TOXICITY OF TITANIUM DIOXIDE NANOPARTICLES (TiO_2) IN *CTENOPHARYNGODON IDELLA*

By

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In

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بسم الله الرحمن الرحيم

CERTIFICATE

It is certified that the contents and forms of the thesis entitled **"Toxicity of titanium dioxide (TiO_2) nanoparticles in *Ctenopharyngodonidella*"** submitted by Ms. Zaib-un-Nisa has been found satisfactory for the requirements of the degree of Master of Science in Environmental Science

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DEDICATED TO

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies.

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ZAIB-UN-NISA

Contents

LIST OF ABBREVIATIONS

mg	Milligram
µg	Microgram
mg/L	Milligram per liter
µl/L	Milliliter per liter
WHO	World Health Organization
APHA	American Public Health Association
TiO_2	Titanium dioxide
NP	Nano particles
FP	Fine particles
TiO_2-NP	Titanium dioxide nano particles
TiO_2-FP	Titanium dioxide fine particles
ENP	Engineered nano particles
NBT	Nitroblue tetrazolium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ENA	Erythrocyte nuclear abnormalities
EtBr	Ethidium bromide
LMPA	Low melting point agarose
NMPA	Normal melting point agarose
ROS	Reactive oxygen species
RBCs	Red blood cells
NADPH	Nicotinamide adenine dinucleotide phosphate
EC_{50}	Effective concentration 50
LC_{50}	Lethal concentration 50

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ABSTRACT

Titanium dioxide (TiO_2) nanoparticles have a wide range of use in various commercial products. Such widespread use of nano sized TiO_2 could lead to significant release of nano-TiO_2 into the environment leading to a potential threat for their increased environmental concentrations. Fish occur at the top of food chain hierarchy in aquatic environment and is valuable bio-monitor of aquatic pollution. In the present study, grass carp (*Ctenopharyngodonidella*) was used as bioindicator for the toxicity potential of nano-TiO_2. The effects were based on acute, sublethal toxicity. Two doses of 1.5 and 3.0 µg of nano- TiO_2 and fine particles of titanium dioxide used as negative control. The exposure periods for the study were 24, 48 and 72 h. Genotoxic effects analyzed through comet assay, suggested morphological changes in erythrocyte nuclei. Nuclear abnormalities including blebbed, segmented and mironulei were observed. The DNA damage was approximately 90 % for nano-TiO_2 exposed group. Nitroblue tetrazolium (NBT) reduction assay for determination of immunological changes provided a clear evidence for increase in reactive oxygen species (ROS) production triggered by the nanoparticles. ROS concentration had reached nearly 50 % at 72 h exposure to nanoparticles. The results suggested a positive correlation (r= 0.7791, 0.7891) between increase in ROS production and DNA damage.

Chapter 1

INTRODUCTION

1.1 Background

Water is a prerequisite for life on Earth. The aquatic environment plays a vital role in functioning of ecosystem. The aquatic biome includes marine and fresh water biomes which approximately cover 75 % of total Earth's surface. Nearly 97.5 % of water exists as oceanic and inland sea salt water and remaining 2.5 % is fresh water. The fresh water biomes cover 1/5 area of Earth not only caters more than 700 species of fish, 1,200 species of amphibians, mollusks, and insects; but is also available for terrestrial species including humans. Only 0.2 % of this fresh water is available for our use, while 80 % exists in form of ice in glaciers and polar ice caps. Freshwater resources are a major requirement for food security, public health, ecosystem protection, and human well-being (Setegn *et al.*, 2014)

Small yet essential for life; fresh water is under potential stress by ever growing needs. The contamination of all aquatic bodies at unprecedented rates, has become a global concern (Ghumman, 2011). Nanoparticles (NPs) are an important example of a new class of environmental pollutants. Increase in production and utilization of NPs has lead to increased human and environmental exposure (Gottschalk *et al*., 2010)

Engineered nanoparticles (ENPs) is a term that refers to a subset of nanomaterial with at least one external dimension in the size range from 1 to 100 nm (ISO, 2008).

In addition to volume of industrial production, possibility of anthropogenic agents ending up in aquatic environments also depends on how they are used. There is increase in concerns of adverse effects of engineered nanoparticles (ENPs) on human health and ecosystem due to their wide use in industrial and consumer products (Alvarez *et al.,* 2009; Klaine *et al.,* 2012; Salieri *et al.,* 2015). Numerous estimates of different quantities of various ENPs presently being produced have been published (Schmid *et al.,* 2010; Hendren *et al.,* 2011; Piccinno *et al.,* 2012; Sun, *et al.,* 2014; Troester *et al.,* 2016).

ENPs exist in various forms including; strongly embedded within a solid material, present in liquid form in emulsions or suspensions or even used as aerosols that are emitted directly to air. Especially the release of ENM contained in liquids, pastes, creams, and powders, and aerosol spray application is expected to play a significant role in contamination of environment (Gottschalk and Nowack, 2011).

Release may also occur unintentionally during any stage in lifecycle of ENPs. This includes release during production of ENP or ENP containing products. They are also released via sewage sludge, wastewater, and waste incineration of products containing ENP (Aitken *et al.,* 2004; Gottschalk *et al.,* 2009; Gottschalk *et al.,* 2011).

Fresh water comprises of various surface water bodies including rivers, lakes or reservoirs. Since these water bodies are direct recipients of waste streams and in direct contact with atmosphere they, are exposed to human and industrial activities and discharges. In view of all multitude release patterns possible for ENPs, surface waters are prone to be recipients of ENPs. Once released into an aquatic environment, significant impact of ENPs depends upon their stability and mobility,

and ultimately on their presence and morphology in water resources used (Troester *et al.,* 2016). Most of these ENPs are found to cause adverse impact in fresh water flora and fauna.

1.2 Present Study

Pollution of fresh water sources is a matter of concern at not only national level but also at international level. Various chemical agents from anthropogenic activities are introduced into aquatic environment through direct release or indirect release. Aquatic systems pose as a sink for various contaminants.

Fish, amongst various aquatic organism, is a valuable bio-monitor of aquatic pollution (Xing *et al.,* 2015). Fish occur at the top of food chain hierarchy in aquatic environment and play an important role in maintaining balance in aquatic ecosystem (Li *et al.,* 2013). Different reactions initiate in biological system when xenobiotics interact with fish, which ultimately result in biochemical changes (Somashekar *et al.,* 2015). Grass carp (*Ctenopharyngodon idella*) is most promising aquatic products because it is commercially valuable and is one of major food sources (Chen *et al.,* 2016). It is a widely distributed mid-lower dwelling fish. Its feeding habits and wide spread distribution exposes it to many different types of environmental contaminants.

Titanium dioxide nanoparticles (NPs) and fine particles (FPs) were the xenobiotic used for the present study. TiO_2-NPs are assumed to be more toxic than their analogous FPs. The release of TiO_2-NPs may occur during various processes and at different life stages of diverse products. Nano-TiO_2 are the most abundant nanomaterials which are dumped to the environment among all engineered nanoparticles, according to latest literature (Lv *et al.,* 2016). Direct evidence of

release of synthetic TiO_2-NPs into aquatic environment from urban applications has been documented (Sharma 2009).

TiO_2-NPs have been reported to cause DNA damages and decreased leukocyte viability, reduction of hatching time, genotoxicity in erythrocytes and altered swimming activity in aquatic organisms (Minetto *et al.,* 2016; Vignardi *et al.,* 2015)

1.3 Objectives

Keeping in view impacts of nanoparticles on fish health, current study was conducted at the Institute of Environmental Sciences Engineering (IESE) to monitor toxicological effects of TiO_2-NPs and to support stance that nanoparticles have a more toxic impact. Fine particles of the same agent were also tested for their toxic effect. Grass carp (*Ctenopharyngodon idella*) was used as bio-indicator for aquatic pollution. Following were the objectives of the study:

1. Investigation of blood for erythrocyte nuclear abnormalities
2. Impact on immunity due to acute nanotoxicity
3. Blood genotoxicity potential of TiO_2 NPs

Chapter 2

LITERATURE REVIEW

The word nanotechnology was first introduced at international conference on industrial production to describe processing of nanometer materials and creation of nano-sized mechanisms, at Tokyo in 1974.

From the second half of 1980s to early 1990s important discoveries and inventions were made which led to further development of nanotechnology. This intensified research in nanotechnology and a sharp increase in number of publications in nanotecnological aspects. Since, practical application of nanotechnology has expanded. Financing of nanotechnology projects has increased and so has involvement of organizations and industries. It is considered as one of the most outstanding innovation since the beginning of industrial engineering (Som *et al.*, 2010).

2.1 Engineered nanoparticles (ENPs)

There are naturally occurring nanoparticles in our surroundings as e.g. soil or salt particles to which humans can be exposed on an everyday basis through air or food, water (Miseljic and Olsen, 2014). Nanoparticles of anthropogenic origin are referred as engineered nanoparticles with its dimensions ranging from 1-100nm (ISO 2008).

2.1.1 ENP Production

The estimated production of engineered nanoparticles in 2004 was 2000 tons (Nowack and Bucheli, 2007). The expected increase in production of engineered nanoparticles by 2020 would be approximately 58,000 tons (Mayland, 2006). The

production scales of engineered nanoparticles (tons) used in consumer products on global market estimated by Wijnhoven *et al.* (2009) show production of paints, coatings, adhesives, food packaging, catalytic converters for motor vehicles to be greater than 10,000 tons. Production of ENPs in motor vehicle interior, cosmetics (mainly UV absorbers) and personal care products, insulation material, hard disk media, photocatalytic coatings, wire and cable sheathing, flat panel display, anti-scratch/stick cleaning products and eyeglass/lens coating was 100-1000 tons. While the production of ENPs in water filtration/ treatment systems was upto 100 tons. It is assumed that bulk material would be totally replaced by nanoparticle form around 2025 (Robichaud *et al.*, 2009)

2.1.2 ENP release in environment

The rapid growth in manufacturing and use of ENPs indicates increase in environmental exposure (Hendren *et al.*, 2011; Keller and Lazareva, 2014). ENPs are inevitably released into environment during the production, transportation, use and disposal of products containing them. Paint and adhesive coating contribute to the largest category of ENPs (TiO_2 mainly) used. Their environmental loading may be high because of large volumes produced/used. Degradation of these paints and adhesives might release ENPs into water. Cosmetics (mainly UV absorbers) and personal care products (titanium oxide and zinc oxide mainly) also have a great potential to contribute to aquatic environment contamination due to direct release of ENPs into wastewaters during use and on disposal. Other categories including water filtration/ treatment systems and fuel additives are also high potential sources for aquatic and atmospheric ENP contamination (Tiede *et al.*, 2012).

2.1.3 ENPs in surface water

Industrial processes and new technologies that are based on nanoparticles can lead to their release in environment and in aquatic ecosystems (Benn *et al.*,2010; Dunphy *et al.*, 2006; European Commission, 2004; Gao *et al.*, 2008; USEPA, 2007; Kammer *et al.*, 2010; Jomini *et al.*, 2015). They get into aquatic environments through main pathways including solid and liquid waste streams, and through diffuse entry resulting from use and/or abrasion of products containing ENPs (Duester *et al.*, 2011; Sun, *et al.*, 2014; Nowack *et al.*, 2012; Keller *et al.*, 2013). Once these are released into the aquatic environment, significance of these particles depend upon their stability, mobility, and ultimately on their morphology in water resources. The pathways of ENPs entering water are depicted in Fig 2.1.

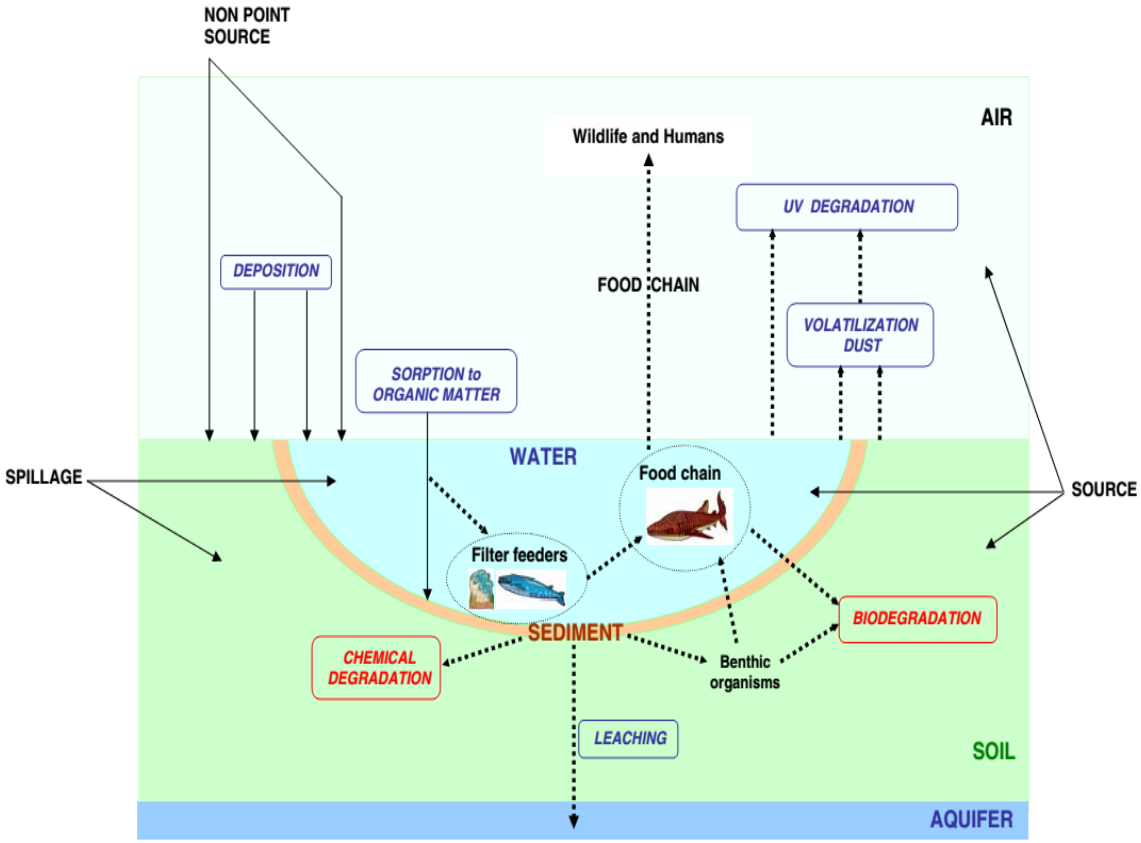

Fig. 2.1: Pathways of ENPs into water (Farré *et al.*, 2009)

2.2 Titanium dioxide (TiO_2)

Titanium dioxide occurs naturally within soils of the Earth. It has a whitish and opaque appearance. This mineral is said to be as old as the Earth itself. According to statistics, it is also one of the top 50 chemicals being produced worldwide. It has been reported in literature that TiO_2 occurs in eleven different polymorphs with distinct structures. Anatase, rutile and brookite are three polymorphs that are abundantly found in nature.

For obtaining pure whitish form of TiO_2, it needs to undergo a series of chemical processes. It is an odorless and absorbent mineral. Due to its innate properties, it has several uses. The paint and cosmetics industry benefit greatly from this naturally occurring mineral. In the field of cosmetics, TiO_2 serves as a white pigment, a sunscreen, and an opacifier.

Table 2.1: Titanium dioxide nanoparticles properties

Properties	
Chemical formula	TiO_2
Molar mass	79.866 g/mol
Colour	White
Appearance	White solid
Odour	Odourless
Density	4.23 g/cm^3 (Rutile) 3.78 g/cm^3 (Anatase)
Melting point	1,843 °C
Boiling point	2,972 °C

Nanoscale TiO_2 that is manufactured for specific applications is by approximately a factor of 100 finer than TiO_2 pigments and has other physical properties. TiO_2-NPs are currently manufactured in large quantities with an estimated 10,000 tons of production per year worldwide because of their applications in a wide range of consumer products (Piccinno *et al.,* 2012). Due to ever-increasing market demand, annual production of nano TiO_2 is predicted to reach 2.5 million tons by 2025 (Zhang *et al.,* 2012). According to market estimation, a production estimated to 2 million tons per year worldwide for period of 2012-2018 (Jomini *et al.,* 2015). It is assumed that nanoparticle form could totally replace bulk material around 2025. Synthesis of phase-pure nano TiO_2 in anatase, rutile and brookite structures, using amorphous titania as a common starting material and may be achieved by hydrothermal treatment at elevated temperatures (Reyes *et al.,* 2008).

TiO_2 for a wide variety of applications, including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water treatment agents and topical sunscreens (Frenzilli *et al.,* 2014). Only cosmetics and sunscreen products alone account for 50 % of nano-TiO_2 usage (Zhang *et al.,* 2012). Currently, they are mainly found in high-factor sun protection creams, textile fibers or wood preservatives. For a long time, sunscreens have been manufactured adding TiO_2 microparticles that gave products a pasty, sticky consistency. Leaving a visible film, application of such sunscreens was not easy and not pleasing to skin. Sunscreens that contain transparent nanoscale TiO_2 can be applied much more easily. In addition, their protective effect against harmful UV radiation is much better. At present, high sun protection factors can only be achieved using nanoscale titanium dioxides.

Such widespread use of nano sized TiO_2 could lead to significant release of nano TiO_2 into environment leading to a potential for increased environmental exposure to TiO_2 nanoparticles (Menard *et al.,* 2011).

2.2.1 Behavior and physical factors affecting TiO_2 nanoparticles in water:

Among the nanomaterials, TiO_2-NPs are predicted to have the highest concentration in surface water (Wang *et al.,* 2016). According to the latest literature, nano TiO_2 is the most abundant nanomaterials dumped to environment among all engineered nanoparticles (Kunhikrishnan *et al.,* 2015). Frequent application at high quantities inevitably results in nano TiO_2-release into aquatic ecosystems for example through wastewater treatment plant effluents, wash-off from facades or major accidents during transport (Nowack *et al.,* 2014; Seitz *et al.,* 2014).

Noticeable nano TiO_2 has been identified in waste waters and natural waters. (Lv *et al.,* 2016). It was estimated in usual estimated environmental concentrations of nano-TiO_2 in aquatic environment are in the range from 0.7 to 24.5 µg L^{-1}. It was predicted that the most frequent concentration of nano-TiO_2 in surface water is 0.02 µg L^{-1}, while same concentration in the sewage treatment water is 4 µg L^{-1} until 2009. Now, predicted environmental concentration for surface water is 0.7-16 µg L^{-1} (Troester *et al.,* 2016).

Physical conditions of water such as ambient temperature and flow velocity, as well as type of water, fresh water and sea water and initial concentration of nano TiO_2 are the critical factors in controlling behavior of these particles in aquatic environment. Higher ionic strength promotes lower stability of nano TiO_2 in water which causes there aggregation. The presence of dissolved of dissolved organic matter is also plays a critical role in particle aggregation (Yang and Cui, 2013). The sedimentation

rate depends upon size of aggregates. These aggregates are reversible and can tend to stay suspended in water due to disturbance and flow rate of water.

Ion strength alone or together with other environment characters can significantly impact aquatic behaviors of nano TiO_2. The hydrodynamics and sedimentation amount of nano TiO_2 both increase with increasing of ambient temperature, indicating a higher mobility of nano TiO_2 in water systems with lower temperatures. Flow velocity also plays an important role in stability of nano TiO_2, with higher velocity leading to smaller aggregates and higher suspension concentrations. The roles of water physical conditions play a great role in stability of nano TiO_2 which can determine different behaviors of nano TiO_2 in aquatic systems (Lv *et al.*, 2016).

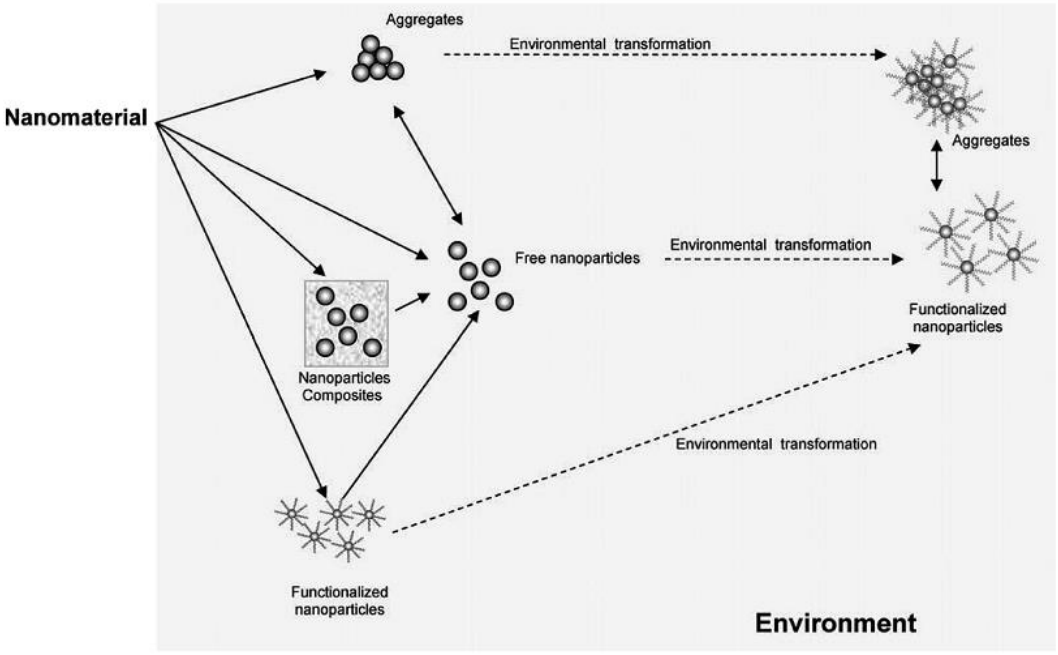

Fig. 2.2: TiO_2 –NP behavior in water (Farré *et al.*, 2009)

2.3 Grass Carp (*Ctenopharyngodon idella*)

The grass carp is one of the largest members of minnow family. Although grass carp are related to both common carp and goldfish, distinct differences exist both in appearance and feeding habits. The grass carp, also known as the White Amur, is a vegetarian fish native to the Amur River in Asia.

Grass carp are native to large rivers in Asia, ranging from the Amur River in China and Siberia south to the West River in China and Thailand. This fish commonly and widely found in Pakistan. Grass carp prefer soft-tissue aquatic plants, filamentous algae (e.g., *Cladophora* and *Pithophora*) and duckweeds (*Lemnaceae*). However grass carp is not an exclusively herbivorous fish species, as it also needs food of animal origin. Suspension-feeding organisms can be considered as a significant target group for effects of different NPs and/or NP agglomerates, and may play an important role in their uptake, biotransformation and transfer through food webs (Barmo *et al.*, 2013). It is an integral part of fish culture, as fish flesh forms an important source of protein for human consumption (Pipalova *et al.*, 2006). Adverse effects of nano TiO_2 in algae have also been studied (Wang *et al.*, 2016). Dietary habit can be one of the main reasons for the exposure to nano TiO_2.

2.4 Nano-TiO_2 toxicity in aquatic organisms

TiO_2 toxicity in aquatic animals in freshwater species, immunotoxicity, cytotoxicity and oxidative stress as well as physiological and reproductive alterations, have been well documented. The sub-acute toxicity of TiO_2-NPs to carp (*Cyprinuscarpio*) resulted in oxidative stress. The extent of depletion of antioxidant enzymes activities and indicating that the liver might be the most susceptible organ to TiO_2-NPs exposure. In addition, carps had gill pathologies including edema and thickening of

gill lamellae as well as gill filaments (Hao *et al.,* 2009). Variable 48-h EC50 and LC50 values were reported for TiO_2 nanoparticles in D. magna, with values ranging from 5.5 mg/L (Lovern and Klaper, 2006) up to 20 000 mg/L (Heinlaan *et al.,* 2008). Results showed an increase of planktonic bacterial abundance at the highest TiO_2-NP concentration. TiO_2-NP exposure to planctinic and sessile bacterial communities, showed a strong decrease of Betaproteobacteria in sessile communities (Jomini *et al.,* 2015). Effect on cumulative production of viable embryos, oxidative stress during 1 mgL^{-1} exposures in zebra fish have also been reported (Ramsden *et al.,* 2013). Nano-TiO_2 exposure has also caused changes in swimming speed due to damage to gill epithelium with a subsequent hypoxia and changes in brain histology with enlarged vasculature (Boyle *et al.,* 2013). Study on genotoxicity and ctotoxicity due to titanium dioxide (TiO_2) nanoparticles exposure have resulted in genotoxicity in erythrocytes of marine fish, *Trachinotuscarolinus* (Vignardi *et al.,* 2015).

2.4.1 TiO_2 induced reactive oxygen species (ROS) and oxidative DNA damage

TiO_2 nanoparticles have been known to generate reactive oxygen species (ROS), with •OH radicals being predominant radical species generated both in aqueous solution as well as in the fish cells. These radicals are likely to play the major role in producing the genotoxic effects in terms of oxidative DNA damage. (Reeves *et al,* 2008).

NP may be actively endocytosed. In phagocytic cells, phagocytosis triggers activation of NADPH oxidase and generation of reactive oxygen species (ROS). NP may enter the cell by passive diffusion and remain nonmembrane bound from where they may enter mitochondria and disrupt normal electron transport leading to oxidative stress. Free particles may also enter the nucleus via the nuclear pore

complex and interact with the genetic material that may lead to genotoxicity and mutagenesis (Lourenço, 2012). This mechanism has been illustrated in Fig 2.3.

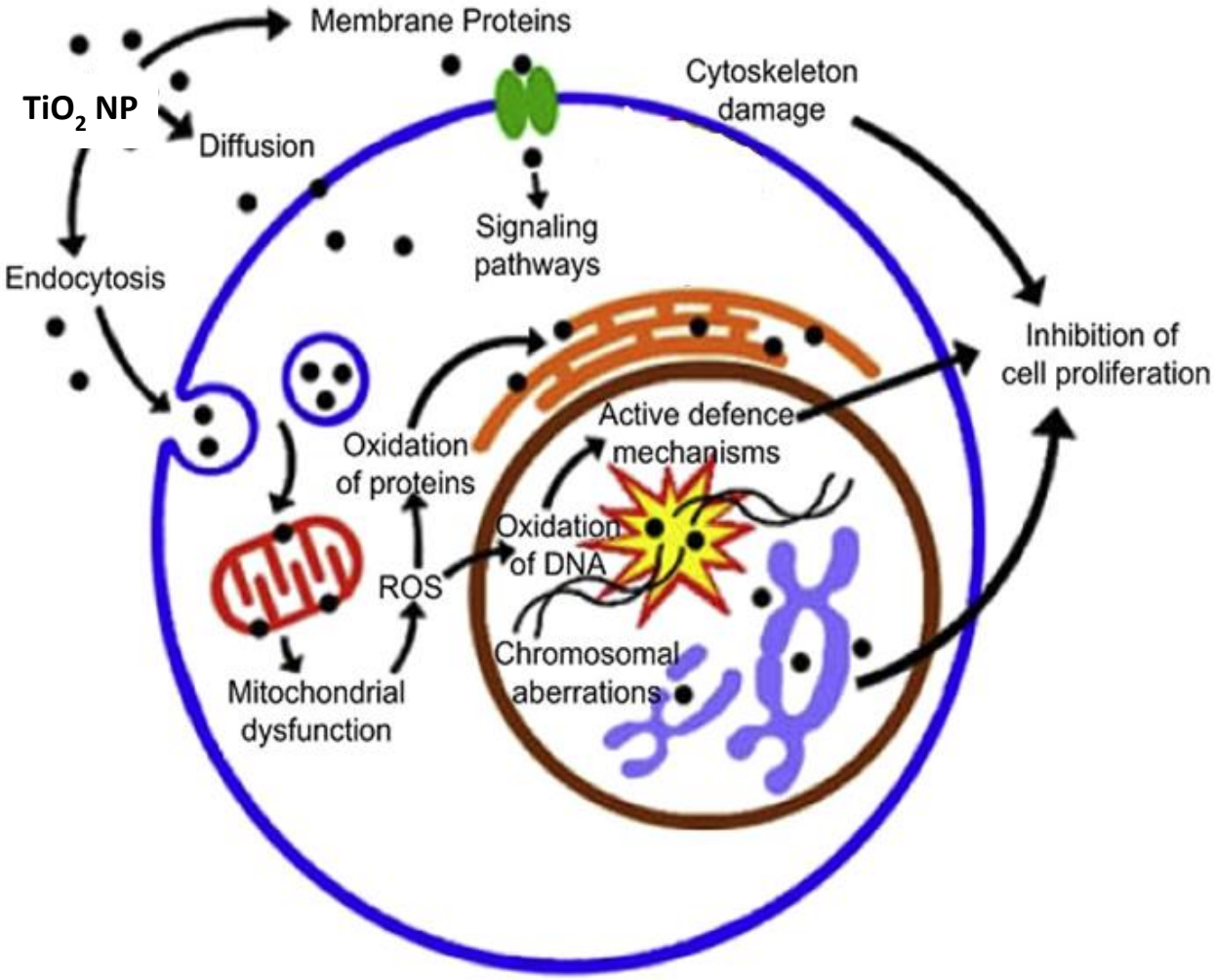

Fig. 2.2: Mechanism of TiO_2 –NP induced toxicity in cell (McShan *et al.*, 2014)

Chapter 3

MATERIALS AND METHODS

Toxicology assays were conducted at Environmental Toxicology Laboratory, IESE, SCEE, National University of Science and Technology (NUST), Islamabad, Pakistan. The experimental study was conducted by intraperitoneal exposure of grass carp (*Ctenopharyngodon idella*) by injecting different concentrations of titanium dioxide nanoparticles (TiO_2-NP) and fine particles (TiO_2-FP), suspensions for different time durations. The study was conducted following the CCAC guidelines on care and use of fish in research, APHA (2012) guidelines.

3.1 Chemicals

Titanium dioxide (Daejung, Korea) was purchased for making dose suspension and for synthesis of pure titanium nanoparticles. Phosphate buffer saline tablets (Oxoid), Normal melting point agarose (Scharlau, Spain), Low melting point agarose (Scharlau, Spain), Sodium chloride, Ethylenediaminetetraacetic acid (Daejung, Korea), Tris(hydroxymethyl)aminomethane (Daejung, Korea), Triton X-100 (Daejung, Korea), Dimethyl sulfoxide (DMSO), Sodium hydroxide (NaOH), Boric acid (H_3BO_3), Tris-hydrochloride (Daejung, Korea) and Ethidium bromide (Et Br) were purchased and used for gel and solution preparation for comet assay. Nitroblue Tetrazolium (Bioworld, USA), and N,N-dimethylformamide (Daejung, Korea) were used for NBT reduction assay. Giemsa stain (Daejung, Korea), absolute Methanol and were used in erythrocyte nuclear abnormalities test.

3.2 Purchase and maintenance of experimental fish

A number of 70, healthy specimen (12-15 cm, yearlings; weight, 87-105 g) of Grass Carp (*Ctenopharyngodon idella*) were purchased from Punjab Hatchery Rawal Town, Islamabad and were transported to the lab in oxygenated polyethylene bags, avoiding mechanical injuries. They were transferred and kept in aerated glass aquarium (dimension 3 X 1.5 X 1.5 ft). Specimen were divided into five batches for each test. Five replicates were placed in each tank, for every dose concentration and exposure duration.

3.2.1 Acclimatization of fish

Initially, fish were acclimatized in laboratory conditions for a period of one week. They were fed commercial dry food pellets. Feed formulations usually contain fishmeal (0-10 percent), soybean meal (10-30 %), rapeseed meal (10-30 %), rice bran, corn, wheat and other agricultural by-products, leaf meal and grass meal. Vitamin and mineral premix and feeding attractants comprise around 2-5 % of the feed. Leftover food and excretory waste in tanks was siphoned off daily. To avoid fouling of tanks, dead fish was removed immediately, if any, due to sudden change in environment at initial stage. Fig.3.1 illustrates the maintenance measure.

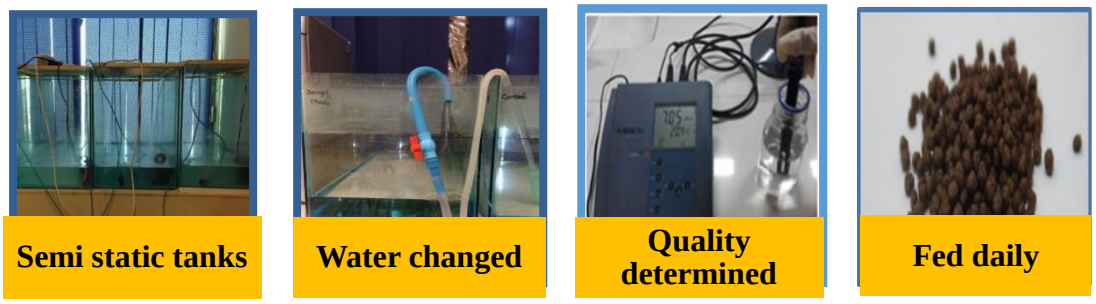

Fig. 3.1: Maintenance of semi static aquatic setup for fish

3.2.2 Water Parameters

The physicochemical parameters were determined according to standard procedures stated in APHA (2012) on regular basis, throughout the study. The water was changed on alternate days. The physicochemical parameters of water are mentioned in Table 3.1.

Table 3.1: Physicochemical parameters of water

Parameters	Units	Mean ± SD	Permissible Limit (APHA, 2012)
pH	----	7.76 ± 0.43	6-9
Dissolved Oxygen	(mg/L)	7.54 ± 0.51	>5
Temperature	(ºC)	21.46 ± 2.31	18-24
Hardness	(mg/L)	234 ± 3	<250
Chlorine	(mg/L)	BDL*	0

*BDL: below detectable level

3.2.3 Experimental design

Using random method the fish were divided in a group of five per tank. The experiment was performed using random selection method for dividing the fish in control and experimental group (Biller *et al.*, 2013). Experiments were performed in batches depending on the type of test and exposure duration. The experimental work was divided into five phases as illustrated in Fig. 3.2.

Fig. 3.2: Experimental design for research work

3.3 Synthesis of TiO_2-NPs

Liquid Impregnation method was performed for the synthesis of pure titanium dioxide nanoparticles (Khan *et al*., 2013). Slurry of TiO_2 was prepared by mixing 50 g of TiO_2 300 mL of distilled water in a beaker. This suspension was constantly stirred for 24 h on magnetic stirrer hot plate after which the solution was allowed to

settle for 24 h. It was oven dried for 12 hours at 105 ^{0}C. After drying, the material was crushed properly using a pestle and mortar. The crushed powder was transferred to china dish which was then, placed in muffle furnace for 6 h at 550 ^{0}C (Sahoo *et al*., 2005) to obtain the pure TiO_2-NP.

3.4 Characterization of Nanoparticles

3.4.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is an advance technique which uses a focused beam of electrons to obtain largely magnified picture. The high-resolution, three-dimensional images produced by SEM provide information including;

a. Topography
b. Morphology
c. Chemistry
d. Crystallography

The topography and morphology of the TiO_2-NP and FP, was carried out using scanning electron microscope (JEOL JSM-6460) at 10,000x magnifications. Scanning Electron Microscopy was used for the examination of sample powders and the direct estimation of particle size. It uses focused beam of electrons to scan a sample for image production with a resolution of 1 nm. X-rays informed us about the elemental and chemical composition of the sample (Lv *et al.*, 2016).

3.4.2 X-Ray Diffraction (XRD)

X-ray Diffraction (XRD, JEOL JDX-II) was used for the identification of crystalline phase and size of nanoparticles. Scherer formula was used to determine the Average

crystaline size of nanoparticles (Younas *et al*., 2014). Principle mechanism of XRD is shown in the following formula.

$$L = K\lambda / \beta \cos\theta$$

Where, L = Average particle size,

K = 0.891; λ = 0.1542,

β = full width of a diffraction line at one half of maximum intensity (FWHM) radian,

θ = the diffraction angle of crystal phase.

3.5 Exposure to TiO_2–NP and FP

Stock suspension of NPs and FPs for two doses 150 and 300 mg/L were prepared. Feed was withdrawn 24 hours before dose administration. Specimen was injected two doses of 1.5 and 3.0 µg–TiO_2–NP and FP in the intraperitoneal region. Before injecting, fish were anesthetized with 33 µl/L of clove oil in a rectangular water container, at 18ºC (Hamackova *et al.,* 2006). After injecting, each fish was transferred to its respective labeled tank in accordance with the dose and chemical agent. The specimen soon recovered after their transfer to the aerated tank water. The exposure period was 24, 48 and 72 hours for each chemical agent and their respective dose. Dosages of TiO_2–NP and FP were prepared and selected in accordance with Scown *et al*. (2009) and Vignardi *et al*. (2015).

3.6 Erythrocyte Nuclear Abnormalities Test

Blood smear slides were prepared by from 20 µL of blood from each fish. These slides were then fixed in absolute methanol for 10 minutes after which they were stained for 40 minutes with freshly prepared 10 % Giemsa solution. These slides were air dried and examined under a light microscope at 100× magnification (Özkan *et al.,* 2011).

The erythrocyte nuclear abnormalities selected for this work were: Segmented nuclear (S), which has a dumbbell shape; Micro nuclei (MN) and blebbed nuclear (B), which has a relatively small evagination of the nuclear envelope (Strunjak *et al.,* 2009).

3.7 Nitroblue Tetrazolium (NBT) Reduction Assay

Stimulated ROS were evaluated using the blood/nitroblue tetrazolium (NBT) method by Smith *et al*. (2013) with minor modification. It is based on the reduction of NBT into the colored compound formazan. For this test, 0.1 ml blood was co-incubated with 0.1 ml of 0.1 % NBT in saline solution for 45 minutes. After incubation period, 1 ml of n,n-dimethylformamide (DMF) was added to the blood/NBT mixture and centrifuged for 10 minutes at 100 x g and the supernatant collected. Evaluation of ROS was done by measuring formazan in the resultant supernatant using a spectrophotometer at 540 nm.

3.8 Comet Assay

Comet assay was performed according to Singh *et al.* (1988) with some modifications in by Tice *et al.* (2000). It is capable of detecting single strand breaks and alkali-labile lesions in the DNA of individual cells. The series of steps in comet assay are as following.

3.8.1 Slides pre-coating

Microscope slides were immersed in 70 % ethanol and burnt over blue flame to remove oil and dust. Slides were then coated with a smooth and thin layer of 1 % normal melting point agarose (NMPA) and laid in a tray on a flat surface to let drying at room temperature.

3.8.2 Sample pouring

A layer (65 µl) of 0.5 % low melting point agarose (LMPA) was added with 20 µl of blood and poured on the previously coated slides. Agarose was allowed to cool in refrigerator until it hardened. Each slide was pre-labeled. After drying a third layer (80 µl) of 0.5 % LMPA poured over the previously hardened layer of agarose to occupy any pores remaining in the second layer. Slides were then transferred in a tray and refrigerated for at least 20-30minutes.

3.8.3 Lysing

After solidifying agarose gel slides were gently immersed in ice cold lysing solution to maintain the stability of agarose gel. Slides were kept in refrigerator for 2-3 hours in a covered dish and radical-induced DNA damage associated with the iron released during lysis from RBCs present in blood sample was prevented by the use of concentrated salts and detergents in lysing solution.

3.8.4 Alkali unwinding

After lysing period, slides were then transferred to highly alkaline buffer (pH>13) for unwinding of damaged DNA fragments for a period of 20-30 minutes.

3.8.5 Electrophoresis

After alkali unwinding the slides were then placed in Horizontal Gel Electrophoresis chamber. The chamber was filled with cold electrophoresis solution to the marked level, making sure that gel on the slides was completely immersed in the solution. The voltage of the chamber was set at 25V and was left to run for 45 minutes.

3.8.6 Neutralization

After electrophoresis, slides were then neutralized drop by drop with neutralization buffer to remove background interferences. Slides were neutralized for 5 minutes and this step was repeated twice or thrice times.

3.8.7 Staining

Neutralized slides were then stained with 80 µl of 1x ethidium bromide at room temperature and left for 5 minutes after which they dipped in chilled distilled water for removal of excess stain. The slides were stained twice with staining solution.

3.8.8 Drying

After staining, comet slides were air dried for 30 minutes to remove moisture. Slides were then prepared to be visualized.

3.8.9 Visual Analysis

Slides were visualized using 40x objective with Trinocular Fluorescent Microscope (Optika- B353FL) equipped with ocular micrometer of 10 µm, camera (AIPTEK:

AHD-Z600) and white LED/12 V 20 W illuminator. Images were obtained for visualization of cells with DNA damage.

3.8.10 Image Analysis

The images obtained from each slide were processed using CASP software. The program works with either color, or gray-scale images of fluorescence-stained comets saved in TIF format. Comets stained with EtBr (red cells on dark background) were analysed. The comets were oriented from left-hand (head) to right-hand side (tail) to be analysed correctly. The image is processed to give values of % DNA damage in numerical form (Końca *et al.,* 2003).

3.9 Statistical Analysis

Statistical analysis was performed for evaluation of data obtained from NBT reduction and comet assay. Data was presented as mean ± SD. One-way ANOVA was applied for determination of significant effect of different dosages of TiO_2-NPs and FPs, for different exposure durations. Pared t-test was applied for significant difference in the effect of same agent (TiO_2-NPs and FPs) at two dosages, for all exposure durations. Significant difference or trend was defined by using the criterion, $p < 0.05$ as significance level. The relationship between ROS concentration and % tail DNA damage was studied by using correlation test. These tests were performed using Graphpad Prism 7.0 (Graphpad Software, Inc., San Diego, CA, USA) and Microsoft Excel 2013.

Chapter 4

RESULTS AND DISCUSSION

The in vivo study was aimed to determine toxicity potential of titanium dioxide nanoparticles. Fine particles were used as a negative control for toxicity assays. *Ctenopharyngodon idella* was selected as biomarker for toxicology study, keeping in view the economic importance of Carps in Pakistan. Acute, sublethal toxicity was determined by in vivo exposure of nano TiO_2 to fish (Jovanovilc *et al.,* 2011). The assays included; erythrocyte nuclear abnormalities test (ENA), nitroblue tetrazolium (NBT) reduction assay and comet assay. All tests were based on hematological analysis for changes in cell nucleus morphology, immunological response and DNA damage. The results were analyzed based on time dependent dose exposure.

4.1 Characterization of TiO_2 particles (fine & nano)

The characterization of nano TiO_2-NPs and FPs was performed scanning electron microscopy (SEM) and x-ray diffraction (XRD) techniques.

4.1.1 Scanning Electron Microscopy (SEM) Analysis

The Scanning Electron Microscopy images show surface morphology of TiO_2-NPs and FPs (Lv *et al.,* 2016). The average particle size of both nano and fine particles, at 50,000 x magnification, was found to be 74.64 and 217.29 nm respectively (Fig.4.1 a & b). This difference in size of both classes of particles predicts different behavior upon exposure.

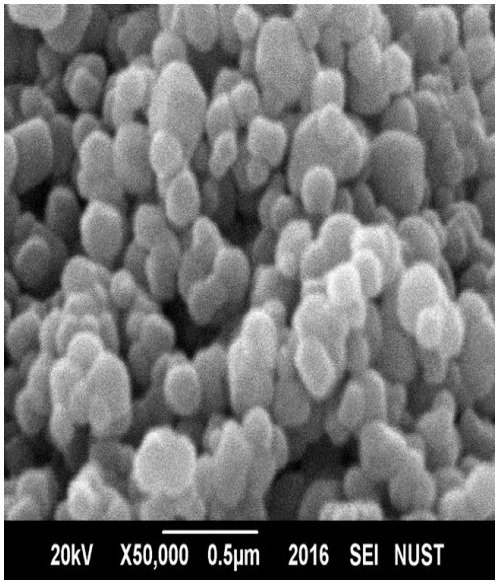

Fig 4.1(a): SEM TiO_2 –NPs

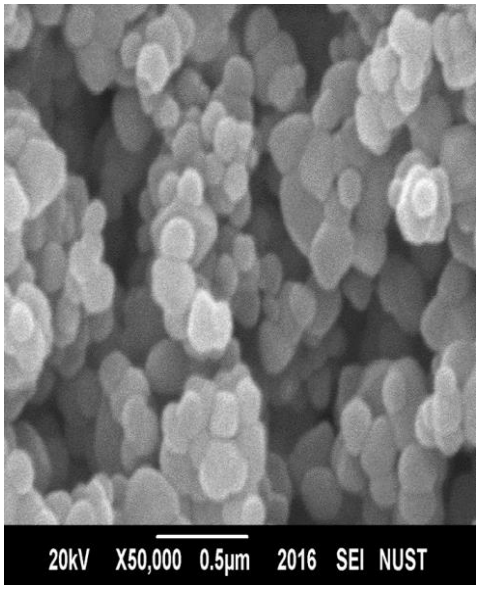

Fig 4.1 (b): SEM TiO_2 -FPs

4.1.2 X-Ray Diffraction (XRD) Analysis

The analysis of titanium dioxide nano and fine particles was performed by X-ray diffraction (XRD) in the range of 2-theta ranging 20º-80º at room temperature. XRD results are illustrated in Fig 4.2 a & b. The crystalline size of nano particles confirmed to be in the range that is less than 100 nm. Peaks of XRD results 25º and 48º revealed that TiO_2-NPs and FPs had anatase crystalline structure (Sirdeshmukh *et al.*, 2006).

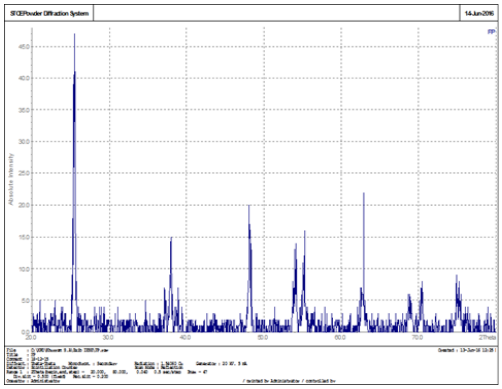

Fig 4.2 (a): XRD TiO_2-NP

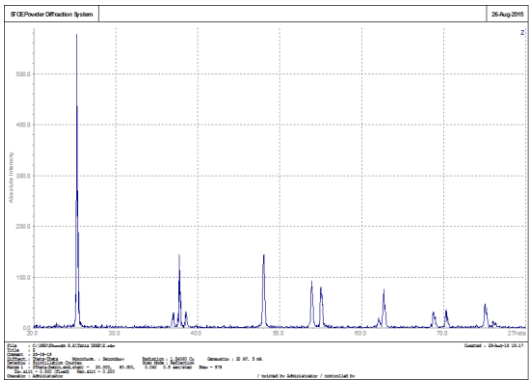

Fig. 4.2 (b): XRD TiO_2-FP

4.2 Erythrocyte Nuclear Abnormalities (ENA) Test

The distinct characteristic of fish erythrocytes is that they possess a nucleus. The interpretation of these nuclei in terms of morphological (structural) changes, is an important bioindicator for pollution. Erythrocyte nuclear abnormalities test is a reliable diagnostic tool for the determination of genotoxic effect caused by pollutants that are present in the aquatic ecosystem. (Walia *et al.*, 2013). In present study, erythrocyte nuclear abnormalities test revealed induction of abnormalities dependent on the toxic impact of the nanoparticles exposure.

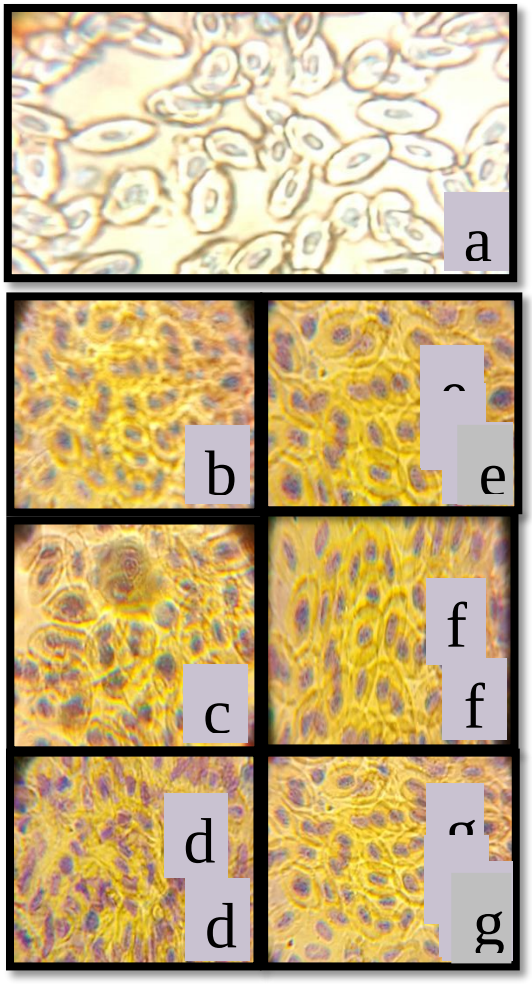

Fig. 4.3: Catalogue of ENA after 1.5 µg TiO_2–NP exposure (a) Control (b) 24 h (c) 48 h (d) 72 h and TiO_2–FP exposure (e) 24 h (f) 48 h (g) 72 h

At exposure concentration 1.5 µg, there was frequent observation of blebbed and segmented nuclei, micronuclei were also observed in TiO_2-NPs exposed group for

all exposure periods (Fig 4.3 b, c & d). While, in FPs exposed group, only a few blebbed and micronuclei were observed (Fig 4.3 e, f, & g).

Similar results were observed at dose 3.0µg where there was a progressive increase in blebbled, segmented and micro nuclei, observed in nano TiO_2 exposed group for different exposure periods of 24, 48 and 72 h respectively (Fig 4.4 b, c & d).

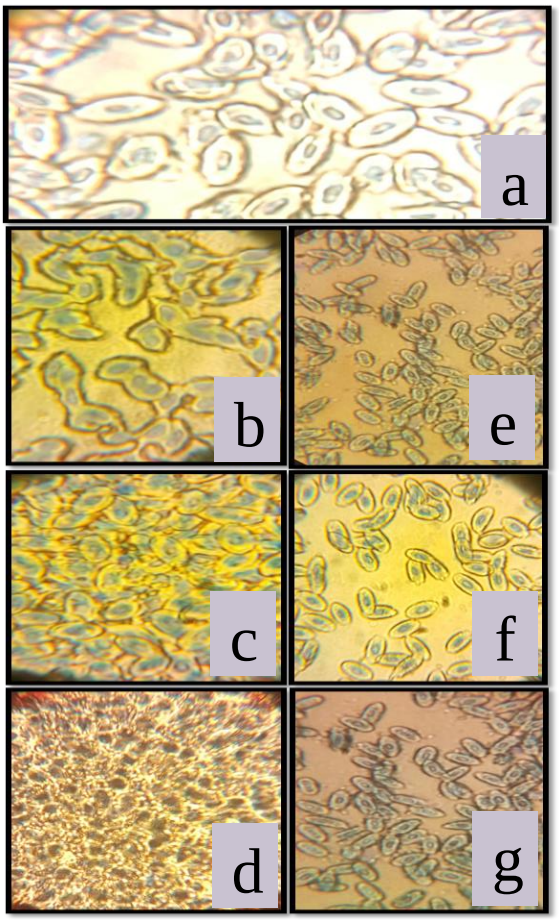

Fig 4.4: Catalogue of ENA after 3.0 µg TiO_2–NP exposure (a) Control (b) 24 h (c) 48 h (d) 72 h and TiO2–FP exposure (e) 24 h (f) 48 h (g) 72 h

The FPs exposed group showed very less number of deformed nuclei for all exposure periods and were similar to control slide observations.

Similar study was conducted by Vignardi *et al.* (2015), in which it was observed that ENA in case of nano TiO_2 exposure was dose and time dependent. Difference between the control group and the exposed group was significant. This suggest that marine fish may be vulnerable to contamination by TiO_2–NPs. The increasing erythrocyte nuclear abnormalities exhibit time and toxic agent dependence. Study by Walia *et al.* (2013) revealed, erythrocytes abnormalities in fishes exposed to the sublethal concentrations of tannery industry effluent. 3.53 and 1.76 % concentration of tannery industry effluent proved to be more toxic concentrations and induced abnormalities which damage erythrocytes completely. The presence of blebbed nucleus indicate budding in interphase. This could be a precursor of micro nuclei (MN) which represents a process of amplified gene elimination from nuclei (Strunjak *et al.,* 2009). These abnormalities caused by damage in genetic material could be a result of free radicals produced under oxidative stress influenced by toxic agent introduced in fish. This lead to further investigation in ROS production rate under influence of oxidative stress caused by exposure to different doses of nano and fine particles of TiO_2.

4.4 NBT reduction assay

Phagocytic cells in fish, like neutrophils, utilize ROS in destroying invading pathogenic microorganisms (Smith *et al.,* 2013). But, these free radicals are deleterious to cells when produced in excess. At high levels, ROS can produce cellular damage of DNA, proteins, lipids, and other macromolecules; this may lead to physiological impairment (Rowe *et al.,* 2008).

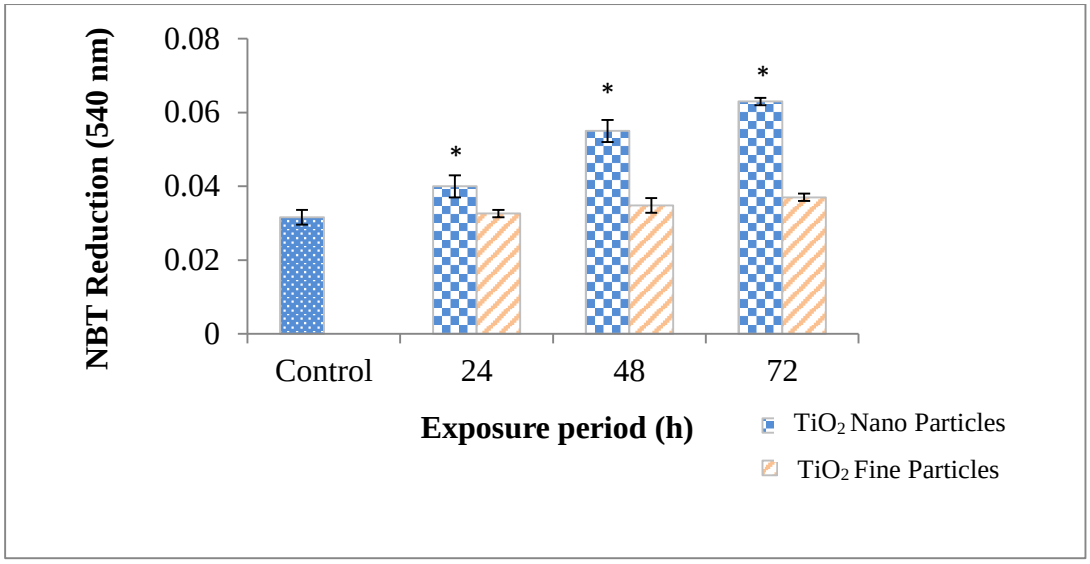

Fig. 4.5: Exposure to 1.5µg of NP & FP

The presence of ROS was measured by their capacity to reduce nitroblue tetrazolium (NBT) into a coloured compound named as formazan. This compound was measured by using spectrophotometer at 540 nm wavelength. When exposed to 1.5 µg dosage, the group exposed to nanoparticles showed a trend with significant increase (*P< 0.05) in ROS concentration. The percentage increase in ROS was 21, 42 and 49 % for 24, 48 and 72 hours respectively. The ROS concentration for fine particles did not deviate much and the control value for all exposure periods (24, 48 and 72 h). The percentage increase was negligible. The results are depicted in Fig 4.5.

Similarly, for 3.0 µg of dosage, the group exposed to nanoparticles showed a trend with significant increase (P< 0.05) in ROS concentration. The percentage increase in ROS was 39, 44 and 49 % for 24, 48 and 72 h respectively. The ROS concentration increased significantly in the fine particles exposed group only at 72 h exposure period, compared to control (Fig 4.6).

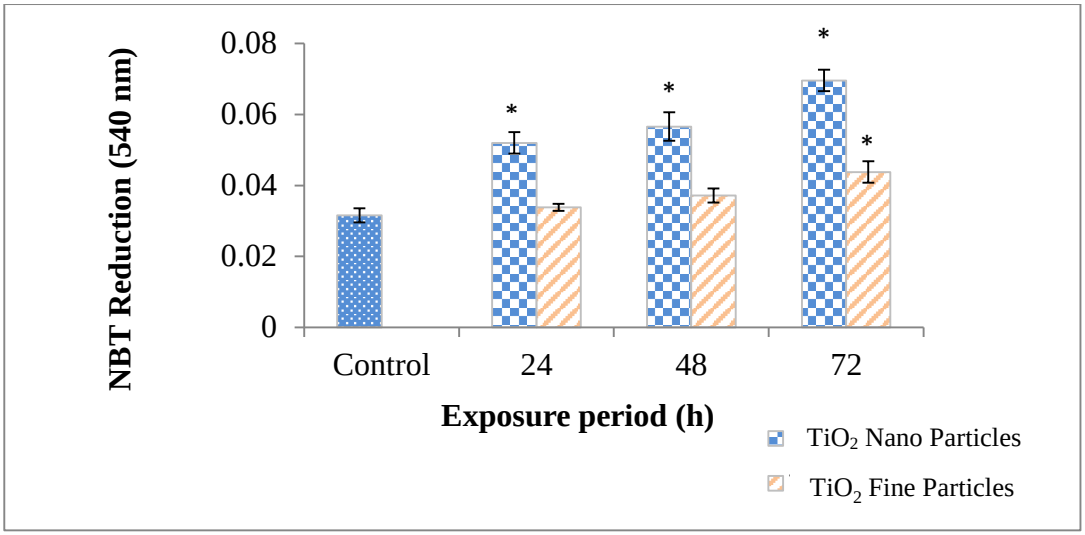

Fig. 4.6: Exposure to 3.0µg of NP & FP

When the values for the same agent at both doses was compared. It was observed that by increasing the dose from 1.5 to 3.0 µg, nanoparticles exposed group showed significant difference in ROS concentration at 24 hour and 72 hour (*P< 0.05), as shown in Fig 4.7. While, for fine particles exposed group significant difference (*P< 0.05) was observed at 72 h exposure period compared to control, as shown in Fig 4.8.

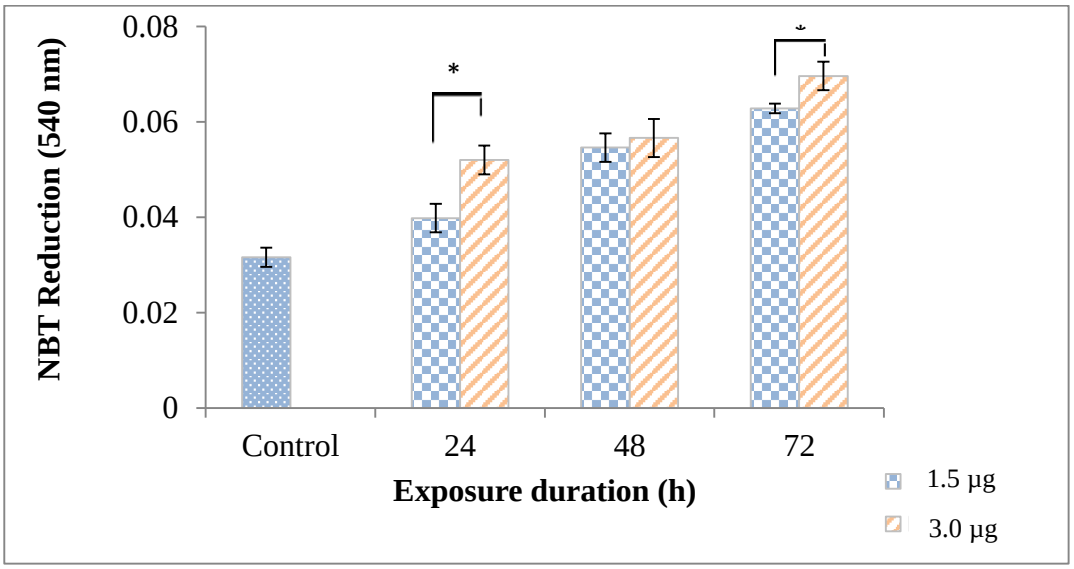

Fig. 4.7: Exposure to NP at doses 1.5 and 3.0 µg

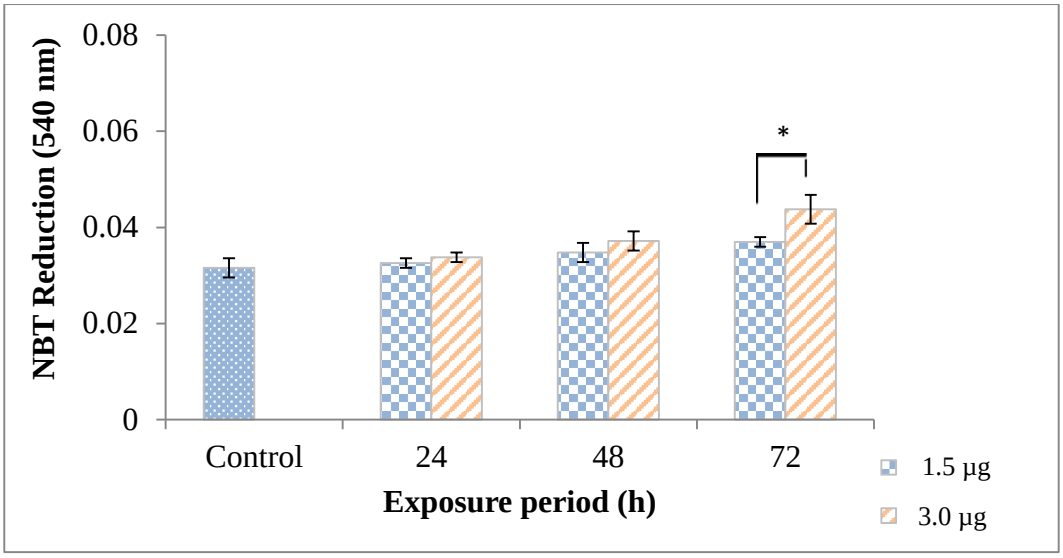

Fig. 4.8: Exposure to FP at doses 1.5 and 3.0 µg

Similar study was performed by Reeves *et al.* (2008) and the results suggested that ROS induced DNA was damaged due to hydroxyl (OH) radicals. Smith *et al.* (2013) studied the decrease in immunity due to astaxanthin in rainbow trout (*Oncorhynchus mykiss*). Nano TiO_2 in *P. tricornutum*, showed significant increase in intracellular ROS level in both the 10 and 50 mg/L nano exposure groups than that of control over 48 hours exposure period (Wang *et al.*, 2016). The results from ENA and NBT reduction assays clearly exhibit oxidative DNA damage.

4.5 Comet Assay

Comet assay has been used for the analysis of DNA damage in various types of eukaryotic cells (Olive & Banáth, 2006). In the present study comet Assay was performed for the determination of percentage tail DNA damage in the blood cells of the control group and the groups exposed to different doses of TiO_2 nano and fine particles.

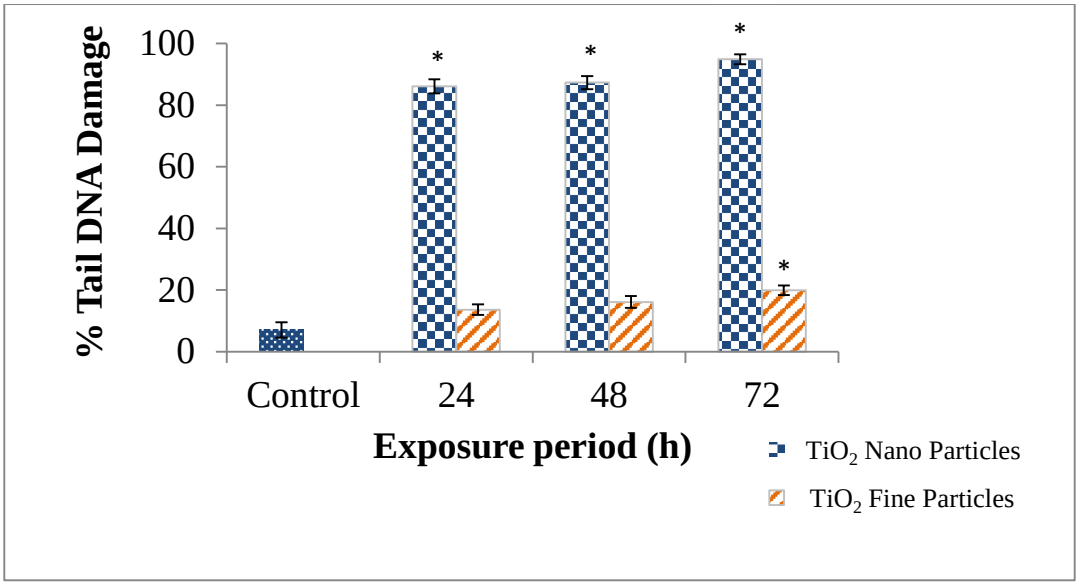

Fig. 4.9: % tail DNA damage by exposure to 1.5µg of NP & FP

There was a significant increase in percentage tail DNA damage of the TiO_2 nanoparticles exposed group (*P< 0.05) from control, at dose 1.5 µg. The percentage increase in tail DNA damage was 91, 92 and 93 % for 24, 48 and 72 h exposure period respectively. The exposure to fine particles had caused negligible percentage tail DNA damage was significant at 72 ho exposure period, compared to control (Fig 4.9).

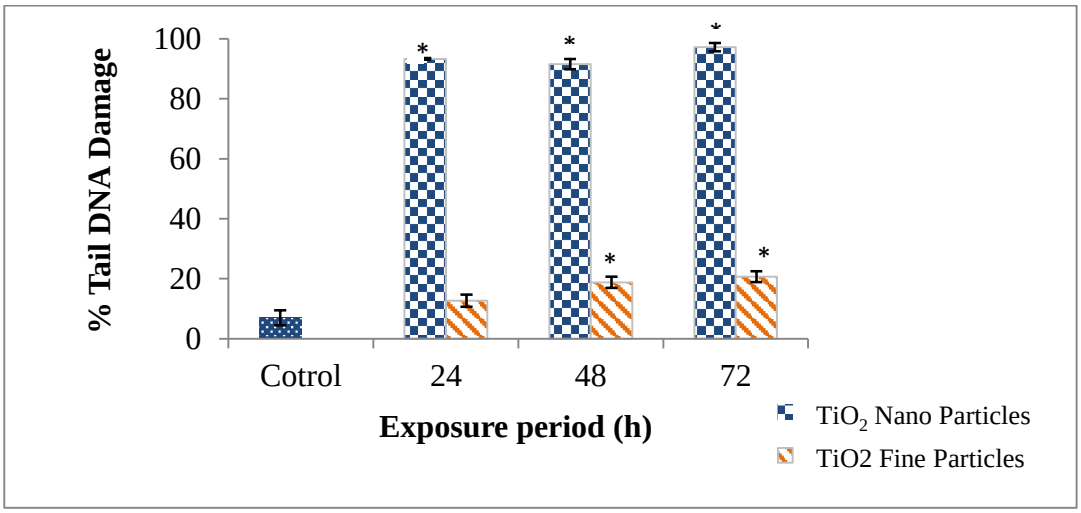

Fig. 4.10: % tail DNA damage by exposure to 3.0µg of NP & FP

Also, at 3.0 µg for exposure to nanoparticles showed a significant increase in the percentage tail DNA damage ($P< 0.05$). For this group the percentage increase in tail DNA damage when compared to control was; 92, 92 and 93 % for 24, 48 and 72 h respectively (Fig. 4.10).

When the percentage tail DNA damage for exposure at both doses of the same agent were compared, depicted that dose increase has not increased the effect on tail DNA damage for both tested treatments. In nanoparticles exposed group, significant difference was observed at 24 and 48 h exposure period and for fine particles, at 48 h exposure period only. This is illustrated in Figs. 4.11 and 4.12.

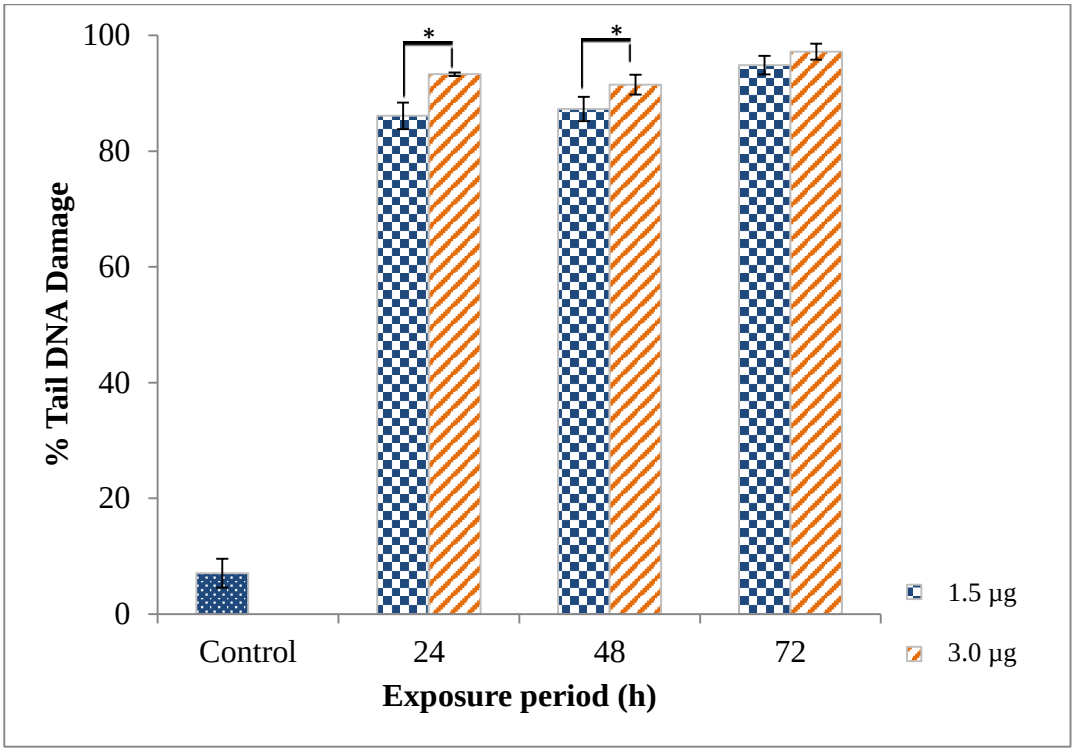

Fig. 4.11: % tail DNA damage by exposure to NP at doses 1.5 and 3.0 µg

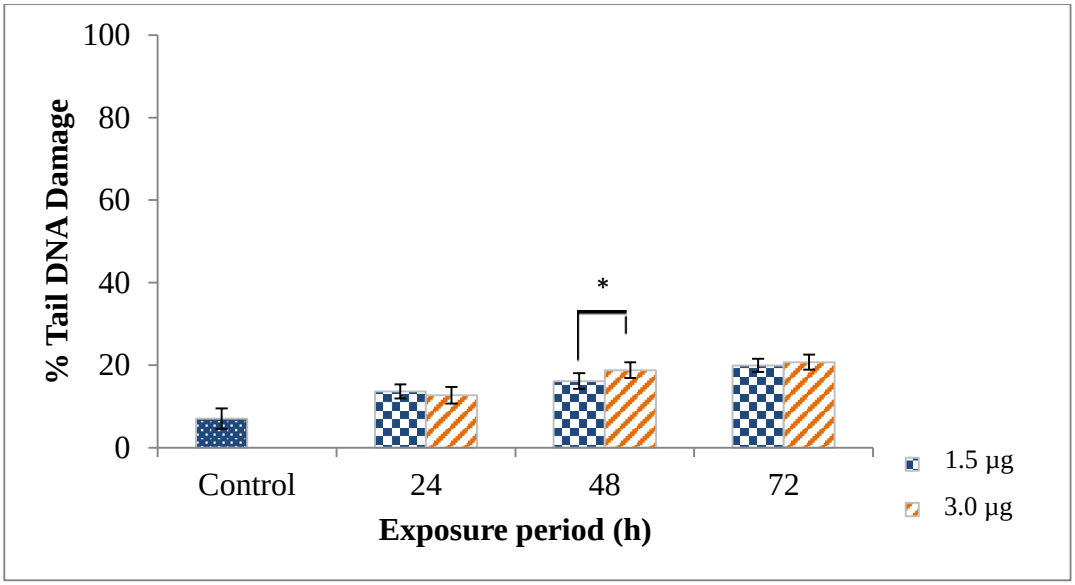

Fig. 4.12: % tail DNA damage by exposure to FP at doses 1.5and 3.0 µg

Reeves *et al.* (2008) studied genotoxicity of TiO_2 nanoparticles in goldfish fish skin cells and results indicated DNA damage at doses 1, 10 and 100 $\mu g ml^{-1}$.In vitro studies on rainbow trout (*Oncorhyncus mykiss*) cells indicated genotoxicity of gonad cells for exposure to nano TiO_2in combination with UV light (Vevers and Jha, 2008).

4.6 ROS v/s % Tail DNA Damage

To conform whether the increase in ROS was resulted in increased % tail DNA damage, correlation factor was applied to the data sets of nanoparticles exposed group for both doses i.e. 1.5 and 3.0 µg. Both exposure dosages showed a positive correlation between ROS and % tail DNA damage with correlation coefficient (r) of 0.7791 and 0.7891, respectively (Fig 4.13 and 4.14).

Results indicate positive, but not a strong correlation between ROS and % tail DNA damage. This suggests, that induction of genotoxicity may be because of direct interaction between DNA and nanoparticles, due to penetration in nucleus (Chen

and von Mikecz, 2005), in combination with indirect damage associated with ROS generation.

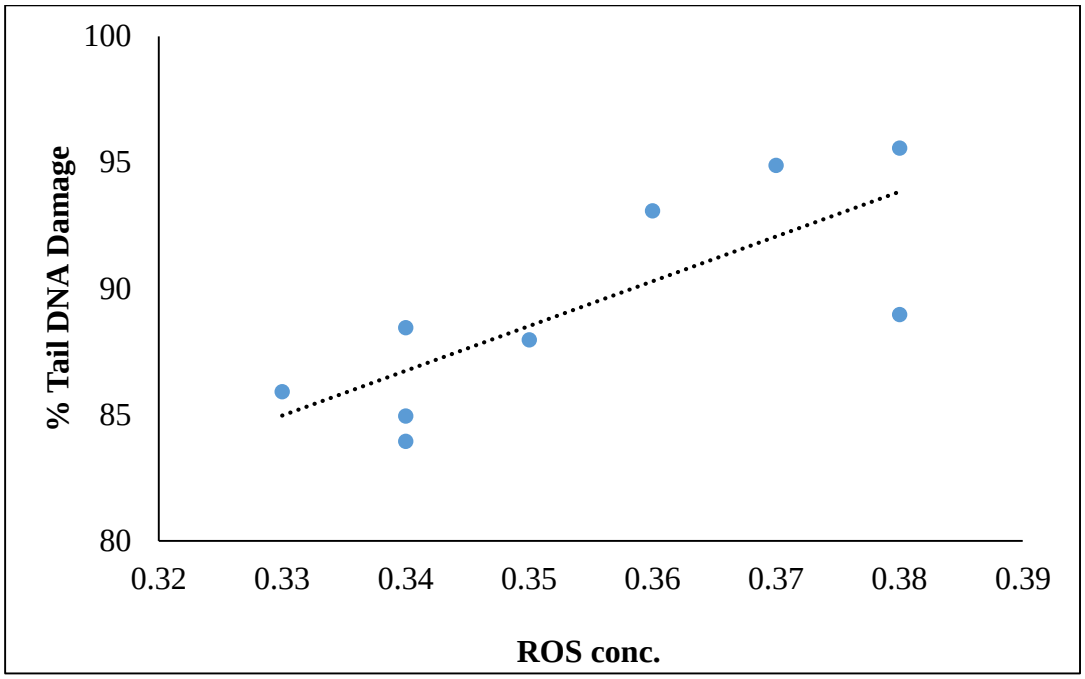

Fig. 4.13: ROS v/s % Tail DNA damage at 1.5 µg nano-TiO_2 exposure

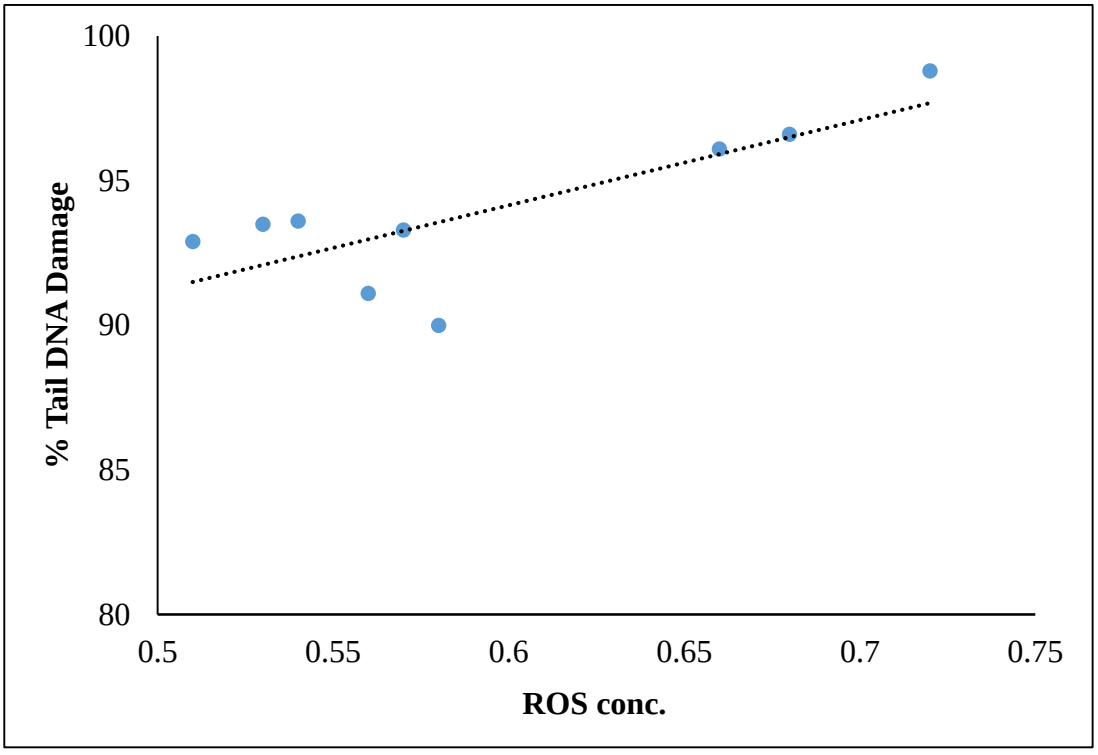

Fig. 4.14: ROS v/s % Tail DNA damage at 3.0 µg nano-TiO_2 exposure

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

Conclusion

The smaller size of nanoparticles ensure large proportion of atoms to be on surface. Thus, surface properties such as; energy level, electronic structure are quite different from interior state. Since, bioactivity of nanoparticles differs from fine size analogue. Fish have been widely used as biosensors for environmental pollutants. In the current study, grass carp was chosen as a model organism to evaluate the effects of titanium dioxide (TiO_2) nanoparticles. Following conclusions were drawn from research work:

1. Nuclear abnormalities observed in erythrocytes were; blebbed, segmented (lobed) and micro nuclei.
2. Exposure to nanoparticles generated more oxidative stress which triggered ROS production, compared to fine particles.
3. ROS concentration reached 50 % increase compared to control value at 72 h, in both (1.5, 3.0 µg) NPs exposed groups.
4. Exposure to NPs has shown greater DNA damage (90 %) at both doses i.e. 1.5 and 3.0 µg, in NPs exposed group.
5. There was a positive correlation (r= 0.7791, 0.7891) between ROS and % tail DNA damage.

Recommendations

Following recommendations are important for further studies:

1. Histopathological study to find morphological alterations in tissues due to environmental xenobiotic.
2. Antioxidant study to evaluate cellular defense mechanism against oxidative stress.
3. Cytotoxicity study for evaluation of cellular uptake and damage caused due to toxicant accumulation.

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ANNEXURE I

Agarose preparation

I. Normal melting point agarose (NMPA) was prepared 1 % by mixing powdered agarose (500 mg) with phosphate buffer saline (PBS, 50 ml) in a glass beaker. It was placed in the 90 °C water bath for 10-15 minutes.

II. 1 and 0.5 % low melting point agarose (LMPA) with PBS in the same way as NMA was prepared.

Lysing solution

To prepare lysing solution of 500 ml following ingredients were used:

• 2.5 M NaCl

• 100 mM EDTA

• 10 mM Trizma base

All the above ingredients were added in 350 ml distilled water and mixed properly. 4 g of NaOH was added to the mixture and allowed it to dissolve for 20 minutes. The pH of the solution was adjusted to 10 with concentrated HCl or NaOH. Solution was made 445 ml with distilled water. Finally at the time of use:

• 1% Triton X-100 (50 ml)

• 10% DMSO (5 ml)

were freshly added to the solution to make up 500 ml solution.

Alkaline solution

Stock solutions of NaOH and EDTA were prepared as follows:

• 10 N NaOH

• 200 mM EDTA

15 ml of NaOH and 2.5 ml of EDTA were mixed with distilled water to make solution 500 ml. The pH of electrophoresis buffer was also adjusted to >13.

TBE Electrophoresis buffer

For 500 ml alkaline solution following ingredients were added.

• Tris base (5.4 g)

• Boric acid (2.7 g)

• EDTA (0.93 g)

All the given ingredients were added to 500 ml distilled water and mixed well. The pH of alkaline solution was maintained to >13.

Neutralization solution

Neutralization solution was prepared by adding 0.4 M Tris to 400 ml distilled water. The pH was adjusted to 7.5 with concentrated HCl (10 M). More distilled was added to make up 500 ml solution.