

LIPID PEROXIDATION AND PROTEIN OXIDATION INDUCED BY DIFFERENT NANOPARTICLES IN ZEBRAFISH ORGANS

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Abstract. The protein oxidation and the lipid peroxidation induced by the presence of five different kinds of nanoparticles were determined in different tissues of zebrafish (*Danio rerio*). Both oxidative stress markers showed to be dose-dependent. At 1 ppm nanoparticle concentration, the five assayed nanoparticles showed higher protein oxidation than the control experiments. Europium-doped yttrium oxide (Y₂O₃:Eu) and carboxylated multi-walled carbon nanotubes (MWCNT-COOH) showed the highest effect, especially in ovaries, testicles and brain, reaching carbonyl content in proteins up to 4-times higher than control, while bismuth germanate (BGO) and MWCNT-COOH nanoparticles induced lipid peroxidation in all analyzed tissues, reaching values up to two-times higher than those from non-exposed control fish. BGO and silver nanoparticles (AgNP) showed less oxidative effect; nevertheless, the protein oxidation still accounted for up to 3-times the control fish values. At 1 ppm, the protein oxidation induced by some nanoparticles reached the values obtained in the experiments with 100 μM H₂O₂. On the other hand, no significant differences were found in gene expression of oxidative stress markers after 48 h treatment. The results from this work clearly indicate that exposure to those nanoparticles may cause significant protein and lipid oxidative damage.

Keywords: *lipid peroxidation, protein oxidation, nanoparticles, oxidative stress, zebrafish, toxicity*

Introduction

Nanotechnology is the origin of a new revolution in material technology with numerous potential applications. The use of nano-structured materials has more than quadrupled in the last 5 years. It is estimated that there are more than 1,000 nano-structured commercial products (Papp et al., 2008; McIntrye, 2012). Nanoparticles (NPs) can be made from almost any kind of materials, and the most used so far are made from carbon, silicon, titanium or other similar elements and materials. Many NPs are also made from metals or metal oxides, containing elements such as cadmium, cerium, copper, germanium, gold, iron, lead, selenium, silver, zinc, or zirconium. In general, the main NPs classes with commercial applications are fullerenes and single-wall/multi-wall carbon nanotubes, quantum dots, metal oxide-based NPs, and polymers (Powell and Kanarek, 2006).

Among the numerous commercial applications of NPs there are antimicrobial preparations, medical materials and devices, medical diagnostics, cosmetics, food packaging, anti-odor textiles, electronics, self-cleaning surfaces, UV-resistant coatings and paints, and lubricants. As the use of NPs increases, it is expected that their presence in aquatic environment will increase. In addition, NPs in aquatic systems can be formed by natural abiotic or biotic processes, induced by human activity (e.g. mining), incidentally introduced to aquatic systems, or intentionally added to these systems

(Delay and Frimmel, 2012; Wigginton et al., 2007). Specifically, NPs have been proposed as potentially useful for water treatment processes (Wiesner, 2006; Wiesner and Bottero, 2007). The physicochemical behavior of NPs in aquatic environments has been reviewed (Lead and Wilkinson, 2006; Nowack and Bucheli, 2007; Christian et al., 2008; Baalousha et al., 2009). These molecular interactions are essential for the understanding of the mobility and transport, bioavailability, ecotoxicity, and reactivity of NPs in the aqueous phase. It has been found that when nanoparticles are dosed to water columns, they tend to precipitate to the subaquatic sediment, but this doesn't reduce its bioavailability since they accumulated in plant and animal biomass (Lowry et al., 2012).

The ecological importance of the NPs presence in aquatic systems has been discussed (Delay and Frimmel, 2012; Baun et al., 2008) and it is clear that the behavior and role of NPs in aquatic systems is very complex. In addition, the information of NPs effects and toxicity mechanisms in aquatic systems and organisms is still scarce, and the specific toxicity of NPs and their long-term impact on environment and human health remains basically unknown (Papp et al., 2008). These are important issues that need to be assessed in order to understand and even forecast the real risk that nanomaterials may pose to the environment and human health (Wiesner and Bottero, 2012).

The current state of knowledge regarding the biological and health effects of NPs has been recently reviewed (Papp et al., 2008; Stark, 2011; Sharifi et al., 2012; Pattan and Kaul, 2012). Several different mechanisms for NPs toxicity have been reported. The increased production of reactive oxygen species (ROS) seems to be an important toxicity mechanism in cells induced by NPs (Nel et al., 2006; Unfried et al., 2007; Moller et al., 2010). These ROS include, among others, hydroxyl radicals, lipidoxyl or peroxy radicals, singlet oxygen, and peroxynitrite formed from nitrogen oxide (NO). The modulation of several cellular events, including signal transduction, proliferative response, gene expression and protein redox regulation requires moderate amounts of ROS. However, high ROS levels induce oxidative stress which can damage cells by peroxidizing lipids, oxidizing proteins, disrupting DNA, interfering with signaling functions, modulating gene transcription, and finally, inducing cancer, renal disease, neurodegeneration, cardiovascular or pulmonary diseases.

In particular, lipid peroxidation is considered highly dangerous as it alters cell membrane properties (Lam et al., 2006). The chemical composition of nanoparticles seems to be the most decisive factor determining the formation of ROS in exposed cells (Limbach et al., 2007). For example, fullerene and its water-soluble derivative caused membrane disruption in rat liver microsomes by inducing significant oxidative damage (Kamat et al., 2000). Fullerenes are lipophilic and insert into lipid-rich regions (liver and gills) in fish. Other nanomaterials have been shown to selectively translocate into the brain via the olfactory bulb in mammals and fish (Oberdörster, 2004). The toxic effect of ROS could be more pronounced in the central nervous system due to the high content of unsaturated fatty acids in the cells, which are more susceptible to peroxidation (Adibhatla and Hatcher, 2010).

On the other hand, the oxidation of proteins in vertebrates has been the subject of several studies (Stadtman, 1991). Among the various oxidative modifications of amino acids in proteins, carbonyl formation may be an early marker for protein oxidation (Stadtman, 2004). TiO₂ NPs showed a dose-dependent linear increase in generating ROS and the generated ROS correlated with the protein oxidation in both *in vivo* and *in vitro* experiments (Han et al., 2012). The loss of membrane bound enzymes was also

detected. Finally, TiO₂ NPs intranasally supplied to mice (Wang et al., 2008) induced a significant oxidative damage in brain tissue. The whole brain was affected, and lipid peroxidation, protein oxidation, increased activities of catalase, and an excessive release of glutamic acid and nitric oxide were detected.

There is a consensus that NPs in environmental systems are of ecotoxicological and public health relevance (Oberdörster, 2004; Ray et al., 2009; Stone et al., 2009). Thus, there is an urgent need for the evaluation of NPs biological effects due to the rapid development and commercialization of nano-structured products. In this work, different kinds of NPs are evaluated for oxidative stress in zebrafish. Different tissue targets are studied and the risk of the NPs presence for aquatic organisms is discussed.

Materials and methods

Nanoparticles

Europium-doped hydroxyapatite powder and bismuth germanate (Bi₄Ge₃O₁₂, BGO) and Y₂O₃:Eu³⁺ (with silica shell) nanoparticles were synthesized by sol-gel method and subsequent annealing (Taxak et al., 2009; Oviedo et al., 2012). The carboxylated multi-walled carbon nanotubes (MWCNT-COOH) were prepared by chemical vapor deposition and purified by refluxing in an aqueous HNO₃ solution (2.6 M) for 6 h, washed several times with distilled water and dried for 12 h at 80°C (Lin et al., 2002). Silver nanoparticles (AgNP) from Sovereign Silver™ (Russia) were a kind gift of Dr. Nina Bogdanchikova (CNyN-UNAM). The physical properties of the different nanoparticles are shown in *Table 1*.

Table 1. Physical properties of the different nanoparticles.

Property	HA:Eu	BGO	MWCNT	AgNP	Y ₂ O ₃ :Eu
Size diameter	50-100 nm	10-15 nm	80-100 nm	10-30 nm	50-100 nm
Zeta potential	-10 mV	ND	-27.3 mV	- 21.2	-25.0 mV
Excitation maximum	280 and 394 nm	284 nm	---	215 nm	237 nm
Emission maximum	575, 613 and 620 nm	485 nm	---	300 nm	611 nm
Melting point	1200 °C	1050 °C	---	810 °C	1600 °C

ND. Not determined

Fish experiments

Adult and healthy zebrafish (*Danio rerio*) of 0.470 ± 0.166 g were obtained from a local fish farm (Granja Acuario Antártico, Cuernavaca, Mexico). Fish were acclimatized in glass tanks at room temperature ($25 \pm 2^\circ\text{C}$) for 2 days with a photoperiod of 12 h light/12 h dark and they were fed twice a day with a commercial diet. Experiments were carried out in a 20 L aquarium containing 16 L of chlorine-free treated water. Ten fish were treated with 0.5 or 1 ppm of NPs and ten fish were used as control without NPs. Every set of treatments and controls were carried out in three independent experiments. After 48 h of treatment, fish were anesthetized on ice, and then dissected to obtain liver, ovaries, testicles, gills and brain tissues. Positive control experiments for oxidative stress were performed treating fish in the presence

of 100 μM hydrogen peroxide for 48 h. All tissues were kept in liquid nitrogen and analyzed immediately.

Protein oxidation

The extent of protein oxidation in the different tissues was determined as the carbonyl content in the protein fraction (Levine et al., 1991). Tissues (150-200 mg) from fish treatments with 0.5 or 1 ppm of NPs, or with 100 μM H_2O_2 (positive control) were placed in an eppendorf tube and 4 volumes of 50 mM HEPES buffer (pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluoride were added, and then grinded with pellet pestle (Kimble-Chase 749521-1590). The homogenized mixture was centrifuged at 10,000 g for 5 min at 4°C. Subsequently, 10 μL of streptomycin (20%) were added to 100 μL of supernatant in order to remove the nucleic acids that may erroneously contribute to higher estimation of carbonyls. The mixture was kept at 25°C for 15 min and the precipitated nucleic acids were removed by centrifugation at 10,000 g for 5 min at 4°C. To a 100 μL aliquot of supernatant, 400 μL of 10 mM 2,4-dinitrophenylhydrazine were added to and kept for 1 hour under gentle shaking. Then, the protein was precipitated by adding 500 μL of trichloroacetic acid 20% and recovered by centrifugation at 10,000 g for 5 min at 4°C. The pellet was washed three times with a ethanol:ethylacetate (50:50 v/v) solution. The pellet was dissolved in 500 μL of 6 M guanidine containing 10 mM dithiothreitol at 37°C. An aliquot of 400 μL of this protein solution were diluted with 600 μL of water and spectrometrically read at 370 nm (Oberdörster, 2004).

Lipid peroxidation

The different tissues from fish treatments with 0.5 or 1 ppm of NPs, or with 100 μM H_2O_2 (positive control) were analyzed for lipid peroxidation. The amount of lipid peroxides was estimated by using the Xylenol Orange reagent (Nourooz Zadeh et al., 1994). The FOX reagent contains 2.5 mM ammonium ferrous sulphate and 110 mM perchloric acid in a methanol:water (90:10 v/v) solution. FOX reagent (900 μL) was added to 100 μL of homogenized tissue supernatant (see previous section) and kept for 30 min at 25°C. Then the reaction mixture was read at 560 nm (Gay and Gebicki, 2003). The molar concentration of lipid peroxides was then estimated comparing the absorbance values with those obtained from a standard curve with different concentrations of hydrogen peroxide.

Protein determination

The amount of protein in the tissue homogenates was spectrophotometrically determined with the BioRad protein reagent using BSA as standard.

Isolation of RNA and reverse transcription

Six fish were reared in 6 L containing 1 ppm of the corresponding nanoparticle (BGO, $\text{Y}_2\text{O}_3\text{-Eu}^{3+}$, MWCNT-COOH and AgNP) in glass flasks at room temperature ($25 \pm 2^\circ\text{C}$). Three independent replicates in separated glass flasks were used for each treatment. After 48 h of exposure, fish were anesthetized on ice and frozen in liquid nitrogen. Liver and brain were dissected out and stored at 4°C in RNA later (Qiagen, USA). After 24 h, RNA later was removed and the tissues were stored at -20°C . The

rest of the body was frozen and stored at -80°C . The different tissues were grounded in a mortar with liquid nitrogen. The tissue was then recovered in $250\ \mu\text{L}$ TRI Reagent (Sigma, USA), and total RNA was isolated according to the manufacturer's instructions. The absorbance ratio 260/280 nm was measured in a Nanodrop. The banding patterns on a 1% agarose gel were used to verify the quality of the RNA in each sample. One μg of total RNA was treated with DNase I, RNase-free (Fermentas, USA) according to manufacturer's instructions. Reverse transcription of mRNA was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). Finally, samples were diluted 4-fold and $1\ \mu\text{L}$ was used for the real-time quantitative polymerase chain reaction.

Gene expression analysis

Real-time quantitative PCR was performed using a StepOne Real Time PCR system (Applied Biosystems, USA). The qPCR reactions were carried out in $10\ \mu\text{L}$ of Maxima SYBR Green /ROX qPCR Master Mix (2X) (Fermentas, USA). The following PCR protocol was used: Denaturation for 10 min at 95°C , followed by 40 cycles of 15 s at 95°C , and 1 min at 60°C . The correct primer amplification was confirmed by agarose gel electrophoresis. Oligonucleotide primer pairs of oxidative stress genetic expression markers; superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1) (Subauste and Burant, 2007), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) (Mougiakakos et al., 2012) are described in *Table 2*. β -actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity. Each experiment was carried out in triplicate.

Table 2. Oxidative stress genetic expression markers. Oligonucleotide primer pairs of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc). β -actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity.

mRNA	Accession number	Forward (5'-3')	Reverse (5'-3')	Position
Gclc	NM_199277.2	ACAGCTATCTGGAGAACATGGAGG	TGTTTGGCAACAACTCCCTCATC	1628-1763
FoxO1a	NM_001077257.2	GACCTCATGCCTTCAGTGGACACT	TGTGTGGGTGAGAAAGAGTGTGAC	1393-1535
Sod1	NM_131294.1	TGAGACACGTCGGAGACCTGGGTA	CCACCCTTCCCCAAGTCATCCTCC	304-460
bact	AF057040	ATGGATGAGGAAATCGCTGCC	CTCCCTGATGTCGGGTCGTC	54-180
Tbp	NM_200096	CGGTGGATCCTGCGAATTAT	GGTTATGAAGCAAAACAACA	51-146
Tub	AF029250	TCTTTGTAGACCTGGAGCCC	TCAATGAGTTCCTTGCCAAT	197-347

Gene expression statistical analysis

Results of gene expression were normalized using the geometric mean of the three control genes using the Biogazelle qbasePlus software (Gent, Belgium). The relative expression values for each gene were calculated taking as reference the value obtained for the control treatment.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA). If the results of the ANOVA indicated significant differences among the mean values, then multiple comparisons of mean values were performed using Tukey's test. Differences were reported as statistically significant at $P < 0.05$. All the statistical analyses were performed using the statistical program SIGMA STAT, 3.1 (Arlington, VA, USA).

Results

Oxidative stress implies a cellular state whereby reactive oxygen species (ROS) production exceeds its metabolism overpassing cellular defenses. The oxidant effect of nanoparticles was estimated in zebrafish (*Danio rerio*) after 48 h exposure to two different NPs concentrations, 0.5 ppm and 1.0 ppm. Five different kinds of nanoparticles were studied including europium-doped hydroxyapatite (HA:Eu), bismuth germanate (BGO), europium-doped yttrium oxide ($Y_2O_3:Eu$), silver nanoparticles (AgNP), and carboxylated multi-walled carbon nanotubes (MWCNT-COOH). In addition, a positive control was performed in which fish were treated with 100 μM of hydrogen peroxide. The H_2O_2 concentration of this positive control was chosen after preliminary experiments with 10, 50 and 100 μM of H_2O_2 , the latter being the one that showed important protein oxidation and lipid peroxidation in the different tissues.

The formation of carbonyl groups is an early marker for protein oxidation (Stadtman, 2004) and was determined in five different tissues of zebrafish after exposure to different NPs. Carbonyl content in liver, ovaries, testicles, gills and brain values were compared with those of a positive control for oxidative stress induced by the presence of 100 μM of hydrogen peroxide (*Fig. 1*). At 0.5 ppm concentration, $Y_2O_3:Eu$ and MWCNT-COOH induced, by far, the highest oxidative stress followed by AgNP, while no protein oxidation was found with HA:Eu and BGO NPs when compared to control experiments (*Fig. 1*). Nevertheless, this increase in carbonyl content in proteins is lower than those found in the positive control with 100 μM H_2O_2 , in which liver, ovaries, testicles and brain are especially prone to protein oxidation. At low NPs concentration all tissues seemed to be equally sensible.

On the other hand, in all cases, when NPs concentration was increased from 0.5 ppm to 1 ppm, an increase was observed on protein oxidation. At high NPs concentration (1 ppm), the five assayed NPs showed higher concentration of carbonyl groups than the control experiments (*Fig. 1*). Here, again the $Y_2O_3:Eu$ and MWCNT-COOH nanoparticles showed the higher effect, specially in ovaries, testicles and brain. The carbonyl group content in these tissues reached up to 4-times higher than in control fish after 48 h treatment. AgNP showed lower but significant oxidative effect, nevertheless, the protein oxidation still accounted for up to 3-times the control fish values. At 1 ppm, the induced protein oxidation by NPs reached the values obtained in the experiments with 100 μM H_2O_2 . These results clearly indicate that exposure to 1 ppm concentration of these NPs for 48 h is able to induce an important oxidative stress on fish demonstrated by the carbonyl content in protein in the different fish tissues.

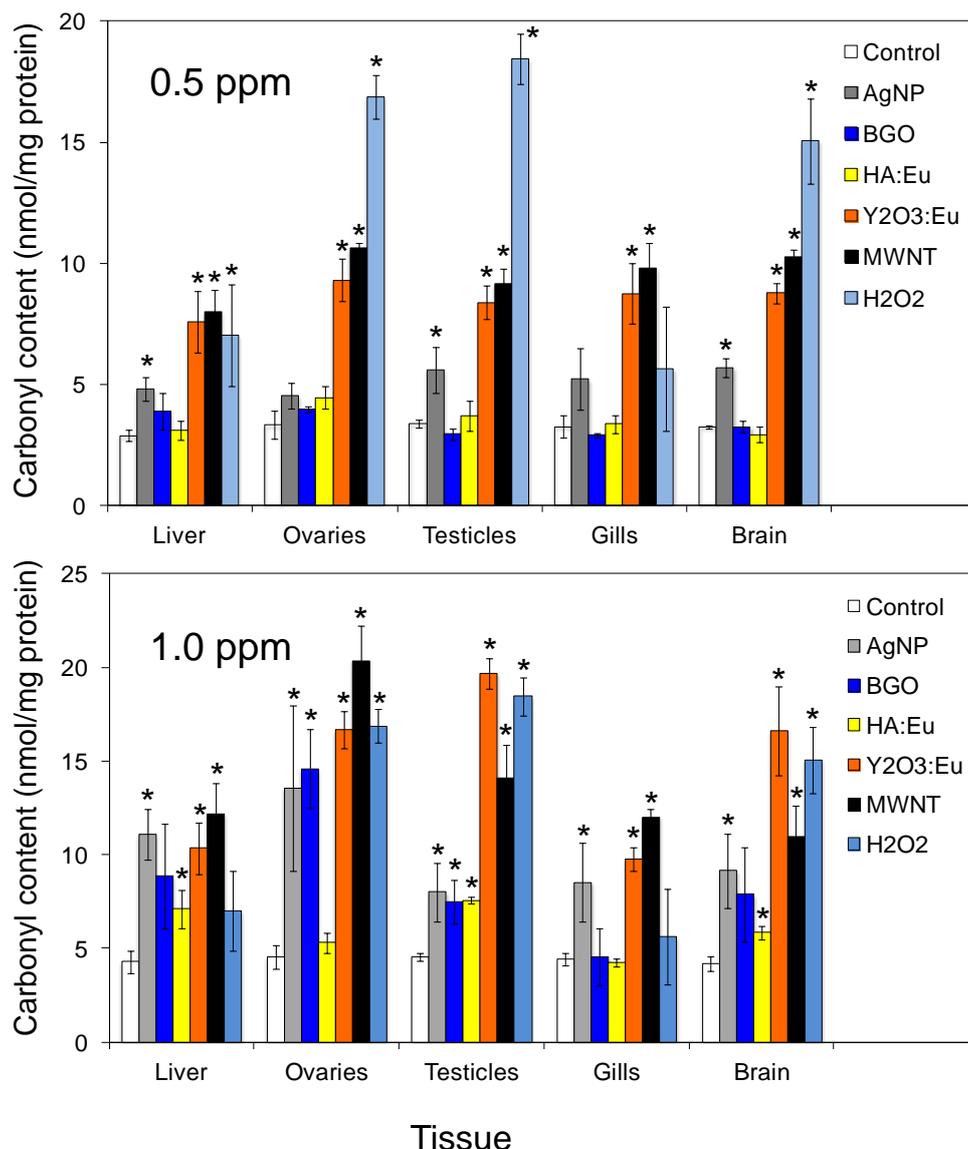


Figure 1. Protein oxidation of different zebrafish tissues estimated as carbonyl content in proteins. a) Fish exposed to 0.5 ppm of nanoparticles for 48 hrs. b) Fish exposed to 1 ppm of nanoparticles for 48 hrs. (*)Values significantly different at $P < 0.05$ with respect to the control.

The concentration of lipid peroxides in the different tissues was estimated after 48 h fish exposure to NPs (Fig. 2). At 0.5 ppm of NPs there is low effect, except with MWCNT-COOH nanoparticles in ovaries, testicles and brain. Lipid peroxidation also showed to be dose-dependent. At high NPs concentration (1 ppm) MWCNT-COOH nanoparticles induced higher lipid peroxides content in all analyzed tissues, reaching values up to two-times higher than those from non-exposed control fish.

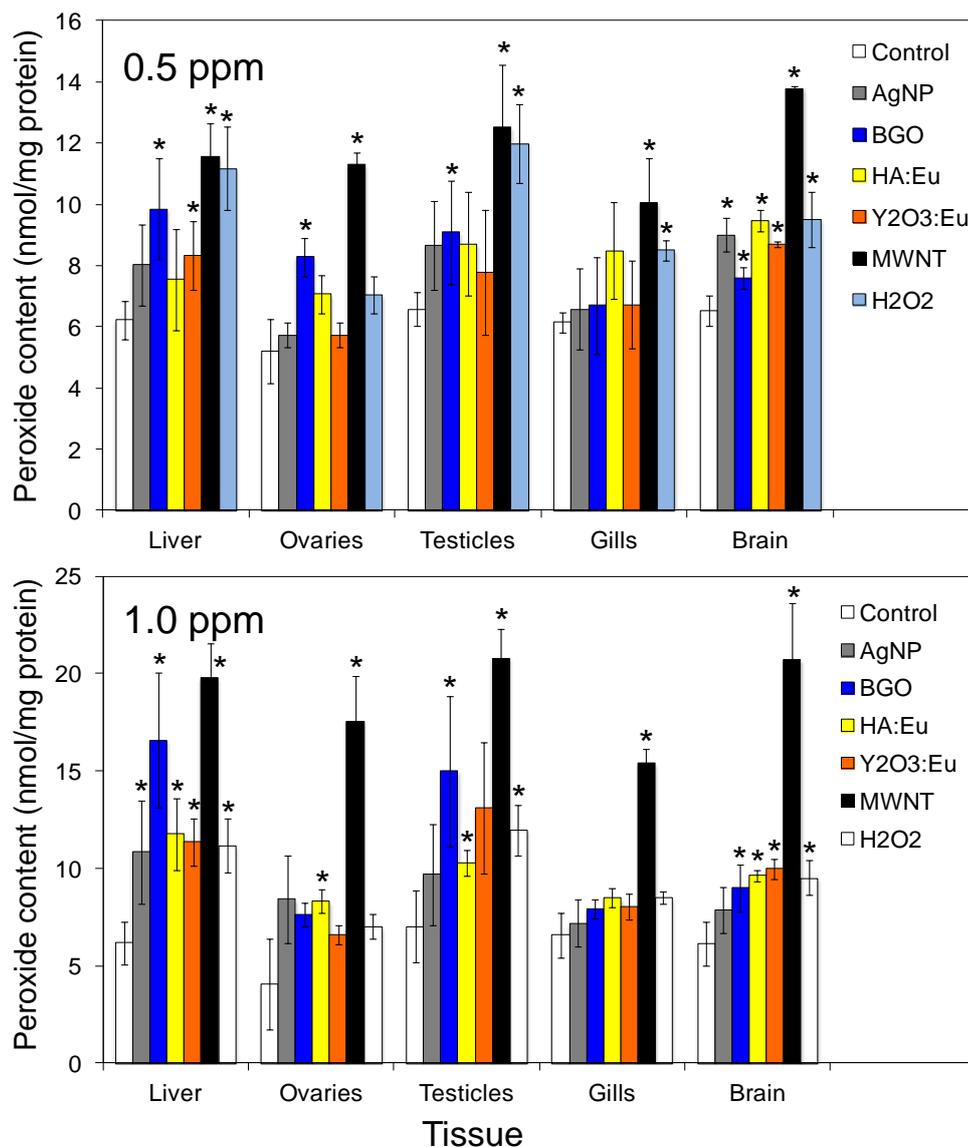


Figure 2. Lipid proxidation of different zebrafish tissues estimated as peroxy content in lipids. a) Fish exposed to 0.5 ppm of nanoparticles for 48 hrs. b) Fish exposed to 1 ppm of nanoparticles for 48 hrs. (*)Values significantly different at $P < 0.05$ with respect to the control.

The genetic expression of oxidative stress markers was measured in treated and untreated fish with 1 ppm of NPs for 48 h (Table 3). Expression of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) was quantified. β -actin (bact), tubulin alpha I (Tub) and TATA-box binding protein (Tbp) transcripts were used to standardize the results by eliminating variations in mRNA and cDNA quantity. No significant differences were found in the gene expression of any marker gene in liver, brain nor whole fish body (Table 3). These results may indicate that 48 h is not enough time for fish to metabolically compensate the accumulation of ROS caused by NPs

exposure by modifying the expression of these genes involved in contending with oxidative stress.

Table 3. Relative mRNA levels of oxidative stress related genes in zebrafish exposed to nanoparticles (mean \pm standard deviation). Values are relative to control fish.

AgNP

Gene	Brain	Liver	Whole body
FoxO1a	0.97 (\pm 0.41)	0.86 (\pm 0.13)	1.04 (\pm 0.45)
Gclc	0.78 (\pm 0.07)	1.05 (\pm 0.09)	1.38 (\pm 0.31)
Sod1	0.68 (\pm 0.17)	0.62 (\pm 0.07)	0.94 (\pm 0.19)

BGO

Gene	Brain	Liver	Whole body
FoxO1a	1.18 (\pm 0.08)	0.64 (\pm 0.43)	0.68 (\pm 0.28)
Gclc	0.83 (\pm 0.06)	1.08 (\pm 0.67)	1.24 (\pm 0.06)
Sod1	0.72 (\pm 0.10)	1.65 (\pm 0.63)	1.04 (\pm 0.26)

MWNT-COOH

Gene	Brain	Liver	Whole body
FoxO1a	0.95 (\pm 0.03)	1.28 (\pm 0.21)	1.29 (\pm 0.41)
Gclc	0.63 (\pm 0.16)	1.02 (\pm 0.13)	1.04 (\pm 0.23)
Sod1	0.95 (\pm 0.27)	1.44 (\pm 0.29)	1.38 (\pm 0.28)

Y₂O₃-Eu³⁺

Gene	Brain	Liver	Whole body
FoxO1a	0.73 (\pm 0.06)	0.70 (\pm 0.18)	0.71 (\pm 0.13)
Gclc	0.94 (\pm 0.08)	0.74 (\pm 0.14)	0.88 (\pm 0.07)
Sod1	0.73 (\pm 0.14)	1.02 (\pm 0.62)	0.82 (\pm 0.07)

Discussion

Our results demonstrated that NPs, after 48 h exposure and independently of its chemical nature, induce protein oxidation and lipid peroxidation of different zebrafish tissues (Figs. 1 and 2). Lipid peroxidation is also one of the earliest indicators of oxidative stress (Hwang and Kim, 2007). Reactive oxygen species (ROS) are able to oxidize unsaturated fatty acids producing lipid peroxides. Lipid peroxidation may play an important role in carcinogenesis (Cai et al., 2012), in the molecular mechanisms of multiple sclerosis (Ferreti and Bacchetti, 2011), in Parkinson's disease (Jenner, 1991), and there is increasing evidence of its role in diabetes (Piconi et al., 2003), among other diseases. In comparison with other nanoparticles, HA:Eu showed the lower oxidative stress levels in the different tissues. However, HA:Eu nanoparticles, depending on the shape and concentration, could induce hatching inhibition in fish and a reduction of metabolic activity of the cells (Zhao et al., 2013). Hydroxyapatite NPs could be aggregated into bigger particles around the membrane protein, inducing low toxicity to development of zebrafish embryos, when compared to SiO₂ and TiO₂ nanoparticles (Xu et al., 2012). Apoptosis could be the possible mechanism of hydroxyapatite NPs toxicity, as reported from *in vitro* cell experiments (Wang et al., 2012).

BGO and AgNP induced moderate protein oxidation and lipid peroxidation. AgNPs have potent antibacterial activity making them interesting for several biomedical applications and they are extensively used in the healthcare, food industries, many domestic applications and as additive in surface materials and textiles. Although there is

abundant literature on their toxic effects, there is little understanding of the AgNPs interactions with microorganisms (Maillard and Hartemann, 2012). Oxidative stress induced by AgNP has been reported from *in vitro* cell experiments (Arora et al., 2008; Carlson et al., 2008; AshaRani et al., 2009; Piao et al., 2011) and *in vivo* experiments including fish (Choi et al., 2010; Gagné et al., 2012; Wu and Zhou, 2012), but the toxicity mechanism is still not fully elucidated. In these studies at AgNP concentrations of 1 to 100 ppm, the hepatic lipid peroxidation, measured as the malondialdehyde generation, increased from 1.5 to 2-fold, results that are in agreement with those obtained in this work.

Bismuth germanate (BGO) has been used as a scintillator in detectors, industry and medicine for the past forty years (Weber and Monchamp, 1973; Chung and Chan, 1994; Macedo et al., 2004). On the contrary to the controversial AgNP, there is not information on BGO toxicity. As far as we know, this is the first report on the oxidative effects of BGO nanoparticles. BGO nanoparticles showed high protein oxidation at 1 ppm in liver, ovaries and brain, while lipid peroxidation was found only in liver and testicles (Figs. 1 and 2).

Yttrium oxide nanoparticles activated with europium ($Y_2O_3:Eu$) showed high oxidative stress, especially in proteins (Figs. 1 and 2). $Y_2O_3:Eu$ is the most red-emitting phosphor widely used in color TV displays and fluorescent lamps. A few studies of the effect of Y_2O_3 nanoparticles are available in literature. Human aortic endothelial cells exposed to Y_2O_3 nanoparticles showed that the NPs were often present within cytoplasmic vesicles, where a transformation is suspected, and their density increased with nanoparticle concentration (Gojova et al., 2007). In addition, Y_2O_3 nanoparticles induced the up-regulation of inflammatory markers observed at the translational level. This effect was concentration dependent, with a statistically significant increase from concentrations of 10 ppm to 50 ppm. As mentioned before, the suggested pathway for nanoparticle-induced inflammation in human aortic endothelial cells is the production of reactive oxygen species (ROS). However, Schubert et al. (2006) suggest that Y_2O_3 nanoparticles could act as antioxidants in neurons.

The carboxylated multi-walled carbon nanotubes (MWCNT-COOH) showed the highest induction of both protein oxidation and lipid peroxidation at the two tested concentrations. The toxicity of MWCNT and their functionalized derivatives has been extensively reviewed (Lam et al., 2006; Schubert et al., 2006; Lacerda et al., 2006; Sargent et al., 2010; Shvedova et al., 2012). Carbon nanotubes (CNT) represent an special case, because relatively high content of transition metals, particularly Fe, Cu, Cr, and Ni (Tessonier and Su, 2011; Valko et al., 2005), are present in some preparations. It is well known that the presence of these metals in single-walled carbon nanotubes (SWCNT) may be important in determining redox-dependent responses of macrophages (Kagan et al., 2006; Pulskamp et al., 2007). Non-purified iron-rich SWCNT induced higher concentrations of intracellular ROS, specifically hydroxyl radicals, determined by EPR spin-trapping analysis, and decreasing the mitochondrial membrane potential, than purified SWCNT. Two main consequences of ROS production induced by the presence of carbon nanotubes have been proposed: Inflammation, since ROS production is thought to be an essential requirement for inflammasome activation, and the activation of the NADPH oxidase. The recognition of MWCNT-COOH by phagocytes could lead to their internalization via endocytosis with subsequent fusion with lysosomes within the cell. Then, the lysosomes disruption may lead to the activation of the inflammasome. However, the contribution of each

ROS generation mechanism (mitochondria-generated and NADPH oxidase-generated ROS) to the activation of the inflammasome induced by carbon nanotubes requires further investigation (Valko et al., 2005).

A state of oxidative stress is created when the generation of ROS is higher than the rate of their elimination. Jones (2006) redefined oxidative stress as “a disruption of redox signaling and control” bringing antioxidant enzymes into the picture and making the point that the deleterious effect is observed when the accumulation of ROS surpasses the cell's defense capacity. In addition to non-enzymatic defenses mediated by some molecules, enzymatic detoxification of ROS constitutes a major component of oxidative stress defense systems and involves enzymes such as superoxide dismutases, catalases and peroxidases. Basal levels of these enzymes work in concert to remove endogenously produced ROS and keep their levels within physiologically safe limits. In our experiments, the expression of superoxide dismutase (Sod1), the transcription factor Forkhead box protein O1 (FoxO1), and the glutamate-l-cysteine ligase catalytic subunit (Gclc) were quantified in different fish tissues. No significant differences were found in the gene expression of any marker gene in liver, brain and fish body (*Table 3*). These results suggest that after only two days of NPs exposure, the fish is not able to metabolically compensate, by the expression of these genes, the oxidative stress caused by the presence of nanoparticles, leading to protein oxidation and lipid peroxidation in the different fish tissues. The exposure of cultured human cells to concentrations 40-times higher than those used in this work of cerium oxide and titanium dioxide NPs of different sizes, induced the expression of oxidative stress-related genes such as heme oxygenase-1, catalase, glutathione S-transferase, and thioredoxin reductase (Park et al., 2008). After prolonged exposures to relatively high concentrations, low cytotoxicity has been seen in cultured cells owing to increases in oxidative stress, most likely due to the increase of antioxidant defenses (Thakor et al., 2011). Recently, Gagné et al. (2012) performed a DNA microarray study of rainbow trout (*Oncorhynchus mykiss*) in order to identify the fundamental mode of action of AgNPs using the gene expression level. They found that 31% of the genes (from 270 studied genes) responded to either nano- or dissolved-silver, while 9.6% were specifically affected by AgNPs. The authors conclude that the increased lipid peroxidation and the reduced level of DNA strand breaks induced by AgNPs could not be explained by the presence of dissolved silver.

It seems that ROS generation by NPs is generated through several mechanisms including redox-cycling pathways, inflammation and protein denaturation. In addition, the activation of lysosomes may trigger a mitochondrial apoptosis, with the subsequent ROS production. Recently it has been demonstrated that the presence of antioxidant (vitamin E) protects cultured cells from the injury induced by SWCNT through the down-regulation of oxidative stress and prevention of mitochondria mediated apoptosis (Wang et al., 2012).

Thus, the oxidative stress is not the sole origin of the toxic effects of NPs. A better understanding of the nature and significance of such oxidative signaling and metabolic responses will offer new opportunities to control the effects of nanoparticles, including their toxicity. The cell could be considered as a collection of “nanomachines” (van den Heuvel and Dekker, 2007) to which engineered nanomaterials may interfere directly with cellular functions. A priority challenge of nanotechnology is to understand the mechanism leading to oxidative stress and its metabolic consequences in order to emphasize in a better nanomaterials design to prevent toxic effects that would put in risk animal health and environmental equilibrium.

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