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Full Length Article

Fish Exposure to Sub-Lethal Toxicity of Nano-Titanium Oxide and Changes in Muscular Antioxidant Enzymes and Protective Role of Vitamins C and E in *Clarias gariepinus*

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Abstract

The ecotoxicology of nano-titanium oxide (TiO_2-NPs) has been initiated recently but still there is no clarity in the results. The objective of this study was to evaluate the response of sub lethal toxicity of nanoparticles of TiO_2 on the muscular antioxidant system in *Clarias gariepinus* and the defensive role of both vitamin C and E. Fishes were at random distributed into 5 groups and a total 50 fish in each group. Fishes of the first and second group were given 1 and 2 mg/L nano-TiO_2 of, respectively. Fishes of the third and fourth group were administered with 1 and 2 mg/L TiO_2NPs and a mixture of vitamins C and E in a dose of 500 mg/kg diet (250 mg of each) and group five was control. After 7 and 15 days of exposure, muscle homogenates were prepared for the biochemical determination of lipid peroxides (LPOs), reduced glutathione (GSH) levels and SOD, CAT, GR, GPx and GST activities. The mRNA expression levels of SOD, CAT, GR, GPx and GST were determined in fish muscle. Fish muscle LPO concentration was significantly decreased while GSH concentration, SOD, CAT, GR, GPx and GST activities were significantly decreased in groups exposed to nano-TiO_2 as compared to control. A significant amelioration in these parameters in groups treated with vitamin C and E. In conclusion, the sub-lethal doses of nano-TiO_2 have the ability to affect the antioxidant system in *C. gariepinus* and it can be used as a bio-indicator for TiO_2 toxicity. © 2017 Friends Science Publishers

Keywords: Nanotoxicity; Antioxidants; *C. gariepinus*; Vitamins C and E; Titanium dioxide

Introduction

Nanotechnology is a rapidly growing field of research. The most widely used nanomaterials are silver (Ag), followed by carbon, titanium (TiO_2), silicon (Si), zinc (Zn) and gold (Au) (Meyer *et al*., 2009; Project on Emerging Nanotechnologies, 2013). Few reports indicated that nanotechnology may open new avenues in the development and the fabrication of products (Roco, 2001; Shah *et al*., 2017). Nanoparticles (NPs) have three dimensions with less than 100 nm (Stone *et al*., 2010). Among the NPs, TiO_2 are one of the most widely used and manufactured in the world (Jomini *et al*., 2015; Mearns *et al*., 2016)

Titanium oxide nanoparticles (TiO_2-NPs) are widely produced in many products (Ahmad *et al*., 2010) and applied for wastewater treatment (Aitken *et al*., 2006; Clemente *et al*., 2012; Dubey *et al*., 2015). The number of industrial TiO_2-NPs is widely increasing in paint industry, sunscreens, coatings, toothpaste and food coloring, which ultimately ends up in the freshwater ecosystem (Ahmad *et al*., 2010; NNI, 2011). Release of nanoparticles (NPs) into aquatic ecosystem have increased through bathing, sewage effluent and other engineering applications of NPs (Bradford, 1976). TiO_2 is available in different forms such as anatase (tetragonal), rutile (tetragonal) and brookite (orthorhombic). The anatase and rutile have different photocatalytic properties (Gaya and Abdullah, 2008). TiO_2 NPs are one of the most abundant materials used in nanotechnology, mainly for their photocatalytic activity and the absorption (Shao and Schlossman, 1999). The mechanisms of NPs toxicity are complex (Bump *et al*., 1992). It may stimulate the reactive oxidative species (ROS) generation through disruption of intracellular reactions, or deteriorate antioxidant system (Culcasi *et al*., 2012), inducing an oxidation of lipids, carbohydrates, proteins and DNA Dorval *et al*. (2003).

TiO_2-NPs was considered as non-harmful to fish, however, many researchers discussed the sub-lethal effects of nano-TiO2 in fish. Literature reports indicated that it may cause oxidative stress and inflammation caused by its sub lethal concentration (Federici *et al*., 2007; Hao *et al*., 2009; Palaniappan and Pramod, 2010). The disproportion between the production of free radicals and the antioxidant system in fish may also cause oxidative stress (Hwang and Kim, 2007). TiO_2-NPs may induce a biochemical and histopathological changes in the liver, gills and intestine

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(Federici *et al*., 2007; Hao *et al*., 2009; Palaniappan and Pramod, 2010). The toxicity of TiO_2 NPs in juvenile carp was documented and manifested by the inhibition of superoxide dismutase (SOD), catalase (CAT) and Glutathione peroxidase (GPx) activities and reduced Glutathione (GSH) content as well as increase in the level of lipid peroxides (LPO) (Brown *et al*., 2004). "Dose dependent increase in DNA damage, lipid peroxidation and protein carbonylation along with a significant decrease in activity of SOD, CAT, GPx levels and total antioxidant capacity after exposure with TiO_2 NPs (Dubey *et al.,* 2015)". Antioxidants such as vitamin E and C protect cells against the effects of oxidative radicals (Hao and Chen, 2009). In the biological system vitamin E is considered as one of the most effective liposolouble antioxidant (Hao *et al*., 2009). The toxic effects of TiO_2 NPs on an antioxidant defense system of *C. gariepinus* are not yet studied, especially at a molecular level.

The study was aimed (i) to assess the disturbance in the antioxidant defense system and activities of Glutathione-S-transferase (GST) activities, gene expression, GSH and LPO levels in response to exposure to TiO_2 NPs and (ii) to test the protective role of antioxidant vitamins C and E was also studied to examine their abilities to eradicate the toxic effect of these nanoparticles.

Materials and Methods

Preparation and Characterization of Nano-titanium Oxide

TiO_2 NPs was obtained in the form of nano-powder (Sigma-Aldrich, 100% anatase, primary particle size b 25 nm, 99.7% purity; Fig. 1). A stock suspension of 1 g/L of TiO_2 NPs in distilled water was prepared by sonication for 10 min (CPX600 Ultrasonic Homogenizer, Cole Parmer, USA) operated at 600 W/L and 100% amplitude (Table 1). The required volume was prepared under static bioassay conditions. The concentration of TiO_2 NPs in the exposure solution was quantified by inductively coupled plasma mass spectrometry (ICP-MS) at zero, 12 and 24 h of exposure to verify the exposure concentration is the same as the prepared concentrations. Water temperature (28 ± 1ºC), pH (8.8–9.5) and electrical conductivity (2.80–2.90 mS/cm) were maintained at optimal conditions. Fish aquaria was continuously aerated, except at the time of feeding, so as the level of dissolved oxygen did not drop below 4.0 mg/L.

Preparation of Fish

Liv*e* 150 *C. gariepinus* (weight 95.7±8.5 g, length 15.9±2.9 cm) were obtained from controlled fish unit. Fishes were stocked in 15 glass aquaria (n = 10 individuals/aquaria) in 70 L glass and water was replaced daily. Fishes were fed twice daily @ 3% body weight. All standard guidelines for animal care were followed.

Fish Grouping and Induction TiO_2-NPs Toxicity

The fishes were at random distributed into 5 groups. Each group was comprised of 30 fishes and stocked in three replicates. The 1st was served as control, while 2nd and 3rd group were administered to TiO_2 NPs of 1 and 2 mg/L, 4th and 5th were administered with 1 and 2 mg/L of TiO_2 NPs and treated with a mixture of vitamins C and E in a dose of 500 mg/Kg diet (250 mg of each) and 5th group was as control. After 7 and 15 days of exposure 20 Fishes of each group were anesthetized on ice. Fishes were fasted 24 h before bioassay. The Muscles were removed, freeze in liquid nitrogen and stored at–80ºC. Standard quality-assurance measures were implemented in the laboratory to control.

Biochemical Assays

Sample preparation: Muscle homogenate was prepared from each sample without pooling by following a method described by Ji *et al*. (2011) for biochemical assays.

Lipid peroxides (LPO), glutathione GSH and antioxidant enzyme activity analysis: Muscle LPO products, GSH were quantized by the methods of Kelly *et al*. (1998) and Bradford (1976). SOD, CAT, GR, GPx and GST activities in fish muscles were detected according to the methods described by Beutler (1969), Esterbauer and Cheeseman (1990) and Lawrence and Burk (1979), respectively.

Gene expression and RT-PCR: Muscle CAT, SOD, *GR and* GPx gene expression were quantified using real time PCR. RNA was isolated from muscles using the RNeasy Mini Kit (Qiagen). For production of cDNA Qiagen Long Range 2 Step RT-PCR Kit was used. Primer3 software was used for primer design (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as per published information, CAT, SOD, GR, GST, GPx and β-actin gene sequences (JF801726.1, JF801727.1, XM_003445184, EU234530, EF206801 and EU887951), respectively. NCBI database for all primers were provided by Sigma Aldrich (Sigma-Aldrich, Germany) are shown in Table 2. PCR reactions were carried out in a thermal cycler (Applied Bio systems Abi Prism-7300 Real Time PCR, USA). The quantitative fold increase in genes was determined in relation to ß-actin mRNA gene and calculated by the $2^{-DD\,CT}$ method".

Statistical Analysis

The data thus obtained was analyzed by one-way analysis of variance (ANOVA) to compare the treated groups with control by SPSS software (Inc., Chicago, IL, Version 20, USA). For inter grouping homogeneity, Duncan's multiple range test was used.

Results

We have observed no mortality in any of the treated groups. The activities of antioxidant enzymes, Glutathione (GSH) and lipid peroxidase (LPO) levels in the muscle of *Clarias gariepinus* are shown in Fig. 2. The exposure of TiO_2 NPs induced a significant inhibition ($P<0.05$) of the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) in fish muscles after exposure to sub lethal concentration of nano-TiO_2 exposed groups (2nd and 3rd) compared to the control. The activity of GST (Fig. 2) showed an effect of TiO2 NPs in 2nd and 3rd group. TiO_2NPs induced the production of LPO and depleted GSH levels in fish muscles. Supplementation of vitamin C and E mixture with nano-TiO_2 induced a significant induction ($p<0.05$) of the antioxidant enzyme activities and GSH levels in all tissues when compared with the respective fishes exposed to TiO_2-NPs only but not affect LPO levels. In this study alteration in CAT, SOD, GPx, GR and GST activities, respectively in the muscle of *C. gariepinus* after exposure to TiO_2-NPs was observed.

The gene expression of SOD, CAT, GR, GPx and GST in fish muscle is shown in Fig. 3. The exposure to nano-TiO_2 caused a significant repression ($P<0.05$) of the relative gene expression of CAT, SOD, GPx, GR and GST in all tissues of TiO_2-NPs exposed groups when compared to their control. Supplementation of vitamin C and E mixture with TiO_2-NPs causes a significant induction ($P<0.05$) in the antioxidant enzymes and relative gene expression in all the tissues as compared with the respective fishes exposed to TiO_2-NPs only. SOD activity was low in control group.

Discussion

The findings of this study showed that TiO_2-NPs have a great effect on the antioxidant enzyme activities and their mRNA expression levels in muscle tissues of *C. gariepinus.* Nanoparticles induce their toxicity through many mechanisms; many of nanoparticles have an oxidant power through the production of ROS or inhibition of cell antioxidant power (Long *et al.*, 2006; Shah *et al.*, 2017). There are great inhibitions in the enzyme activities in exposed groups 2nd and 3rd compared to control. This proved the oxidative stress generated in fish tissues after the exposure to TiO_2-NPs. The results also showed high levels of LPOs in the fish muscles of exposed fishes. Our data disagreed with the data obtained from (Moreno *et al*., 2005). Although there is an agreement that NPs in general and TiO_2-NPs specially induce an oxidative stress in the tissues of fish. The antioxidant enzymes were higher in *C. gariepinus* NPs exposed groups, while the reverse antioxidant activities were inhibited in exposed groups. In our opinion this inhibition was due to the exhaustion of the enzyme by the huge quantity of the oxidants generated due to exposure of fish to nano-TiO_2.

Table 1: Actual Nano-TiO_2 concentrations (mg/L) used in the exposure water

Concentrations (mg/ L)	Time (hours)		
	Zero	12	24
control	ND	ND	ND
1	1±0.003	0.96±0.003	0.92±0.001
2	2±0.005	1.98±0.006	1.95±0.004
1+vitamins	1±0.003	0.93±0.003	0.90±0.001
2+vitamins	2±0.004	1.96±0.006	1.93±0.004

ND= Not detected

Table2: oligonucleotides sequences of primers for Catalase, superoxide dismutase, glutathione peroxidase, glutathione reducatse, glutathione-S-transferase and ß-actin genes

Gene	Forward 5'->3'	Reverse 5'->3'	Amplicon size (pb)
CAT	tcctgaatgaggaggagcga	atcttagatgaggcggtgatg	232
SOD	ggtgccctggagcccta	atgcgaagtcttccactgtc	377
GPx	ccaagagaactgcaagaga	caggacacgtcattcctacac	180
GR	cattaccgagacgcggagtt	cagttggctcaggatcatttgt	420
GST	taatgggagagggaagatgg	ctctgcgatgtaattcagga	640
ß atin	caatgagaggttccgttgc	aggattccataccaaggaagg	280

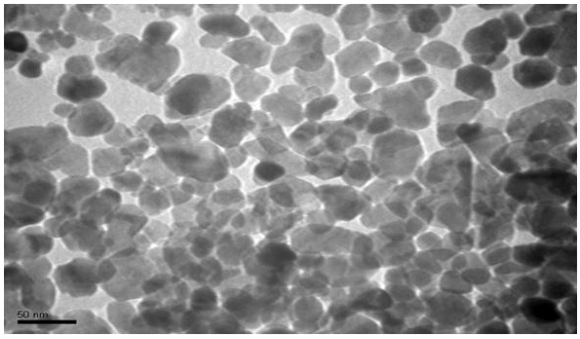

Fig. 1: TEM photomicrograph of nano TiO_2, which shows that the APS is 30±5 nm

The mechanism of oxidant particles as the NPs convert the endogenous hydrogen peroxides with free hydroxyl radicals, which are the cause of ROS generation in fish tissue (Musalmah *et al*., 2002; Jomini *et al*., 2015). The tended toxicity of NPs to its ability to generate oxidative stress. SOD is a potent marker for early detection of environmental oxidative pollution; its activity was significantly reduced in exposed groups when compared to the control group. The reduction in SOD activity may be used as an indicator for removal of oxidants from muscle tissues of *C. gariepinus* (Moreno *et al*., 2005). Hoa *et al*. (2009) and they reported a significant decline in SOD activities in the brain and gills of carps. Xiong *et al*. (2011) reported a reduction in SOD in liver tissues after exposure to TiO_2NPs. In the same line, a significant decrease in SOD activity was reported in the liver of adult Japanese medaka after exposure to nano iron (Pabst *et al*., 1974; Dubey *et al*., 2015).

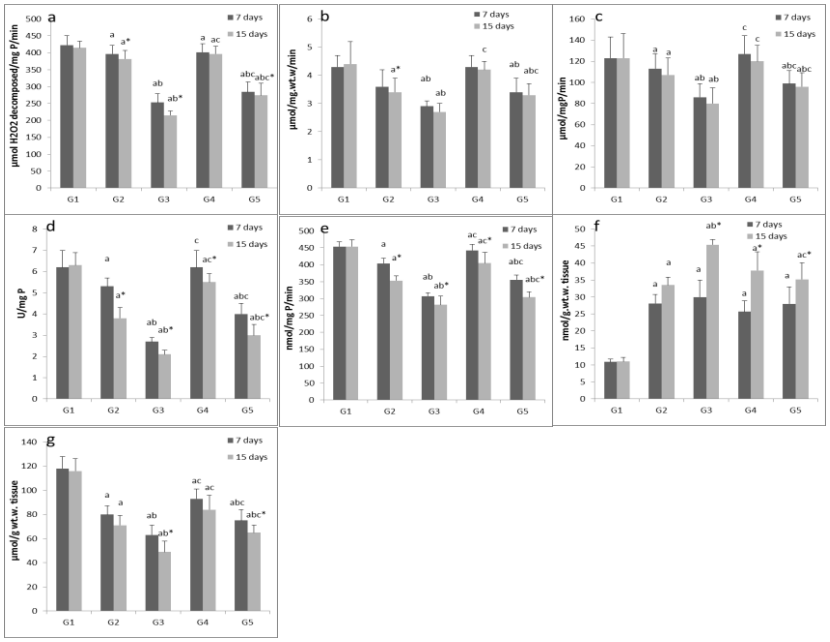

Fig. 2: Activities of muscle antioxidant enzymes, a; Catalase, b; Superoxide dismutase, c; Glutathione peroxidase, d; Glutathione reductase, e; Glutathione-s-treansferase, f; Lipid peroxide and g; Reduced glutathione in control group. (G1), TiO_2 NPs groups (G2 and G3) and TiO_2 NPs with vitamin mixture groups (G4 and G5). Values are expressed as mean ± SD (n 20). Significant levels ($p<0.05$) observed are: a= in comparison to control group, b= when 2 mg TiO_2NPs groups versus 1 mg TiO_2NPs groups are compared, c= when TiO_2NPs + vitamins groups versus their respective TiO_2NPs groups are compared.* = when 15 days treated groups compared with their respective 7 days treated groups

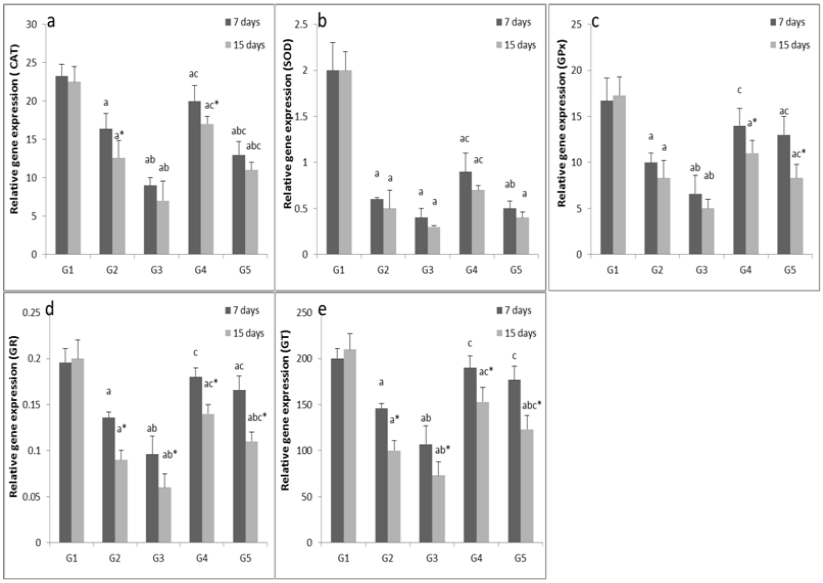

Fig. 3: Muscular relative gene expression of Catalase (a); superoxide dismutase (b); Glutathione peroxidase (c); Glutathione reductase (d) and Glutathione-s-transferase (e). Control group (G1), TiO_2 NPs groups (G2 and G3) and TiO_2 NPs with vitamin mixture groups (G4 and G5). Values are expressed as mean ± SD (n 5). Significant levels ($p<0.05$) observed are: a= in comparison to control group, b= when 2 mg TiO_2NPs groups versus 1 mg TiO_2NPs groups are compared, c= when TiO_2NPs + vitamins groups versus their respective TiO_2NPs groups are compared.* = when 15 days treated groups compared with their respective 7 days treated groups

CAT and GPx are two enzymes found in peroxisomes and are responsible for the removal of H_2O_2 and preventing an accumulation of more oxidants in tissues, their reactions come at the expense of GSH (Pasceo *et al*., 1987). GR act as a regenerator of GSH to close the cycle and remove H_2O_2.

The activities of CAT, GPx and GR, GST activities and gene expression were reduced in nano-TiO_2 exposed groups when compared with control (Fig. 3). GPx was found more sensitive to H_2O_2 than CAT (Puerto *et al*., 2009). GSH is as an antioxidant that eradicates many toxic oxidant agents through its SH group and H_2 donor of GPx catalyzing H_2O_2 reduction bio-reactions (Ramesh *et al*., 2013). Our results indicated a significant decrease in GSH concentration in the muscles of fishes exposed to TiO_2 NPs. Similar findings were reported by Li *et al.* (2009) and Xiong *et al.* (2011). It estimated through measuring the content of Malondialdehyde (MDA) (Puerto *et al*., 2009). LPOs level was increased in the muscles of the nano-TiO_2 exposed fish groups, suggesting that under the stress induced by TiO_2 NPs. The activities of enzymes SOD, CAT, GPx GR and GST were reduced the matter, which reduces the antioxidant power in the cells and generate more and more oxidants lead to a massive increase in LPO. LPOs products of lipid oxidative damage may be used as bio-indicator for the oxidative stresses (Sayeed *et al*., 2003). In fact, the increase in LPO is occurring gradually according to the exposure manner and dose. Tian *et al*. (2010) found that exposure to TiO_2 NPs led to increase in LPO level in zebra fish embryos. Xiong *et al*. (2011) and Wise *et al*. (2010) argued that it does takes place in two ways; first by unsaturated fatty acid interaction and second by preserving the protein peptide chains. In addition, it scavenges O_2, H_2O_2 and (OH^-) radicals and (O^-) radicals in the same way, vitamin C scavenges O_2 and (OH^-) and (O^-) radicals (Xiong *et al*., 2011). Vitamin E protects all cell wall, nucleus, endoplasmic reticulum and mitochondria, while vitamin C acts in the cytoplasm and lysosomes (Zhang *et al*., 2008). A significant neutralization in the antioxidant system *C. gariepinus*, activities of SOD, CAT, GPx and GR and GST started to be returning to normal activities as in control with an amelioration in GSH level and a reduction in LPO level in all fishes supplemented with vitamin E and C mixture. This confirms the ability of two vitamins to fight with an oxidative damage caused by TiO_2 NPs exposure.

Conclusion

Sub-lethal doses of TiO_2 NPs have the ability to affect the antioxidant system in *C. gariepinus*. The results of this study indicated that the specific activity of CAT, GPx and GR, GST could be used as a biomarkers, since they exhibit biochemical and genetic changes in fish exposed to TiO_2 NPs. Vitamin C and E have the ability to ameliorate the toxic effects of TiO_2 NPs on the antioxidant system in *C. gariepinus*.

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