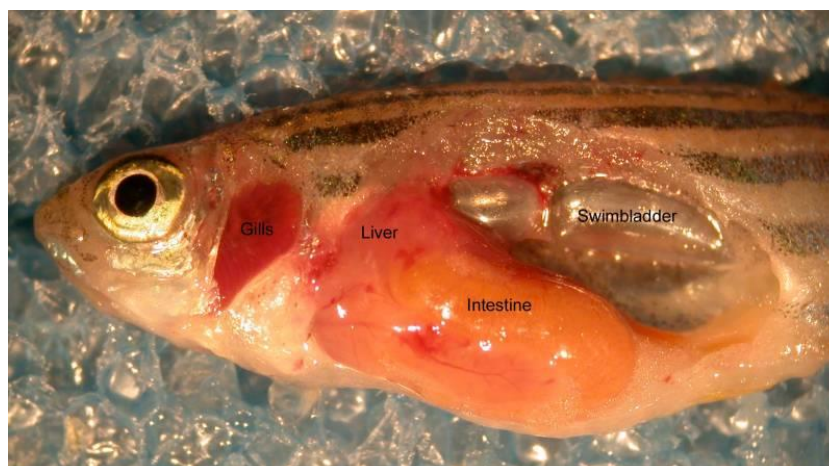


Fig 4.2.5: Dissected zebrafish showing gills, liver, intestine. Image is taken from (<https://www.google.com/url?sa=i&url=https%3A%2F%2Fzebrafish.>)

4.3 CHANGES IN BODY AND LIVER WEIGHT:

After the treatment, changes in body and liver weight has been seen between control and treated animals.

4.4 PROTEIN SAMPLE PREPARATION:

Proteins are extracted from liver. Beta-Mercaptoethanol used for breaking of disulfide bonds of protein, 2X sample buffer were brought to room temperature before use. After that, 950µl of 2X sample buffer were added with 50 µl of Beta-Mercaptoethanol (stock 1). In a fresh 1.5ml tube tissues are added with 200 µl of stock 1 and homogenized manually by homogenizing bud . The tubes are kept in dry bath at 100°C for 5 mins and centrifuged for 5 minutes at 10000 rpm. The supernatant was transferred to fresh 1.5ml tube and stored at -20°C.

4.5 AGAROSE GEL ELECTROPHORESIS:

4.5.1 Principle:

The principle of agarose gel electrophoresis is based on the fact that DNA, RNA, and proteins are negatively charged molecules that can be separated based on their size and charge by applying an electric field.

The gel matrix used in agarose gel electrophoresis is made of agarose, a polysaccharide extracted from seaweed that forms a porous structure when mixed with water.

The agarose gel acts as a sieve, allowing smaller molecules to pass through more easily than larger ones. The electric field is applied by placing the gel in a buffer solution and applying an electrical current across the gel using two electrodes.

The DNA, RNA, or protein sample is mixed with a loading buffer, which contains a tracking dye that allows the progress of the electrophoresis to be monitored.

The sample is loaded onto the gel, and the electric current is turned on. The negatively charged molecules in the sample migrate through the gel towards the positively charged electrode.

The smaller molecules migrate more quickly through the gel and reach the end of the gel first, while the larger molecules take longer to migrate and form distinct bands on the gel. The bands can be visualized using a staining agent, such as ethidium bromide, which binds to the nucleic acid molecules and fluoresces under UV light.

4.5.2 EXTRACTION OF DNA:

CHEMICAL PREPARATION :

Chloroform-Isoamyl alcohol solution:

Chloroform - 12ml

IAA - 50µl

4M NaCl:

NaCl - 0.234 mg

Distilled water - 1 ml

Homogenise buffer:

EDTA - 186 mg

NaCl - 450 mg

Distilled water - 50 ml.

pH was adjusted to 8.0 with 10N NaOH.

20% SDS:

SDS - 200 mg

Distilled water - 1 ml.

4.5.3 METHODOLOGY:

200 mg of fish body is taken and 2 ml of homogenise buffer is added. The tissue was well homogenized with motor and pestle and transferred in 1.5 ml tube. Then centrifuged at 10000 rpm for 2 minutes. 300 µl of supernatant is taken in another tube. 25 µl of 20% SDS and 300 µl of phenol and 300 µl of chloroform –IAA mixture was added into the tube and shaken vigorously to mix the added solutions. Then centrifuge at 11000 rpm for 5 minutes. Collect the aqueous layer and add 9 µl of 4M NaCl and 2 volumes of 100% ethanol and centrifuge at 12000 rpm for 5 minutes and wash twice with 60% ethanol. Air dry the tube and dissolve in 50 µl of Milli-Q water.

4.5.4 PREPARATION OF TBE BUFFER:**TBE (10X) – For 250 ml:**

Tris base - 27 g

Boric acid - 13.75 g

Distilled water - 225 ml

0.5M EDTA - 1.46 ml (pH was adjusted to 8.0 with 10N NaOH)

The solutions were mixed and make upto 250 ml.

TBE (1X) – For 500 ml:

Take 50 ml of 10X TBE and then mix it with 450 ml of distilled water.

4.5.5 PREPARATION OF THE GEL:

30 ml of 1X TBE buffer is taken and mixed with 0.36 g of Agarose and 10 µl of ethidium bromide are added to create a gel matrix.

The concentration of the agarose gel can vary depending on the size range of the molecules being analysed. Higher concentrations of agarose are used for the separation of smaller molecules, while lower concentrations are used for larger molecules.

The gel matrix is poured into a casting tray and allowed to solidify. Once the gel has solidified, it is removed from the casting tray and placed in a gel electrophoresis chamber.

4.5.6 PREPARATION OF THE SAMPLE:

The sample to be analysed (DNA) is prepared by mixing it with a loading buffer, which contains a tracking dye (Coomassie blue) that allows the progress of the electrophoresis to be monitored.

The sample is loaded into wells at one end of the gel using a micropipette. Care should be taken not to overload the wells, as this can lead to distortion of the bands.

4.5.7 RUNNING THE GEL:

Once the sample has been loaded onto the gel, the electric current is turned on, and the gel is allowed to run for a specific period of time.

The length of time required for the electrophoresis run depends on the size range of the molecules being separated and the concentration of the agarose gel. The gel is typically run until the tracking dye reaches the end of the gel.

4.5.8 VISUALIZATION:

Once the tracking dye reaches the end the current is turned off and the gel is separated from the case and observed using ChemDoc from Advanced Biomaterials Lab in Sathyabama IRC.

4.6 ANTIOXIDANT ASSAY:

4.6.1 ANTIOXIDANT:

The process of oxidation in the human body produces unstable chemicals called free radicals, which damage cell membranes and other structures. Free radicals have been linked to a variety of diseases (including heart disease and certain cancers). Antioxidants are the compounds in foods that scavenge and neutralise free radicals.

4.6.2 CATALASE:

Catalase is one of the crucial antioxidant enzymes that mitigates oxidative stress to a considerable extent by destroying cellular hydrogen peroxide to produce water and oxygen. Deficiency or malfunction of catalase is postulated to be related to the pathogenesis of many age-associated degenerative diseases like diabetes mellitus, hypertension, anemia, vitiligo, Alzheimer's disease, Parkinson's disease, bipolar disorder, cancer, and schizophrenia.

4.6.3 SUPEROXIDE DISMUTASE:

SOD is used in cosmetics and personal care products as an anti-aging ingredient and antioxidant due to its ability to reduce free radical damage to the skin, therefore preventing wrinkles, fine lines, and age spots, and it also helps with wound healing, softens scar tissue, protects against UV rays, and reduces other signs of aging. It has been reported that SOD has an important link in several human health problems including RBC-related disorders, cystic fibrosis (CF), postcholecystectomy pain syndrome, malignant breast disease, steroid-sensitive nephrotic syndrome, amyotrophic lateral sclerosis, neuronal apoptosis, AIDS, and cancer.

4.6.4 VITAMIN E:

Vitamin E is the major lipid-soluble component in the cell antioxidant defence system and is exclusively obtained from the diet. It has numerous important roles within the body because of its antioxidant activity. Oxidation has been linked to numerous possible conditions and diseases, including cancer, ageing, arthritis and cataracts; vitamin E has been shown to be effective against these. Platelet hyperaggregation, which can lead to atherosclerosis, may also be prevented by vitamin E; additionally,

it also helps to reduce the production of prostaglandins such as thromboxane, which cause platelet clumping.

4.6.5 GLUTATHIONE S-TRANSFERASE (GST):

Glutathione is a powerful antioxidant that may help protect the body from disease, slow cancer progression, and improve insulin sensitivity, among other benefits. Some people swear by its anti-aging properties, while others say that it can treat autism, increase fat metabolism, and even prevent cancer.

4.6.6 GLUTATHIONE L-GLUTAMYL-L-CYSTEINYL-GLYCINE (GSH):

Glutathione is an antioxidant in plants, animals, fungi, and some bacteria and archaea. Glutathione is capable of preventing damage to important cellular components caused by sources such as reactive oxygen species, free radicals, peroxides, lipid peroxides, and heavy metals.

4.6.7 SAMPLE PREPARATION FOR ANTIOXIDANT ASSAY:

PREPARATION OF 1X TRIS BUFFER:

Tris - 0.605 g.

NaCl - 0.876 g

H₂O - 80 ml

Adjust pH to 7.5 with 1M HCl and make the solution upto 100 ml.

Liver is the most important organ concerned with the biochemical activities in the human body. It has great capacity to detoxicate toxic substances and sythesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. There is an ever increasing need of an agent, which could protect it from such damage. A number of pharmacological and chemical agents act as hepatotoxin and produce variety of liver ailments. Therefore liver is taken to study changes in antioxidant enzymes level in zebrafish

The liver tissues from control and treated animals were taken in 1.5 ml tubes and 1X tris buffer was added in it. The tissues were homogenized with homogenizing bud and centrifuged at 2000 rpm for 5 minutes. The supernatant were transferred in fresh 1.5 ml tube and stored in -20°C. The samples were kept in antioxidant auto analyser and readings were taken for control and treated animals.

4.7 HISTOLOGY

4.7.1 MATERIALS:

Tissue, screw cap bottle, clean albumenized glass slides, coupling jars, cover slip, forceps, formaldehyde, isopropyl alcohol, xylene, paraffin wax, haematoxylin and acidic dye eosin.

4.7.2 REAGENT PREPARATION:

50% Formaldehyde (Fixation)

Add 50ml of formaldehyde in 50ml of distilled water.

Isopropyl alcohol (Dehydration)

60% Isopropyl alcohol- 60ml of Isopropyl alcohol + 40ml of distilled water.

70% Isopropyl alcohol- 70ml of Isopropyl alcohol + 30ml of distilled water.

80% Isopropyl alcohol- 80ml of Isopropyl alcohol + 20ml of distilled water.

90% Isopropyl alcohol- 90ml of Isopropyl alcohol + 10ml of distilled water.

100% Isopropyl alcohol- 100ml of Isopropyl alcohol.

4.7.3 WAX PREPARATION:

For preparation of wax, the melting temperature of wax should be between 58 – 60°C.

4.7.4 ALBUMIN COAT:

Egg white + equal amount of glycerol.

4.7.5 TISSUE PROCESSING FOR HISTOLOGY:

After the head was dissected from zebrafish , the samples were stored in 10% formalin. Then after 1 day, the samples were washed with distilled water. Then for fixation, the samples were fixed with 50% formaldehyde solution for 24 hours at 50°C. Then wash the tissue with distilled water. After washing, the tissues were transferred into 60% isopropyl alcohol at 50°C for 1 hour. Again transfer the tissues into 70% isopropyl alcohol at 50°C for 1 hour. Then at 80% isopropyl alcohol at 50 °C for 1 hour, 90% isopropyl alcohol at 50°C for 1 hour, 100% isopropyl alcohol at 50°C for 1 hour and finally repeat again in 100% isopropyl alcohol at 50°C for 1 hour. Then, transfer the tissue into a cleaning solution, xylene for 30 – 45 minutes. Prepare wax-1, wax-2, wax-3. The tissues were immigrated in wax-1 overnight (Temperature supposed to be maintained in 58-60°C). After incubation tissues rottenly transferred to the second wax for 3 hours and after that tissues were placed on the final wax for 3 hours. Then the tissues were transferred into the boxes for sectioning by preparing the blocks by melting the paraffin wax and keeping the sample in horizontal position. After the preparation of wax blocks, the sectioning of the tissues can be done by using a microtome which is of 7-micron size.

The 7µm sections were made and the ribbon model tissue with wax is kept in hot water for 5 seconds at 60°C. Then take off the tissue by albumin coated slide, and keep the slide in the slide warmer for 10 minutes to remove the water.

4.7.6 TISSUE STAINING FOR HISTOLOGY (H&E STAINING) :

First take the slides and keep it in xylene – 1 and 2 for 5 minutes to remove the water. Then, transfer the slide into a 100% isopropyl alcohol for 3 minutes to remove xylene – 1 and 2. After that, wash the slide with tap water for 5 minutes. Then transfer the slide into haematoxylin stain for 7 - 10 minutes. Wash the slide with tap water for about 10 minutes. Then wash the slides containing the tissue sections with acid solution (99ml of 70% isopropyl alcohol + 1ml of distilled water). After that keep the slide in blooming tap water 21 overnight. Then dip the slide in eosin stain for 30

seconds and after that wash it with tap water for a few seconds. Next step is to decolourize the slide by keeping it in 100% isopropyl alcohol for 5 – 10 seconds then repeat the xylene wash step. Finally, mount the slides containing the tissues with synthetic resin mounting media, DPX mount (a mixture of distyrene, plasticizer and xylene)

CHAPTER-5

RESULTS AND DISCUSSION

5.1 MORPHOLOGICAL AND BEHAVIOURAL CHANGES IN ANIMALS:

The swimming capacity is in steady and regular manner before treatment . The fishes tend to swim fast and in an irregular manner after the treatment. The resulted act may be due to neurotoxicity changes because of the chemical.

The animal have bluish and greyish stripes on the body and it is clearly visible as seen in figure 5.1(A). Whereas the pattern is slightly diminished after the treatment as seen in figure 5.1(B).



Fig 5.1 (A): Zebrafish before treatment



Fig 5.1 (B): Zebrafish after treatment

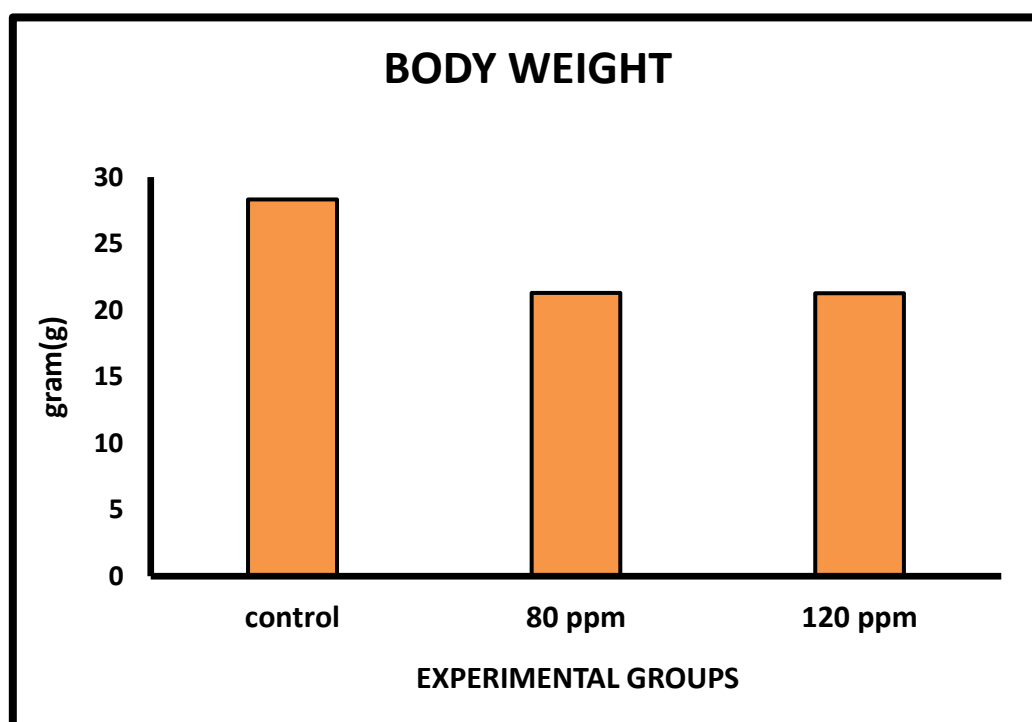
5.2 CHANGES IN BODY AND LIVER WEIGHT BETWEEN CONTROL AND TREATED ANIMALS:

The body weight of fishes has been recorded between the control and treated animals. The weight tends to decrease in the fishes which have undergone the chemical exposure.

Simultaneously, liver weight has been recorded since liver has been used for protein estimation and for determining antioxidant assay. The weight decreases between the control and treated animals.

Table 5.1: Changes in body weight between control and treated animals

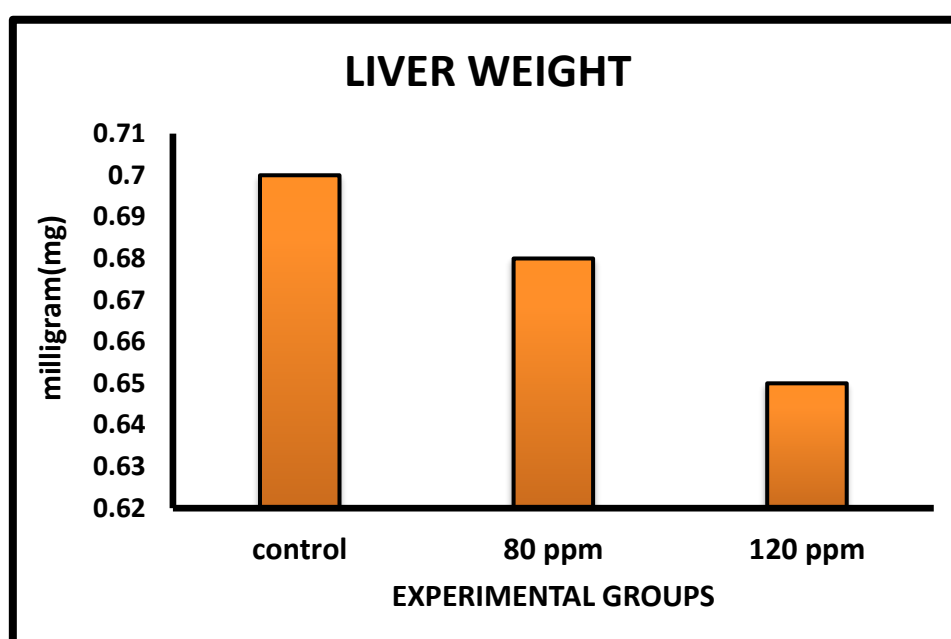
CONTROL	CUSO ₄ TREATED 80ppm	CUSO ₄ TREATED 120ppm
0.5 g	0.45 g	0.36 g



Graph 5.1: Graphical representation of body weight between control, 80 ppm and 120 ppm groups.

Table 5.2: Changes in liver weight between control and treated animals

CONTROL	CUSO ₄ TREATED 80ppm	CUSO ₄ TREATED 120ppm
0.7 mg	0.68 mg	0.65 mg



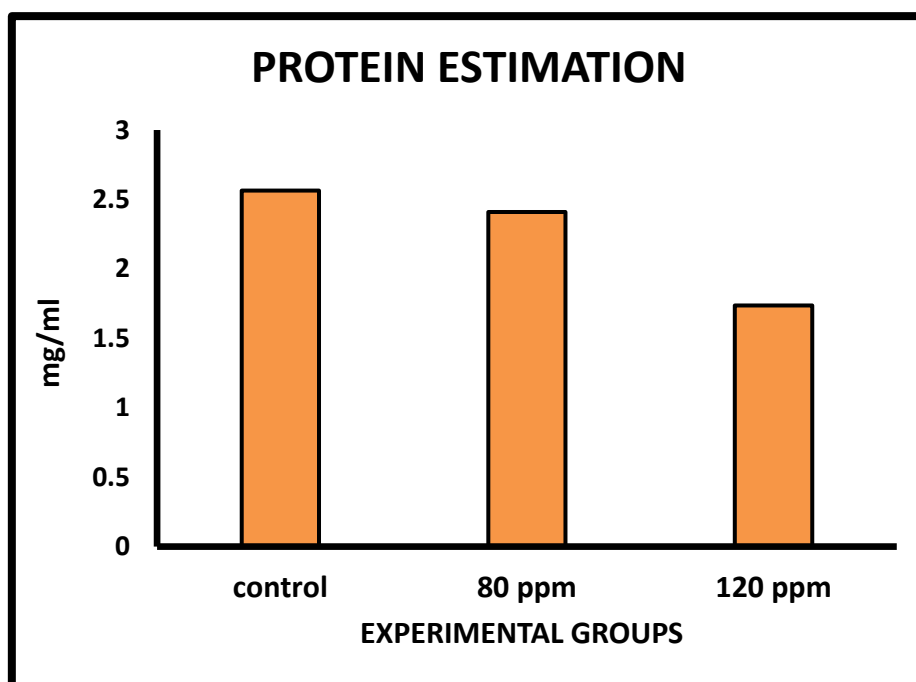
Graph 5.2: Graphical representation of liver weight between control ,80 ppm and 120 ppm groups.

5.3 PROTEIN ESTIMATION BETWEEN CONTROL AND TREATED ANIMALS:

The protein sample was prepared as per the procedure mentioned above in 4.4. The protein content decreases gradually as the dose of the copper sulphate increases. The protein content was estimated with the use of nanodrop from Neuroscience lab in Sathyabama IRC. Liver was chosen to estimate protein value because it is an important organ for protein synthesis, degradation and detoxification as well as amino acid metabolism.

Table 5.3: Protein estimation between control and treated animals

CONTROL	CUSO ₄ TREATED 80ppm	CUSO ₄ TREATED 120ppm
2.564 mg/ml	2.409 mg/ml	1.737 mg/ml



Graph 5.3: Graphical representation of protein estimation between control , 80 ppm and 120 ppm groups.

5.4 DNA FRAGMENTATION ANALYSES USING AGAROSE GEL ELECTROPHORESIS:

The prepared DNA was quantified using nanodrop and it was estimated with 2000ng as the standard. The amount of DNA present has been decreased from 1.8 (control) to 1.57 (80 ppm) . The sample loaded was 10.8 μ l and 15.06 μ l of control and 80 ppm respectively . In addition , 2 μ l of Coomassie Blue has been added in each sample.

The resulted DNA fragmentation has been obtained from ChemDoc instrument. The DNA of 80 ppm (Fig 5.4B) was slightly disturbed when comparing control sample (Fig 5.4A).

This concludes that DNA can be disturbed further when the dose is elevated.

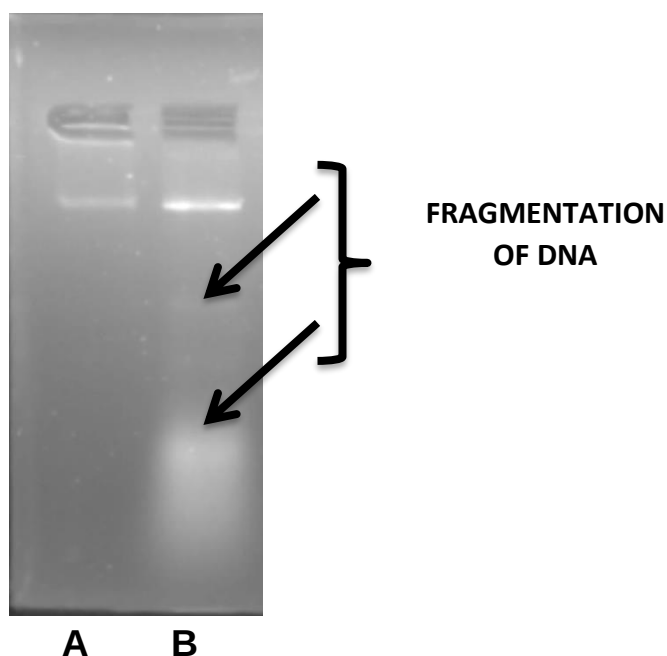


Fig 5.4: Agarose gel electrophoresis

(A)- Control sample showing no DNA fragmentation. **(B)-** 80 ppm showing fragmentation in the DNA

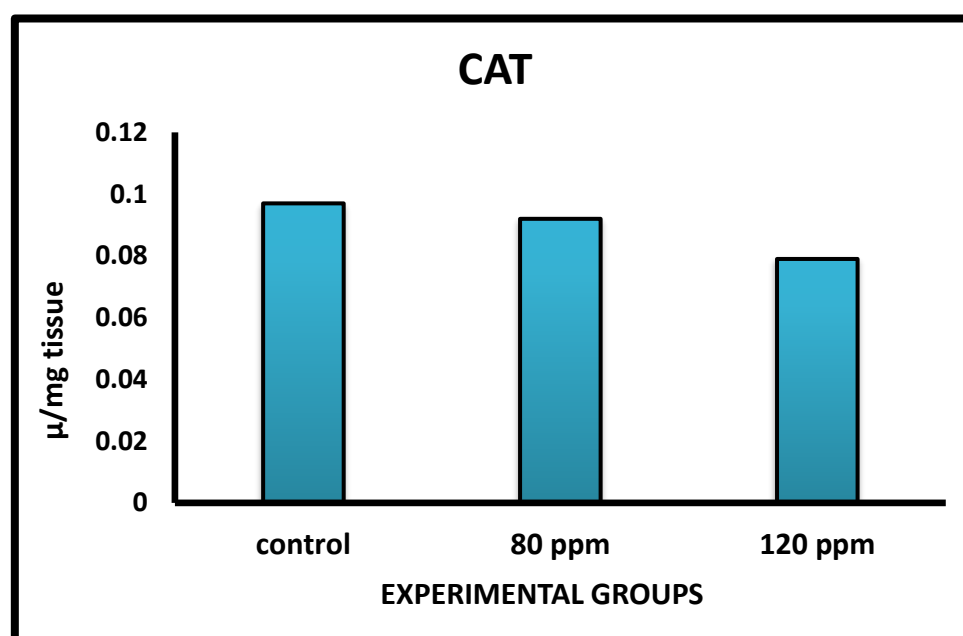
5.5 ANTIOXIDANT ENZYME ANALYSES BETWEEN CONTROL AND TREATED ANIMALS:

5.5.1 CATALASE:

Decreased activity of catalase enzyme has been observed in treated animals. the values are tabulated as follows:

Table 5.4: Changes in CAT enzyme between control and treated animals

CONTROL	CUSO ₄ TREATED 80ppm	CUSO ₄ TREATED 120ppm
0.097 μ /mg tissue	0.092 μ /mg tissue	0.079 μ /mg tissue



Graph 5.4: Graphical representation of changes in CAT enzyme between control, 80 ppm and 120 ppm groups.

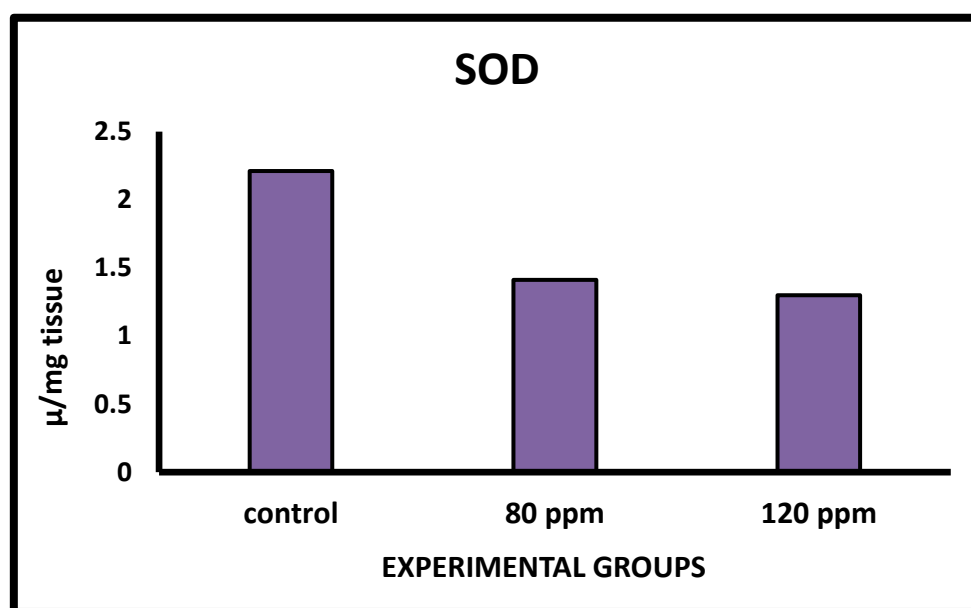
A shortage of this enzyme can allow hydrogen peroxide to build up to toxic levels in certain cells. For example, hydrogen peroxide produced by bacteria in the mouth may accumulate in and damage soft tissues, leading to mouth ulcers and gangrene.

5.5.2 SUPEROXIDE DISMUTASE:

Decreased activity of superoxide dismutase enzyme has been observed in treated animals. the values are tabulated as follows:

Table 5.5: Changes in SOD enzyme between control and treated animals

CONTROL	CUSO4 TREATED 80ppm	CUSO4 TREATED 120ppm
2.211 μ /mg tissue	1.411 μ /mg tissue	1.300 μ /mg tissue



Graph 5.4: Graphical representation of changes in SOD enzyme between control, 80 ppm and 120 ppm groups.

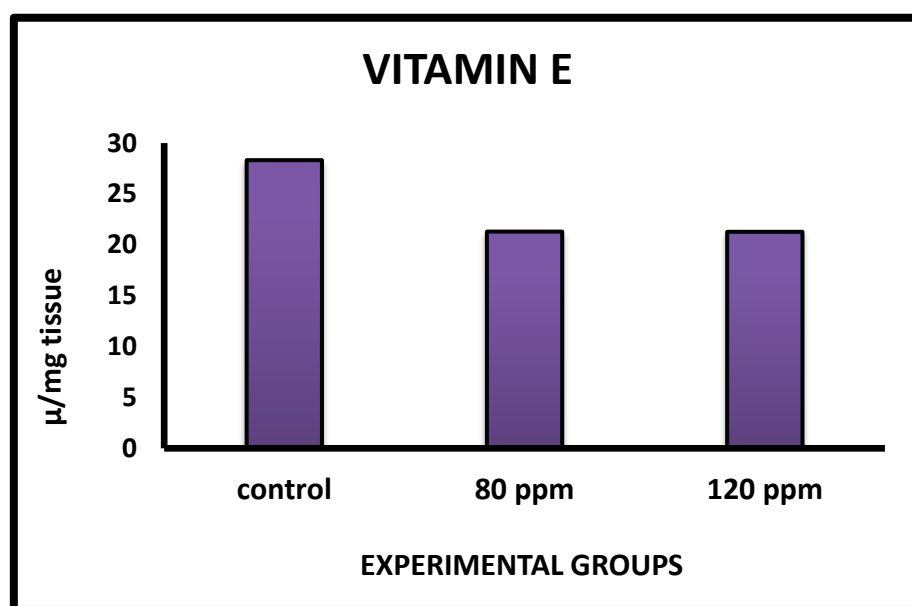
SOD deficiency contributes to several neurological diseases, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and stroke (Flynn and Melov, 2013).

5.5.3 VITAMIN E:

Decreased activity of vitamin E has been observed in treated animals. The values are tabulated as follows:

Table 5.6: Changes in Vitamin E between control and treated animals

CONTROL	CUSO4 TREATED 80ppm	CUSO4 TREATED 120ppm
7.96 μ /mg tissue	6.69 μ /mg tissue	5.96 μ /mg tissue



Graph 5.4: Graphical representation of changes in Vitamin E between control, 80 ppm and 120 ppm groups.

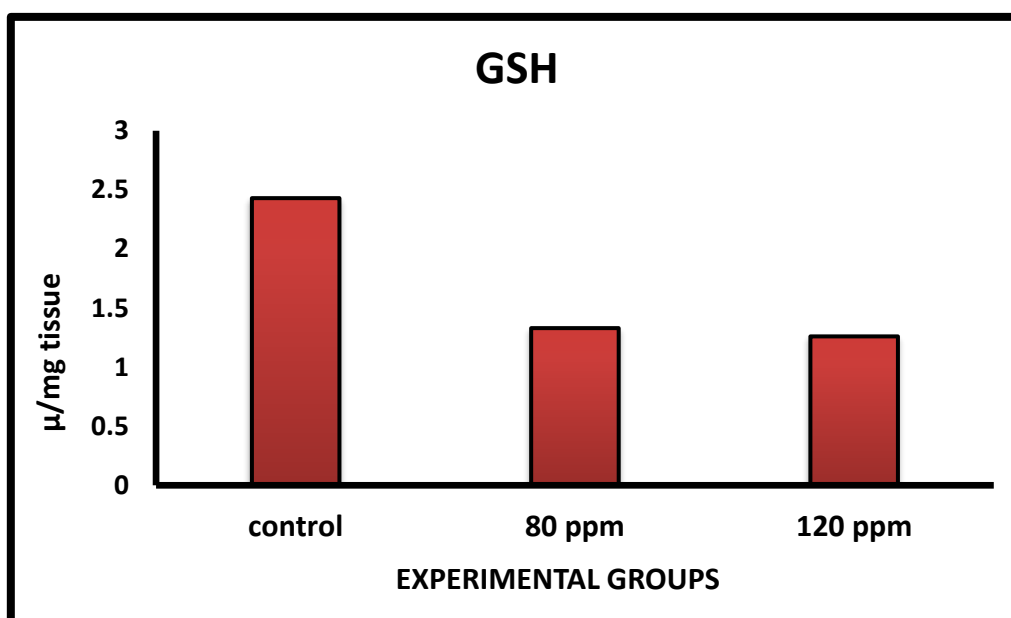
Vitamin E deficiency can cause nerve and muscle damage that results in loss of feeling in the arms and legs, loss of body movement control, muscle weakness, and vision problems. Another sign of deficiency is a weakened immune system.

5.5.4 GLUTATHIONE L-GLUTAMYL-L-CYSTEINYL-GLYCINE (GSH):

Decreased activity of GSH enzyme has been observed in treated animals. the values are tabulated as follows:

Table 5.7: Changes in GSH enzyme between control and treated animals

CONTROL	CUSO4 TREATED 80ppm	CUSO4 TREATED 120ppm
2.43 μ /mg tissue	1.33 μ /mg tissue	1.26 μ /mg tissue



Graph 5.5: Graphical representation of changes in GSH enzyme between control, 80 ppm and 120 ppm groups.

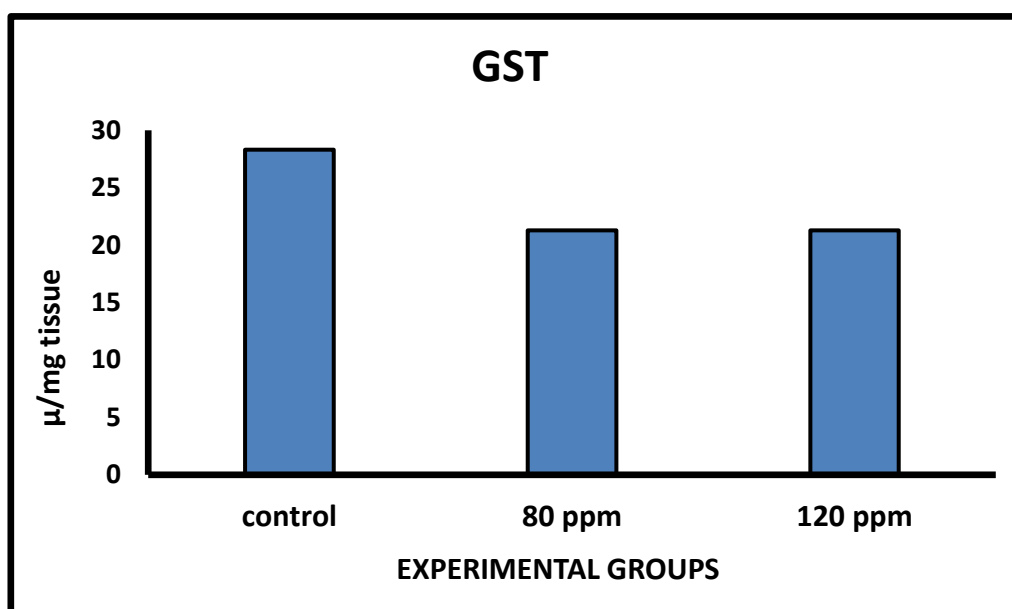
The decreased levels of GSH and/or the activities of antioxidant enzymes in the peripheral blood of the patients may indicate the occurrence of a systematic reaction that may cause oxidative stress in the brain of schizophrenic patients as is the case in other disorders of the central nervous system .

5.5.5 GLUTATHIONE-S-TRANSFERASE (GST):

Decreased activity of GST enzyme has been observed in treated animals. the values are tabulated as follows:

Table 5.8: Changes in GSH enzyme between control and treated animals

CONTROL	CUSO4 TREATED 80ppm	CUSO4 TREATED 120ppm
28.32 μ /mg tissue	21.3 μ /mg tissue	21.28 μ /mg tissue



Graph 5.6: Graphical representation of changes in GST enzyme between control, 80 ppm and 120 ppm groups.

Glutathione synthetase deficiency is a disorder that prevents the production of an important molecule called glutathione. Glutathione helps prevent damage to cells by neutralizing harmful molecules generated during energy production.

5.6 HISTOPATHOLOGICAL ANALYSIS OF HEAD OF ZEBRAFISH:

The mobilization of these heavy metals to the aquatic ecosystem alters the physicochemical property of water which is hazardous for aquatic organisms. Heavy metals mainly enter the fish body through gills, body surface and digestive tract during ingestion of metal accumulated food materials.

The histological section of the treated head shows obvious impact. In the treated head (Fig. 5.6B) the gills got highly disturbed. Sameway, the eye region got collapsed and the head was not in the proper structure. But in control group (Fig. 5.6A) the gills were in proper and healthy pattern surrounding the eye area and the head is well formed. From the above observation it is clear that treatment with copper sulphate pentahydrate causes changes in head of zebrafish.

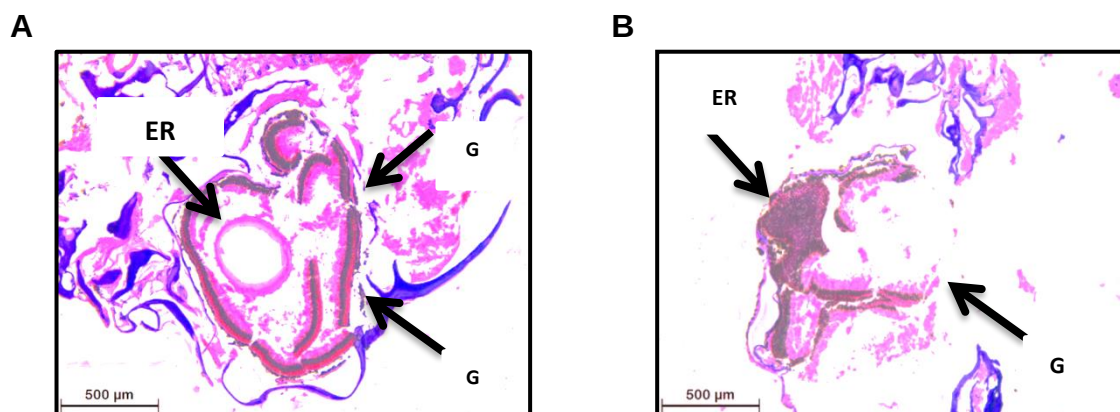


Fig 5.6: Histological section of the normal and treated head of zebrafish.

(A)- Histological section of normal head of the animal. **(B)-** Histological section of treated (120 ppm) head of the animal. **G-** Gills. **ER-** Eye Region

CHAPTER – 6

SUMMARY AND CONCLUSION

Zebrafish are a valuable tool for Environmental Health researchers as evidenced by a rapidly expanding body of research using zebrafish. A PubMed search using the terms “zebrafish environmental health” reveals that the use of zebrafish in this field has been steadily increasing over the past few decades. Zebrafish is a widely used animal model in biomedical sciences and toxicology. Although evidence for the presence of phases I and II xenobiotic defense mechanisms in zebrafish exists on the transcriptional and enzyme activity level.

Almost all the metals are toxic at higher concentrations and some are lethal even at a very low concentration. The priority list of pollutants compiled by the Environmental Protection Agency (EPA) of the United States contains eight widespread heavy metals, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc. In the present study was to assess the histopathology, agarose gel electrophoresis in DNA, protein estimation and antioxidant enzymes in copper sulphate pentahydrate exposed in Zebrafish (*Danio rerio*).

The following conclusion obtained from the study

- In the present experiment study, the copper sulphate pentahydrate toxicity causes the physical and behavioural changes in the animal.
- The decreased in body and organ weight (liver) of copper sulphate treated zebra fish were noticed due to the chemical stress.
- A reduction in the protein content in the present investigation in zebra fish suggests that the tissue protein might have undergone proteolysis, during the stressful situation in the copper sulphate intoxicated fishes.
- The DNA fragmentation was observed in 80 ppm copper sulphate intoxicated fish.
- The values has been decrease by performing antioxidant assay between the control and treated animals.

- Histological observation showed The head region has been resulted in degeneration of gills and particles are collided around the eye area in 120ppm dose.

Overall, it can be concluded that copper sulphate pentahydrate induced the toxicity in dose dependent manner via oxidative stress confirmed by biochemical, antioxidants and histological studies.

CHAPTER – 7

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