Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 27 issue 2, 2017, pp 127-133

BIOMARKERS OF OXIDATIVE STRESS, LIPID PEROXIDATION AND ROS PRODUCTION INDUCED BY TiO_2 MICROPARTICLES ON SNAILS *HELIX ASPERSA*

Lyes Khene[1*], Houria Berrebbah[1], Amina Yahyaoui[1] , Tayeb Bouarroudj[2], Sabrina Zouainia[1], Houssem Kahli[1], Chiraz Bourayou[1]

[1]Laboratory of Cellular Toxicology, Department of Biology, Faculty of Science, Badji Mokhtar University, Annaba, Algeria
[2]Laboratory of Chemistry Inorganic Materials (LCMI). Département of Chemistry. Faculty of Science, Badji Mokhtar University, Annaba, Algeria

ABSTRACT: The increasing production of nanomaterials will in turn increase the release of nanosized by products to the environment. The aim of this study was to assess toxicity of TiO_2 microparticle on the bioindicator model snail *Helix aspersa*. Gastropods are exposed to microparticle TiO_2 with Semi-static method at several concentrations (31.25; 62.5; 125; 250; 500; 1000 mg/kg of soil) mixed to ISO soil (ISO11268-1) for 28 days. Oxidative stress biomarkers: Antioxidant enzymatic activities (CAT and GST) were investigated, level of Glutathione (GSH), Lipid peroxidation indicator malondialdehyde (MDA), and free radical was quantified. TiO_2 microparticle exposure has led to a significantly induction of antioxidant enzymes Catalase (CAT), glutathione-S-transferase (GST) which might be associated with decreased level of Glutathione (GSH) and lipid peroxidation supported by a significant increase in (MDA) level. It can be hypothesized that by the triggering of oxidative stress by TiO_2 microparticles, level of free radicals was quantified on snail and the measuring of ROM's on snails hemolymph confirm the high production of ROS.

Keywords: Helix aspersa, TiO2 microparticle, Biomarkers, Oxidative stress , ROS.

INTRODUCTION:

According to recent developments in nanotechnology, exposure to micro- and nano-sized particles or debris has increased (Veranth *et al.*, 2007). Microparticles are of considerable interest because of their extensive applications (Petros *et al.*, 2008). These applications depend on the chemical and physical properties of the particles, such as wettability, size, and shape, which are also important for their self assembly (Dendukuri *et al.*, 2009).Materials with micro-and nano-sized dimensions have attracted considerable attention of the researchers due to their exponential promises in almost all fields (Santhoshkumar *et al.*, 2014). Smaller size and higher surface area lead to enhance the reactivity and modification of various properties opening the field of many new applications in imagin g, biology and medicine (Le Trequesser *et al.*, 2014).

TiO_2-based microparticles and films are the subject of an extensive research aiming at the applications as photocatalysts for environmental purification, photoinduced superhydrophilic coatings, and electrodes of dye-sensitized photochemical solar cells (Yamashita *et al.*, 1996; Choy *et al.*, 1998). The performance of their photocatalytic reactions depends on the characteristics of the TiO_2 crystallites, such as the size and surface area (Matsuda *et al.*, 2006). With decreases in the size of materials, the chemical reactivity of the materials/ particles is generally increasing. This increased reactivity sometimes stimulates cells or tissue. In other words, micro/nano particles potentially have a high level of toxicity depending upon the size (Abe *et al.*, 2009; Tamura *et al.*, 2004). The increase in surface area is usually considered to be a nanosizing effect, and the toxicity of materials is observed when the particle size drops to below 10 μm. Even biocompatible materials such as Ti and TiO_2 show stimulus to cell or tissue with a decrease in particle size (Watari *et al.*, 2008).

The toxicity of UFPs is particularly evident through mechanisms related to inflammation and the formation of free radicals that can damage biological tissues, if the antioxidant systems of the body are not sufficient to inactivate (Falfushynska *et al.*, 2015). Several xenobiotics, and among them ultrafine particles (UFPs), can produce oxidative stress, providing a mechanistic basis for their observed toxicity. Chronic oxidative stress induces deleterious modifications to DNA, lipids and proteins that are used as effective biomarkers to study pollutant-mediated oxidative stress (Poletta *et al.*, 2016).

Face to the growing industrial use of nanomaterials, ultrafine particles (UFPs) are inevitably expected in aquatic and terrestrial environments. For that, it's essential to carry out studies on toxicity of micro/nano materials. Among terrestrial invertebrates, the gastropods *Helix aspersa* have the capability to accumulate different classes of chemicals and serve as pertinent species for monitoring trace metals, agrochemicals, urban pollution, and electromagnetic exposure (Viard *et al.*, 2014; Regoli *et al.*, 2006). In the present study it was examined oxidative stress responses and lipid peroxidation of TiO_2 microparticles, in single exposures of these

Correspondence*: Lyes Khene, Laboratory of Cellular Toxicology, Department of Biology, Faculty of Science, Badji Mokhtar University, Annaba, Algeria. e-mail: khene.lyes@yahoo.com , Tel: +213550857818.*

contaminants on the gastropod *Helix aspersa*. When some of oxidative stress biomarkers has been characterized in digestive gland and kidney.

MATERIALS AND METHODS:

Biological material:

Terrestrial gastropods *Helix aspersa* (8±0.5 g) were collected at region of Guelma (Northeast Algeria) and maintained in the laboratory with optimal environmental conditions (photoperiod 18h light/24h, temperature (20±2°C), humidity 80 to 95%)(Coeurdassier *et al.*, 2001). Snails have been acclimated to laboratory conditions for 15 days before being exposed to microparticles of TiO_2.

Chemical material characterization and suspension:

In this study, TiO_2 Microparticles whose size is 155nm was used. These microparticles are provided by Laboratory of Chemistry inorganic materials (LCMI) Badji Mokhtar University, Annaba. TiO_2 powder was characterized by X-ray powder diffraction (XRD) using X'Pert PRO PANalytical system with Cu radiation at wavelength λ=1.5405980 Å at 2θ values between 10° and 90°. Microparticles were first suspended in 18 MΩ deionized water through sonication to prevent aggregation. The stocks were sonicated for 30 min in a temperature controlled sonication bath (150 W, 40 Hz, 25 ºC)(Wang *et al.*, 2016), the resultant suspension were applied to soils (Shoults-Wilson *et al.*, 2011).

Treatment of snails:

Selected snails were divided into 7 lots at 15 snails / lot in plastic boxes. They are exposed to TiO_2 microparticle with Semi-static method at several concentrations (31.25; 62.5; 125; 250; 500; 1000 mg/kg of soil) to ISO soil (ISO11268-1) for 28 days. After 4 weeks of exposure, the snails were sacrificed after freezing (-80°C), the digestive gland and kidney was removed for evaluate some of biochemical and enzymatic parameters. Hemolymph was withdrawn from the visceral hemocel (Regoli *et al.*, 2006) and immediately use for Measuring of the Reactive Oxygen Metabolites(ROM's).

Biochemical assay:

Estimation of Glutathione (GSH) content: GSH content was quantified using the method of Wechbeker *et al.* (1988). Cells are mixed in 1ml EDTA (0.02M). ASS 0.2ml was added to 0.8ml of homogenate. After agitation, the homogenate was centrifuged. The assay mixture contains 1ml tris/EDTA buffer(0.02M, pH 9.6), 0.025ml of 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) and the sample. The reaction was monitored at 412nmand the amount of GSH was expressed asμmol/mg of proteins.

Determination of Glutathione S transferase (GST) activity:

The GST activity was measured according to the method of Habig *et al.* (1974). The homogenization of samples was done in 1ml of phosphate buffer (0.1M, pH6) and centrifuged (14000rpm/30min)(SIGMA 3-16K, United Kingdom). The final reaction contain 1.2ml CDNB (1mM)/GSH (5mM) and the sample. The absorbance was measured spectrophotometrically (JENWAY 6305, United Kingdom) at 340nm. The result was expressed as μmol/min/mg of proteins.

Determination of Catalase (CAT) activity:

The CAT activity was determined spectrophotometrically at 240nm by calculating the rate of degradation of H_2O_2 (Regoli *et al.*, 1995) Samples are mixed in 1ml of phosphate buffer then centrifuged at 15000g. At 0.025ml of supernatant we added 0.75ml of phosphate buffer and H_2O_2. The result was expressed as μmol/min/mg of proteins.

Determination of MDA levels:

According to the method of Draper *et al.* (1990), the extent of lipid peroxidation in terms of malondialdehyde (MDA) formation was measured. The samples were ground with Tris HCl (50 mM, pH 7.5), after homogenization, samples were centrifuged. 500 μl of supernatant was added to 2.5 ml of TCA and heated at 100 ° C for 1 h. After cooling, the precipitate was removed by centrifugation. 2 ml of supernatant was added at 1 ml of TBA. After a second heating and cooling, 1.5 ml of Butanol was added. The absorbance of the sample was measured at 532 nm using a blank containing all the reagents except the sample. The result was expressed in μmol/mg proteins.

Quantification of ROM's:

Measuring of ROM's by using (FRAS Evolvo System (H&D srl-Parma Italy) diagnostic kits (Pasquini *et al.*, 2008). The assay was adapted to our working conditions and biological model.

Statistical analysis:

The obtained results are represented by the average ± Standard Error. Statistical analysis of data was performed by the Student t test (Dagnelie, 1999). The probability were considered significant when *p < 0.05; very significant when **p < 0.01; and very high significant when ***p < 0.001

RESULTS:

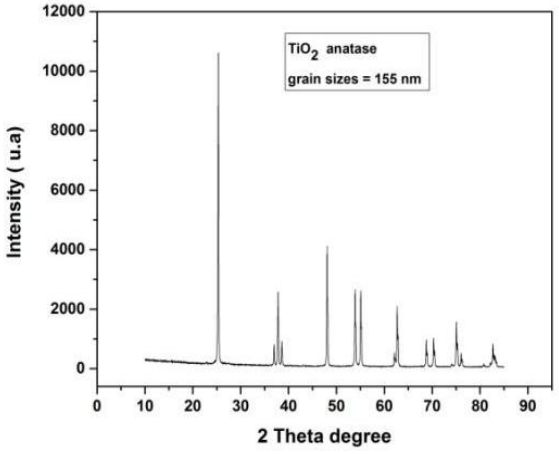

Fig 1. XRD pattern of the TiO2

Structural Properties by XRD:

The XRD pattern of the product is shown in figure 1. The peaks pointed at 2θ valuescan be associated to anatase TiO_2 structure crystallite, which are in good agreement with the literature values(JCPDS card no. 41–1445). The average grain size of TiO_2 particles

can be estimated by Scherrer equation (Patil *et al.*, 2012). D= kλ /β cos θ, where D is the crystallite size, K= 0.9, λ is the X-ray wavelength, β is the full width at half maximum of the diffraction peak, and θ is the Bragg diffraction angle of the diffraction peaks.

Oxidative stress biomarkers:

The measurements of catalase activity on snails treated with different concentrations of TiO_2 Microparticles (Fig.2) showed a significant induction in digestive gland and kidney compared to controls. Indeed, after 28 days of treatment, the level of catalase increased from (0.01 µmol/min/mg prot) and (0.0021µmol/min/mg prot) in controls to (0.019 µmol/min/mg prot) and (0.0067 µmol/min/mg prot) in digestive gland and kidney, respectively for the highest concentration .

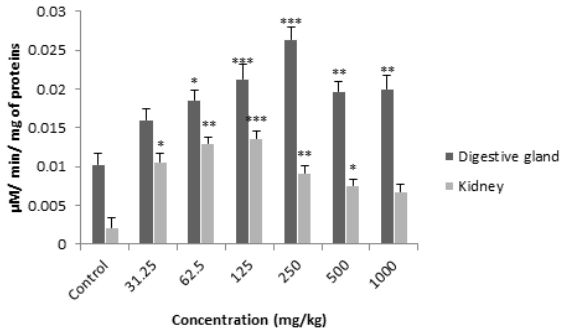

Fig. 2. Level of catalase activity in digestive gland and kidney after treatment of snails with increasing concentrations of TiO_2 Microparticles.

Results are presented as mean±S.E (Standard Error); Significantly different from the control: *p<0.05, **p< 0.01, ***p<0.001.

GST activity was significantly increased in snails exposed to TiO_2 Microparticles with a dose-dependent manner in both of digestive gland and kidney (fig.3). This result revealed a significant induction of the activity of this enzyme particularly for the highest concentration. This increase is in order of 201.25% and 216.66% for the highest concentration 1000mg/kg, respectively for digestive gland and kidney.

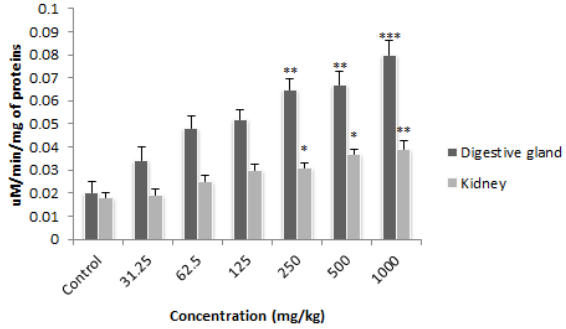

Fig. 3. Level of GST activity in digestive gland and kidney after treatment of snails with increasing concentrations of TiO_2 Microparticles.

Results are presented as mean±S.E (Standard Error);Significantly different from the control: *p<0.05, **p< 0.01, ***p<0.001.

The effects of the increasing concentrations of TiO_2Microparticles on the GSH rate as illustrated in figure 4.The result showed that this non-enzymatic antioxidant decrease in digestive gland and kidney after 28 days of treatment.

The treatment of snails caused an important dose-related decrease (18.09% -42.70%) for digestive gland and (9.03% - 65.19%) for kidney, compared to the controls.

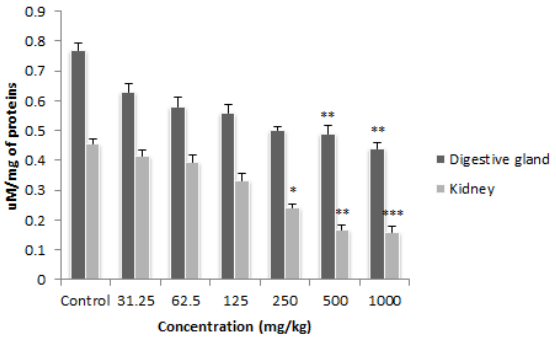

Fig. 4. Level of GSH in digestive gland and kidney after treatment of snails with increasing concentrations of TiO_2 Microparticles.

Results are presented as mean±S.E (Standard Error); Significantly different from the control: *p<0.05, **p< 0.01, ***p<0.001.

Lipid peroxidation:

MDA level in digestive gland and kidney was dose-related increased at different treatment concentrations when compared with the controls as shown in (fig.5).The maximum increase (206.95%) and (188.88%) was observed at the highest concentration(1000mg/kg) on digestive gland and at concentration (500mg/kg) on kidney, respectively.

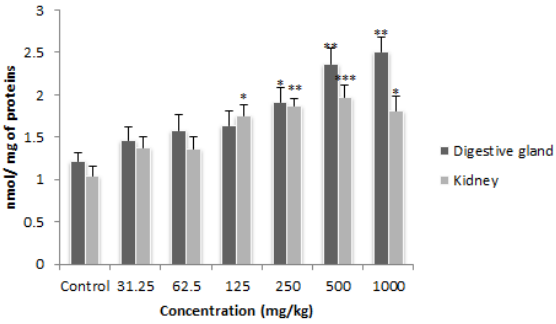

Fig. 5. Level of MDA in digestive gland and kidney after treatment of snails with increasing concentrations of TiO_2 Microparticles.

Results are presented as mean±S.E (Standard Error); Significantly different from the control:*p<0.05, **p< 0.01, ***p<0.001.

Quantification of ROM's:

The effect of TiO_2 Microparticles on production of Reactive Oxygen Metabolites (ROM's) in hemolymph of snails treated as shown in table 1. This production was significant and dose-related increased particularly for the highest concentration compared

with the controls. This increase is in order of 253.33% and 206.66% for two highest concentration 500mg/kg; 1000mg/kg, respectively.

Table 1.
Level of Reactive Oxygen Metabolites (ROM's) in snails treatment with TiO_2 Microparticles.

Concentrations (mg/kg)	ROM's (U.CARR)
Control	15±2
31.25	22±4
62.5	31±5*
125	35±4**
250	34±6***
500	38±5***
1000	31±3**

Results are presented as mean±S.E (Standard Error); Significantly different from the control: *p<0.05, **p< 0.01, ***p<0.001.

DISCUSSION:

Oxidative stress is believed to occur when there is an imbalance in the biological oxidant-to antioxidant ratio; this can result in oxidative damage to lipid, proteins, carbohydrates and nucleic acids. In most cases, the abnormal generation of ROS, which can result in significant damage to cell structure, is considered an important signal of oxidative damage (El-Demerdash, 2007). The main mechanisms described about the mode of action of ultrafine metal particles are chiefly by the generation of reactive oxygen species (ROS) and direct interaction with biological target s (Ma *et al.,* 2013). The consequent loss-of-function and structural integrity of modified bio-molecules through oxidative stress can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage (Pamplona,2008).

In the present study, it was investigated a battery of biomarkers of oxidative stress, including enzymatic (CAT and GST), non-enzymatic (GSH) antioxidants, representative product damage to lipids (MDA), and quantification of Reactive Oxygen Metabolites (ROM's). The antioxidant defense systems are present in all aerobic cells and neutralize the intermediate chemical reactions produced endogenously and/or metabolism of xenobiotics. The antioxidant system activity may undergo an increase or depletion under the effect of a chemical stress (Ojha *et al.,* 2011).

Beginning with catalase, is an essential enzyme in the detoxification mechanisms, catalyses the conversion of H_2O_2 to molecular water and oxygen (Sanchezcasas *et al.,* 1994), the significant induction on the catalase activity observed in this work at level of kidney and digestive gland is an indicator of cellular lesions and can be explained by the activation of an anti-oxidant mechanism to prevent the accumulation of ROS. These results are in line with those of Ali *et al.* (2014; 2015)recorded a significant induction of catalase activity in *Lymnea luteola* treated with TiO_2 NPs, the same results was observed by Tedesco *et al.* (2008) and FarKas *et al.*(2015) in the digestive gland for a bivalve molluscs *Mytilus edulis* exposed to TiO_2 NPs. Boucena (2016) have also recorded induction in *Helix aspersa* treated with ETM. GST is an important enzyme involved in catalyzing the conjugation of a variety of electrophilic substrates to reduce glutathione and protect the cell against the effects of xenobiotics (Ferrari *et al.,* 2007). In the present study, GST activity was significant and dose-related induced in kidney and the digestive gland. This result is in agreement with Mouneyrac *et al.*(2014), observed induction GST in bivalve molluscs*Scorbicularia plana*exposed to ultrafine metal particles of (Ag, Au, Cds, and ZnO). Canesi *et al.* (2010) have also observed an increase of GST activity in digestive gland of *Mytilus galloprovincialis* treated with differents NPs of titanium dioxide (TiO_2), fluorene (C60) and silica (SiO_2). The same results have been recorded by Amamra *et al.* (2015) on freshwater ciliate *Paramecium tetraurelia* exposed to cypermethrin, a pyrethroid insecticide.

About non-enzymatic biomarker, the GSH who is probably the most active antioxidant in biological systems, and is especially effective at neutralizing the destructive hydroxyl radicals against which there is no enzymatic neutralizatio n (Finkel *et al.,* 2000). Higher levels of GSH can not only function as an "extra" non-enzymatic antioxidant defense, but can also increase the in situ activities of glutathione-S-transferase (GST), Se-glutathione peroxidase (Se-GPX) and some peroxiredoxins, as GSH is a substrate of these enzymes (Hermes-Lima *et al.,* 2012). In this work, it was noted a significant depletion in total GSH level with dose dependent manner. The decrease in total GSH level may be caused by to the presence of free radicals produced by microparticle of TiO_2 and or by the direct bonding of glutathione to the metal, as described by Barillet (2007), ETM and ultrafine particles are characterized by their high affinities with the thiol groups where they form complexes with these chemical entities (Xiong *et al.,* 2011; Fahmy *et al.,* 2014). Chandran *et al.* (2005), recorded a decrease in total glutathione (GSH) level in gastropod *Achatina fulica* treated with cadmium and zinc. The same results are observed by Ramsden *et al.* (2013) in brain, gill and liver tissues of zebrafish exposed to bulk TiO_2.

Lipids are one of the principal targets of oxidative stress. Lipid peroxidation gives rise to a number of secondary, highly damaging products, known to further perpetuate ROS production. The two major used as biomarkers of lipid peroxidation are F2-isoprostanes and MDA. Both are important secondary decomposition products of polyunsaturated fatty acid (PUFA)(Liu *et al.,* 2013). Membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from exogenous and endogenous sources (Furtado-Filho *et al.,* 2007; Monaghan *et al.,* 2009). For this reason the MDA concentrations can indicate the rate and intensity of lipid peroxidation within the organism. In this study, it was recorded dose-related increase in MDA level in kidney and the digestive gland. Fahmy *et al.* (2014) also recorded a significant increase in MDA level in the hemolymph and tissue of freshwater Snail *biomphalaria alexandrina* exposed to zinc oxide nanoparticles. The same results have

observed in liver, gill and brain tissues of carps (*Cyprinus carpio*) exposed to TiO_2-NPs suspensions (Linhua *et al.*, 2009), and in liver of mice treated with titanium dioxide nanoparticles (Abdel-Azim *et al.*, 2015). Grara *et al.* (2012) showed an induction of MDA in *Helix aspersa* exposed to Metallic dust.

Moreover, the production of ROS do not necessarily result in oxidative stress if this can be balanced by an up regulation of defenses. So the individuals having relatively high levels of antioxidants are not necessarily in a better redox state than those with lower levels, this will depend on the level of ROS that these defenses have to dealwit h (Poletta *et al.*, 2016). For this purpose the Reactive Oxygen Metabolites (ROM's) was quantified. The results of present study show a high levels and dose-related production of Free Radicals in snail hemolymph treated whith TiO_2 micoparticle. This result is in agreement with Atailia (2016) who observed a high production of ROS on hemolymph of snail *helix aspersa* exposed to Chronic Metal Pollution.

The main mechanism of toxicity of ultrafine particle of TiO_2 is caused by a declenchement of oxidative stress and production of reactive oxygen species (ROS) (Kohen *et al.*, 2002). This mechanism of action was be confirmed by the measurement of the ROMs on the hemolysis of snails.

CONCLUSION:

The results obtained allow us to conclude that ultrafine particle of TiO_2 affected some toxicological parameters of *Helix aspersa*. Antioxidant enzymes system CAT ,GST was induced, Glutathione in kidney and the digestive gland was decreased by scavenging residual free radicals and increase in product of lipid peroxidation MDA. The different biomarkers of toxicity previously measured confirm the triggering of the antioxidant metabolism in organism of *Helix aspersa*.

That can be hypothesized by the triggering ofoxidative stress by TiO_2 microparticles, and caused a lipid peroxidation of membrane phospholipids. Therefore, release of microparticles of TiO_2 into the environment may pose highly potential risks to the different ecosystems.

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