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Impact of Exposure to Titanium Dioxide Nanoparticles on Oxidative Stress in common carp, Cyprinus carpio

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***Abstract*— Titanium Dioxide Nanoparticles (TiO2NPs) are considered one of the top five nanoparticles mostly used in fields of veterinary, agriculture, medicine and industry. High levels of TiO2NPs are found in aquatic environment, which result in harmful effects on living organisms with public health concerns. This study was designed to evaluate the effect of TiO2NPS on oxidative stress in common carp, Cyprinus carpio. A total of 100 fish (weight 50 g) were divided randomly into four treated groups (10 fish/tank/ 2 replicate) as follows: T1, T2, T3, and T4 exposed to TiO2NPs at (50,100, 200 and 300 mg/L) respectively. The control group (C) received a diet without treatment. Following 21 days, blood samples were collected from the caudal vein for determination of DNA damage using comet assay. Also, liver samples were collected for analysis of catalase and glutathione peroxidase (GPx) enzymes activity and for measuring lipid peroxidation using thiobarbituric acid reactive substance (TBARS), which measured the Malondialdehyde (MDA). Results showed a significant increase (P≤0.05) in catalase and GPx activities at 50mg/l of TiO2NPs (T1) with a decrease in these activities in (T2, T3 and T4) of nanoparticles, respectively. lipid peroxidation marker (MDA) significantly increased at 300 mg/L (T4). Results showed the highest DNA damage in RBCs was seen at concentrations of 200 and 300 mg/L of the TiO2NPs (T3 and T4) respectively. This study concluded that TiO2NPs induced potent oxidative stress in common carp, indicated by decreased antioxidant enzyme activity and increased lipid peroxidation with DNA damage in erythrocytes.**

***Keywords* — Nanoparticles, Lipid Peroxidation, Oxidative Enzymes, Comet Assay**.

INTRODUCTION

Nanotechnology deals with the small nanoparticles (NPs) or molecules ranging in size from 1 to 100 nm. Nanotechnology is a field of engineering and science that designs, manufactures and uses different devices, systems and structures at the nanoscale level (1).

Nanotechnology has been recognized as a quickly developing field with many scientific, biological and medical applications. In medical field, it has been widely used in new diagnostic devices, drug delivery, imaging diagnostic tools, cancer treatment, tissue engineering, disease monitoring, therapeutic products, biosensors, and personalized medicine (2). In recent years, nanotechnology applications have begun making their way into the veterinary medicine sector (3).

The potential uses of nanotechnology in veterinary medicine include: the sector of animal food products, pathogen recognition, animal waste modification, novel instruments for genetic and molecular breeding, disease diagnostics, drug delivery systems, and with other possible uses in animal agriculture and veterinary care (4). Titanium Dioxide Nanoparticles are considered one of the top five nanoparticles mostly used (5). Titanium Dioxide Nanoparticles are produced in three crystalline forms: Anatase, rutile, and brookite (6).

The accumulation of TiO_2NPs in aquatic environments can be attributed to their discharge into wastewater, raw sewage and industrial effluents (2,7-11). In spite of the strict laws controlling their discharge into the water, these nanoparticles are still found at high concentrations in the aquatic environment, which may result in harmful effects on living organisms with potential public health concerns (12). The mechanisms of TiO_2NPs toxicity in living tissues can be described in three classes: oxidative stress and production of reactive oxygen species (ROS) by TiO_2-NPs; lipid peroxidation of the cell membrane which occurs when NP-cell attachment via electrostatic force resulting in cell wall disintegration; and TiO_2NPs adhering to biological macromolecules and intracellular organelles after the breakdown of cellular

membranes (13,14). The extensive production and application of TiO_2NPs are contributing to bioaccumulation and pollution in aquatic environments (15).

Additionally, the common carp provides importance from both ecological and economic points of view (16,17). Therefore, this study was designed to investigate the toxicity of TiO_2NPs using the common carp *Cyprinus Carpio* species as a model for aquatic animal toxicity. This work aims to investigate the effect of sub-lethal exposure to titanium dioxide nanoparticles on oxidative stress in common carp. The intermediate lethal concentration of TiO_2NPs in fish was 720.76 mg/L (18).

MATERIALS AND MTHODS

TiO_2NPs characterization and treatment

TiO_2NPs powder was purchased from a US research nanomaterials company. The characterization of TiO_2NPs was studied using Fourier transform infrared spectroscopy (FTIR) and X–ray diffraction (XRD) analysis (19-22). The concentrations of TiO_2NPs (50, 100, 200, 300 mg/L) were selected according to a previous study by Simonin et al. (23) with some modifications by adding new higher concentrations of 300mg/L. Fourier Transform Infrared (FTIR) Spectra Analysis is regarded as one of the most effective methods for analysis of chemicals due to its easiness, sensitivity, diversity, and quick analysis (24,25). FTIR technique was performed by a device (Vectors 22, Brucker) with a single beam range (4000-400) cm^{-1}. The powder (sample) was measured by mixing KBr with TiO_2NPs as a pellet. This pellet was put into the holder of the sample, and then the spectrum was recorded in the FTIR spectroscopy. This device uses Michelson interference in the spectrum analysis (26). XRD method was performed using an X-ray diffraction device (XRD-7000 Shimadzu). The nanoparticles were examined at 40 kV voltage and with 20 mA and a Cu tube that has a wavelength of (1.54 A˚). (27). The principle of the XRD technique includes scattering of X-rays as a result of electrons' movement in the nucleus of an atom when the waves of rays strike the nanoparticles. Then, the scattering X-rays were reflected in several directions, and led to interference patterns and diffraction (28).

Experimental design

All procedures in this study were reviewed and approved by the local Committee on Animal Use and Care, College of Veterinary Medicine, University of Baghdad (328 at 2022/0208). This work was carried out in fish diseases laboratory at the College of Veterinary Medicine /University of Baghdad. Common carp (n=100) were obtained from a local cages farm from Babylon province/Iraq. Fish were acclimatized for 14 days in glass tanks before starting the experiment (29,30,31) The temperature of the water was ranged from 23 - 26°C (32,33), dissolved oxygen was ranged 6.4-7.5 mg/L and pH ranged from 7.4-7.8 (34,35). Fish were fed on commercial carp diet which consisted of: crude protein:36%, crude fat: 9%, crude fiber: 5%, moisture:10%, carbohydrate: 29%, phosphorus: 1%, and ash: 10%. Fish (weighing 50±10 g; length 20± 2 cm (n = 100) were then categorized into 5 equal groups (10 fish for each tank/ two replicates for each group). One group (2 tanks) served as control (C) without any treatment, and four groups (8 tanks) served as TiO_2NPs groups that were exposed to variable concentrations as follows: T1 TiO_2NPs 50 mg/L; T2 100mg/ l^{-1}; T3 200mg/L; T4 300 mg/L (23). This exposure is sufficient to induce disturbance and oxidative stress. The tanks were cleaned every day with partial water change and monitoring (36). After 21 days, blood samples were immediately obtained from the caudal vein from five fish /treatment for determination of DNA damage in red blood cells (37). Liver samples were collected (n=5) at the end of the experimental period for the determination of catalase and glutathione peroxidase activity and also for measuring lipid peroxidation using thiobarbituric acid reactive substance (TBARS), which depends on the liberation of MDA.

Determination of DNA damage using Comet assay

Determination of DNA damage was achieved using comet assay/single-cell gel electrophoresis (SCGE) 38. Blood samples were immediately obtained from the caudal vein from five fish /treatments (n=5). Determination of DNA damage in red blood cells was done in the Iraqi Ministry of Science and Technology according to the protocol of the Industrial Toxicology Research Centre (ITRC), according to the procedure (39,40). The percentage of tail DNA was chosen as a measure of single-strand DNA break/ alkali labile sites. DNA Damage was counted as the comet tail moment test, which represents the degree of DNA damage in individual cells. The scoring technique was achieved by using the computer comet 5 image analysis software. The percentage of damage was calculated automatically via the computer software system, with an average of 50 cells chosen for the measurement (41).

Measurement of catalase and glutathione peroxidase activity and lipid peroxidation

For catalase and glutathione peroxidase tests, liver tissue samples were dissected from five fish /treatment (n=5) and kept in the phosphate-buffered saline (PBS) directly after dissection. While lipid peroxidation test, liver samples were kept in 10% formalin. After that, CAT, and GPx activity were measured by using kits bought from Elabscience company (USA) according to the protocol of the company. The GPx kit was performed as follows: plate has been pre-coated with GPx antibody. When the liver sample was added, it would bind with the antibodies that coated on the wells. Then, biotinylated GPx antibody was added and binds with GPx in the sample. Streptavidin-HRP was added and binds to the Biotinylated GPx antibody. Incubation unbound Streptavidin-HRP was then washed away during a washing step. Substrate solution was added and the color develops in proportion to the amount of GPx. Finally, the reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm (42). Catalase activity in the sample: the liver sample was added to freshly prepared reagents according to the steps of the company with measure the OD values were measured at 405 nm with a 0.5 cm optical

path cuvette. After that the amount of CAT in 1 mg of tissue protein that decompose 1 µmol H_2O_2 per minute at 37°C was express as U/ml. lipid peroxidation was performed by measuring Malondialdehyde (MDA) using the thiobarbituric acid reactive substance (TBARS) kit was bought from Elabscience company (USA). In this kit, which can work in hepatic tissues, MDA combines with thiobarbituric acid (TBA) to produce fluorescent adduct that can be distinguished at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Then, the results were articulated as MDA equivalents (43,44).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism version 8; software. All data were analyzed using one-way analysis of variance (ANOVA). The data were expressed by mean± standard error (SE) with least significant differences LSD. The level of P<0.05 was considered as a significant difference (7,45,46) .

RESULT AND DISCUSSION

TiO2NPs characterization

FTIR spectra of sol-gel-prepared TiO2NPs in the 500–4000 cm range are displayed in Figure 1. The vibrations of TiO2NPs bond are found at 643.99 cm-1 (which means TiO2NPs connection) and 1634. 72-1cm. The vibration of hydroxyl group (-OH) was observed at 3429.51-1. The band at 1634.72-1cm was related with the presence of OH. That means the samples consist of titanium and oxygen (pure TiO2NPs sample). These results are in agreement with (47, 48). Furthermore, X-ray diffraction is an essential analysis for understanding the solid crystalline structures and the atomic arrangements, demonstration the network spacing, phases, and symmetry (49). Figure 2. showed the diffraction peaks for TiO2NPs at (24.6˚, 37.1˚, 37.8 ˚, 47.3 ˚, 53.2 ˚, 54.5 ˚, 62.2 ˚, 67.9 ˚, 69.5 ˚, 69.8 ˚, 74.3 ˚), these results are in agreement with the results of Theivasanthi *et al.* (50) who recorded the nano crystalline powder form of TiO2 by using a Bruker make diffractometer, and data was taken for 2θ range of 10° to 70°. XRD proved the crystalline small size structure of TiO2NPs powder samples.

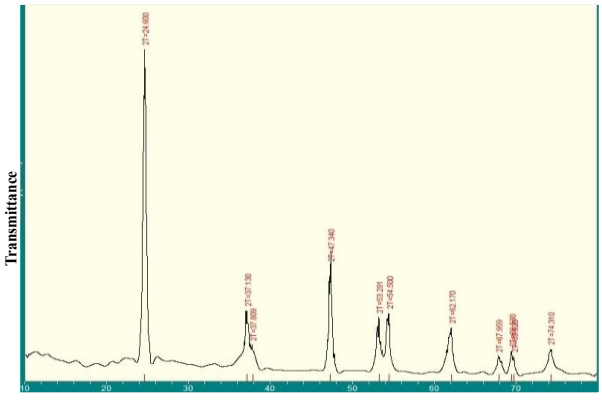
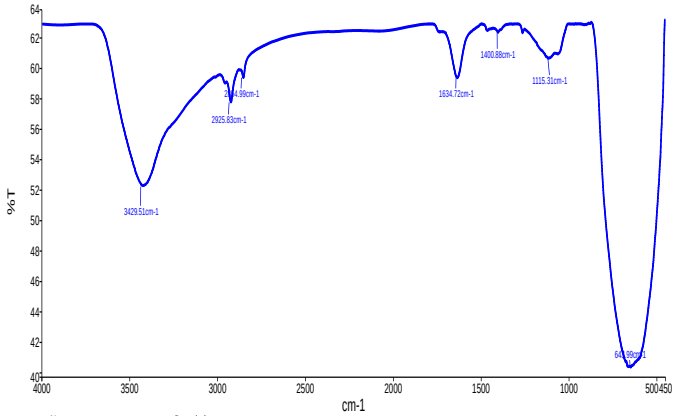


Figure 1. FTlR spectrum of titanium dioxide nanoparticles



Degree (2 Theta)

Figure 2.XRD spectrum of titanium dioxide nanoparticles

Determination of Catalase and glutathione peroxidase activity

Results of CAT and GP x activity are illustrated in Table 1. in common carp exposed to 50, 100, 200 and 300 mg/L after 21 days of the exposure. The highest activity of catalase was observed in T1 at a concentration of 50 mg/L of TiO2NPs. However, catalase activity was significantly decreased P≤0.05 in T2, T3, and T4, respectively, compared with the control and T1 groups. Catalase is a vital part of an organism's antioxidant defense system. It eliminates free radicals to reduce their damaging effects on the host and enhances healthy immunological function (51). A study conducted by Linhua *et al.* (52), they found that CAT activity increased in fish exposed to low concentrations of TiO2NPs (10 mg/L) and decreased when carp exposed to high levels of TiO2NPs (100 and 200 mg/L). In contrast, Lee *et al* (53) reported a reduction in CAT activity when common carp were treated with relatively higher levels of TiO2NPs (80-200 mg/L). Also, the results are similar to dos Santos Santana et al. (54) observed an increase in CAT in the liver of tilapia fish exposed to low concentrations of TiO2NPs (1, 5, 10 and 50 mg/L).

Titanium dioxide nanoparticles mostly change the activity of the catalase enzyme. It is well known that this enzyme is considered the first line of defense of the antioxidant system, so any defect in this enzyme results in disturbances of metabolism. Nevertheless, the exact mechanism of TiO2NPs on this enzyme is not well understood (55). Zhang *et al* (56) demonstrated that TiO2NPs bind with catalase via the electrostatic and hydrogen bonding forces, leading to the instability of catalase. Additionally, TiO2NPs functioned as a catalase inhibitor at higher molar concentrations and as an activator of catalase at low concentrations. The excessive production of ROS (•OH, O2) may disrupt the organism's defense against oxidative stress, causing cell toxicity and damage to the redox system in zebrafish, Danio rerio (57-60).

Results of glutathione peroxidase activity showed there was significant increase in P≤0.05 in T1 compared with control, T2, T3, and T4, respectively. Also, there was a significant decrease in glutathione peroxidase P<0.05 in T2, T3 and T4 compared with the control and T1 groups. GPx is a broad term for an

enzyme family having peroxidase activity whose primary biological function is protecting the organism against oxidative damage. GPx converts lipid hydroperoxides to alcohols and free hydrogen peroxide to water (61). The current study reported that GPx concentrations increased in liver of carp at low TiO2NPs concentration 50 mg l-1) and decreased at higher concentrations (over 100 mg/L). The low dose of TiO2NPs causes increase in antioxidative enzymes concentrations due to the synthesis of new antioxidative enzymes or stimulation the pre-existing enzymes under the effect of lower nanoparticle levels (62). However, Liang *et al.* (63) reported that the high levels of TiO2NP lead to a significantly decreased level of GPx activity in the liver tissue (64). High concentrations of TiO2NPs produce a lot of oxidant particles by converting the endogenous hydrogen peroxides with liberation of higher hydroxyl radicals, which finally lead to accumulation of ROS within the fish tissue (65,66) with inhibition the activity of antioxidant enzymes mainly GPx.

Table 1. CAT, GPx, TBARS activity (U/mL) and tail DNA damage of C. carpio liver exposed to 50, 100, 200 and 300 mg/l of TiO2NPs for 21 days

Groups	CAT (U/ml)	GPx (U/ml)	TBARS activity (U/ml)	% Tail DNA Damage
Control	127.29± 15.31 b	84.74± 5.42 b	0.13±0 .04 c	11.10± 1.09 d
T1 50mg/L	450.17± 67.87 a	343.14 ±107.0 4 a	1.14±0 .21 c	21.03± 1.92 c
T2 100mg/L	77.65±7. 12 b c	76.35± 7.98 b	1.73±0 .44 c	35±4.8 0 b
T3 200mg/L	37.23±4. 37 c	44.87± 5.77 b	3.16±0 .72 b	68.90± 3.09 a
T4 300mg/L	25.15±2. 01 c	29.26± 1.23 b	4.51±0 .59 a	71.22± 3.07 a
LSD	92.5	142.01	1.4	9.05

Means having different letters in same column indicated significantly different (P<0.05, n=5)

Measurement of Lipid peroxidation (Thiobarbituric acid reactive substance TBARS)

As displayed in Table 1, TiO2NPs at higher concentration (300mg/L) caused the highest significant elevation P≤0.05 in the levels of malondialdehyde (MDA) marker of lipid peroxidation compared with control group. Furthermore, there was a significant increase P≤0.05 of TBARS in T3 compared to T1, T2, and the control group. However, there were no significant differences P≤0.05 between T1 and T2 groups.

TBARS are assay of lipid peroxidation which based on the reaction between malondialdehyde and the thiobarbituric acid (TBA) in an acidic medium, which finally measures malondialdehyde (MDA) levels at a wavelength 535 nm. Malondialdehyde is one of the final substances that form during decomposition of lipid peroxidation products (67). The findings of the present study are in line with Bobori *et al.* (68) reported that TiO2NPs exposure for 8 days causing significant rise in MDA levels in liver of Danio rerio and C. gibelio. In a similar manner, following exposure to high doses of TiO2NPs, rainbow trout, juvenile C. carpio, and chinook salmon cells showed increased peroxidation of lipids (69-71).

Correspondingly, Clarias gariepinus fish exposed intravenously to TiO2NPs (3µg/ g) for 96 hours led to elevated levels of MDA in the liver (72). Besides, Xia *et al.* (73) found that TiO2 (1 mg/L) for two weeks resulted in a significant increase of MDA in marine scallop Chlamys farreri. Generally, TiO2NPs are considered to be toxic due to the production of reactive oxygen species (ROS), which can lead to cellular injury, inflammation, and alterations in expression of genes in multiple animal organs (74). These ROS oxidizes polyunsaturated fatty acids that exist in the plasma membrane, leading to the breakdown and production of malondialdehyde (MDA) compounds from the peroxidation of lipids (75). This occurs when free radicals remove electrons from molecules of lipids. Nanomaterials also can impair normal function of cells via lipid peroxidation, and reactive oxygen species have been established to be the cause of membrane damage, which results in cell degeneration (76).

Determination of DNA damage using comet assay

As shown in Table 1, figure 3, each TiO2NPs treated groups (T1, T2, T3 and T4) showed a significant rise (P≤0.05) in tail length values compared to the control group. Table 1 shows that higher exposure doses of TiO2NPs 200 and 300 mg/L cause more DNA damage. However, there were no significant differences between T3 and T4, which were exposed to high concentrations of TiO2NPs.These results were in accordance with results of previous study that demonstrated TiO2NPs at a low dose of 0.1 mg/L induced DNA damage in erythrocytic of Nile tilapia after 7 days of exposure (77). In addition, another study discovered that Nile tilapia exposed to low concentrations of nano-TiO2 (0.1, 0.5 and 1 mg/L) for three weeks led to DNA damage and genotoxicity (78).

Besides, the data of Girardello, Leite (79) showed that TiO2NP at (1, 5, 10 and 50 µg/mL) potentially induced DNA damage in hemocytes of golden mussels. Several studies reported that TiO2NPs were able to bind with phosphate and/or nitrogenous base residues of the DNA, which led to modifications of DNA structure. Also, these nanoparticles bind with some molecules which are necessary for stabilizing and repairing DNA led to an inhibition mechanism of DNA repair (80,81). In addition, TiO_2NPs physically bind to the p53 protein or alter its conformation through oxidative damage. This interaction can disturb p53's ability to recognize and bind to DNA at damage sites. Inhibiting transcription of downstream genes is critical for DNA repair (e.g., p21) and apoptosis (e.g., bax) (81). Similarly, other studies have revealed that the main mechanisms of TiO2NPs leading to damage of DNA were intracellular ROS production, lipid peroxidation, and oxidative stress (83-86). Lipid peroxidation led to the production of MDA, which is toxic and have the affinity to interact with nucleic acid bases of DNA, resulting in DNA damage, toxicity, and mutagenicity (87).

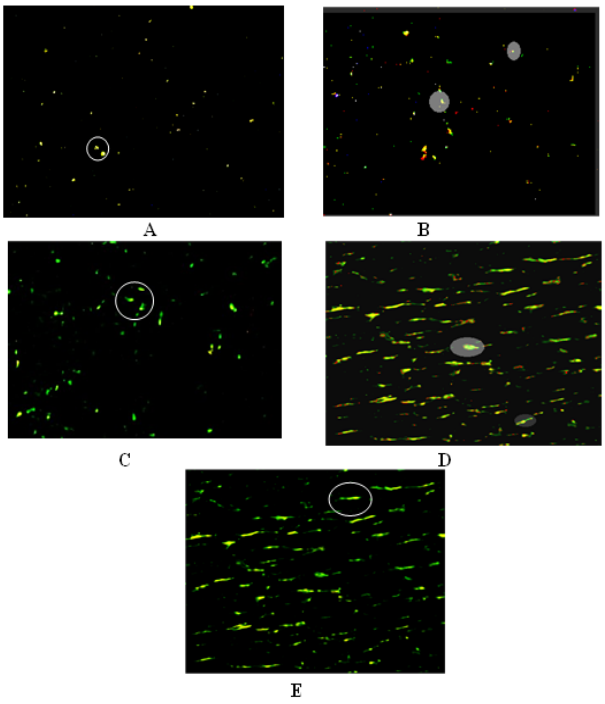


Figure 3. Photomicrographs of red blood cells DNA of C. carpio. A: control group, B: exposed to 50 mg/L TiO2NPs, C: exposed to 100 mg/L TiO2NPs, D: treated with 200 mg/L, E: received 300 mg/l of TiO2NPs.

CONCLUSION

The results of this work demonstrated the sub-lethal exposure of Titanium dioxide nanoparticles cause a significant depletion in the fish antioxidant defense system by affecting the activity of antioxidant enzymes mainly catalase, glutathione peroxidase. Furthermore, titanium dioxide nanoparticles induce lipid peroxidation in common carp due to the generation of ROS. This results correspondingly confirm that Titanium dioxide nanoparticles considered as genotoxic and cause DNA damage due to oxidative stress.

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N/A

Conflict of Interest
The authors declare no conflict of interest.

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