

EXPLORING EXOSOMES ROLE IN ZEBRAFISH LARVAE NEURODEGENERATION

PROJECT WORK

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Science in Biotechnology

By

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INSTITUTE OF SCIENCE AND TECHNOLOGY

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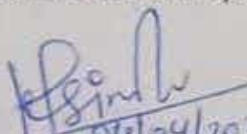
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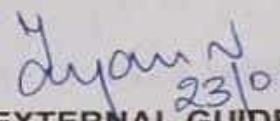
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This is to certify that this Project Report is the bonafide work of
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LARVAE NEURODEGENERATION**" under our supervision from
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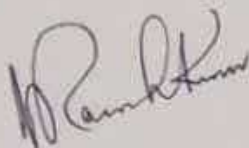
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We **R. Mahalakshmi (41770015)** and **S. Ramya (41770024)** hereby declare that the Project Report entitled “**EXPLORING EXOSOMES ROLE IN ZEBRAFISH LARVAE**” done by us under the guidance of **Dr. JAYSHREE NELLORE** (External) at the department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfillment of the requirements for the award of **Bachelor of Science Degree in Biotechnology**

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A handwritten signature in black ink, appearing to read 'Mahalakshmi', is written over a faint, light blue rectangular stamp. The stamp contains some illegible text and a circular emblem.

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ABSTRACT

Background of the Study:

Tartrazine (TTZ) is a synthetic yellow azo dye commonly used as a food coloring agent. Despite its approval by regulatory agencies at low concentrations, concerns persist regarding its potential adverse effects, particularly its impact on developmental health. Zebrafish (*Danio rerio*) embryos provide an excellent model for investigating developmental toxicity due to their rapid development and genetic similarity to humans.

Methods:

Zebrafish embryos were exposed to TTZ at 5.5 ppm to assess its effects on survival rate, morphological characteristics, locomotor activity, exosome morphology, and miR-132 expression. Survival rate was monitored throughout the experimental period, while morphological characteristics were observed using a stereo zoom microscope. Locomotor activity was analyzed by tracking tail movements. Scanning electron microscopy (SEM) was utilized to examine exosome morphology, and miR-132 expression was evaluated through real-time PCR analysis of RNA extracted from exosomes.

Results:

Exposure to TTZ did not significantly alter survival rates compared to the control group, indicating no acute lethal toxicity. However, TTZ exposure induced morphological abnormalities such as bent tails and curved spine structures, suggesting potential developmental toxicity. Locomotor activity analysis revealed no significant differences between TTZ-exposed groups and controls, indicating minimal impact on early-stage neurodevelopment.

SEM analysis showed alterations in exosome morphology and cargo content in TTZ-treated embryos, indicating disruptions in intercellular communication pathways. Quantification of RNA extracted from exosomes demonstrated differences in quantity and quality between TTZ-treated and control samples. Real-time PCR analysis

revealed downregulation of miR-132 in TTZ-treated embryos, suggesting potential molecular impacts of TTZ exposure on gene expression.

Conclusions:

While TTZ exposure did not lead to acute mortality events, it induced morphological abnormalities and molecular alterations in zebrafish embryos. These findings imply potential developmental toxicity and molecular dysregulation associated with TTZ exposure. Further research is essential to elucidate the underlying mechanisms and implications for developmental health.

Keywords: Exosomes, Neurodegeneration, Tartrazine, Zebrafish

LIST OF CONTENTS

S. NO.	CONTENTS	PAGE NO.
	ABSTRACT	i
	LIST OF FIGURES	ii
	LIST OF TABLES	iii
	LIST OF ABBREVIATIONS	iv
1.	INTRODUCTION	1
	1.1 Exosomes	1
	1.2 Biogenesis, composition and cargo sorting of exosomes.	2
	1.2.1 <i>Biogenesis</i>	2
	1.2.2 <i>Composition</i>	3
	1.2.3 <i>Cargo sorting</i>	3
	1.3 Physiological functions of exosomes	4
	1.4 Application of exosomes	5
	1.5 Exosomes in neural communication and neurodegeneration	5
	1.6 miR- 132	6

1.7 Zebrafish (<i>Danio rerio</i>)	7
1.8 Purpose of study	8
1.9 Hypothesis	8
2. LITERATURE SURVEY	9
2.1 Biogenesis of exosome, composition and functions.	9
2.2 Role of exosomes in disease pathogenesis	9
2.3 Effects of tatrazine	12
2.4 Behaviourial changes in children by tartrazine	12
2.5 Zebrafish as model organism	13
2.6 Role of miR - 132 in neurodenegeration.	14
3. AIM AND OBJECTIVES	15
3.1 Aim	15
3.2 Objectives	15
4. MATERIALS AND METHODS	16
4.1 Materials required	16
4.2 Methodologies	16
4.2.1 Preparation of Phosphate buffer saline	16
4.2.2 Preparation of embryo medium(E3 medium)	17

4.2.3 Zebrafish animal husbandry and breeding	18
4.2.4 Tartrazine exposure procedure	18
4.2.4.1 Locomotor Analysis	20
4.2.5 Perfusion buffer	20
4.2.6 Isolation of Exosomes from zebrafish larvae	20
4.2.7 Lyophilization of exosomes	21
4.2.7.1 Principle of lyophilisation	21
4.2.7.2 Steps involve in lyophilization	22
4.2.8 Scanning electron microscope (SEM)	23
4.2.8.1 Principle of SEM	23
4.2.8.2 Working of SEM	24
4.2.9 Isolation of RNA from exosomes	24
4.2.9.1 Principle	24
4.2.9.2 Procedure	25
4.2.10 Quantification of RNA using microplate reader	26
4.2.11 cDNA transcription	26
4.2.12 Real time PCR	27
4.2.12.1 Real time PCR for miR-132	28

4.2.13	Statistical Analysis	29
5.	RESULT AND DISCUSSION	30
5.1	Zebrafish toxicity induced by tartrazine exposure: survival rate and developmental abnormalities	30
5.1.1	Survival rate	30
5.1.2	Morphological characteristics	31
5.1.3	Locomotor activity	32
5.2	Characterization of exosomes using SEM	34
5.3	miR - 132 Expression in exosome	35
5.3.1	Quantification of RNA	35
5.3.2	Real time PCR	36
6	SUMMARY AND CONCLUSION	39
6.1	Summary	39
6.1.1	Zebrafish Toxicity Induced by Tartrazine Exposure	39
6.1.2	Characterization of Exosomes using SEM	39
6.1.3	miR-132 Expression in Exosomes	39
6.2	Conclusion	40
	REFERENCES	41

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ADEVs	Astrocyte Derived Extracellular Vesicles
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
BBB	Blood Brain Barrier
CNS	Central Nervous System
cDNA	Complementary Deoxyribo Nucleic Acid
CT	Cycle Threshold
CRISPR	Clustered Regularly Interspaced Short palindromic Repeats
EVs	Extracellular Vesicle
E3	Embryo Medium
EMT	Epithelial Mesenchymal transitions
ESCRT	Endosomal sorting complexes required for transport
ESEs	Early sorting Exosomes
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	Hours post- fertilization
ILVs	Intra Luminal Vesicles
LSEs	Late Sorting Endosomes
LAPTM	Lysosome associated Protein Transmembrane
miRNA	Micro Ribo Nucleic Acid
MHC	Major Histo compatability Complex
MVBs	Multi vesicular bodies
PBS	Phosphate Buffer Saline
PD	Parkinson's Disease
RNA	Ribo Nucleic Acid
rtPCR	Real Time Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
TGN	Trans Golgi Network
TTZ	Tartrazine

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
1.1	Structure and Composition of exosomes	4
4.1	E3 medium	17
4.2.1	18hpf zebrafish embryos obtained from Aquatic research laboratory.	19
4.2.2	24hpf larvae as control in E3 medium	19
4.2.3	24 hpf larvae as TTZ induced in E3 medium	19
4.2.4	48hpf <i>larvae as control</i> in E3 medium	19
4.2.5	48hpf larvae as TTZ induced in E3 medium	19
4.2.6	96hpf larvae as control/ in E3 medium	19
4.2.7	96hpf larvae as TTZ induced in E3 medium	19
4.2.8	Crude exosome pellet from 96hpf control larvae by ultracentrifugation.	21
4.2.9	Crude exosome pellet from 96hpf TTZ induced larvae by ultracentrifugation.	21
4.2.10	Lyophilizer	23
4.2.11	Lyophilized exosomes	23
4.2.12	Phase separation of RNA from 96hpf control exosomes	25
4.2.13	Phase separation of RNA from 96hpf TTZ induced exosomes	25

4.2.14	Obtained RNA pellet from 96hpf control exosomes	25
4.2.15	Obtained RNA pellet from 96hpf TTZ induced exosomes	25
4.2.16	cDNA Transcription	22
5.1	Survival rate of zebrafish embryos during exposure to TTZ (5.5ppm).	31
5.2	Morphological characteristics of zebrafish embryos assessed from 24hpf to 96 hpf following exposure to Tartrazine using stereo zoom microscope	32
5.3	Locomotor Changes in Zebrafish Exposed to TTZ (5.5ppm)	33
5.4	SEM images of exosomes derived from zebrafish embryos (A-B) Control and (C-D) TTZ treated.	34
5.5	Expression of miR - 132 in rtPCR	38

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
4.1	Primer pairs used for quantifying the miR – 132 level	29
5.1	Quantification Of RNA	36

CHAPTER – 1 INTRODUCTION

Artificial food colorings, derived from petroleum and coal, find extensive application across various industries such as food, bakery, textile, pharmaceuticals, and cosmetics (Benkhaya, S., et al., (2020)). Despite their prevalent use, these synthetic dyes, including tartrazine (E102), pose significant health risks, particularly among children. Only a limited number of artificial food colorings are approved with regulatory limits by authorities like the Food Safety and Standard Authority (FFSAI) in India (Adeel et al., 2024). Tartrazine, a water-soluble synthetic food colorant, is permitted under an acceptable limit of 100 mg/kg but exhibits mutagenic, carcinogenic, and toxic effects when consumed regularly (Subhashish Dey, Bommu Hema Nagababu, 2022).

Neurodegenerative disorders, like Parkinson's and Alzheimer's disease, stem from the progressive loss of neurons (Alsalman, N., et al., (2019)). Studies indicate that artificial food colorings, like tartrazine, possess neurotoxic properties, impacting brain function and contributing to behavioral changes such as hyperactivity and autism in children (Ismail, O.I., Rashed, 2022). These dyes contain harmful chemicals like lead and chromium, which affect mental health, increasing the prevalence of conditions like autism spectrum disorders (ASD) and attention deficit hyperactivity disorder (ADHD).

Now, we can see the impact of exosomes on neurodegeneration. (Bakthavachalu, et al., (2020)).

1.1 EXOSOMES

Extracellular vesicles (EVs) are released by both prokaryotes and eukaryotes as a natural aspect of their physiology and in the event of acquired disorders. EVs can be generally classified into two categories: exosomes and ectosomes. Ectosomes are microvesicles or giant vesicles. that range from ~50 nm to 1 μ m in diameter, and they pinch off the surface of the plasma membrane by outward budding. Exosomes are endosomal-derived EVs that range in size from around 40 to 160 nm (average: about 100 nm) in diameter (Kalluri, R., & LeBleu, V. S. (2020)). Researchers have discovered that exosomes include proteins, lipids, cytokines, transcription factor receptors,

nucleic acids, and other bioactive materials. Exosomal protein components fall into two main categories: public components and specific components. The production and secretion of vesicles are facilitated by the public components. These consist of integrins, heat shock proteins (HSP70, HSP90), proteins linked to the ESCRT complex (such as Tsg101, Alix), etc. The other type has specific components. They are derived from antigen-presenting cells and are also cell-specific (Zhang, Y., et al., (2020)). Exosomes, on the other hand, are not very harmful to nearby cells and were once thought to be either cellular waste from cell injury or a byproduct of cell homeostasis. These extracellular vesicles have only lately been discovered to be functional vehicles. Therefore, exosomes are a unique form of intercellular communication that may be important for a variety of cellular functions, including immune regulation, signal transduction, tissue repair, tumor diagnosis and treatment, and pathological functions like metabolic disorders, neurodegeneration, cardiovascular diseases, etc (Zhang, Y., et al., (2019)).

1.2 BIOGENESIS, COMPOSITION, AND CARGO OF EXOSOMES

1.2.1 *Biogenesis*

Every cell is capable of producing unique extracellular vesicles (EVs), and these are classified based on their origin, function, effect on recipient cells, and so on. Each exosome is a ~30–150 nm-diameter vesicle formed by the fusion of several multi-vesicular bodies (MVBs) with the cell's plasma membrane (Zhu, X. (2021)). The formation of MVBs generally starts with the inward budding of the plasma membrane and the formation of early sorting endosomes (ESEs). The sorting of cargo is initiated, and constituents from the Endoplasmic Reticulum (ER), Trans Golgi Network (TGN), and Mitochondria are sorted into ESEs and later into Late Sorting Endosomes (LSEs) (Yuan, Y., et al. (2021)). They eventually lead to the formation of intraluminal vesicles (ILVs) to generate multi-vesicle bodies (MVBs). The outward release of ILVs as exosomes is carried out by the fusion of mature MVBs with plasma membranes. MVBs can also fuse with lysosomes or with autophagosomes to degrade and release their products, which can be further recycled by cells (Baruteau, J et al. (2021)).

1.2.2 Composition

The exosome contains various cargoes, such as proteins, lipids, nucleic acids (DNA and RNA), cytoskeletal proteins, and other metabolites (Zhou, Y. (2021)). The membrane of exosomes is rich in lipid-related proteins such as cholesterol, phosphatidyl serine, ceramide, and sphingomyelin, tetraspanins, lysosome membrane-associated proteins (LAPTH M4B), MVB-associated proteins (ALIX-1), adhesion molecules, antigen-presenting molecules (MHC- I and MHC- II), membrane transporters, and fusion proteins (Rab GTPase and Annexin) (Llorente, A. (2019)).

1.2.3. Cargo Sorting

The contents of intraluminal vesicles (ILVs) depend on the cytoplasmic composition and type of cell where they originate from. The content of the host cell determines the dynamic and functional properties of exosomes. The mechanism of sorting cargo is done through ESCRT-dependent and independent pathways. Other factors such as stress, inflammation, the presence and absence of cytokines, and growth factors also influence the cargo sorting of exosomes (Mittelbrunn, M. (2014)).

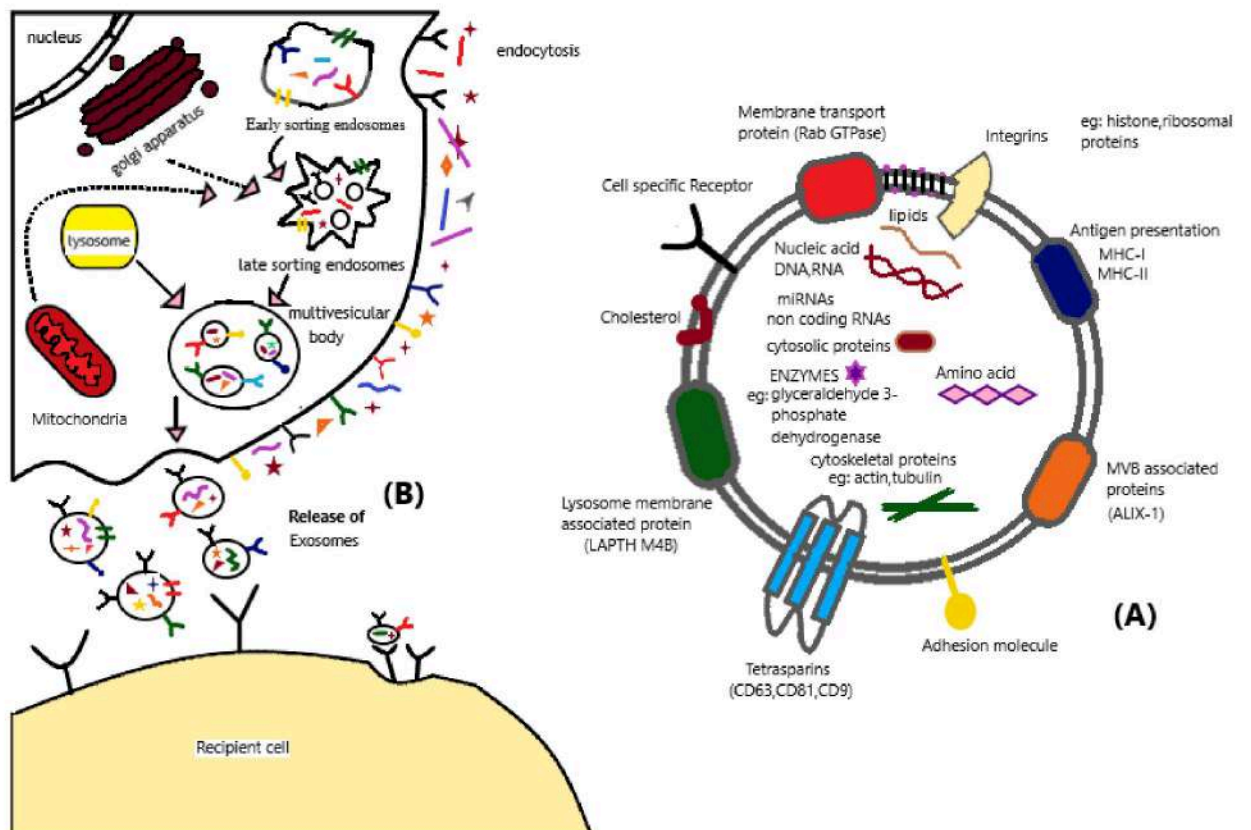


FIG 1.1 (A) Structure and composition of exosome

(B) Schematic representation of the intracellular communication between exosome and the recipient cell.

1.3 PHYSIOLOGICAL AND PATHOLOGICAL FUNCTIONS OF EXOSOMES

Exosomes play a significant role in various physiological processes, contributing to intercellular communication, tissue homeostasis, and overall health. Their ability to transport bioactive molecules, including proteins, nucleic acids, and lipids, makes them key mediators in these functions. Here are some of the physiological functions of exosomes:

- Immune Regulation
- Tissue Regeneration
- Neural Communication
- Drug delivery, etc.

Exosomes are implicated in the pathogenesis of numerous diseases. They are also considered to be an important factor in tumor progression and immunosuppression. Some of the diseases are:

- Cancer
- Cardiovascular Diseases
- Neurodegenerative Disorders etc

1.4 APPLICATIONS OF EXOSOMES

Some of the clinical applications of exosomes include

- Therapeutic Agents: Cancer, Cardiovascular Diseases,
- Neurological disorders
- Diagnostic Biomarkers: Liquid Biopsy
- Immunotherapy
- Skincare Products: Facial Serum, etc.

1.5 EXOSOMES IN NEURAL COMMUNICATION AND NEURODEGENERATION

Exosomes derived from various cell types assume crucial roles not only in the development of the central nervous system (CNS) but also in the maintenance of the adult brain. The interplay between exosomes originating from glial cells and neurons is pivotal in both the development and upkeep of neural circuits. This interaction fosters neurite outgrowth in hippocampal neurons and augments the survival of cortical neurons (Zhang, G., & Yang, P. (2018)). In addition to controlling synaptic activity, astrocytes are essential for preserving the blood-brain barrier's (BBB) integrity. Exosomes produced by astrocytes are essential in mediating a number of processes. They enhance the delivery of Apolipoprotein D to neurons, thereby boosting neuronal survival and enabling neuroprotective responses in neurons grown in hypoxic settings (Yuyama, K., & Igarashi, Y. (2016)). In an in vitro model simulating cerebral ischemia, where neurons were cultured under conditions of heightened stress, exosomes originating from oligodendrocytes demonstrated a protective effect on these cells. They actively bolstered neuron survival amidst oxygen and glucose deprivation. These findings

suggest that exosomes derived from oligodendrocytes have the potential to influence neuronal gene expression, either through the initiation of distant signaling pathways or via the selective transfer of mRNA and miRNA (Pascual, M., Ibáñez, F., & Guerri, C. (2020). Exosomes not only involve the protection of neurons but also play a role in neurodegeneration. In neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and CNS injury, the exosomes have a role in the spreading of neuropathology and neurodegeneration (Saeedi, S., Israel, S., Nagy, C., & Turecki, G. (2019)). ADEV's involved as a carrier of aggregated proteins such as amyloid beta oligomers (amyloid β) and protofibrils (A β) in AD pathogenesis. Prolonged exposure to aggregated A β protofibrils will induce increased production of inflammatory components, leading to progressive synaptic and neural injury that ends in neural dysfunction and dementia. A study demonstrated that the EVs shredded from astrocytes increase the cell's A β exposure in the presence of the NSMase- II enzyme. This shows that the increased production of EVs containing these toxic protein aggregates by astrocytes results in the progression of Alzheimer disease (AD). Parkinson's disease (PD) is characterized by the loss of nerve cells in the part of the brain called the substantia nigra, which are responsible for the production of the happy hormone 'Dopamine'. Astrocytes are also involved in the pathogenesis of PD through the ingestion of aggregated neurotoxic α -synuclein (α -syn) proteins. When astrocyte cells are stimulated by inflammatory lipopolysaccharides (LPS), miR-34a-5p (also referred to as miR-34a) is seen to be enriched in EVs shredded by them. This enhances the sensitivity of dopaminergic neurons to the neurotoxin. It is shown that ADEVs containing miR-34a-5p, when entered into dopaminergic neurons, negatively regulate the anti-apoptotic Bcl-2 protein. This elevated expression of miR-34a-5p results in the progression of PD. However, PD can be silenced by miR-34a-5p inhibitors, and ADEVs rich in miR-34a-5p reduce the neural vulnerability to neurotoxin (Saeedi, S., Israel, S., Nagy, C., & Turecki, G. (2019)).

1.6 miR-132

MicroRNA plays a major role in the progression of neurodegenerative disorders caused by the effects of neurotoxins. MicroRNAs are short, non-coding

RNAs that have 21 to 25 nucleotides. MicroRNAs are important for gene regulation because they negatively regulate the production of specific proteins during the post-translation process (Qian, Y., et al.,(2017)). A special concern is made for mir-132, which is found in the brain tissue of humans, mice, zebrafish, and cattle (Roy, B., et al., (2022)). miR -132 has two homologous RNAs, such as mir-132-3p and mir-132-5p. Dysregulation of miR-132 results in the amplification of neural development, degenerative disorders, neural infections, and psychiatric disorders such as consciousness, depression, and schizophrenia (Mu, C., et al., (2024)). For an effective study of the role of mir-132 in neurodegenerative disorders due to exposure to tartrazine, zebrafish larvae are used as a model organism as they have similar genetics to humans (Li, S., Lei, Z., & Sun, T. (2023).

1.7 ZEBRAFISH (*Danio rerio*)

A small freshwater tropical fish, Zebrafish (*Danio rerio*), is used for our studies. Zebrafish belongs to the family Cyprinidae and is widely used as a model organism for various human diseases, such as cardiovascular diseases, genetic mutations, and neurodegenerative diseases, because they bear resemblance to human neuroanatomical and neurochemical pathways (Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014)). Zebrafish have the great advantage of being used as a powerful toxicological model system and can be used to explore neurotoxic effects on humans. Zebrafish embryos are transparent, convenient to study their development, easy to handle, small, and require less space for maintenance, which makes them low-cost compared with other model organisms such as mice and cell lines (d'Amora, M., & Giordani, S. (2018)). They display a well-developed central nervous system (CNS) by 72 hours post-fertilization (that is, by 3 days), and they also have a similar neuromediator system, including neurotransmitter receptors, enzyme systems, and metabolism as humans. Because of their small embryonic size, they can be easily stored in glass petri plates and grown in cell culture flasks along with embryo medium (E3 medium). Toxin induction can be done simply by adding toxin chemicals directly to the medium. Toxin chemicals can penetrate easily into the transparent membrane by the diffusion process, making them suitable for neurotoxicity screening. Expression of miRNA-132 is analyzed by isolating exosomes from zebrafish larvae because microRNAs are

one of the components of exosomes (Chia, K., Klingseisen, A., Sieger, D., & Priller, J. (2022)).

1.8 PURPOSE OF THE STUDY

The primary objective of this study is to investigate the role of exosomes in neurodegenerative processes using the zebrafish model system. Specifically, we aim to characterize the cargo carried by exosomes in zebrafish experiencing neurodegeneration. Our study will focus on analyzing the composition of exosomes, including mir-132, in order to identify potential biomarkers associated with neurodegeneration.

1.9 HYPOTHESIS

Our hypothesis proposes that tartrazine, an artificial food coloring, could potentially impact the nervous system of zebrafish larvae. Specifically, we suggest that exposure to tartrazine might lead to neurodegeneration by affecting the contents of exosomes, tiny vesicles involved in intercellular communication. We hypothesize that tartrazine could disrupt the normal release and content of microRNA-132 (miRNA-132) within these exosomes, consequently disturbing communication between cells and contributing to neuronal dysfunction. To test this, we plan to investigate the formation, composition, and sorting of exosomes in tartrazine-exposed zebrafish larvae. Additionally, we aim to measure the levels of miRNA-132 within these exosomes and examine its correlation with markers of neurodegeneration. By doing so, we hope to gain insight into how tartrazine might influence neurotoxicity and potentially identify miRNA-132 as a biomarker for neurodegenerative conditions.

CHAPTER 2 - LITERATURE SURVEY

2.1 EXOSOME BIOGENESIS, COMPOSITION AND FUNCTIONS

Exosome originate from the endocytic pathway. The process of exosome production starts by the formation of Early Sorting Exosomes (ESEs) which invaginate from the plasma membrane to form intracellular vesicles. These ESEs eventually mature into Late Sorting Exosomes (LSEs). Small intraluminal vesicles (ILVs) are formed inside the cavity of LSEs which together lead to the formation of Multivesicular Bodies (MVBs). These MVBs will either fuse with lysosomes for degradation or with plasma membrane, releasing their contents as exosomes into extracellular space. The mechanism of exosome biogenesis, composition, cargo sorting mechanism and their effects on other recipient cells were explained by (Huiying Liang, et al 2020).

The cargo sorting mechanism plays a major role in many physiological functions of exosomes. Cargo sorting of exosome is a critical process which significantly influence their physiological functions. Their particular packing of specific molecules into the exosome allows them to carry out accurate functions such as intracellular communication, tissue homeostasis and disease modulation. The knowledge on exosome biogenesis and release was discussed by Hessik. N and Llorente. A (2018). The cargo consists of lipids, proteins, nucleic acids and other regulatory factors. Exosomes have been implemented in various physiological functions such as inter-cellular communication, cellular signalling pathway, stress response, immune regulation, disease pathogenesis and many applications in variety of areas. The underlying mechanism of exosome biogenesis and secretion was explained by Thery.C, Witer.K et al (2018).

2.2 ROLE EXOSOME IN DISEASE PATHOGENESIS

Exosomes plays a major role in pathogenesis of various diseases. As they act as a mediator for inter cellular communication, they can easily transfer biomolecules from one cell to another cell. Exosomes has role in cancer pathogenesis, particularly by involving in promotion of metastasis through the transfer of bioactive molecules (Li. Y, Yin Z et al 2018) . Recent studies show that exosomes play an important role in growth, invasion, and metastasis of lung

cancer and controlling cell to cell signal transduction (Wv.X, Luo. H et al 2018). The Epithelial- Mesenchymal transition (EMT) is a process where the epithelial cells of liver loses cell polarity and adhesion properties and acquire motility and invasive property of mesenchymal cells. Throughout this process, E-cadherin (Tumor suppressor protein) will get decreased or absent and N- cadherin (Adhesion molecule) and Vimentin (structural protein) are over expressed. This EMT process plays an important role in the expression of highly aggressive mesenchymal tumor cells to promote proliferation and metastasis of tumor.

A study made by Zhi Xian Tang et al.,2023 shows us EMT transcriptional factors (SNAIL-1, SNAIL-2 OR SLUG) contribute significantly to the development of EMT, induces the expression of genes such as Vimentin (VIM), N- cadherin (CDH2). Exosomes obtained from sera of patient with advanced lung cancer have high expression of VIM which shows that exosomes can trigger EMT and induce lung cancer

Exosomes derived from neural tissues and CNS have great potential in pathogenesis of neurodegenerative diseases. ADEV's involved as a carrier of aggregated proteins such as amyloid beta oligomers (amyloid β) and protofibril ($A\beta$) in AD pathogenesis (Yuyama, K and Igarashi Y 2016). Prolonged exposure to aggregated $A\beta$ protofibrils will induce increased production of inflammatory components leading to progressive synaptic and neural injury which ends in neural dysfunction and dementia. A study demonstrated that the EVs shedded from astrocytes increase the cell to $A\beta$ exposure in the presence of NSMase- enzyme. This shows that the increased production of EVs containing these toxic protein aggregates by astrocytes results in progression of Alzheimer disease (AD) (Kapogiannis. D et al 2019).

Parkinson's disease (PD) is characterized by loss of nerve cells in the part of brain called as the substantia nigra which are responsible for the production of happy hormone 'Dopamine'(Paola Feraco et al 2021). Astrocytes also involve in the pathogenesis of PD by ingestion of aggregated neurotoxic α - synuclein (α -syn) protien (Jennifer R, Pinell et al 2021). When astrocyte cells are stimulated by inflammatory Lipopolysaccharides(LPS), miR- 34a-5p(also referred as miR-34a) seen to be enriched in EVs shedded by them. This enhances the sensitivity of dopaminergic neurons against the neurotoxin. It is shown that Astrocyte Derived Extracellular Vesicle (ADEVs) containing miR-34a-5p, when entered into the

dopaminergic neurons, negatively regulates the Anti -apoptotic Bcl-2 protein. This elevated expression of miR-34a-5p results in progression of PD (Haiyang yu et al 2020). However, PD can be silenced by miR-34a-5p inhibitor within ADEVs rich in miR-34a-5p reduce the neural vulnerability towards neurotoxin. Exosomes also involved in inflammatory disease. Inflammation is a kind of immune response when the cells are subjected to any kind of infections (Bardon Dow Chan et al 2019). The inflammation was triggered by active production of neutrophils and monocytes where they produce chemokines which was derived from exosomes triggered by infected tissue. The underlying mechanism was explained by Yuan Tian et al., 2022.

Exosomal cargo contains DNA and RNA along with other associated proteins. Therefore, exosomes from infected cells tend to have genetic materials of particular micro-organism (Rajesh kumar et al 2021). In viral infection ,diseases such as HIV, members of Flaviviridae family- Dengu, West Nile virus (WNV), Hepatitis C virus (HCV) ,the infection are mediated through the production of ILVs with the viral genome which is dumped into the cell's cytoplasm.(Cheng Peng et al., 2020).

Bacterial components are most known to involve in triggering the biogenesis and release of exosomes as immune response during any bacterial infection (Sang Ho Choi et al., 2020). Among many bacteria *Mycobacterium tuberculosis* is the best example to study about exosomes and its effects on immune system (Chiranjit Mukhopadhyay et al., 2020). Mtb infection can stimulate production of various kinds of exosome which triggers immune cells such as macrophages, DC, T-cells and B-cells (Neeraj K. Saini et al., 2019).

In parasitic infection such as Malaria, Leishmaniasis, Toxoplasmosis, Trypanosoma and more are spread through blood(Xin Zhanget al.,2023). The pathogenesis of infection is mediated by many kinds of EVs. Several parasites such as *Plasmodium falciparum*, *Leishmania infantum*, *Toxoplasma gondii* and *Trypanosoma cruzi* are tend to produce exosomes during infection.(Yue Yuan et al.,2021)

Study made by Daniel L Klotz et al (2020) shows that the further spreading of infectious disease caused by bacteria, virus, fungi and parasites can be easily progressed by exosomes.

2.3 EFFECTS OF TARTRAZINE

Tartrazine, a potentially harmful dye which has been associated with allergic reaction in some people including skin rashes, hives, itching, swelling and difficulty in breathing (Nema Allah Hosieny et al., 2021). The effect of tartrazine and its reaction may differ from person to person and can range from mild to severe and may need medical attention. In rare cases, tartrazine may trigger asthma symptoms or respiratory issues who are most sensitive to dye. It is also known that it may trigger migraines and headaches in some individuals (Amina E Essawy et al., 2022). Some also experience digestive issues such as nausea, vomiting, abdominal pain, and diarrhoea after consumption of tartrazine containing food.

Tartrazine have genotoxic and cytotoxic effects especially in human peripheral blood lymphocytes. In some In-vitro studies suggests that tartrazine causes DNA damage and alteration in cell viability making them a potential carcinogenic agent under certain conditions (Diksha Bhatt et al., 2018). It also acts as a neurotoxin and pose their neurotoxic effects on spatial memory, motor coordination and oxidative stress on human neural system. The neurotoxic effect of tartrazine on humans was explained by Abdel Wahab et al., 2020.

2.4 BEHAVIORAL CHANGES IN CHILDREN BY TARTRAZINE

Mc Cann, Cooper et al 2007., investigating the effects of food additives, artificial colorings on hyperactivity behavior in children. The test trial was made with 3 year old and 8/9 year old children. The study found out that consuming of dye containing benzoate create hyperactivity among kids.

The relationship between dietary factors including addition of food colourings and neurodevelopmental condition such as ADHA and ASD was explained by Munger. KL, O'Reilly et al (2013). This study provide us that some evidents supports a possible link between food colourings and behavioral symptoms and there is a need for further research to understand the mechanism and potential impact of food colourings for clinical practise.

The risk of ASD is raising due to the environmental exposure to neurotoxic compounds such as heavy metal and pesticides. As tartrazine like azo dyes contains heavy metals in them, it's significant role of prenatal and early- life

exposure to these compounds in increasing ASD susceptibility was studied by a meta analysis made by Rossignol DA et al (2014).

The consumption of beverages containing artificial food colourings especially tartrazine will increase the risk of behavioural complication such as Inattention which lead to decreased attention and difficulty in focusing, agitations, mood swings, aggressiveness, anxiety and sleeplessness in children (Daniel more MD 2022)

2.5 ZEBRAFISH AS A MODEL ORGANISM

Zebrafish serves as a valuable model for studying various human diseases, including cancer, cardiovascular disorders, neurological disorders, and metabolic syndromes (Kristin M kwan et al., 2021). Classic studies have elucidated key developmental pathways, including embryogenesis, organogenesis, and tissue regeneration. Research often involves genetic manipulation to induce disease phenotypes or screening for small molecules that modulate disease progression (Yu bo shi et al., 2021).

Zebrafish is used to assess the toxic effects of environmental pollutants, pharmaceuticals, and chemicals (Xianhong Meng et al., 2018). Their rapid development and transparency allow for real-time observation of toxicological endpoints, such as embryonic development, organ morphogenesis, and behavioral changes (Hui zhang et al., 2020). The key research involves screening of effects of heavy metals and pesticide. (Yuanyuan Liu et al., 2022)

Researchers use zebrafish to screen chemical libraries for compounds with therapeutic potential, particularly in the fields of oncology, infectious diseases, and neuropharmacology (Zhibin Guo et al., 2023). Zebrafish provide an efficient platform for drug discovery and high-throughput screening due to their small size, high throughput capabilities, and physiological relevance to humans (Randall J Nonneman et al., 2019).

The field of zebrafish research continuously evolves with the development of novel techniques and technologies (Gorge E Ekker 2020). Advances in genome editing (e.g., CRISPR/Cas9), live imaging, optogenetics, and microfluidics enhance the utility of zebrafish as biomedical models. (Feng zhang et al., 2022).

2.6 ROLE OF MIR- 132 IN NEURODEGENERATION

MicroRNA-132 (miR-132) plays a crucial role in neurodegeneration by regulating various cellular processes involved in neuronal survival, synaptic plasticity, inflammation, and neuro inflammation. Dysregulation of mir-132 production will lead to various neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and Huntignton's disease (Liang Cet al.,2019).

CHAPTER – 3 AIM AND OBJECTIVES

3.1 AIM

The aim of the research is to investigate the role of exosomes in zebrafish larvae (*Danio rerio*) neurodegeneration .

3.2 OBJECTIVES

In order to accomplish the above aim, the following objectives are outlined:

- To obtain and maintain zebrafish embryos into larvae at specific developmental stages (e.g.,24 hours post-fertilization(hpf),48 hpf,72 hpf,96hpf etc..)
- To induce neurodegeneration in the larvae exposing to an appropriate neurotoxin between 18hpf to 96 hpf and observe their abnormalities.
- To isolate the exosomes from the Zebrafish larvae at a specific developmental stage 96 hpf.
- To extract the miRNA from the isolated exosomes and perform RTPCR to analyse the expression of miRNA-132.

CHAPTER 4 MATERIALS AND METHODS

4.1 MATERIALS REQUIRED

Duran Bottles, Tissue culture flasks, Droppers, 18 gauge needles, Pipette and pipette tips, Centrifuge tubes (2ml, 15ml, 50ml), Zebrafish larvae, Phosphate buffer saline, Perfusion buffer, E3 medium, Tartrazine

4.2 METHODOLOGIES

4.2.1 Preparation of Phosphate Buffer Saline (PBS)

Phosphate buffer saline, also called as PBS is one of the most commonly used buffer in different biological researches especially for maintaining pH of the solutions. It is an isotonic solution, that is the ionic content of PBS mimics the human body fluids. So it will prevent any cell damage during cell culturing, prevent toxicity and unwanted precipitation in biological, medical and biochemical solution. Generally the buffer contains sterile water, sodium hydrogen, phosphate, and sodium chloride. Depending on our purpose we can make 1X, 5X and 10X PBS. Here we prefer 10X PBS for our research.

80.1g of sodium chloride, 14.4g of disodium hydrogen phosphate, 2g of potassium chloride and 2.7g of potassium dihydrogen phosphate was measured and added into a duran bottle containing 800ml of sterile distilled water. The solution was mixed well and the pH was adjusted to 7.4 using diluted Hydrochloric acid. Finally, the total volume was made up to 1 litre by the addition of sterile distilled water. Thus, PBS was prepared.

4.2.2 Preparation of E3 medium (embryo medium)

E3 medium, also called as embryo medium is used for growing zebrafish embryos. The purpose of the medium is to provide essential nutrients, growth factor and they can control environmental variables such as pH, temperature, osmolarity and prevent any bacterial and fungal infections as it contains methylene blue dye which act as anti-bacterial and anti-fungal agent.

1.74g of Sodium chloride, 0.08g of Potassium Chloride, 0.29g of Calcium chloride dehydrate and 0.489g of Magnesium dichloride dehydrate was measured and added into 100ml of sterile distilled water. The solution was mixed well and this solution is known as E3 Stock solution. To prepare E3 working medium, 33 ml of the stock solution was measured and added to 2 litres of sterile distilled water. Finally, 200 μ l of Methylene blue was added. Thus, the E3 medium was prepared for the development of embryos (Fig: 4.1)



Fig: 4.1: E3 Medium

4.2.3 Zebrafish animal husbandry and breeding

- Adult Zebrafish were maintained at a temperature of 28 ± 0.5 °C using the Submersible heater, lighting conditions as 14 hours-light and 10 hours-dark cycle at pH between 7.0 to 8.0.
- The zebrafish were bred at Aquatic research laboratory, Sathyabama Institute of Science and Technology, Chennai.
- The Female zebrafish were distinguished from males with large silver blue streaks underneath whereas males were yellowish.
- Breeding was performed by transferring two males and four females to mating tank, a transparent barrier was placed in the tank to separate male and females and the tanks were left overnight and the barriers were removed the following morning and fishes were left to spawn for 0.5-0.6 hours.
- Fertilized eggs were collected from the breeding tank with the help of sieve. The collected embryos were immediately rinsed with water and transferred into culture flask with E3 medium and incubated at 28 °C. Dead individuals were removed and E3 medium was changed on a daily basis.

4.2.4 Tartrazine Exposure Procedures

- The 18 hours post fertilization embryos were obtained and grown in E3 medium. 30 embryos were added to 40ml of E3 medium in each cell culture flask. (Fig:4.2.1)
- Embryos were grown into several developmental stages such as 24hpf, 48hpf, 72hpf and 96hpf larvae which could be used as control.
- For obtaining neurotoxin induced larvae, 5.5ppm tartrazine (8.25µl) was added into the medium containing 18hpf embryos.
- In each stage, the media was regularly changed and the fresh media was added.
- Total number of live and death larvae were counted until 96 hpf larvae was obtained.

- Larvae of each developmental stage were monitored using microscope for any abnormal growth.
- 96 hpf larvae (both control and tartrazine induced) were finally obtained and used for exosome isolation.(Fig:4.2.2 – 4.2.7)

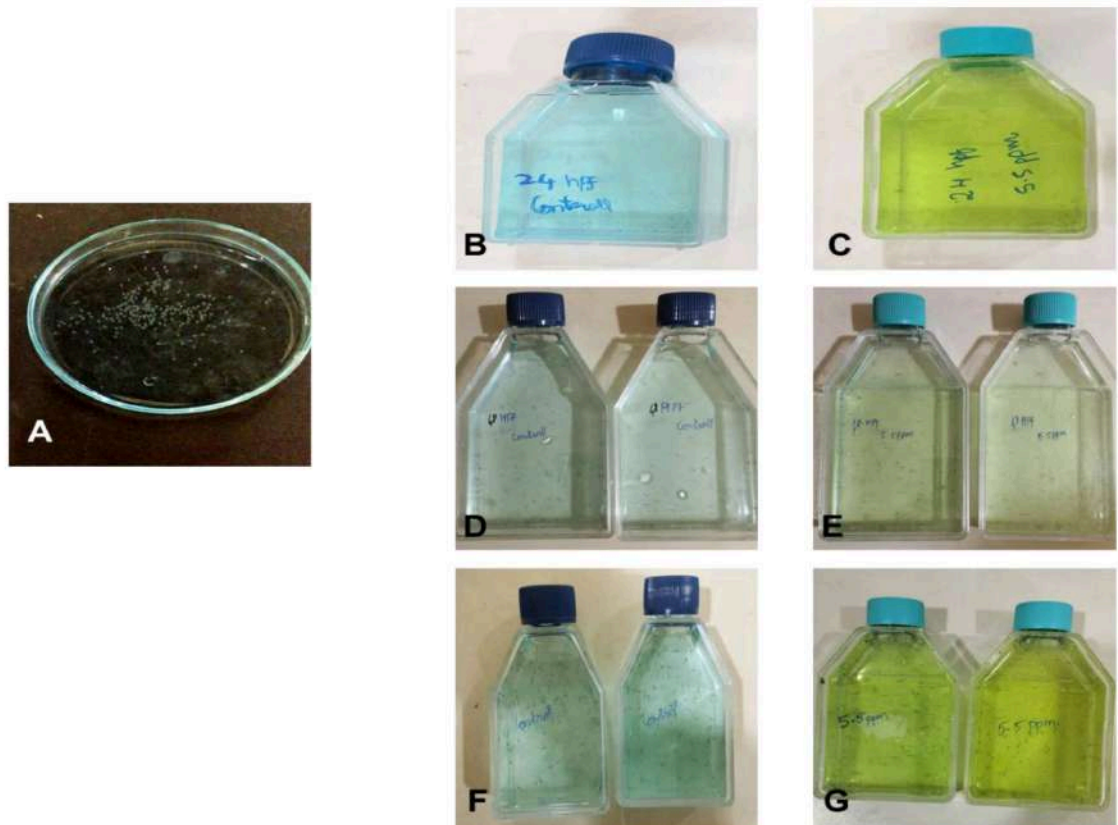


Fig 4.2.1: (A) 18hpf zebrafish embryos obtained from Aquatic research laboratory.

Fig 4.2.2 (B) and Fig 4.2.3 (C): Showing 24hpf larvae as control and TTZ induced in E3 medium respectively

Fig 4.2.4(D) and Fig 4.2.5 (E): Showing 48hpf larvae as control and TTZ induced in E3 medium.

Fig 4.2.6(F) and Fig 4.2.7(G): Showing 96hpf larvae as control and TTZ induced in E3 medium.

4.2.4.1 Locomotor analysis

The locomotor activity of zebrafish embryos at 24 hpf was assessed by observing tail movements under a stereo zoom microscope. For embryos and larvae between 48 hpf and 96 hpf, locomotor activity was measured in a marked petri dish with grid lines, and the distance traveled by the embryos or larvae was calculated (Xia, J., Niu, C. and Pei, X. (2010)).

4.2.5 Perfusion Buffer

2ml of 1M HEPES Buffer was diluted with 198 ml of distilled water to prepare 200ml of 10mM/L HEPES Buffer solution. Then, 200ml of HEPES Buffer solution and 0.9g of glucose was added to 500ml of 10XPBS Buffer solution. The pH was checked using a pH paper and the pH was 7(neutral). Finally, the volume was adjusted to 1l using a 10XPBS Buffer solution. Then, the Perfusion Buffer was autoclaved, cooled and was stored in a refrigerator to maintain its stability.

4.2.6 Isolation of Exosomes from Zebrafish larvae

Using ultracentrifugation, exosomes are separated according to their size, shape, and density using strong centrifugal forces. Exosomes are separated from other extracellular vesicles and impurities using a series of procedures that include ultracentrifugation. (Scott, A.,et al.,(2021))

- The control medium larvae and the tartrazine induced larvae were allowed to react with Perfusion buffer, 0.25% of Trypsin and 12.5 $\mu\text{mol/L}$ of Calcium chloride solution for the dissociation of cells.
- The samples were incubated at room temperature in the dark conditions for 1 hour.
- The reaction was stopped by adding PBS buffer, 10% (v/v) Fetal Bovine Serum and 12.5 $\mu\text{mol/L}$ of Calcium chloride solution.
- The cells were then centrifuged at 4°C for
 - 300g (2*10 minutes)

- 1200g (2*10 minutes)
- 10000g (30 minutes)
- Only the supernatant was taken and the pellet was discarded.
- The crude exosomes were obtained by centrifuging at 118000g for
- 1 hour 54 minutes at 4°C.
- The pellet obtained was resuspended in 300 µl of PBS solution.
(Fig: 4.2.8, 4.2.9)
- The isolated exosomes were stored at -20°C to maintain its stability

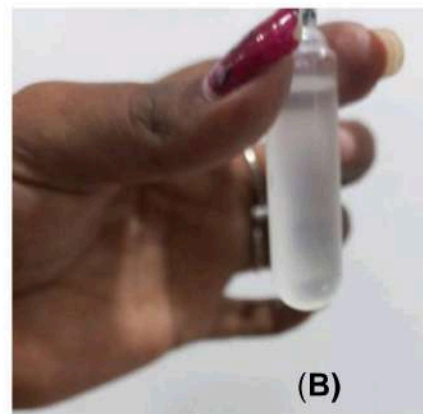
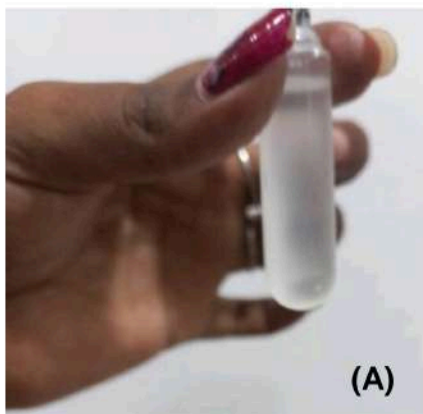


Fig 4.2.8 (A): Showing obtaining of crude exosome pellet from 96hpf control larvae by ultracentrifugation.

Fig 4.2.9 (B): Showing obtaining of crude exosome pellet from 96hpf TTZ induced larvae by ultracentrifugation.

4.2.7 Lyophilization of Exosomes

4.2.7.1 Principle of lyophilization

Lyophilization is one of the important technique, also called as freeze drying is a process of elimination of moisture or water content from sample by sublimation process. The sample undergoes a series of stages where the moisture is slowly removed without involving into liquid phase. Lyophilization of samples made them

more liable and stable than non lyophilized samples. Lyophilized samples are stored in room temperature for many months when compared with the non lyophilized samples. This process enables minimal contamination of sample as it is undertaken in controlled environment.

4.2.7.2 Steps involved in Lyophilization

1. Pre-treatment

This involves in preparation of sample before primary freezing. This process involves in improving concentration of product, formulation revision such as increasing stability, decreasing a high vapour pressure, increasing surface area. Samples needed to be lyophilized were filled inside a glass vial or in petriplate.

2. Freezing and annealing

Freezing also referred as pre - freezing where the sample is frozen below to its 'Eutetic point' or safe freezing point. The temperature here ranges from -40°C to -60°C. Once the freezing point of the product is determined by thermal analysis, the required rate of freezing also needed to be determined.

3. Primary drying

Primary drying begins when sublimation process starts (I.e the ice turn into vapour) under low pressure typically below to 0.01 hPa (m Bar) depending on the pre-treating of sample. The vacuum will help to improve the speed of drying process by removing air molecule from chamber and allowing easier removal of vapour molecule from sample.

The temperature of sample was under monitored by small sensors into the glass vials inside the chamber. The temperature here will be upto -40°C to -20°C. Primary drying is long and time consuming steps in overall steps until the sample reaches its eutetic points.

4. Secondary drying

Once the sample reaches its eutetic point, the secondary drying will start. In this step the low pressure created by vacuum enable the removal of solvents and make

products dry. This step of whole process is typically represent only half of time of cycle but it was very important for removal of final moisture content.

Once the products entered into at the end of cycle, the sample were removed. Usually glass vials were closed under vacuum and appropriately stored.



Fig: 4.2.10: Lyophilizer



Fig: 4.2.11: Lyophilized Exosomes

4.2.8 Scanning Electron Microscope

4.2.8.1 Principle of scanning electron microscope

SEM is a kind of an electron microscope that scans the surface of microorganism by using a beam of electron moving at low energy to scan the thin specimens enables a high resolution power.

The principle of SEM works by a beam of electron which was formed by electron source and accelerate toward the specimens. The electron beam is then condensed by metal aperture and magnetic lenses into a focused monochromatic beam. The electron in the beam engage with the atoms of specimens and produce signals which contains the information about the surface topography, composition and other properties of the specimens. The signals obtained were transformed into image by system connected with it.(Relucenti, M.,. (2021))

4.2.8.2 Working of SEM

- The source of the electrons and the electromagnetic lenses were from tungsten filament lamps which was placed at the top of the column ,similar to those of the transmission electron Microscope.
- The electrons were emitted after thermal energy is applied to the electron source and allowed to move in a fast motion towards anode, which act a positive charge.
- This beam of electrons activates the emission of primary scattered (Primary) electrons at high energy levels and secondary electrons at low-energy levels from the specimen surface. The beam of electrons engage with the specimen to produce signals which have information about the surface topography and composition of the specimen.
- Microbial specimens need fixation, dehydration, drying and lyophilization in order to maintain the structural features of the cells and to prevent rupturing of the cells when exposed to the high vacuum of the microscope.
- The samples are mounted to the carbon tags and coated with thin layer of conducting metal elements such as gold to allow spatial scattering of electric charges on the surface of the specimen allowing better image production, with high clarity.

4.2.9 Isolation of RNA from Exosomes

4.2.9.1 Principle

Trizol reagent is a ready-to use reagent used for RNA isolation from cells and tissues. Trizol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cell and cell components. Addition of chloroform, after the centrifugation separates the solution into aqueous and organic phase, RNA remains only in the aqueous phase.(Wijenayake,S., et al.,(2021)).

4.2.9.2 Procedure

Zebrafish embryos (50) were taken into 2 mL microcentrifuge tubes with Pasteur pipette and homogenized with 300 μ l of Trizol and were vortexed, followed by incubating for 5 minutes at room temperature. To the solution, 200 μ l of chloroform was added and it was mixed and incubated for 15 minutes at room temperature. Later, the samples were subjected to centrifuged for 12,000 rpm for 10 minutes at 40 °C and the aqueous layer was transferred to the new tube. To the tube, 1ml of ice-cold isopropanol was added and mixed for 10 seconds. Later, the sample tube kept at -20 °C for overnight. On the next day, tubes were centrifuged for 12,000 rpm for 10 minutes at 40 °C.

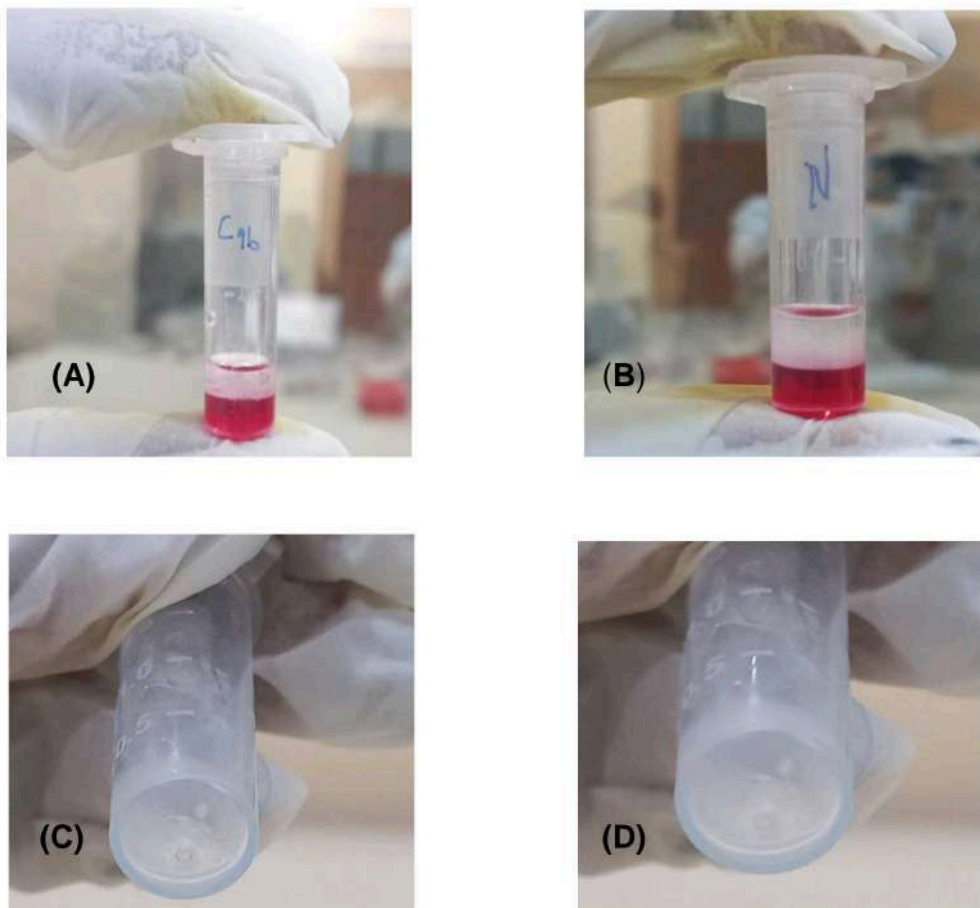


Fig 4.2.12 (A) and Fig 4.2.14(C): Showing phase separation of RNA from 96hpf control exosomes and obtained RNA pellet.

Fig.4.2.13 (B) and Fig 4.2.15(D): Showing phase separation of RNA from 96hpf tartrazine induced exosomes and obtained RNA pellet.

4.2.10 Quantification of RNA Using Micro Plate Reader

Quantification of RNA is a crucial step in numerous biological and biochemical assays, as it provides essential information about the concentration and purity of RNA samples. One common method for quantifying RNA is using a microplate reader, which allows for the simultaneous measurement of multiple samples in a high-throughput manner. It is commonly used in molecular biology and genetics studies to determine the concentration of RNA for downstream applications such as reverse transcription, real-time PCR, and RNA sequencing. The principle of quantifying RNA using a microplate reader is based on the measurement of absorbance at specific wavelengths. RNA has a characteristic absorbance peak at around 260 nm due to the presence of nucleotide bases, which allows for its quantification based on the Beer-Lambert law. When RNA samples are placed in a microplate reader, the absorbance at 260 nm is measured, and the concentration of RNA is determined based on a standard curve generated using known concentrations of RNA. Additionally, the absorbance at 280 nm is also measured to assess the purity of the RNA sample, as it provides information about the presence of contaminants such as proteins and phenol.

4.2.11 cDNA Transcription

Complementary DNA transcription, or cDNA transcription, is a basic molecular biology process with several scientific and therapeutic implications. Reverse transcriptase, an enzyme, is used in this procedure to create a single-stranded DNA molecule from a single-stranded RNA template. The generated cDNA molecule may be applied to several types of research and diagnostics as it is complementary to the original RNA template. Studying gene expression and regulation is one of the main goals of cDNA transcription. Researchers can examine the transcriptome of a cell or tissue—the entire collection of RNA molecules generated in that specific biological sample—by turning RNA into cDNA. This information can provide insights into which

genes are actively being transcribed and how their expression levels change under different conditions. (Mohr, G., et al., (2023))

- 15µl of control RNA was mixed with 4µl of iscript reaction mix and 41µl of tartrazine induced RNA was mixed with 8 µl of iscript reaction mix.
- Then, 1µl of iscript Reverse transcriptase enzyme were added to both the samples.
- The samples were made to run in a thermal cycler.
- The steps involved in a thermal cycler includes :
 - Priming – 5 minutes at 25°C
 - Reverse Transcription – 20 minutes at 46°C
 - Reverse Transcription Inactivation – 1 minute at 95°C
 - Hold at 4°C.
- Therefore, the final volume of control cDNA is 20µl and the tartrazine induced cDNA is 50µl. They were stored at -20°C for further usage.



Fig: 4.2.16: cDNA Transcription

4.2.12 Real Time PCR

Real-time PCR (also known as quantitative polymerase chain reaction) is a molecular biology technique which is used to amplify and quantify a targeted nucleic acid in real-time. Real-time PCR is based on the principles of traditional PCR, but with the incorporation of a fluorescent reporter molecule that allows for the real-time detection and quantification of amplified nucleic acids. This is achieved by using

fluorescent dyes or probes that emit fluorescence as the amplification of the target nucleic acid progresses.

The working principle of real-time PCR involves several key steps. First, the sample containing the target nucleic acid is mixed with the PCR reagents, including primers, nucleotides, and DNA polymerase. The primers are designed to specifically anneal to the target sequence, and they define the region of DNA to be amplified. The DNA polymerase then extends the primers, synthesizing new DNA strands complementary to the target sequence. During the amplification process, the fluorescence signal from the fluorescent dye or probe increases as the amount of amplified DNA also increases. The real-time PCR instrument continuously monitors the fluorescence signal, and the data is used to generate a quantification curve. This curve is used to determine the initial amount of the target nucleic acid in the sample based on the cycle threshold (Ct) value, which represents the cycle number at which the fluorescence signal exceeds the background level.

Real-time PCR offers several advantages over traditional PCR and other DNA quantification methods. One of the main advantages is its high sensitivity and specificity, allowing for the accurate detection and quantification of target DNA molecules. It also has a wide dynamic range, capable of detecting a broad range of DNA concentrations. Additionally, real-time PCR is a rapid and efficient technique, providing results in a relatively short amount of time. It is also less prone to contamination, as it is a closed-tube system, reducing the risk of false-positive results. Furthermore, real-time PCR is highly adaptable and can be used for a variety of applications, making it a versatile tool for researchers (Artika, I. M., et al., (2022))

4.2.12.1 Real-Time PCR for miR-132

Quantitative RT-PCR analysis of miR-132 was performed using Mir-X miRNA. qRT-PCR SYBR Kit (Takara) with a specific forward primer and an adaptor PCR primer and along with universal Reverse primer (Xcelris). RU5 used as an internal control. Real-time PCR was performed at 95°C for 10 min, followed by 40 cycles at

95°C for 5 s and 60°C for 30 s. The test was conducted in duplicates with 25µL reaction. Q-PCR data was analyzed quantitatively by using the formation of $2^{-\Delta Ct}$.

Table: 4.1: Primer pairs used for quantifying the miR132 level

miR-132	Stem-loop	5' - GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AGTAAC - 3'
	Forward primer	5' - GTTTG ACCGTGGCTTTTCGA - 3'
	Universal reverse primer	5' - GTGGGTGCATTGTAGTTGC - 3'

4.2.13 Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) for the assessment of survival rate and locomotor activity. Additionally, statistical significance was determined for miR-132 expression using p-values obtained from comparisons between control and Tartrazine-treated groups.

CHAPTER 5 RESULTS AND DISCUSSION

5.1 Zebrafish Toxicity Induced by Tartrazine Exposure: Survival Rate and Developmental Abnormalities

In our analysis, we assessed the adverse effects of TTZ (5.5ppm) exposure on zebrafish, specifically focusing on survival rate and developmental abnormalities (Figures 5.1, 5.2 and 5.3).

5.1.1 Survival rate

Zebrafish embryos exposed to Tartrazine at a concentration of 5.5 ppm did not exhibit any significant impact on survival rates compared to the control group.

Survival rates remained comparable between the control and experimental groups throughout the experimental period, with no observable mortality events recorded (Figure 5.1). Data analysis using ANOVA relative to control showed no statistically significant differences in survival rates (Mean $\pm$ SEM) between the control and experimental groups (n=100).

This finding suggests that acute mortality events were not induced by Tartrazine at the tested concentration, highlighting a lack of immediate lethal toxicity under these experimental conditions.

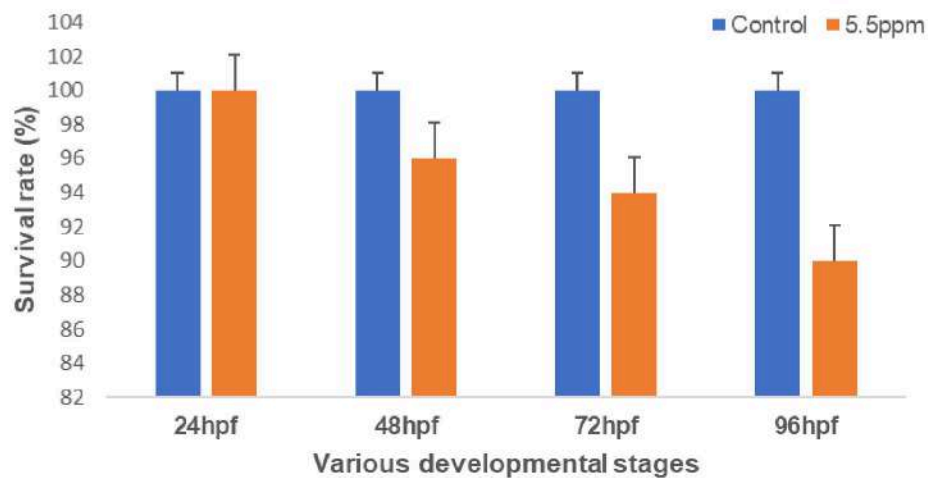


Fig: 5.1: Survival rate of zebrafish embryos during exposure to TTZ (5.5ppm). Data are presented as Mean $\pm$ SEM (ANOVA relative to control) n=100.

5.1.2 Morphological characteristics

Morphological characteristics of zebrafish embryos were evaluated from 24 hours post-fertilization (hpf) to 96 hpf following exposure to Tartrazine using a stereo zoom microscope (Figure 5.2). Specific features assessed included cardiac edema, bent tail, curved spine, body pigmentation, and yolk sac accumulation. Morphological changes such as a bent tail and curved spine structure were notably observed in zebrafish embryos exposed to 5.5 ppm Tartrazine at 72 hpf and 96 hpf.

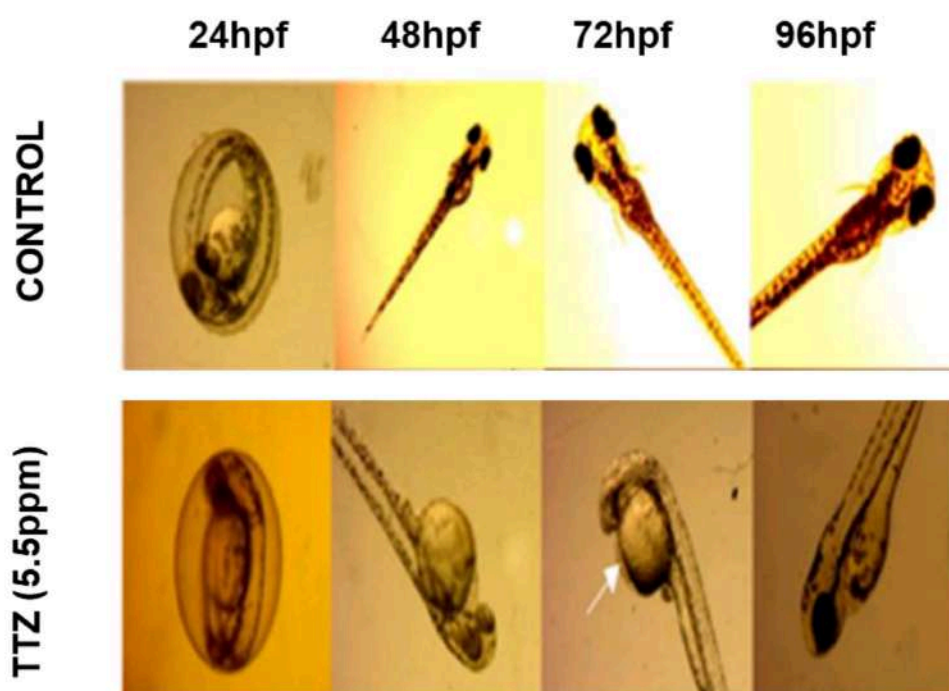


Fig: 5.2: Morphological characteristics of zebrafish embryos assessed from 24hpf to 96 hpf following exposure to Tartrazine using stereo zoom microscope

5.1.3 Locomotor Activity

The locomotor behavior of zebrafish embryos and larvae serves as a sensitive indicator of neurodevelopmental effects induced by chemical exposures. In this study, we evaluated locomotor changes in zebrafish embryos and larvae following exposure to Tartrazine (TTZ) at various developmental stages. At 24 hours post-fertilization (hpf) and 96 hpf, zebrafish embryos exposed to Tartrazine did not exhibit significant differences in spontaneous tail movements compared to the control group. Statistical analysis of tail movement frequencies did not reveal any significant differences between the Tartrazine-exposed groups and the control group at either developmental stage.

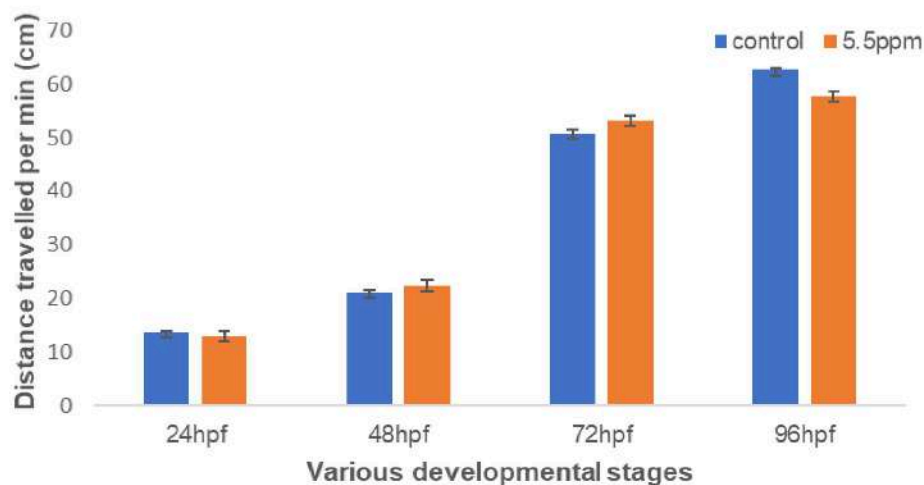


Fig: 5.3: Locomotor Changes in Zebrafish Exposed to TTZ (5.5ppm). This figure depicts the locomotor changes observed during exposure TTZ. Bars indicate SEM values. Statistical analysis was performed using ANOVA, relative to the control group. $n = 5$.

The results of our study provide insights into the toxicological effects of Tartrazine exposure on zebrafish embryos. Despite the absence of acute mortality events, exposure to Tartrazine at 5.5 ppm induced notable morphological abnormalities, including a bent tail and curved spine structure, indicating potential developmental toxicity. These findings align with previous studies suggesting that Tartrazine may disrupt normal embryonic development. The presence of bent tails and curved spine structures in Tartrazine-exposed embryos is consistent with previous findings linking Tartrazine to musculoskeletal defects and spinal deformities in zebrafish. These morphological abnormalities may reflect disruptions in the normal development of the axial skeleton and musculature, potentially attributable to Tartrazine-induced teratogenic effects. (Joshi, V., & Katti, P. (2018))

The lack of significant differences in locomotor activity between Tartrazine-exposed groups and the control group suggests that Tartrazine exposure did not elicit

pronounced effects on early-stage locomotor behaviour in zebrafish embryos. However, it is essential to note that locomotor activity is just one aspect of neurodevelopmental effects, and further investigations are warranted to assess other aspects of neurotoxicity induced by Tartrazine exposure.

5.2 CHARACTERIZATION OF EXOSOMES USING SEM

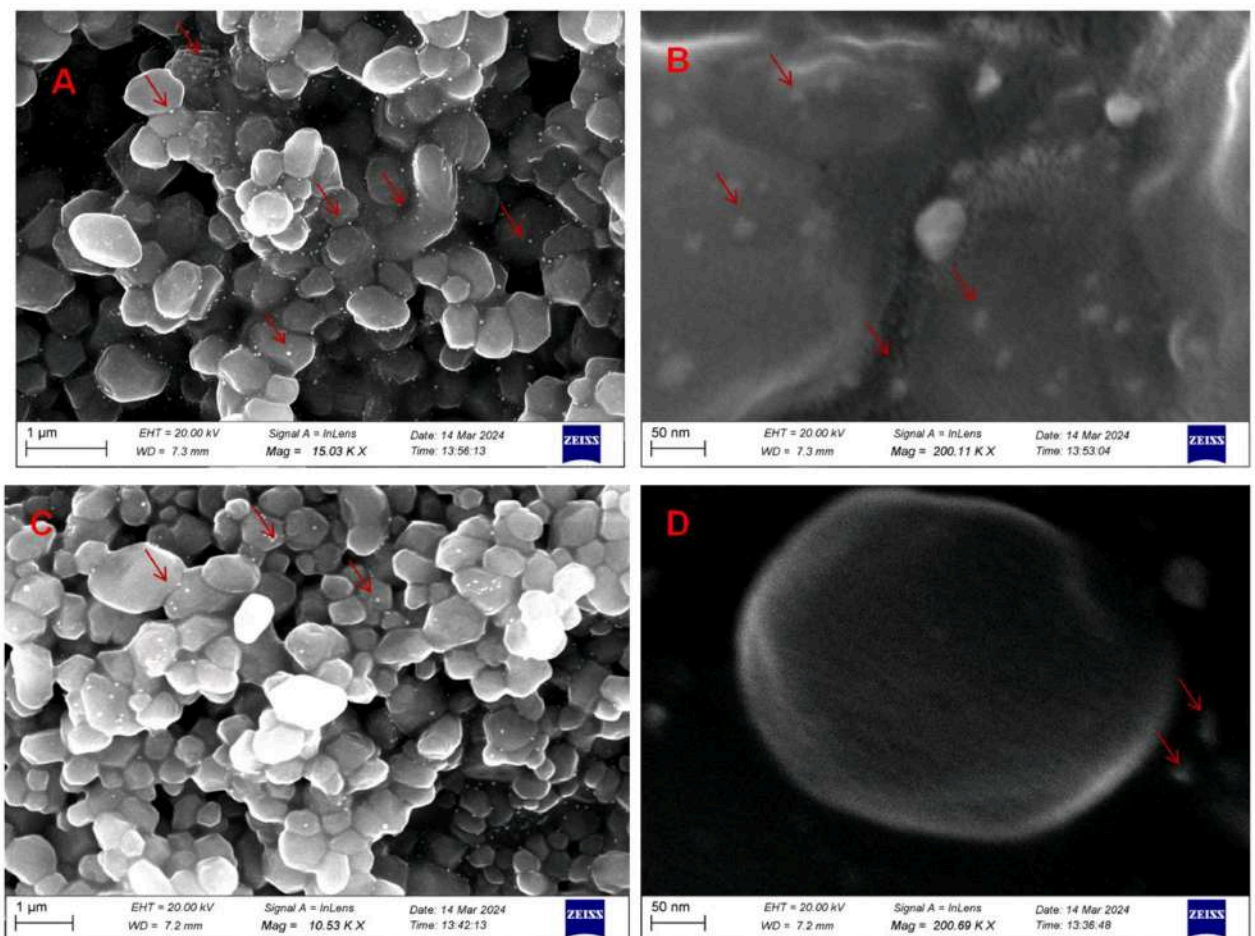


Fig: 5.4: SEM images of exosomes derived from zebrafish embryos (A-B) Control and (C-D) TTZ treated. Red arrows represent nodules/cargoes on the surface of exosomes.

Scanning electron microscopy analysis of exosomes exhibited a characteristic spherical shape, with an average diameter of approximately 100 nm (Figure 5.4). Furthermore, examination of the surface morphology of vesicles revealed the presence of small spherical nodules, potentially representing cargos such as proteins and miRNA.

In the control group, these nodules were prominently observed, suggesting the normal functioning of the exosomes and their cargo transportation mechanisms. However, in embryos treated with TTZ (food additive), these nodules were notably less, indicating an impact on the functionality of exosomes.

The absence of cargo-containing spherical nodules in exosomes from TTZ-treated embryos implies disruptions in intercellular communication pathways, potentially compromising cellular signaling and homeostasis. This alteration may extend to developmental processes, raising concerns about abnormalities and growth defects (Zhao, C et al., (2020)).

5.3. miR-132 EXPRESSION IN EXOSOMES

5.3.1 QUANTIFICATION OF RNA

The quantification of RNA extracted from exosomes isolated from 96-hpf zebrafish embryos revealed varying concentrations and quality metrics for the samples (Table 5.1). The control exhibited an absorbance at 260 nm (A₂₆₀) of 0.087 and an absorbance at 280 nm (A₂₈₀) of 0.051, resulting in a 260/280 ratio of 1.703. The concentration of RNA in this sample was determined to be 69.68 ng/μl. Conversely, TTZ treated displayed lower absorbance values, with an A₂₆₀ of 0.032 and an A₂₈₀ of 0.024, yielding a 260/280 ratio of 1.367. The concentration of RNA in this sample was measured at 25.76 ng/μl.

These results indicate differences in RNA quantity and quality between the two samples, suggesting potential variations in RNA profiles within exosomes extracted from TTZ-treated and untreated embryos. Further analysis of miR-132 profiles will

provide insights into the role of miRNAs in mediating communication and influencing changes within TTZ-treated embryos.

Table 5.1. This table presents the quantification data for RNA extracted from exosomes isolated from 96-hour post-fertilization (hpf) zebrafish embryos, with samples labeled as Control and TTZ treated. The table includes measurements of absorbance at 260 nm (A260) and 280 nm (A280), as well as the calculated 260/280 ratio and RNA concentration (ng/μl) for each sample.

S.NO	Sample	260	280	260/280	ng/μl
1	Control	0.087	0.051	1.703	69.68
2	TTZ treated	0.032	0.024	1.367	25.76

5.3.2 REAL TIME PCR

After normalization using RU5 as a reference gene, the relative expression of miR-132 in the test group was found to be approximately 0.026, while in the control group, it was approximately 0.034. The fold change of miR-132 in the test group compared to the control group was calculated to be approximately 0.765. (Fig: 5.4)

The observed downregulation of miR-132 in the test group compared to the control group ($p \leq 0.05$) suggests a potential impact of the treatment (TTZ) on miR-132 expression levels.

The observed downregulation of miR-132 in TTZ-treated embryos aligns with previous studies linking miR-132 dysregulation to developmental defects and highlights the potential role of miR-132 as a biomarker for TTZ-induced toxicity (ref). Additionally, disrupted exosome cargo transportation observed in TTZ-treated embryos may contribute to the dysregulation of miR-132 and other critical signaling

molecules, further emphasizing the importance of exosomal communication in developmental processes (Zhang, M., & Bian, Z. (2021)). Overall, these findings underscore the need for further research to elucidate the molecular mechanisms underlying TTZ-induced toxicity and its implications for developmental health.

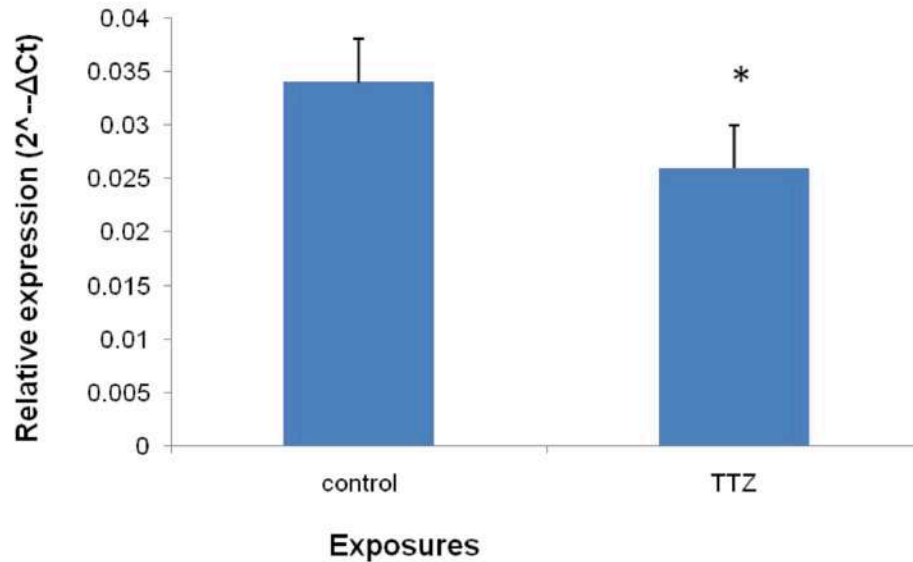
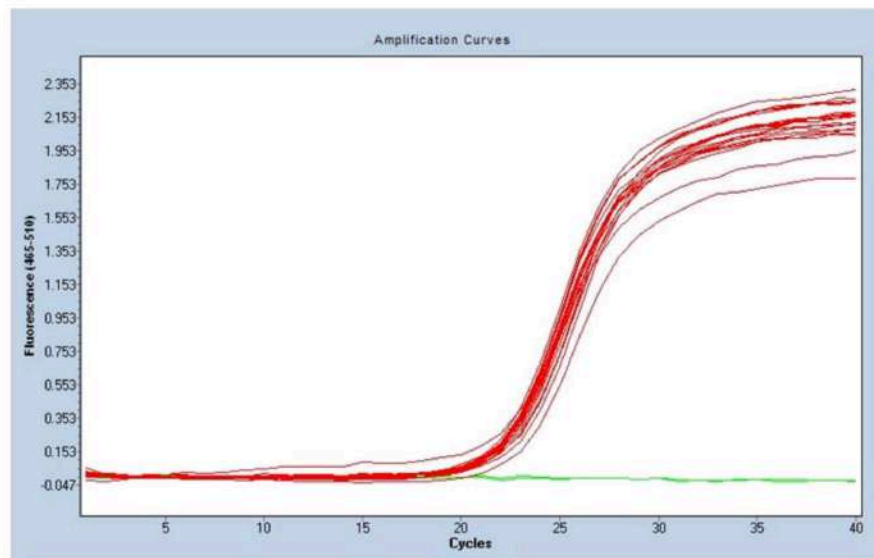
A**B**

Fig: 5.5: miR-132 is significantly decreased in TTZ exosomes isolated from 96hpf zebrafish embryos. A) miR-132 was significantly different between control and TTZ group [$p = 0.05$]. B) The RT-PCR amplification plot displays the fluorescence signal (typically measured as cycle threshold, Ct) against the number of PCR cycles. Each curve represents the amplification of miR-132 in a specific sample or condition.

CHAPTER 6 SUMMARY AND CONCLUSION

6.1 SUMMARY

In this study, we investigated the toxicological effects of Tartrazine (TTZ) exposure on zebrafish embryos, focusing on survival rate, developmental abnormalities, and miR-132 expression in exosomes. Here's a summary of our key findings and conclusions:

6.1.1 Zebrafish Toxicity Induced by Tartrazine Exposure

- Tartrazine exposure at 5.5 ppm did not significantly affect the survival rate of zebrafish embryos compared to the control group.
- Morphological analysis revealed notable abnormalities such as bent tails and curved spine structures in TTZ-exposed embryos, indicating potential developmental toxicity.
- Despite the absence of significant locomotor changes, further investigations are warranted to assess other neurodevelopmental effects induced by Tartrazine exposure.

6.1.2 Characterization of Exosomes Using SEM

- SEM analysis showed characteristic spherical exosomes with cargo-containing nodules.
- TTZ-treated embryos exhibited fewer nodules on exosomes, suggesting disrupted cargo transportation and potential disruptions in intercellular communication pathways and developmental processes.

6.1.3 miR-132 Expression in Exosomes

- RNA quantification revealed differences in quantity and quality between control and TTZ-treated samples, indicating potential variations in RNA profiles within exosomes.

- Real-time PCR analysis normalized to RU5 reference gene showed significant downregulation of miR-132 in TTZ-treated embryos compared to controls ($p \leq 0.05$).

6.2 CONCLUSION

- Our study provides insights into the toxicological effects of Tartrazine exposure on zebrafish embryos. Despite no acute mortality events, TTZ exposure induced morphological abnormalities and downregulated miR-132 expression in exosomes.
- These findings highlight the potential developmental toxicity of Tartrazine and the importance of miR-132 dysregulation in mediating its effects.
- Further research is needed to elucidate the molecular mechanisms underlying TTZ-induced toxicity and its implications for developmental health. Additionally, investigating the role of exosomal communication in mediating these effects may provide valuable insights for future studies.

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