

DEVELOPMENTAL TOXIC EFFECTS OF FOOD ADDITIVE INS 171 ON ZEBRA FISH AND EMBRYO'S

(PROJECT – REPORT)

Submitted in partial fulfillment of the requirements for the award of
Master of Science degree in medical biotechnology and clinical research

by

JOY. R (41101003)



DEPARTMENT OF MEDICAL BIOTECHNOLOGY AND CLINICAL RESEARCH

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

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MAY– 2023



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **JOY.R (41101003)** and who carried out the project entitled "**Developmental toxic effects of food additive INS 171 on zebra fish and embryo's**" under our supervision from December 2022 to May 2023.

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ABSTRACT

Food additives are commonly used in the food industry to improve the texture and appearance of food products. However, some additives have been reported to have potential negative effects on human health and the environment. This study aims to investigate the potential developmental toxic effects of food additive INS171 (Titanium dioxide (TiO₂)) on zebra fish and their embryos, in the presence of TiO₂ nanoparticles. Zebra fish embryos were exposed to different concentrations of INS171 (TiO₂), and their growth, deformities, gene expression changes, and oxidative stress markers were monitored over time. The results indicate that exposure to INS171 (TiO₂) may cause delayed growth, deformities, decreased heart rate, and malformations in zebra fish embryos. These findings highlight the potential risks associated with the use of food additives and their interactions with environmental toxins. Further studies are needed to better understand the mechanisms underlying the observed effects and their implications for human health and the environment.

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CHAPTER «1

INTRODUCTION

1.1 TOXICOLOGICAL EFFECTS

The term "toxicological effects" refers to a substance's detrimental or unfavourable impacts on living things, such as people, animals, and the environment. Exposure to harmful substances such chemicals, pesticides, medications, and toxins created by microbes can have toxicological effects. A substance's toxicological effects can change based on a number of variables, such as the dose, route of exposure, duration of exposure, age, and overall health of the exposed person or organism. Acute toxicity, chronic toxicity, mutagenicity, carcinogenicity, reproductive toxicity, neurotoxicity, and immunotoxicity are a few frequent toxicological effects. Toxicologists study the toxicological effects of substances to determine their potential risks to human health and the environment. They use various methods, including animal testing, cell cultures, and computer models, to evaluate the toxicological effects of substances and develop strategies to mitigate or prevent their harmful effects (Abbas et al., 2019).

1.2 TiO₂

The most popular metal oxide nanoparticle, TiO₂ (TiO₂ NPs), has been used more and more in various industrial applications. These result in increased exposure to humans and have an impact on both their health and the environment. For instance, TiO₂ NPs are often employed in the cosmetics, sunscreen, pharmaceutical, and electronics industries, making their discharge into the environment inevitable. Numerous recent studies on the possible danger of TiO₂ NPs revealed that these particles had certain toxicological issues. According to several in vitro experimental findings, TiO₂ NPs greatly increased oxidative stress, which led to DNA damage and cell death. According to studies on the acute toxicity and oxidative effects of nanoscale TiO₂ on zebra fish, the acute toxicity of TiO₂ NPs was higher than that of bulk TiO₂. TiO₂ NPs show higher toxicity as compared to bulk TiO₂. By Servin et al., the potential route by which TiO₂ NPs enter the food chain from the soil was also investigated (Zhang et al., 2014).

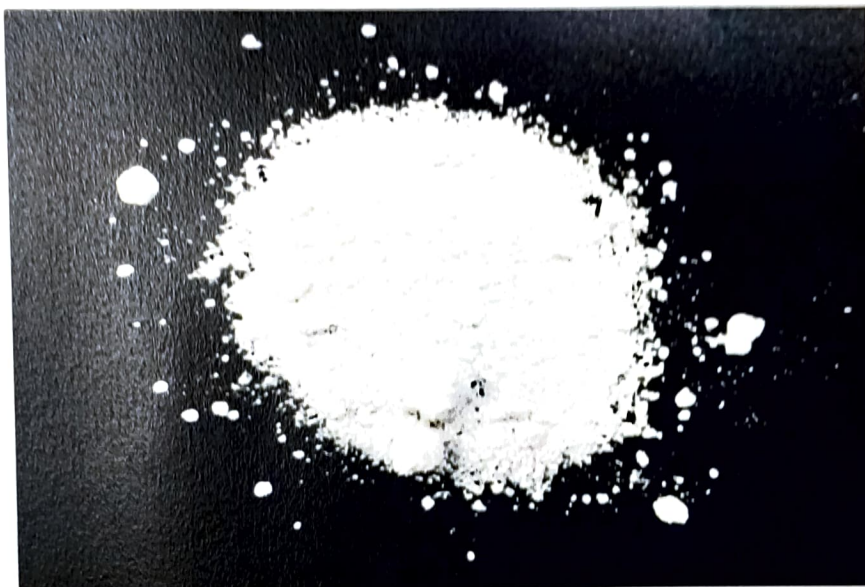


Figure 1.1-Food grade TiO₂ powder

1.3 ONGOING CONTROVERSY ON TITANIUM DIOXIDE

There is currently debate regarding the use of technology food additives because of possible negative effects on human health. This Titanium dioxide (TiO₂) and specifically, its usage as an E171 food additive are the main topics. France is the first nation in Europe to ban the sale of foods containing certain substances for a year, starting on January 1, 2020 (Boutillier et al., 2020).

1.4 GROWTH AND HISTOLOGICAL PARAMETERS OF ZEBRAFISH

Due to its lack of obvious toxicity, high functionality, and potential usage in a variety of coatings, titanium dioxide (TiO₂) has been dubbed "the environmental white knight". Titanium dioxide (NPs) is photocatalytic, which means that when it is subjected to ultraviolet (UV) light, it turns into a catalytic substance. TiO₂ in its standard form is thought to be physiologically inert, provide little risk to people, and can be used as a food additive (colourant). But when TiO₂ is created at the nanoscale (particle size 100 nm), its consequences on biology and the environment need our growing attention. Further

evidence points to the need for longer-term exposure in order to determine the risk of nanomaterials in aquatic organisms. There is very little information on the long-term ecotoxicity to aquatic organisms, despite the fact that these studies have raised concerns regarding the chronic toxicity of TiO₂ NPs to aquatic life. However, there aren't many research that have looked into the long-term chronic toxicity of NPs on aquatic organisms. Numerous studies on acute toxicity revealed that TiO₂ NPs are either non-toxic or harmless for aquatic creatures. However, a recent study found that zebrafish ribosomal function genes' expression was affected by nano-TiO₂ exposure (Chen et al., 2011).

1.5 GENOTOXIC EVALUATION OF TiO₂

For the purpose of determining the risk of probable carcinogens, genes (such as gene mutations, DNA damage, and chromosomal damage) play a significant role in cancer research. The International Agency for Research on Cancer (IARC) has classed TiO₂ as probably carcinogenic to humans (class 2B) because inhaled or intratracheally injected nano and fine-sized TiO₂ has been shown to produce lung cancer in rats. Titanium dioxide (TiO₂) is a well-known food ingredient that is frequently used to whiten and brighten meals, particularly confectionery, white sauces and dressings, and some powdered foods. According to recent research on food-grade TiO₂ (also known as E171), 36% of the particles had a minimum diameter of 100 nm or smaller. The current study's objective was to assess TiO₂ NPs' genotoxicity using in vivo and in vitro test systems. Different genetic endpoints were thoroughly examined to find the sensitive genetic endpoints for the detection of TiO₂ NPs-induced genotoxic effects under the guidance of the OECD standard for the testing of chemicals (Chen et al., 2014).

1.6 IMPACT OF TiO₂

For consumers, oral contact to TiO₂ NPs appears unavoidable. Investigating the impact of TiO₂ NPs on the intestine is urgently required for the safety assessment of TiO₂ NPs because the gastrointestinal tract is the first stop for TiO₂ NPs once they enter the human body orally. In addition, TiO₂ NPs have to endure and pass through saliva, gastric and intestinal juices before reaching the intestine. Under these conditions, the physiological

parameters of TiO₂ NPs such as pH, ionic strength, as well as protein content and composition change greatly. These contradictory in vitro results might come from the different cells and culture conditions, and tested TiO₂ NPs with various physicochemical properties, such as size and surface property. These further indicate that more investigations on the toxicity of TiO₂ NPs to the intestine are needed. The Caco-2 cells and monolayer, which are the standard intestinal in vitro models generally accepted by pharmacological businesses and regulatory authorities, were used for toxicity, cellular uptake and translocation experiments using native and pretreated TiO₂ NPs (Song et al., 2015).

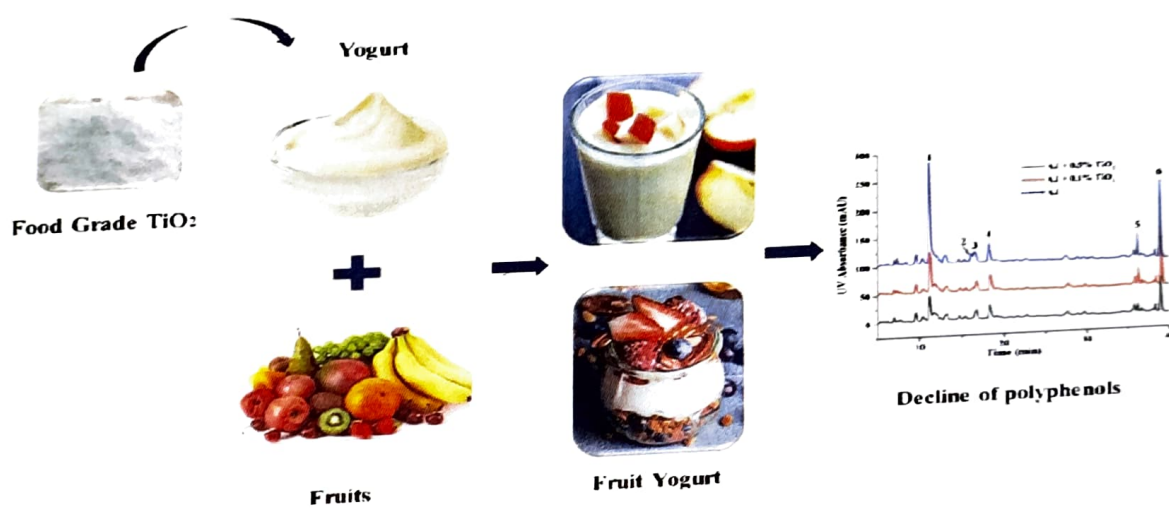


Figure 1.2- Impact of food additive titanium dioxide on the polyphenol content and antioxidant activity of the apple juice (Li et al., 2022).

1.7 BEHAVIOURAL CONSEQUENCES OF E171

The behaviour and consequences of E171 and nano-sized TiO₂ after consumption have been the subject of an increasing number of research in recent years. These investigations have found potential negative effects, such as the stimulation of inflammation, the creation of reactive oxygen species (ROS), and co-genotoxic effects. It is still unclear what the observed toxicity and danger classification after inhalation mean for oral toxicity. The Netherlands Food and Consumer Product Safety Authority (NVWA)'s

Office of Risk Assessment and Research (BuRO) organized a workshop in July 2018 to discuss "potential health effects of the food additive titanium dioxide (E171)," on which BuRO based its opinion that was released in 2019. In accordance with the CLP Regulation (EC No 1272/2008), the EU announced its classification of TiO₂ as a suspected carcinogen (category 2) by inhalation of powder containing at least 1% particles with an aerodynamic diameter of less than 10 µm on February 18, 2020, replacing ECHA's assessment. After an 18-month transition period, the classification will take effect on October 1, 2021 (Winkler et al., 2018).

1.8 ENVIRONMENTAL EFFECTS OF TiO₂

The environmental effects on aquatic animals and toxicity mechanisms of TiO₂ on aquatic ecosystems have been the subject of several studies, although they still remain poorly understood. In particular, carbon-based NPs have been the main focus of toxicity experiments on adult fish. Additionally, studies on the toxicity of metal oxide nanoparticles (TiO₂) to fish have focused on the first phases of development. The toxicity of TiO₂ to adult fish has not received much attention from researchers. Contradictory findings, however, have been found in recent investigations, raising the notion that toxicity may depend on size in addition to the negative effects brought on by the presence of dissolved ions. According to Govern and Klaper (2006), the tiny size and high specific surface area of NPs are generally related to their toxicity. Due to their increased surface reactivity, capacity to enter and aggregate inside of cells and animals, and higher surface area than their bulk counterparts, nano materials are theoretically predicted to be more harmful than their counterparts. The majority of the aggregates formed by NPs in suspensions will settle out after a few hours, potentially reducing their toxicities. NPs often form big particles in suspensions. In contrast, reactive oxidative species (ROS) produced by a variety of mechanisms, including illumination of nanoparticles (NPs) (Jiang et al., 2008) and disruption of intracellular metabolic processes, may interfere with the anti-oxidant system, resulting in damage to lipids, carbohydrates, proteins, and DNA (Xiong et al., 2011).

1.9 ECOTOXICITY OF TiO₂

Numerous acute and short-term ecotoxicity tests demonstrated that nTiO₂ exerted no or low toxicity to testing species, ranging from bacteria, algae, Daphnia, to fish, which may have contributed to the widespread use of nTiO₂. These investigations suggest that determining the possible toxicity of nTiO₂ may need considering longterm exposure. Aquatic organisms would be exposed to nTiO₂ over a lengthy period of time given the variety of uses for it and the possibility that it could be released into the aquatic environment. As a result, it is important to carefully assess the negative effects of chronic nTiO₂ exposure, particularly at low doses that are thought to be non-toxic. We discovered that nTiO₂ exposure could affect zebrafish reproduction at levels as low as 0.1 mg L⁻¹, which is an environmentally relevant dose range. According to our research, when nanotechnology is developed, the long-term effects of manmade nanomaterials on the aquatic ecosystem should be taken into account. In order to further address this problem, we carried out a chronic toxicity study in which zebrafish were exposed to nTiO₂ for 91 days. The goal of this study was to evaluate the potential effects of this exposure on the zebrafish reproductive system, including egg production, embryo survival, ovary histology, nTiO₂ accumulation in ovaries, and overall gene expression in ovarian tissues by microarray analyses. Concerns have been expressed about the release of engineered nanomaterials (ENMs) into the environment and its possible effects on human and environmental health as a result of their extensive manufacturing and use (Wang et al., 2011).

1.10 TiO₂ TOXICITY IN ZEBRAFISH EMBRYOS

Tropical freshwater fish called zebrafish (*Danio rerio*) serve as helpful model organisms in scientific studies. Their continued appeal can be attributed to their humorous nature, as well as to their quick reproduction, low cost, and wide availability. Previous zebrafish investigations have shown that differing exposure settings and times to NPs result in a variety of pathogenic and gene expression alterations. applied 10-1000 mg/L Ni-NP to zebrafish embryos, we noticed the aggregation of Ni, thinner intestinal epithelium, and separation of skeletal muscle fibre. However, it is still unknown how low dosage and

subchronic exposure to TiO₂ NPs causes zebrafish neurotoxicity. TiO₂ nanoparticle residues have been shown to have a deleterious impact on the functioning of digestive enzymes. TiO₂ NPs showed little toxicity after a 14-day exposure, however they had an impact on zebrafish reproduction. The difference between this piece and earlier research is in its concentration (mg/L versus mg/L in earlier research) and length (45 days versus 5-14 days). Our research will serve as a crucial developmental foundation for assessing aquatic creatures' neurotoxicity to low dosage and subchronic TiO₂ NP exposures. showed that the behavioural endpoints (such as hatchability and survival) were more sensitive than the other endpoints to identify the toxicity of TiO₂ NPs on developing zebrafish for 120 hours after fertilisation.

Environmental Toxicology [DOI 10.1002/tox]

1.11 INVESTIGATIONS ON THE IN VIVO TOXICITY

Due to oxidative stress and apoptosis, TiO₂ and reduced graphene oxide (RGO) cause genotoxicity and cytotoxicity in a dose- and size-dependent way. Bacteria, aquatic ecosystems, and mammalian cells are all cytotoxic as a result of reactive oxygen and hydroxyl species produced by photoactivation of TiO₂ and its nanocomposites. Particularly, Amora et al. Revealed the toxicity of GO-based nanocomposites, oxidised carbon nano-horns (oxi-CNHs), and oxidised carbon nano-onions (oxi-CNOs) in zebrafish (*Danio rerio*). They amply revealed that less than 85% of larvae and embryos survived in the presence of 50 g mL⁻¹ of GO. Predictable environmental concentrations indicate that TiO₂ and graphene-based nanocomposites may carry a greater risk to aquatic ecosystems than the most widely utilised nanomaterials, reaching the maximum concentration in surface waters. Additionally, the sizes and aggregation of graphene and its nanocomposites will have an impact on how zebrafish embryos develop. The goal of the current study is to examine and provide a thorough understanding of the toxicity of RGO/TiO₂ based nanocomposites on zebrafish embryos (Prakash et al., 2019).

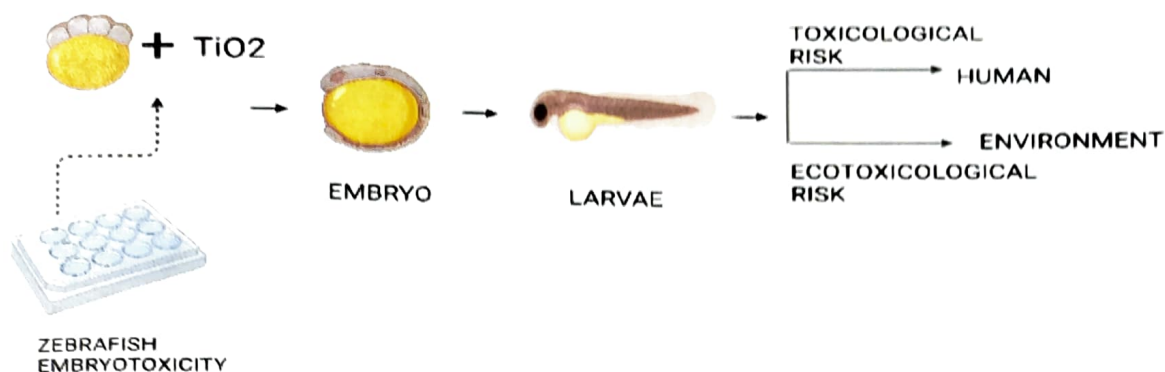


Figure 1.3- Zebra fish Embryo toxicity

1.12 CAUSES OF TiO2

Both with and without exposure to ultraviolet A (UVA) radiation, nano-TiO₂ has been shown to have oxidative stress effects in mammals and fish, causing inflammation, cell damage, and genetic damage. Invertebrates exposed through water may exhibit sub-lethal toxicity at concentrations between 5 and 50 mg L⁻¹, according to the available evidence. Nowadays, nano-silver is widely employed in consumer goods, mainly due to its powerful antibacterial capabilities and affordable manufacture. Silver concentrations have been found to reach 100 mg L⁻¹ in wastewater treatment facilities that receive influents from businesses that use silver nanoparticles (Ag NPs). This level of silver exceeds the tolerable limit for some bacteria, which may have a negative effect on bacterial communities (Marambio-Jones & Hoek 2010). The simplicity of seeing developmental effects through a transparent chorion is just one of the many experimental conveniences that zebrafish embryos provide. As endpoints for evaluating the impacts, mortality rates, aberrant development, apoptosis, and targeted (in situ) gene expression were used. Ag NPs may have harmful consequences in a variety of animals, including fish, according to several studies (Osborne et al., 2012).

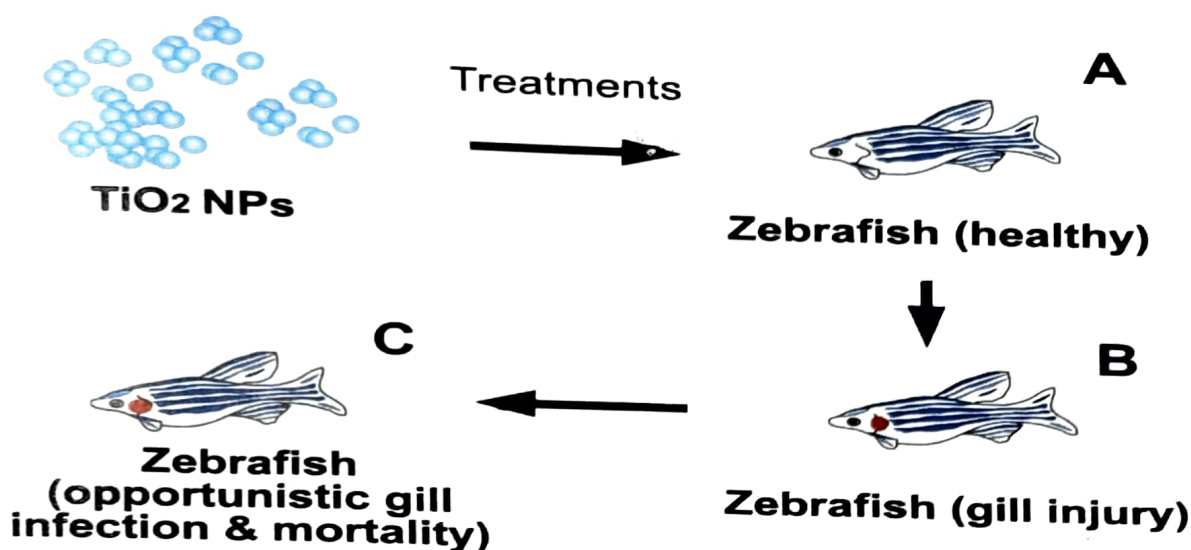


Figure 1.4 A model for putative role of opportunistic gill infection in TiO_2 -induced mortality in zebra fish (Chiao-Yi Huang et al., 2021)

1.13 GOAL OF CURRENT STUDY

The goal of the current study was to compare TiO_2 nanoparticle phototoxicity with zebrafish at various life stages under ultraviolet (UV) radiation that was relevant to the environment and to determine which life stage was the most sensitive. Embryo (within 2 hours of fertilisation), yolk-sac larvae (within 48 hours posthatch), freeswimming larvae (21 days posthatch), and juvenile (50 days posthatch) were the four developmental phases that we examined. Zebrafish embryos have become a well-liked animal model for researching the toxicity of nanomaterials [5-8]. Zebrafish's oviparity is one of the many benefits of utilising them as a representative model species in toxicity studies. Recent research using artificial or real sunlight has shown extremely noticeable increases in the toxicity of TiO_2 nanoparticles when certain wavelengths of energy are present. Zebrafish's oviparity is just one of the many benefits of utilising them as a representative model species in toxicity research. This reproductive approach has benefits for determining how sensitive developing embryos, freshly emerged larvae, and later life stages are to toxins (Ma & Diamond, 2013).

1.14 FOOD GRADE OF TiO₂

Food, pharmaceutical, and personal care goods for stabilisation or optical qualities are some consumer-based applications for ENMs. In human bronchial epithelial cells, anatase TiO₂ was more hazardous than rutile, causing DNA damage, lipid peroxidation, and the production of micronuclei. TiO₂ ENMs introduced to an in vitro intestinal model had the ability to transcytose through the epithelial lining, however these effects weren't fatal. In a second investigation, five different types of food-grade TiO₂ were procured from international food suppliers and characterised for comparison to industrial grade (Aeroxide P25; Evonik Degussa), indicating significant discrepancies between characteristics related to environmental destiny and toxicity. Each experimental condition was tested over a 5-day period known as an experimental "week," including baseline (control), food, and industrial grade TiO₂ exposures. Each condition was tested in triplicate, consisting of three baseline weeks, three industrial grade exposure weeks, and three food grade exposure weeks. Utilising a variety of food sources, the average daily consumption of TiO₂ ENMs in food and personal care items was determined. It was discovered that adults in the US may consume up to 1 mg TiO₂/kg of bodyweight daily.

Furthermore, consumption would mostly consist of food grade forms of TiO₂, which are more frequently found in food and personal care items, as opposed to industrial versions, which are more frequently used in environmental health and safety research. Food grade TiO₂ should be used in addition to industrial grade TiO₂ in research looking at the destiny and toxicity of ENMs since there are intrinsic distinctions between the particle types that can affect how they behave in biological and environmental systems. The different effects of food- or industrial-grade TiO₂ ENMs on the microbial community structure and cellular function were specifically discussed in this work (Waller et al., 2017).

1.15 POTENTIAL RELEASE OF TiO₂

The potential for the release of TiO₂NPs into the environment grows along with TiO₂NP production rates and applications. The oxidative stress theory of toxicity was supported by the in vivo production of superoxide, increased expression of an antioxidant response-element driven reporter, and enhanced DNA damage with concomitant light

exposure. The suspension stability of unfunctionalized TiO₂NPs is improved by DOM adsorption. Increased water column residence periods, easier advective movement in aqueous environments, more exposure to free-swimming aquatic creatures, and decreased delivery to benthic species are all anticipated effects of increased stability of TiO₂NP suspensions. We investigated how HA affected the stability of TiO₂NP solution, zebrafish uptake, and toxicity. Because Degussa P25 TiO₂NPs are used commercially, we decided to study them. Zebrafish are an ideal animal model for testing the toxicity of nanomaterials because of their quick, well-studied development, small size, and high fecundity. In order to achieve this, we subjected embryonic and larval zebrafish to a commercial TiO₂NP preparation in both the absence and presence of illumination that mimicked sunshine (Yang et al., 2013).

1.16 EXPOSURE OF E171

E171 is used in products such as candy, coffee creamer, chewing gum, sauces, nutritional supplements, toothpaste, and pharmaceuticals. Although both the anatase and rutile forms of TiO₂ are authorized for foods, the characterization of European and American food samples showed that anatase is the predominant TiO₂ crystalline structure used as food additive E171 and thus the main source of exposure for the general population. The intake of E171 varies between different age groups and countries, while children, in general are the most highly exposed group, due to their lower body mass and disproportionally higher consumption of E171-containing products (Nicolaj S. Bischoff, 2021)

1.17 FISH EMBRYO TEST STUDY

The Fish Embryo Test (FET), which uses fish embryos for testing, falls between the usual 52 studies that use cell cultures and those that use mammalian models. There is proof that nano-TiO₂ can stick to the embryo's chorion, where it may be absorbed and dispersed equally throughout the fish's tissues without any tissue-specificity. Nano-TiO₂'s toxicity to aquatic species such as fish, microcrustaceans, and hydra has been found to be quite low. TiO₂ is a semiconductor with the crucial ability to be photoactivated, making it particularly appealing for application in processes based on heterogeneous

photocatalysis to degrade a range of organic and inorganic chemicals. Reactive oxygen species (ROS), which have the potential to harm 78 biomolecules, are produced when TiO₂ is exposed to UV light with a wavelength range of 300-388 nm. When TiO₂ is present as nanoparticles, its photocatalytic activities are improved, which can increase its toxicity to aquatic species when exposed to environmental factors such as sun UV radiation. Our research team has previously found that depending on the crystal phase, concentration, and lighting conditions, exposure to nano-TiO₂ can have sublethal effects on fish and microcrustaceans. However, there are still quite a few unknowns, therefore more research is necessary to thoroughly assess the toxicity of nano-TiO₂ in the fish embryos, particularly its sublethal effects. The study of biomarkers linked to this effect, such as antioxidant enzymes, should be used in the case of creatures exposed to the substance because oxidative stress is likely the primary cause of the toxicity of nano-TiO₂. On the other hand, less attention has been paid to the effects of nano-TiO₂ on similarly significant enzymes such as phosphatases, which are involved in a variety of transphosphorylation events and can be influenced by metals and ROS (Aoyama et al., 2003). According to reports, exposure to nano-TiO₂ can have an impact on the size and growth of fish and microcrustaceans (Clemente et al., 2014).

1.18 HAZARDS AND RISKS ASSOCIATED WITH TiO₂

Understanding how solar radiation interacts with TiO₂, how this interaction is reduced in natural settings, and how life stage may affect exposure and susceptibility to this mode of toxicity associated with the materials' photoactivity are all necessary for assessing the hazards and risks associated with TiO₂ nanoparticles. (UV) radiation and to determine the stage of life that is most vulnerable.

Embryo (within 2 hours of fertilization), yolk-sac larvae (within 48 hours post hatch), free swimming larvae (21 days post hatch), and juvenile (50 days post hatch) were the four developmental phases that we examined. Recent research using artificial or real sunlight has shown extremely noticeable increases in the toxicity of TiO₂ nanoparticles when certain wavelengths of energy are present. Zebrafish's oviparity is just one of the many benefits of utilizing them as a representative model species in toxicity research. One of

the numerous advantages of using zebrafish as a representative model animal in toxicity research is its oviparity. These results suggest that post hatch life stages should be examined to determine the entire potential hazard and risk of nanomaterials. Since they may be more susceptible to toxicity (Ma & Diamond, 2013).

1.19 STUDY OF SOD, CAT AND HEAT SHOCK PROTEINS

TiO₂ nanoparticles' (nano-TiO₂) widespread use in nanotechnology and low toxicity are the main reasons for their popularity. Recent research, however, has indicated that the photocatalytic properties of this substance can cause changes in their behaviour in the environment, leading to impacts that are still not entirely understood. Utilising an experimental methodology consistent with the three Rs of alternative animal experimentation (reduction, refinement, and replacement), the goal of this study was to assess the toxicity of two nano-TiO₂ formulations under various illumination settings.

Danio rerio fish embryos were subjected for 96 hours to various concentrations of nano-TiO₂ in the form of anatase (TA) or an anatase/rutile mixture (TM), while being either exposed to visible light alone or to both visible and ultraviolet light (UV). Survival rates, malformation, hatching, equilibrium, and total length of the larvae were among the acute toxicity and sublethal parameters assessed, along with biochemical indicators (specific activities of catalase (CAT), glutathione S-transferase (GST), and acid phosphatase (AP)). The larvae's hatching was accelerated by TA and TM both. Larvae from the groups exposed to TM died more frequently under UV irradiation than those exposed to TA. The balance of the larvae was disrupted when exposed to TM under UV radiation. Although no distinct dose-response association was seen, changes in the activities of CAT and GST were suggestive of oxidative stress. The type of formulation and the lighting situation both seemed to affect how nano-TiO₂ behaved. The findings help build standards for risk assessments of nanotechnology and shed light on the factors influencing the toxicity of these nanoparticles. (Z. Clemente ,et.al, 201

HSP (HEAT SHOCK PROTEINS)

Zebra fish express heat shock proteins (HSPs) in particular spatial patterns when they are not under stress. For controlling transgenic expression in tiny aquatic creatures like

zebra fish embryos, the heat shock promoter is helpful. It is possible to significantly increase downstream gene expression throughout the body by increasing the temperature from 28.5°C to 38.0°C. In zebra fish, routine heat shock can change neutrophil function. Inhibiting HSP90 function in the embryo encourages phenotypic flexibility in zebra fish. Embryos are commonly treated with heat shock by submerging them in water that is 38°C for 30 minutes (Krone et al., 2003).

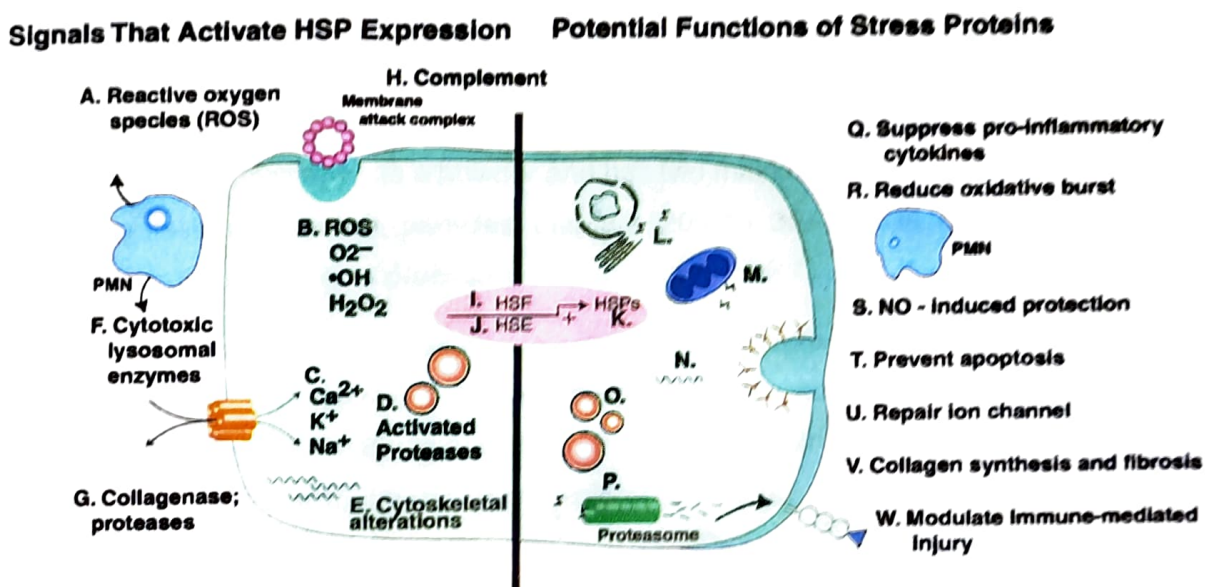


Figure 1.5-Signals that activate HSP Expression, Potential functions of stress proteins

CHAPTER-2

REVIEW OF LITERATURE

2.1 TiO₂

At atmospheric pressure, titanium dioxide (TiO₂) is the only naturally occurring oxide of titanium. Rutile, anatase, and brookite are examples of TiO₂ crystals. It exists as microparticles or nanoparticles since its size ranges from a few dozen to several hundred nanometers (i.e., particles with at least one dimension less than or equal to 100 nm according to the European definition of nanomaterials). The crystal phase, particle size, and shape all affect TiO₂'s properties. With a mean primary particle size of 10 to 50 nm, TiO₂ is primarily employed as a powder and has two main uses: either as a pigment (light scattering is maximised in particles that are 200 to 300 nm in diameter) or as a photocatalyst in a variety of processes.

Human and other organism exposure to nanoparticles is exponentially rising. It is crucial but challenging to foresee the biological effects of these exposures. It was proposed that the distinctive chemical features that give nanoparticles their utility may also hold the key to understanding how to foretell their biological effects. We used growing zebrafish embryos and titanium dioxide nanoparticles (TiO₂NPs) as model systems to look at this. Reactive oxygen species (ROS), which are cytotoxic, are created when water and oxygen react with electron-hole pairs that are created when TiO₂NPs absorb photons. Here, we demonstrate how exposure to TiO₂NPs causes deformity and death in zebrafish embryos, but only when the fish are also lighted. TiO₂NPs are taken up by the growing fish, however until the embryos, the egg chorion acts as a barrier to uptake. (Ofek Bar-Ilan, et.al, 2012)

2.2 HISTORY OF (E171)

The current debate surrounding the food ingredient titanium dioxide (E171) is examined in this article highlighted by the French government's decision to temporarily halt the sale of foods containing it, effective January 1, 2020. It seeks to comprehend the technological and scientific aspects of the debate, which entails a better evaluation of the influence on

health of E171. Through the identification and examination of stakeholder viewpoints, we also investigate its nature. Our third goal is to help people understand how the public makes decisions. This study, which is based on interviews and the examination of secondary data, integrates ideas from organisational routines and sociology of dispute studies (controversy sociology). (Sophie Boutillier ,et.al,2020)

2.3 TOXICITY OF TiO2

Previous investigations have shown that titanium dioxide (TiO₂) nanoparticles (NPs) would be of modest to In short-term experiments, the ecological toxicity was moderate. However, it is yet uncertain what concerns TiO₂ NPs pose over the long run. The zebrafish (*Danio rerio*) was utilised as an in vivo model in this work to determine the chronic toxicity of NPs of TiO₂. Our findings showed that zebrafish suffer from clear negative effects, including concentration- and time-dependent growth suppression and a reduction in the liver weight ratio.

Only the gills, however, showed histopathologic alteration at the time, including thickening of the oedema and the gill lamellae. ICP-MS analysis revealed that TiO₂ NPs had accumulated and been dispersed in the brain, liver, heart, and gills. Our current research revealed that the TiO₂ nanoparticles might move between organs and

following prolonged exposure, cross the blood-heart and blood-brain barriers. Due to the ubiquitous usage of TiO₂ NPs, a methodical, logical, and tried-and-true framework for regulating the hazy health and environmental effects of TiO₂ NPs is required.(Jinyuan Chen, et al.,2010)

2.4 GROWING DISCUSSION OF TiO2 IN FOOD INDUSTRY

There is growing discussion about the potential harm connected with exposure to titanium dioxide (TiO₂) nanoparticles (NPs) as a result of the widespread use of TiO₂ NPs in the food industry. This study's objective is to assess TiO₂ NPs' genotoxicity using in vivo and in vitro test systems. In an in vivo experiment, adult male Sprague-Dawley rats were given intragastric doses of anatase TiO₂ NPs (75 15 nm) every day for 30 days at 0, 10, 50, and 200 mg/kg body weight. TiO₂ NPs could cause DNA double strand breaks in bone marrow cells after oral administration, according to the -H2AX assay. However, the

micronucleus test showed that in rat bone marrow, oral exposure to TiO₂ NPs did not result in damage to chromosomes or the mitotic apparatus. Chinese hamster lung fibroblasts were studied in vitro. TiO₂ NPs were administered to (V79 cells) at concentrations of 0, 5, 10, 20, 50, and 100 g/mL. After 24 and 48 hours of treatment, significant reductions in cell viability were found in each of the treated groups. Substantial DNA damage was only detected using the comet assay after 24 hours at a concentration of 100 g/mL. Using the hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene mutation test, the evident gene mutation was discovered at the concentration of 20 and 100 g/mL after 2 h of treatment. TiO₂ NPs were thoroughly evaluated for their potential genotoxicity in this study, and both in vivo and in vitro experiments revealed that TiO₂ NPs were genotoxic. DNA strand breakage and gene mutations appear to have higher sensitivity as genetic endpoints for the identification of genotoxic effects caused by TiO₂ NPs. (Zhangjian Chen, et al, 2014)

2.5 IMPACT OF TiO₂

Consumer goods that are related to food frequently contain titanium dioxide nanoparticles (TiO₂ NPs). For the safety evaluation of orally administered TiO₂ NPs, it is crucial to comprehend the impact of TiO₂ NPs on the intestinal barrier and absorption. In this study, the cytotoxicity and translocation of two native TiO₂ nanoparticles as well as these two TiO₂ NPs that had been prepared with either bovine serum albumin or a digestion simulation fluid were examined in undifferentiated Caco-2 cells, differentiated Caco-2 cells, and Caco-2 monolayer. After 24 hours of exposure, differentiated cells and a Caco-2 monolayer did not exhibit any toxicity when exposed to TiO₂ NPs at concentrations lower than 200 g/mL. However, undifferentiated Caco-2 cells' proliferation was decreased when TiO₂ NPs were pretreated with digestion simulation fluids at a concentration of 200 g/mL. The fact that undifferentiated Caco-2 cells readily ingested native TiO₂ NPs but not pretreatment NPs suggests that the protein coating on the NPs prevented cellular uptake. Differentiated cells had a substantially reduced capacity to absorb these TiO₂ NPs than did undifferentiated cells. The passage of TiO₂ nanoparticles through the Caco-2 monolayer was equally minimal.

Therefore, we conclude that there is a very little likelihood that TiO₂ NPs will pass through the colon of an animal or a human following oral ingestion. This study offers important data for the evaluation of the danger of TiO₂ NPs in food. 2015 John Wiley & Sons, Ltd. copyright. (Zheng-Mei Song,et.al,2015)

2.6 USES OF TiO₂

Frequently used in meals, coatings, pigments, and paints, titanium dioxide (TiO₂) makes up a significant portion of the designed nanoparticles emitted from items when they are utilised. To understand systemic perturbations to the microbial community, researchers looked at the effects of diet and industrial grade TiO₂ on the composition and phenotype of a human gut microbiota, representing an upstream anthropogenic system. TiO₂ exposures may not have much of an impact on the stability of the microbiome, according to similar patterns in the hydrophobicity and electrophoretic mobility of the baseline, food, and industrial grade microbial communities. The findings of this study suggest that the two forms of TiO₂ may indeed cause different microbial reactions, which is significant when taking environmental exposure and risk into account as well as when planning investigations on environmental destiny and toxicity. (Travis Waller, et.al, 2017)

2.7 EFFECT OF TiO₂

The adsorption of dissolved organic matter (DOM) can change the stability of a suspension of titanium dioxide nanoparticles (TiO₂NP). Their bioavailability and environmental fate are anticipated to be impacted by this. The effect of DOM on TiO₂NPs' toxicity to aquatic animals has not yet been documented. In the dark and with artificial sunlight lighting, we looked at how Suwannee River humic acid (HA) affected the toxicity of TiO₂NPs to developing zebrafish (*Danio rerio*). Adsorption of HA improved suspension stability and reduced exposure to TiO₂NP. In the presence of HA, TiO₂NPs were more poisonous. Fish treated to TiO₂NPs in the presence of HA showed a slight but considerable increase in lethality in the absence of simulated sunlight. The photocatalytic breakdown of HA lowered suspension stability under artificial sunlight irradiation. Under simulated sunshine illumination, median lethal concentrations were lower and oxidative DNA damage was increased compared to fish exposed to TiO₂NPs in the absence of HA, despite the lower Ti concentrations associated with fish in the treatments including HA.

This work underlines the significance of taking environmental parameters (such as sunlight exposure and DOM absorption) into account when evaluating the possible threats that manmade nanomaterials bring to the environment. (Sarah P. Yang,et.al,2013)

2.8 TOXICITY TEST OF TiO₂

Due to its photocatalytic activity and ability to absorb certain wavelengths of ultraviolet light, titanium dioxide nanoparticles (TiO₂ NPs) are now a commonly utilised nanomaterial. This study examined the embryos and adults of zebrafish to determine the harmful effects of rutile TiO₂ NPs. Exposure to 100 mg/L TiO₂ NPs had no effect on the rate of zebrafish embryos hatching in the embryo acute toxicity test, and there was no evidence of deformation. The effects of TiO₂ NPs on oxidative damage in liver, gut, and gill tissue were investigated in the adult toxicity test. The three antioxidant enzymes were identified using ELISA and fluorescence-based quantitative real-time reverse transcription PCR (qRT-PCR) methods. Superoxide dismutase (SOD), catalase (CAT), and glutathione S transferase (GSTs) at the protein and gene levels in the aforementioned zebrafish organs. The findings demonstrated that long-term exposure to TiO₂ NPs can harm organisms through oxidative stress, and they also revealed that, in comparison to the control group, the three different types of enzyme activity had somewhat decreased at the protein level. In order to counter the negative reaction, prolonged exposure to TiO₂ NPs may also result in increased expression of CAT, SOD, and GSTs in three adult zebrafish organs. The liver and gill of adult zebrafish showed more evident impacts from long-term exposure to TiO₂ NPs.(Tianle Tang ,et.al,2019)

2.9 RISK OF TiO₂ IN ZEBRAFISH EMBRYOS

More and more scientists are testing the toxicity of nanomaterials on zebrafish embryos. The 96-hour standard toxicity assay was used in the current work to assess the phototoxicity of TiO₂ nanoparticles with zebrafish at four different life stages (embryos, yolk-sac larvae, free-swimming larvae, and juvenile) under simulated sunshine. The most sensitive to TiO₂ phototoxicity were discovered to be yolk-sac larvae, indicating that It's possible that the widely used zebrafish embryo test can't completely or precisely forecast the danger and risk that these nanoparticles pose to little fish.(HONGBO MA,et.al,2013)

2.10 EXPOSURE OF TiO₂

A growing body of literature describes the toxic effects of produced nanoparticles (NPs) on zebrafish eggs. Zebrafish (*Danio rerio*) have historically been used to assess the toxicity of ambient and aquatic toxicants. The neurotoxicity of subchronic exposure to TiO₂ NPs on adult zebrafish is the subject of relatively few studies, nevertheless. This investigation examined the morphological changes, changes in the neurochemical composition, and changes in the expression of genes associated to memory and behaviour in zebrafish brains after 45 days of exposure to 5, 10, 20, and 40 µg/L TiO₂ NPs. Our findings showed that after low dose exposures to TiO₂ NPs, overgrowth of glial cells, neuron apoptosis, and TiO₂ NP aggregation were seen. Additionally, levels of norepinephrine, dopamine, and 5-hydroxytryptamine were significantly decreased, and NO levels were markedly elevated. Additionally, the low doses of TiO₂ NPs considerably upregulated the expressions of the genes for C-fos, C-jun, and BDNF while dramatically downregulating the expressions of the genes for p38, NGF, CREB, NR1, NR2ab, and GluR2. These findings suggest that low doses of TiO₂ NP exposures may cause brain damage in zebrafish, offer a developmental framework for assessing the neurotoxicity of subchronic exposure, and raise concerns about the use of TiO₂ NPs in aquatic environments. (Lei Sheng, et al, 2014)

2.11 EXAMINATION OF TiO₂ IN ZEBRAFISH

TiO₂ nanoparticles (nTiO₂) are typical manmade nanomaterials that are typically regarded as non-toxic and have previously been extensively used in numerous goods and applications. Some short-term studies that showed no or minimal toxicity of nTiO₂ to cells and eco-relevant organisms may have contributed to this notion. The long-term effects on the environment and human health are still a concern and require additional research because nTiO₂ will eventually be released into the aquatic environment, which can serve as a sink for manufactured nanoparticles. Here, we show for the first time that zebrafish reproductive can be considerably hampered by persistent exposure to 0.1 mg L⁻¹ nTiO₂. For instance, 13 weeks of exposure to nTiO₂ resulted in a drop of 29.5% in the total number of zebrafish eggs. We therefore provided timely information indicating a serious risk of reproductive impairment of aquatic organisms in environments

contaminated with low levels of nTiO_2 , which could change population dynamics and the balance of the aquatic ecosystem. This calls for a careful examination of nTiO_2 toxicity assessment, especially their long-term impact (Jiangxin Wang, et.al, 2010).

2.12 CROSS-SECTIONAL DATASETS OF BRANDED FOODS

As a food colouring ingredient, food-grade titanium dioxide (TiO_2 ; E171) is used. The European Food Safety Authority published a scientific opinion in May 2021 that stated TiO_2 could no longer be regarded as a safe food additive. Our goal was to look into the usage patterns of TiO_2 in the food supply. Two nationally representative cross-sectional datasets of branded foods were used in a case study in Slovenia. An analysis of $N = 12,644$ foods from 15 food subcategories where TiO_2 was discovered as a food ingredient was done (6012 in 2017 and 6632 in 2020, respectively). TiO_2 usage was seen to have significantly decreased (3.6% vs. 1.8%; $p < 0.01$). In 2017 and 2020, the chewing gum (36.3%) and chocolate and sweets (45.9%) categories, respectively, were the ones that used TiO_2 the most. The chewing gum category had the highest percentage of foods containing TiO_2 in 2017, at 70.3%, and these goods represented more than 85% of the market. Only 24.6% of chewing gums in 2020 contained TiO_2 , making up just 3% of the market. Even though TiO_2 hasn't been formally taken off the list of permitted food additives, we were able to demonstrate a general decline in TiO_2 use. (Urška Blaznik, et.al, 2021)

2.13 POSSIBLE ADVERSE EFFECT OF FOOD ADDITIVE

As a food additive (E171), titanium dioxide (TiO_2) is utilised in a variety of foods, including sauces, icings, chewing gum, toothpaste, and medication tablets. Concerns have been raised concerning TiO_2 's use in commercially available items in addition to the material's pervasiveness and fresh discoveries regarding its possibly dangerous characteristics. Particularly the nano-sized particle fraction of TiO_2 (100 nm) calls for a more thorough analysis of potential harmful health consequences upon consumption. Insecurities and knowledge gaps regarding the gastrointestinal absorption of TiO_2 , its distribution, the potential for accumulation, and the induction of harmful health effects like inflammation, DNA damage, and tumour promotion were identified during a workshop organised by the Dutch Office for Risk Assessment and Research (BuRO). In order to postulate an Adverse

Outcome Pathway (AOP) after ingestion, this review intends to locate and assess recent toxicological investigations on food-grade TiO₂ and nano-sized TiO₂ in ex-vivo, in-vitro, and in-vivo tests along the gastrointestinal tract.(Nicolaj S. Bischoff,et.al,2020)

CHAPTER – 3

AIM AND SCOPE

3.1 AIM

To check the developmental toxic effect of INS 171 (Titanium dioxide) during developmental phase of Zebra fish embryos. The study aims to determine the impact of INS171 (TiO₂) exposure on various endpoints, including growth, deformities, gene expression changes, and oxidative stress markers. The present study seeks to provide insights into the potential risks associated with the use of food additives and their interactions with environmental toxins, and to inform the development of safer food products and environmental policies.

3.2 SCOPE

Evaluation of toxic effect on the food ingredients by using in vivo zebra fish model system. The dose dependent effect of TiO₂ on developmental zebra fish embryos. The study will involve exposing zebra fish embryos to different concentrations of INS171 (TiO₂), and monitoring their growth, deformities, gene expression changes, and oxidative stress markers over time. The study will also explore the potential mechanisms underlying the observed effects and their implications for human health and the environment. The project will contribute to our understanding of the potential risks associated with the use of food additives and their interactions with environmental toxins, and inform the development of safer food products and environmental policies. The study is limited to the effects on zebra fish and their embryos and may not be directly applicable to other organisms.

CHAPTER-4

MATERIALS AND METHODS

MAINTENANCE AND SPAWNING OF ZEBRAFISH (DANIO RERIO)

Fish were fed live brine prawns once daily and dry prawn flakes twice daily while being kept at a constant temperature of 28°C with a photoperiod of 14 hours of light and 10 hours of darkness and a pH of 7.0-7.2. For spawning, a 1:2 ratio of mature acclimatised female and male zebrafish was used. In order to prevent cannibalism of the released eggs, zebrafish were placed in a plastic mesh enclosure and covered with black plastic for 12 hours to create a dark environment. Eggs were exposed to light after being incubated in the dark, and spawning began as soon as the light was turned on and finished in 30 minutes. Following fertilization, embryos in the segmentation phase were gathered, rinsed, and put in a watch glass, where their uniformity was checked by looking through a stereomicroscope. Prior to its usage in subsequent trials, the embryos that had not fertilized or were necrotic were removed (Karanam et al., 2021).

4.1 EXPERIMENTAL PLAN

4.1.1 EMBRYO TOXICITY

- Embryo toxicity is an in vivo toxicity test using zebrafish embryos to evaluate the toxic nature of the TiO₂
- Embryos were transferred into 96 well plate, each well containing 5 embryos and treated with different concentrations of TiO₂ (1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml, 5mg/ml, 6mg/ml)

Control(embryo water E3 medium containing (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 10-5% Methylene Blue) placed into each well of the 96- well plate and the plate was incubated at 26°C±1°C (Karanam et al., 2021).

4.1.2 TERATOGENICITY

The teratogenic activity was examined using a stereomicroscope after 0, 24, 48 and 72 Hours of incubation

- Morphological examination of zebra fish embryos was observed in the following parameters of coagulation, tail not detached and no heart-beat
- Malformation of head and tail, deformity of yolk and growth retardation were examined (Karanam et al., 2021).

4.1.3 HEART RATE ASSESSMENT

Similar to the human heart, the embryonic zebra fish heart demonstrates a pattern of electrical excitation that involves pulses produced. Humans typically have a resting heart rate of 60 to 90 beats per minute. The heart rate is noticeably different in many of the more conventional models. Normal embryonic heart rate in zebra fish ranges from 120 to 180 beats per minute, which is substantially closer to that of humans-32. To guarantee the perfusion of all tissues in the developing embryo, zebra fish heart contraction increases throughout time¹². Zebra fish embryos were subjected to the blood pool procedure. Zebrafish heart contraction increases as embryonic development proceeds to make sure that all tissues of the developing embryo are adequately perfused¹². Under standard circumstances (28°C), we used the blood pool method on zebra fish embryos at various stages of development (De Luca et al., 2014).

4.1.4 HATCHABILITY

In zebrafish, hatchability can be influenced by various factors such as water quality, temperature, genetic background and maternal nutrition

- The hatchability percentage was recorded as number of hatched embryos after 48Hour and it is calculated by the formula;

$$\% \text{ Hatchability} = \frac{\text{Number of hatched embryos}}{\text{Total embryos}} \times 100$$

4.1.5 MORTALITY

The percentage of mortality was recorded as number of dead embryos using the formula;

$$\% \text{ Mortality} = \frac{\text{Number of dead embryos}}{\text{Total embryos}} \times 100$$

4.1.6 MORPHOLOGICAL OBSERVATION OF ADULT ZEBRAFISH TREATED WITH TiO₂

After maintenance in ex-vivo condition, the morphological changes of adult Zebrafish have been observed using Leica M165 FC Stereo-microscope and all the changes were documented.

4.1.7-HISTOPATHOLOGICAL ANALYSIS OF ADULT ZEBRAFISH GILLS AFTER ADMINISTRATION OF TiO₂

In the laboratory, the second right gill arch was removed, fixed in 10% formalin for 48 hour and then decalcified in 10% nitric acid, dehydrated in increasing concentrations of alcohols, cleared in xylene, impregnated and embedded in paraffin. Five µm thick sections were stained with hematoxylin and eosin for histological description. Gill histological changes were evaluated semi-quantitatively by calculating the Histological Alteration Index (HAI), based on the severity of each lesion (Santos et al., 2014).

4.1.8-HISTOPATHOLOGICAL ANALYSIS OF ADULT ZEBRAFISH LIVER AFTER ADMINISTRATION OF TiO₂

After the experiment, zebrafish were collected, the fish were put down in 10% buffered formalin, and the tissues were preserved in cassettes for 48 hours using Davidson's Fixative, which contains 95% ethanol, acetic acid, formalin, and deionized water. After that, 70% ethanol was used to keep the tissues. Additionally, the tissues were dehydrated using a graded ethanol series of 80%, 90%, 95%, and 100% ethanol for 40 minutes each, followed by an additional 30 minutes in 100% ethanol, 60 minutes in Xylene, and another 40 minutes in fresh Xylene. The tissues were then embedded in paraffin after the carcasses were moved to a tissue embedding system. Using a microtome, the tissue blocks were divided into ribbons that were 6 m thick. The ribbons were then put into a

water bath that was preheated to 45 °C. Selected tissue samples were mounted on slides, which were then placed vertically on a slide heater set to 45 °C to dry entirely. The slides were then stained using the Shehand and Hrapchak²⁹ method of hematoxylin and eosin (H&E) staining. A coverslip was placed over the mounted, stained slides. Finally, an Olympus BX40 microscope was used to view the slides under a light microscope, and an Olympus BX53 digital camera was used to take photomicrographs of the slides (Min Zheng, et al.,2018).

4.1.9 Quantitative PCR (RT- PCR)

After TiO₂-treatment (at 96 hpf), total RNA was extracted from 20 larvae using the TRIzol reagent (affyclone laboratoris, Chennai, India). The quality of extracted RNA was evaluated on the basis of OD260/OD280 ratio. Then, cDNA was synthesized using the HiScript II Q RT SuperMix (Vazyme, Nanjing, China), and quantitative real-time polymerase chain reaction (RT-PCR) was performed with an RT-PCR system (Biorad, CA, United Status) using the SYBR Green mix (Takara, Dalian, China). The primer sequences of the genes that were detected are sod1 forward 5'-GGCCAACCGATAGTGTTAGA-3' ; reverse 5'-CCAGCGTTGCCAGTTTTTAG-3' cat forward 5'-AGGGCAACTGGGATCTTACA-3'; reverse 5'-TTTATGGGACCAGACCTTGG-3' hspa5 forward 5'-CAGATCTGGCCAAAATGCGG-3' ; reverse 5'-GGAACAAGTCCATGTTGAGC-3'.

CHAPTER-5

RESULTS AND DISCUSSION

5.1-SOLUBILITY TEST

TiO₂ is a food grade product, which was purchased from sigma aldrich company Bangalore, India. 1mg TiO₂ was weighed and dissolved in 1ml of sterile water and obtained clear solution. Figure 5.1 showed the solubility analysis of TiO₂, where the white colour solution indicated the TiO₂ solubility.

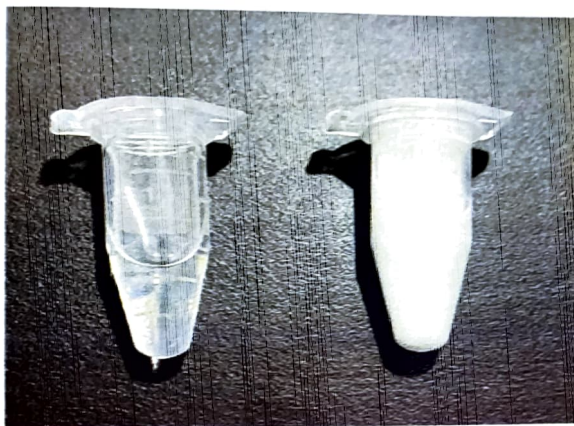


Figure 5.1-Solubility test of TiO₂

5.2- ZEBRAFISH GROWTH

5.2.1- Effect of TiO₂ on developmental embryo in different time intervals.

Embryo-toxicity is an *in vivo* toxicity test using zebra fish embryos at segmentation phase were transferred into 96- well plate and each well containing 5 embryos. Each well was treated with five different concentrations as control, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml and 150 µg/ml of TiO₂ respectively and it is viewed after 0, 24, 48 and 72 hours of incubation and it was examined under stereo microscope for the morphological observation of zebra fish embryos. In our study, the zebra fish embryos were lethal due to higher concentration which as coagulation, tail not detached and no heart rate. In teratogenic effects the zebra fish embryos shows malformation of head, tail and heart, deformity of the yolk and growth retardation was observed (Karanam et al., 2021).

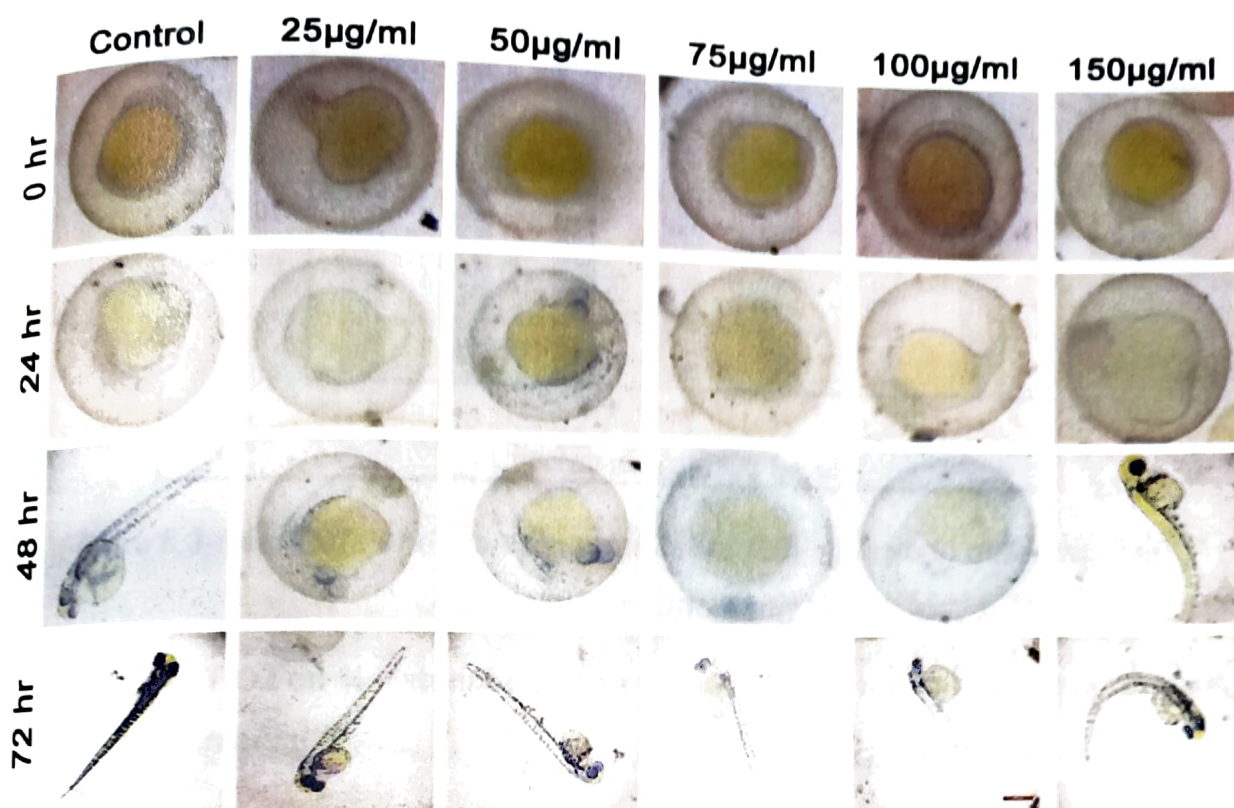


Figure 5.2-Growth factor of effect of TiO₂ on developmental Zebra fish embryo was seen in different time intervals

5.2.2-Effect of TiO₂ on Heart rate of developmental embryo on different time intervals

In our study, we found that the higher concentration of TiO₂ influences the Zebrafish embryo heart rate, which was found to decrease gradually.

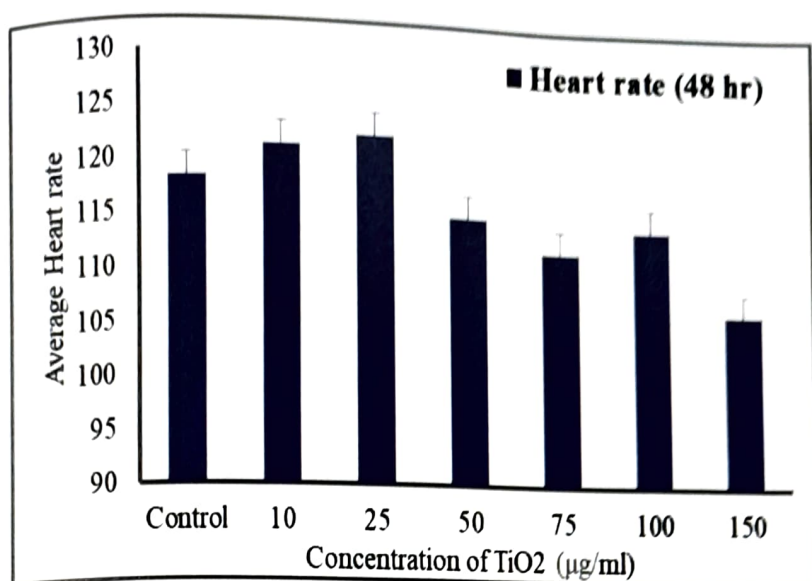


Figure 5.3-Heart rate of developmental Zebra fish embryo in different time intervals (24hr and 48hr)

5.2.3-Effect of TiO₂ on % Hatchability rate on developmental Zebra fish embryo of different time interval

In our study, we recorded that the higher concentration of TiO₂ is increased. So the hatchability of zebrafish embryo was delayed. And the hatchability rate was decreased.

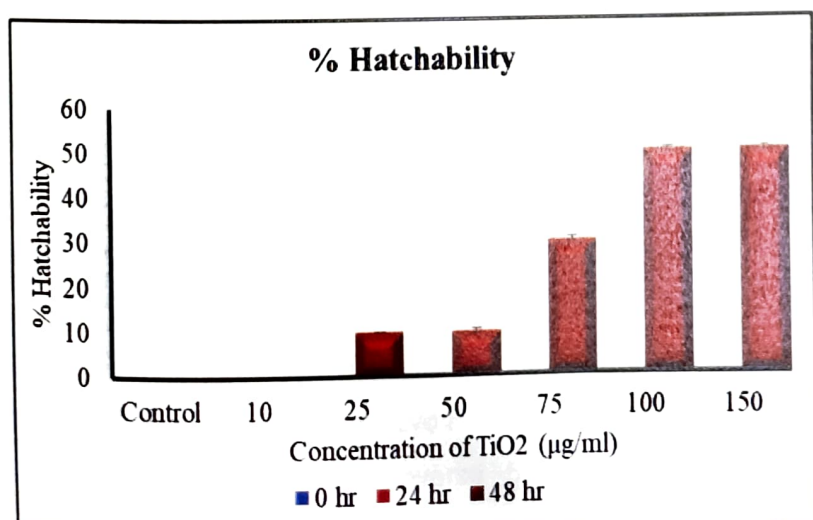


Figure 5.4 -Hatchability rate on developmental zebra fish embryo in different time intervals (24hr and 48hr)

5.2.4-Effect of TiO2 on % mortality rate on developmental embryo of different time intervals

In our study, we found that the higher concentration of TiO2 showed the increased dead rate of zebrafish embryo. Also found, later time interval the zebrafish embryos were started degrade slightly. That shows the toxic effect of TiO2 on higher concentration with zebrafish embryos on developmental stages.

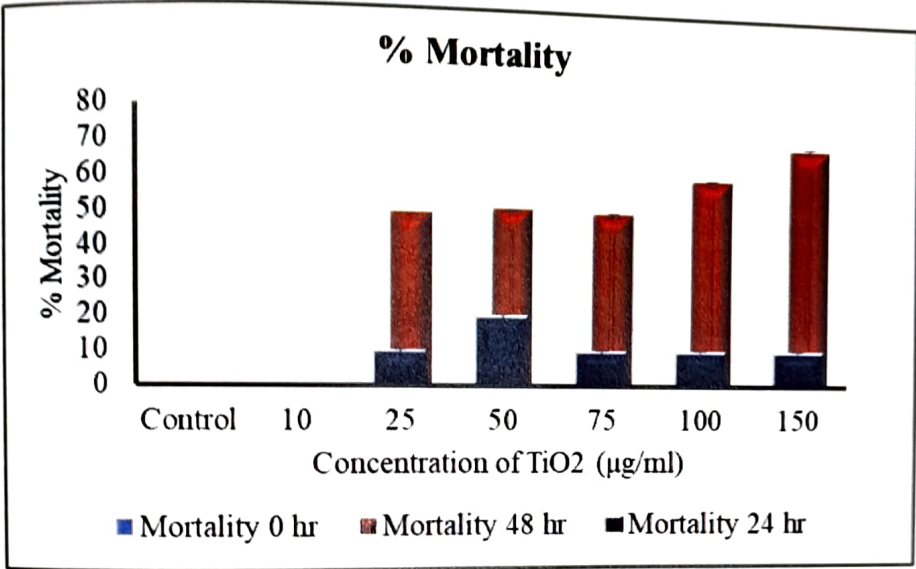


Figure 5.5 -Mortality rate on developmental zebra fish embryos in different time intervals (24hr and 48hr)

5.3 The Impact on Antioxidant Related Gene Expression on Zebrafish embryos

The zbrafish embryos were treated with in 100ug/ml concentrations of TiO2 for 48 hrs and the embroys were collected. The total RNA was isolated from the embryos by using trizol method. The RNA was quantified and cDNA conversion was done using Reverse polymerase conversion kit, Thermo scientific, India. The cDNA was further amplified for the gene expression by using Quantitative PCR. The sod1 forward 5'-GGCCAACCGATAGTGTTAGA-3' ; reverse 5'-CCAGCGTTGCCAGTTTTTAG-3' cat

forward 5'-AGGGCAACTGGGATCTTACA-3'; reverse 5'-TTATGGGACCAGACCTTGG-3' hspa5 forward 5'-CAGATCTGGCCAAAATGCGG-3'; reverse 5'-GGAACAAGTCCATGTTGAGC-3'.

The gene expressions of three antioxidant damage-related genes (SOD1, CAT and HSPa5) was assessed. The antioxidant genes SOD and CAT was decreased with 100µg/ml concentration of TiO₂ treatment, whereas a gene HSPa5 was significantly increased with TiO₂ treatment. The obtained results from RT-PCR analysis showed the TiO₂ induces the oxidative damage when exposure for 15 days (Tang et al., 2019).

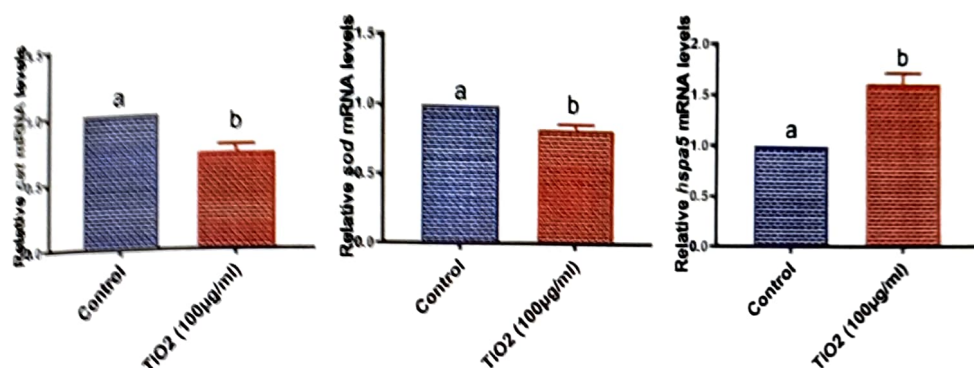
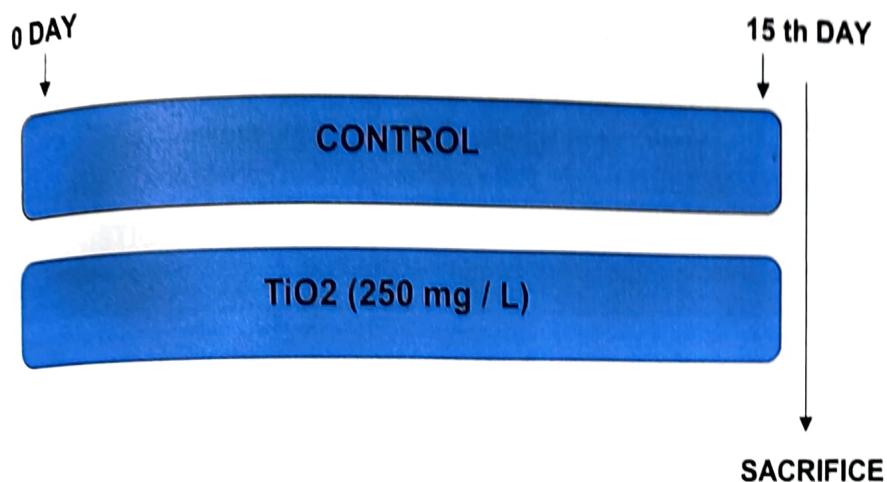


Figure 5.6 CAT, SOD and HSP activities in zebra fish tissues

The addition of TiO₂ to adult zebra fish may have various effects on the activities of antioxidant enzymes and heat shock proteins (HSPs) such as SOD (superoxide dismutase), CAT (catalase), and HSP70. TiO₂ nanoparticles have been reported to cause oxidative stress in fish, which can lead to changes in the activities of antioxidant enzymes. Studies have shown that exposure to TiO₂ can increase the activity of SOD and CAT in fish, indicating that the fish are experiencing oxidative stress and attempting to counteract it by increasing antioxidant enzyme activity. However, prolonged exposure to TiO₂ may cause a decrease in antioxidant enzyme activity due to damage to the enzymes themselves. As for HSPs, exposure to TiO₂ has been shown to induce the expression of HSP70 in fish, indicating a stress response to the nanoparticles. However, the extent and duration of the response may depend on the concentration and duration of exposure to TiO₂. Overall, the effects of TiO₂ on SOD, CAT, and HSP activities in adult zebra fish may

vary depending on the concentration and duration of exposure to the nanoparticles. It is important to note that TiO₂ can have negative impacts on fish health and that the use of TiO₂ should be carefully monitored and regulated to minimize potential harm to aquatic organisms.

5.4-EXPERIMENTAL DESIGN FOR ADULT ZEBRAFISH STUDY



5.4.1-Morphology

For developmental biologists, the Zebrafish (*Danio rerio*) has gained popularity as a model organism. After maintenance of the TiO₂ treated with zebrafish in ex-vivo condition, the morphological changes have been observed. The changes observed as embryos are small, develop quickly, and are optically transparent. These benefits, together with the efforts of other labs, have allowed for the detection of thousands of mutations that alter development (Hernandez, 2002).



Figure 5.7- Morphology of adult zebra fish treated with TiO₂

5.4.2-HISTOPATHOLOGICAL ANALYSIS OF ADULT ZEBRAFISH GILLS AFTER ADMINISTRATION OF TiO₂

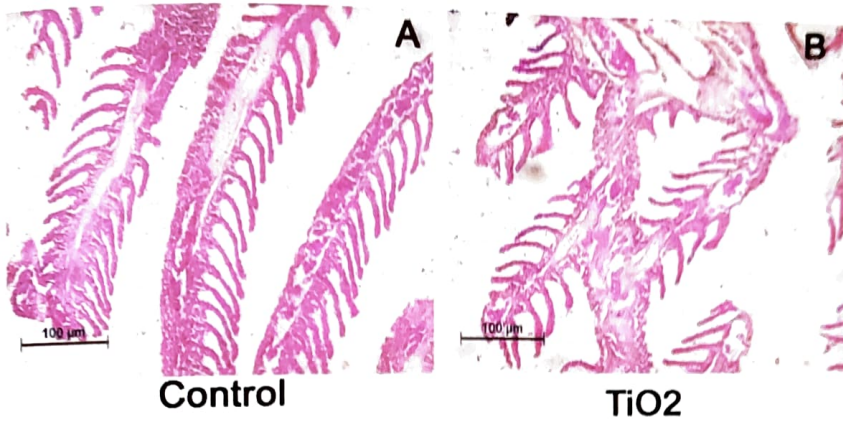


Figure 5.8 Histological alterations in the gills of adult zebra fish treated with TiO₂

5.4.3-HISTOPATHOLOGICAL ANALYSIS OF ADULT ZEBRAFISH LIVER AFTER ADMINISTRATION OF TiO₂

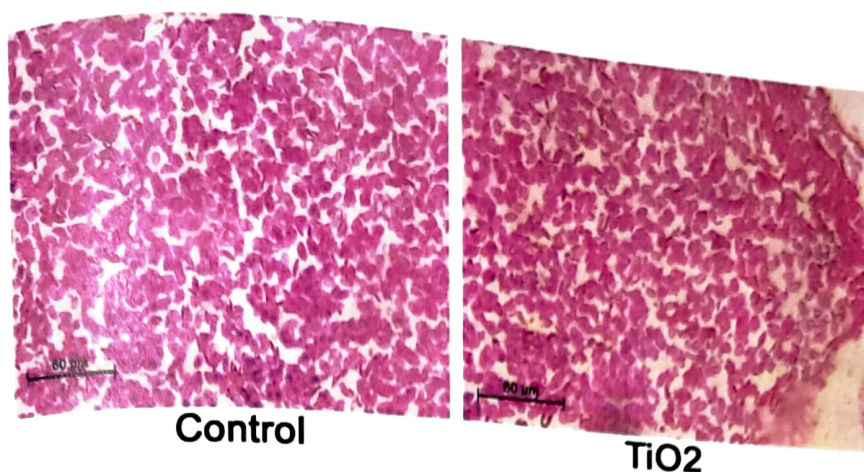


Figure 5.9 *Histological alterations in the liver of adult zebra fish with TiO₂*

The histology of zebrafish have also been investigated in several studies. These studies have examined changes in tissue morphology and structure in various organs, including the liver and gill. The study found that exposure to TiO₂ nanoparticles caused at 250mg/L concentration for 15 days exposure influences the liver histological morphology, including alterations in the arrangement of hepatocytes and the presence of vacuoles and lipid droplets. Similarly, the exposure to TiO₂ nanoparticles caused at 250mg/L concentration for 15 days exposure affects the architecture of gills of adult zebrafish (Hou et al., 2015).

DISCUSSION

The food additive INS 171, also known as titanium dioxide (TiO₂), is commonly used as a whitening agent and as an additive in a variety of food products. However, recent studies have shown that TiO₂ can have developmental toxic effects on zebrafish and embryos. One study published in the journal *Environmental Pollution* exposed zebrafish embryos to different concentrations of TiO₂ nanoparticles and found that they caused developmental abnormalities and increased mortality rates. The study concluded that TiO₂ nanoparticles have the potential to cause developmental toxicity in zebrafish embryos (Wang et al., 2016). Another study published in the journal *Aquatic Toxicology* investigated the effects of TiO₂ nanoparticles on the development of zebrafish larvae. The study found that exposure to TiO₂ nanoparticles caused a range of developmental abnormalities, including reduced hatching rates, delayed development, and abnormal

swimming behavior. The study also found that exposure to TiO₂ nanoparticles caused oxidative stress and DNA damage in zebrafish larvae (Chen et al., 2016). As exposure to toxic food ingredients can have negative effects on development and health, it is crucial to take into account the potential toxic effects of TiO₂ on zebrafish and zebrafish embryos. According to studies, exposure to TiO₂ during the first 48 hours of incubation a zebrafish embryo's development can result in delayed hatching, lower survival rates, and physical malformations and deformities of the body and organs are observed. TiO₂ exposure in adult zebrafish can result in oxidative stress and organ and tissue damage, which may impair immune system and reproductive function.

The previous studies suggest that TiO₂, as a food additive, could potentially have negative effects on the development of zebrafish embryos and larvae. However, further studies are needed to determine the specific mechanisms by which TiO₂ causes developmental toxicity and to investigate the potential long-term effects of exposure to TiO₂ in food products. The study also found that exposure to TiO₂ nanoparticles caused changes in the zebrafish's skin, including inflammation, edema, and hyperplasia (Zhu et al., 2017). Another study published in the journal *Aquatic Toxicology* investigated the effects of TiO₂ nanoparticles on zebrafish larvae and found that exposure to TiO₂ nanoparticles caused dose-dependent reductions in body length and weight. The study also found that exposure to TiO₂ nanoparticles caused changes in the zebrafish's eyes, including abnormal pigmentation and retinal degeneration (Chen et al., 2017). A third study published in the journal *Chemosphere* investigated the effects of TiO₂ nanoparticles on the morphology and behavior of adult zebrafish. The study found that exposure to TiO₂ nanoparticles caused changes in the zebrafish's behavior, including reduced swimming activity and increased anxiety-like behavior. The study also found that exposure to TiO₂ nanoparticles caused changes in the zebrafish's morphology, including decreased body weight and altered fin morphology (Huang et al., 2018). Taken together, these studies suggest that exposure to TiO₂ nanoparticles can cause a range of morphological changes in zebrafish, including changes in body length, weight, skin appearance, eye pigmentation, and fin morphology. These changes may have implications for the overall health and survival of zebrafish populations exposed to TiO₂ nanoparticles.

The previous studies have examined changes in the expression levels of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as changes in the expression levels of heat shock proteins (HSPs). One study published in the journal *Aquatic Toxicology* investigated the effects of TiO₂ nanoparticles on zebrafish embryos and larvae. The study found that exposure to TiO₂ nanoparticles caused dose-dependent increases in the expression levels of SOD and CAT in zebrafish embryos. However, the study found that exposure to TiO₂ nanoparticles caused dose-dependent decreases in the expression levels of SOD and CAT in zebrafish larvae. The study also found that exposure to TiO₂ nanoparticles caused dose-dependent increases in the expression levels of HSP70 in zebrafish embryos and larvae (Chen et al., 2016).

CHAPTER 6

SUMMARY AND CONCLUSION

"Developmental Toxic Effects of Food Additive (INS 171) on Zebrafish and Embryos with the Addition of TiO_2 " suggests that TiO_2 exposure may have negative effects on zebrafish and zebrafish embryos. Specifically, results indicate that TiO_2 exposure may cause developmental abnormalities in zebrafish embryos, such as delayed hatching, decreased survival rate, or morphological abnormalities. While the toxic effects of TiO_2 on humans are not yet fully understood, some studies have suggested that exposure to high levels of TiO_2 may have negative health effects, such as respiratory problems and potential impacts on the reproductive and immune systems. Additionally, exposure to TiO_2 may lead to oxidative stress in zebrafish, as evidenced by changes in the activity of antioxidant enzymes like SOD and CAT. Study found that exposure to TiO_2 may lead to tissue damage and morphological abnormalities in the gills and liver of zebrafish. Additionally, TiO_2 exposure may cause oxidative stress in these organs, as evidenced by changes in the activity of antioxidant enzymes like SOD and CAT. The negative effects of TiO_2 exposure on zebrafish and zebrafish embryos may be concentration- and time-dependent, with higher concentrations and longer exposures leading to more severe effects. Furthermore, TiO_2 exposure may cause tissue and organ damage in adult zebrafish, which can negatively impact reproductive capacity and immune function. Overall, project suggests that TiO_2 exposure may have developmental and physiological effects on zebrafish and zebrafish embryos. In conclusion, your project highlights the potential risks associated with TiO_2 exposure in aquatic ecosystems and human health. It underscores the importance of continued research into the potential risks associated with nanoparticles in food and other applications, and the need for careful monitoring and regulation to protect the health of both aquatic life and humans.

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