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Research Article

Toxic and Genotoxic Effects of Silver Nanoparticles in Drosophila

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The *in vivo* model *Drosophila melanogaster* was used here to determine the detrimental effects induced by silver nanoparticles (AgNPs) exposure. The main aim was to explore its interaction with the intestinal barrier and the genotoxic effects induced in hemocytes. The observed effects were compared with those obtained by silver nitrate, as an agent acting *via* the release of silver ions. Larvae were fed in food media containing both forms of silver. Results indicated that silver nitrate was more toxic than AgNPs when the viability "egg-to-adult" was determined. Depigmentation was observed in adults including those exposed to non-toxic concentrations, as indicative of exposure action. Interestingly, AgNPs were able to cross the intestinal barrier affecting hemocytes that show significant increases in the levels of intracellular reactive oxygen species. Additionally, significant levels of genotoxic damage, as determined by the comet assay, were also induced. When the expression of different stress-response genes was determined, for both AgNPs and silver nitrate, significant upregulation of *Sod2* and *p53* genes was observed. Our results confirm for the first time that in an *in vivo* model as Drosophila, AgNPs are able to cross the intestinal barriers and produce primary DNA damage (comet assay) *via* oxidative stress induction. In general, the effects induced by silver nitrate were more pronounced than those induced by AgNPs what would emphasize the role of silver ions in the observed effects. Environ. Mol. Mutagen. 60:277–285, 2019.

Key words: genotoxicity; silver nanoparticles; *Drosophila melanogaster*; reactive oxygen species; comet assay

INTRODUCTION

The increase of engineered nanomaterials in the environment requires a systematic assessment of their harmful consequences (Zhao and Castranova 2011; Pal et al. 2015). With this aim, a large amount of *in vitro* studies have been already published using different cell-targets and biological end-points (Ajdary et al. 2018; Missaoui et al. 2018). Nevertheless, these *in vitro* approaches do not completely reflect the real events occurring in whole organisms (Lewinski et al. 2008). This means that *in vivo* approaches are required to support *in vitro* findings. In this scenario, *Drosophila melanogaster* is considered as a powerful model widely used in many fields due to its multiple advantages. The fact that around 75% of the genes involved in human diseases have related sequences in *D. melanogaster* (Reiter et al. 2001) supports the use of this model organism to study different human pathologies (Pandey and Nichols 2011; Gonzalez 2013). A complementary advantage of using Drosophila is that it rides out from the ethical limitations associated to the use of more complex organisms such as mammals. It must be indicated that Drosophila has already been used in studies addressed to determine the potential harmful effects of nanomaterials, including silver nanoparticles (AgNPs), as recently reviewed (Alaraby et al. 2016a).

Due to the attributed properties of AgNPs, mainly as antimicrobial agent (Maillard and Hartemann 2012), this engineered nanomaterial is placed among the most often incorporated in nanofunctionalized consumer products (Zhang et al. 2016). With regard to their potential harmful effects, different studies have been conducted to understand the underlying mechanisms of action (Stensberg

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et al. 2011; Schluesener and Schluesener 2013; McGillicuddy et al. 2016; Cameron et al. 2018). The obtained results suggest that AgNPs penetrate cell membranes in a size-dependent ratio. Once inside the cells high levels of silver ions are produced causing toxic and genotoxic effects and affecting also the antioxidant defenses (Stensberg et al. 2011).

As indicated, Drosophila has already been used to determine, in a fractionated way, different aspects related to AgNPs exposure. Delays in the development from egg-to-adult have been detected when eggs or larvae were exposed (Gorth et al. 2011; Philbrook et al. 2011; Vecchio et al. 2013; Han et al. 2014; Vega-Alvarez et al. 2014; Raj et al. 2017a). Interestingly, these effects were translated to the emerged adults who present shortened life span, mating success decrease, and less fertility (Key et al. 2011; Panacek et al. 2011; Posgai et al. 2011; Armstrong et al. 2013; Tian et al. 2013; Mao et al. 2018). The observed loss of fertility was associated with a decreased number of germline stem cells (Ong et al. 2016), and also a reduced pigmentation was observed in adults, mainly depending on AgNPs concentration (Panacek et al. 2011; Avalos et al. 2015; Phatak et al. 2016). From the genotoxic point of view oxidative stress and DNA damage induction has been reported, measured indirectly as changes in the expression of different genes regulating such processes (Ahamed et al. 2010; Raj et al. 2017b). In addition, significant increases in the frequency of somatic mutated clones resulting from both mitotic mutation and recombination were reported (Demir et al. 2011). No studies using the comet assay have been carried out until now.

In spite of the reported studies, there are many questions still unsolved that prevent the completely understanding the mode of action of AgNPs in Drosophila. In this study, we have focused in determining the effects associated to the uptake of AgNPs through the larval intestine. It must be pointed out that the intestine of Drosophila's larvae has been proposed as a suitable *in vivo* model to evaluate the interferences of nanomaterials with the intestinal barrier (Pompa et al. 2011; Alaraby et al. 2016b). In addition, the effects on internal hemocytes have been evaluated by measuring reactive oxygen species (ROS) and DNA damage using the comet assay. In this context, the use of the comet assay using hemocytes as a targeted cells is relevant since indicate the translocation of AgNPs through the intestinal barrier. Results using AgNPs have been compared with those obtained using silver nitrate to determine the relevance of the nanoparticulated form versus the ionic form.

MATERIAL AND METHODS

Chemicals

AgNPs were supplied by NanoComposix (Prague, Czech Republic). These nanoparticles have not any type of coating. The ionic form of silver (silver nitrate, $AgNO_3$, 99.0 purity, CAS 7761-88-8), and all of the other compounds used in the different tests, were supplied by Sigma Chemicals (St. Louis, MO).

Commercial AgNPs were suspended in Milli-Q water. According to the manufacturer, the physical characteristics of the supplied nanoparticles are: diameter (5.0 ± 1.0 nm), coefficient of variation (19.0%), surface area (105.8 m^2/g), mass concentration (5.43 mg/mL), and particle concentration (7.7E+15 particles/mL). To confirm such characteristics, a further characterization was carried out by using transmission electron microscopy (TEM; JEOL JEM-2011) to determine size and morphology. In addition, the hydrodynamic size and the zeta potential were determined by using a Malvern Zetasizer "Nano-ZS zen3600" instrument.

The doses used were prepared by using Milli-Q water, which was used as negative control. Ethyl methanesulphonate (EMS) and hydrogen peroxide (H_2O_2) were used as a positive control in genotoxicity and ROS studies, respectively. Silver nitrate was used to compare the effects of the ionic versus nanoparticulated Ag form.

Drosophila Strain and Toxicity Studies

The *wild-type* Canton-S strain was used for all the experiments. This strain has been maintained in the lab for many years. It was raised on standard Drosophila food media and maintained at 25°C, 60% humidity, and light/dark daily cycle of 12/12 hr.

The potential toxicity of AgNPs and silver nitrate was determined by measuring viability (e.g., g-to-adult). Fly adults were placed in vials with black medium (normal medium with carbon powder) in order to collect eggs for 6 hr periods. After that, samples of 50 eggs were picked-up and placed in plastic vials containing 4 g of Drosophila instant medium (Carolina Biological Supply, Burlington, NC). This medium was previously wetted with 10 mL of different doses of AgNPs or silver nitrate (0.016, 0.08, 0.4, 1, and 2 mM). Five replicates of each concentration were established. Milli-Q water was used as control solution. After 10 days, emerged adults were collected and counted to calculate the percentage of viability with regard to the control. To facilitate the comparisons, equal concentrations were used of both Ag compounds.

Uptake of AgNPs Through the Intestinal Barrier

To detect the presence of AgNPs in the intestine of the larvae, both in lumen and enterocytes, 4-day-old larvae (exposed, as indicated in the above section) were dissected and midguts extracted. The followed procedure was previously described by our group (Alaraby et al. 2015). Briefly, larvae were cleaned of food particles, dissected in phosphate buffer (PB; 0.1 M, pH 7.4), and fixed for 2 hr in a solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.15 M PB, pH 7.4. Midgut tissues were postfixed for 2 hr with 1% (w/v) osmium tetroxide containing 0.8% (w/v) potassium hexacyanoferrate (prepared in PB), followed by four washes with deionized water and sequential dehydration in acetone. Finally, samples were embedded in Epotane 12™ resin (Ted Pella, Redding, CA) and polymerized at 60°C for 48 hr. Semi-thin sections (1 μm thick) where obtained with a Leica ultracut UCT microtome (Leica Microsystems GmbH, Wetzlar, Germany) and stained with 1% (w/v) aqueous toluidine blue. Ultrathin sections (70 nm in thickness) were cut with a diamond knife (45°, Diatome, Biel, Switzerland), placed on noncoated 200 mesh copper grids and contrasted with conventional uranyl acetate (30 min) and Reynolds lead citrate (5 min) solutions. Sections were observed with a Jeol1400 (100 kV) TEM equipped with CCD Gatan ES1000w Erlangshen Camera (Gatan, Pleasanton, CA).

ROS Induction

The presence of intracellular ROS was measured in hemocytes using the 6-carboxy-2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA) assay, following the procedure previously described (Alaraby et al. 2015).

Newly hatched larvae were treated with two different doses of AgNPs and silver nitrate (0.5 and 1 mM) for 3 days, besides negative control (water). Hemocytes were exposed to 5 μM DCFH-DA for 30 min at 24°C. The fluorescence in cells was investigated using a fluorescent microscope with an excitation of 485 nm and an emission of 530 nm (green filter). Hydrogen peroxide (H_2O_2, 0.5 mM) was used as a positive control. The ImageJ program was used for the quantitative evaluation of fluorescent images from both control and treated larvae.

Genotoxicity: The Comet Assay

To determine the induction of DNA damage, third-instar larvae were placed in plastic vials containing 4 g of Drosophila instant medium wetted with different doses of AgNPs and silver nitrate (0.5, 1, and 2 mM) for 24 hr. Purified water and 4 mM EMS were used as negative and positive controls, respectively. All experiments were performed at 25 ± 1°C and at ~60% relative humidity. *D. melanogaster* hemocytes were collected according to Carmona et al. (2011). Ten microliters of cell samples (≈10,000 cells) were carefully resuspended in 90 μL of 0.75% low melting agarose at 37°C, mixed and dropped in triplicate on the hydrophilic surface of Gelbond films (GBFs). The GBFs were left for 2–3 min at 4°C and immersed in cold, freshly made lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100 and 1% N-lauroylsarcosinate, pH 10) for 1 hr at 4°C in a dark chamber. GBFs were washed for 5 min with cold electrophoresis buffer (0.001 M EDTA, 0.3 M NaOH, pH 13.2) and directly placed for 25 min in a horizontal gel-electrophoresis tank filled with cold electrophoresis buffer to allow DNA unwinding. Electrophoresis was carried out in the same buffer for 20 min at 20 V and 300 mA. The unwinding and electrophoresis were done at 4°C. After electrophoresis, the GBFs were neutralized with two washes for 5 min with phosphate-buffered saline (PBS) followed by 1 min in distilled water. The GBFs were rinsed in 100% ethanol for 5–10 min, then they were left to dry all night before being stained with 25 mL of TE-buffer pH 7.4 containing 10,000× diluted SYBERGold fluorochrome for 20 min. Finally, the GBFs were washed with water to remove excess staining and allowed to dry. The images were examined at 400× magnification with a Komet 5.5 Image-Analysis System (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide-band filter and a 590-nm barrier filter. Triplicates of 100 randomly selected cells were analyzed per treatment. The percentage of DNA in the tail (% DNA tail) was used to measure DNA damage.

Gene Expression by Real Time Reverse Transcription (RT) PCR

Changes in the expression of different genes were determined. The selection included genes related to general stress: *Heat-shock-protein-70* (*Hsp70*, NM_169441.2), *Catalase* (*Cat*, NM_080483.3), *Superoxide dismutase 2* (*Sod2*, NM_057577.3), and *p53* (NM_206544.2). In addition, a gene related to the response of the intestinal barrier to physical stress, *Dual oxidase* (*Duox*, NM_001273039.1) was included to detect the effect of AgNPs. The expression of the selected genes in treated (0.1, 0.5, and 1 mM) and untreated larvae were detected through homogenized groups of 30 third-instar larvae (~50 mg) in TRIzol® Reagent (Invitrogen, Carlsbad, CA). The mRNA was extracted according to the manufacturer. RNase-free DNase I (DNA-freeTM kit; Ambion, Paisley, UK) was used to remove DNA contamination. The quantity of mRNA in each sample was determined using a Nanodrop device. The cDNA was synthesized using 2 μg of total RNA and the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer instructions and stored at −20°C until further use. The resulting cDNA was subjected to real-time RT-PCR analysis on a Light Cycler 480 (Roche, Basel, Switzerland) to determine the relative expression of the selected genes, using *β-actin* as the housekeeping control. For each one of the selected genes, 20 μL of reaction volume were used containing 1 μL of cDNA (400 ng/μL for gene stress group and 800 ng/μL for the *Duox* gene) mixed with 10 μL of 2×SYBER Green mix, 2 μL of 10 μM gene specific primers mix and 7 μL of water. Reaction conditions for all genes were: preincubation for 5 min at 95°C, 1 cycle, and the amplification was repeated 45 times (10 sec at 95°C, 15 sec at 61°C, 72°C for 25 sec). Three independent experiments were carried out to get the mean level of expression.

Quantification of the Ions Releases Using ICP-MS

Dissolution to the ionic form of AgNPs was evaluated at different time intervals using inductively coupled plasma mass spectrometry (ICP-MS). Initial concentration of 10 μg/mL AgNPs was dispersed in Milli-Q water for 24, 48, 72, and 96 hr. Subsequently, the supernatant from the respective samples was collected and ultracentrifuged for 10 min at 10,000*g* to precipitate undissolved NPs. After centrifugation, 0.25 mL of the resultant supernatant containing Ag ions was carefully transferred to a new vial and digested with concentrated nitric acid (HNO_3; Merck) at 150°C for 30 min on a heating block. The quantity of released ions was determined by averaging three independent experiments.

Statistical Analysis

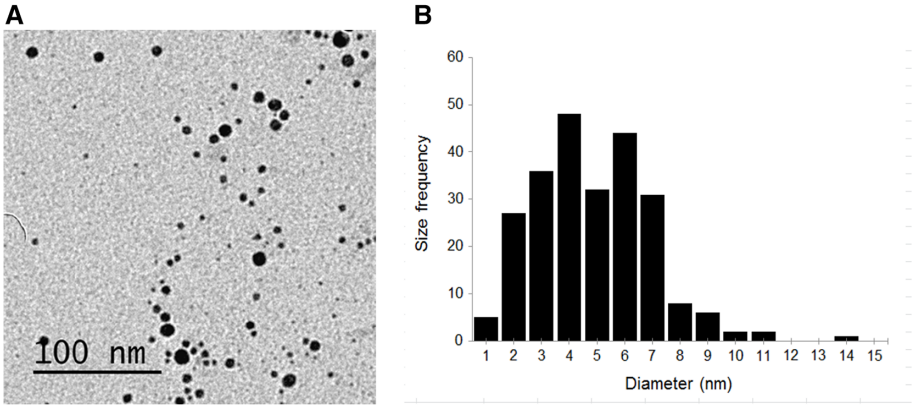
Previous to the statistical analysis, we checked the normality (Kolmogorov–Smirnov and Shapiro–Wilk test), and the homogeneity of variance (Levene's test) of the data. Data presenting normal distribution and equal variance were analyzed with one-way Analysis of Variance (ANOVA) followed by *post hoc* multiple comparisons. Data following unequal variance or skewed distribution were analyzed with nonparametric analysis (Mann–Whitney *U*-test). Data were calculated as mean ± standard error and significant differences were considered at $P \leq 0.05$.

RESULTS

TEM was used to characterize both the morphology and agglomeration status of AgNPs. The obtained images showed that AgNPs had a diameter of 3.44 ± 2.1 nm, as assessed by observing over 200 particles measured in random fields of view (Fig. 1A). The average of hydrodynamic radius and the zeta potential of 50 μg/mL AgNPs dispersion in water were 9.720 ± 1.571 nm and −20.14 ± 0.611 mV, respectively (Fig. 1C). The low-hydrodynamic radius and the high-zeta potential value confirm that these AgNPs were well dispersed in solution and they do not be likely to being agglomerated. However, to avoid potential further aggregations, fresh dispersions were used immediately after preparation in all the experiments.

Viability experiments were carried out to determine the range of doses to be used in the different studies. Viability was determined as the ability to reach the adult stage. Results indicate that silver nitrate was more toxic that AgNPs, mainly at the dose of 2 mM (Fig. 2). The concentration of 10 mM was completely toxic for both compound forms (data not showed). It must be indicated that in both cases, depigmentation was observed in many adults, including those exposed to concentrations where no toxic effects were observed.

The potential internalization of AgNPs in Drosophila larvae was evaluated by using TEM. The uptake throughout the intestinal barrier supposes its translocation into the



C

DLS		LDV	
Polydispersity Index (PDI)	Average hydrodynamic size (nm)	Z-potential ζ (mV)	Electrophoretic mobility (μm·cm/V·s)
0.495 ± 0.014	9.720 ± 1.571	-20.14 ± 0.611	-1.602 ± 0.048

Fig. 1. Characterization of AgNPs. (**A**) Typical TEM image and (**B**) size distribution histogram using such images. (**C**) Different parameters on AgNPs in dispersion.

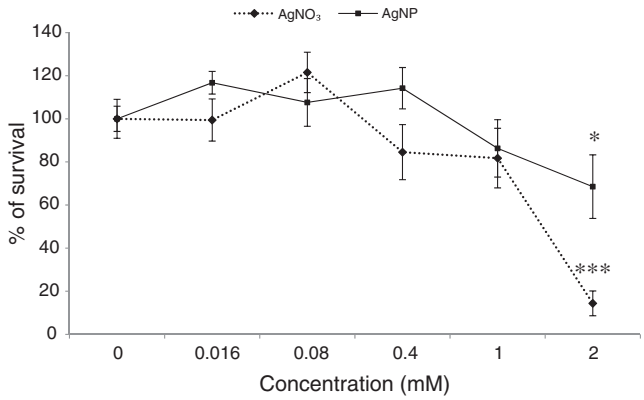


Fig. 2. Toxic effects of silver nitrate and AgNPs. Two hundred fifty eggs per concentration were seeded and the emerged adults were counted. Statistical significances were established according to the control data. $*P < 0.05$, $***P < 0.001$ (t-test).

internal compartment (fluid hemolymph) interacting with hemocytes. TEM images showed that in AgNPs exposed larvae vacuoles were observed in both the microvilli area and inside the cytoplasm of midgut cells (Fig. 3). Nevertheless, the use of Energy Dispersive X-rays microanalysis methodology did not permit to identify silver inside these structures. We assume that this was due to the small size of AgNPs.

Since ROS induction is usually an associated effect related to NPs exposure, the intracellular levels of ROS were measured in hemocytes of third instar larvae exposed to AgNPs and silver nitrate. ROS detection was based on the conversion of unfluorescent 6-carboxy-2,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA) to the fluorescent oxidized DCF inside the cells. Using fluorescent microscopy, and the ImageJ software for the quantitative evaluation of fluorescent images, we found that both AgNPs and silver nitrate exposures (at 0.5 and 1 mM) induced significant levels of intracellular ROS (Fig. 4). As observed, the obtained levels of intracellular ROS are even greater than the observed in the positive control, but with greater variability as indicated by the size of the significance bars. Interestingly, the effects of silver nitrate were more marked than those induced by AgNPs.

The comet assay was used to address the potential genotoxic effect of both AgNPs and silver nitrate. This assay detects primary DNA damage, mainly single and double DNA strand breaks. The results obtained in the comet assay clearly indicate that both compounds act as genotoxic, increasing the levels of DNA in the tail of the observed comets significantly (Fig. 5). As occurs with the ROS induction, silver nitrate exposures induced slightly higher levels of DNA damage than AgNPs.

Exposure to NPs can activate different metabolic or physiological pathways according to their ways of action. As indicators of a general stress response, we have used *Hsp70; Cat*, and *Sod2* as indicators of oxidative stress, and *p53* as indicator of genotoxicity. In addition, the *Duox* gene was used to detect physical effects on the intestinal barrier, but only in the larvae exposed to AgNPs. Changes in the expression of defined genes have been observed after the exposure to AgNPs (Fig. 6). Interestingly, an upregulation of *Sod2* and *p53* was observed after the exposure of Drosophila larvae to both silver nitrate and AgNPs, being higher in larvae

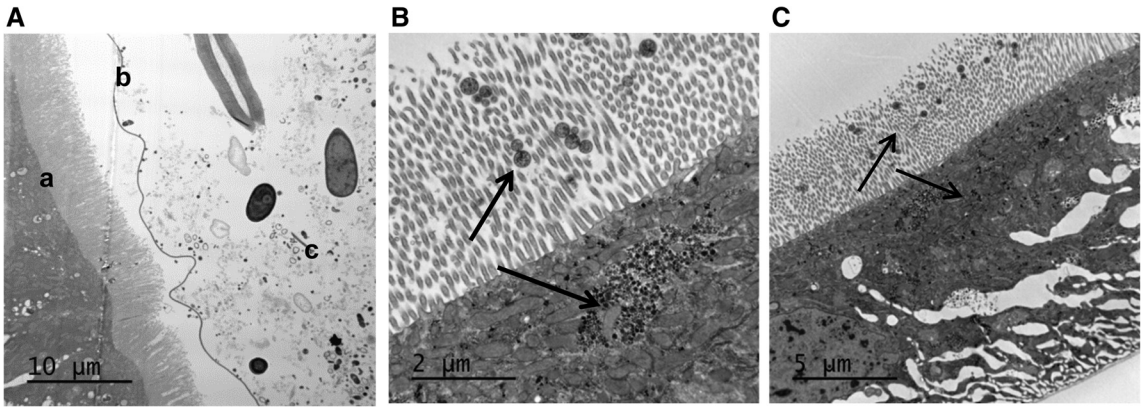


Fig. 3. Transmission electron microscopy images of larvae midguts. (**A**) General observation with the midgut tissue cells (a), the peritrophic membrane (b), and the lumen, with intestinal microbiota (c). Images from AgNPs treated larvae (**B**) and (**C**); arrows indicate vesicles in both microvilli and cytoplasm associated with the exposure to AgNPs.

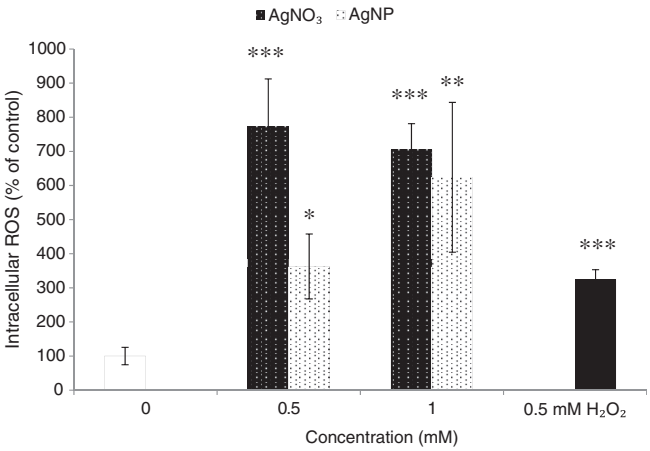


Fig. 4. Reactive oxygen species production in the hemocytes of control and 96 hr larvae, treated with different doses of AgNPs and silver nitrate. Hemocytes were incubated with 5 µM DCFH-DA at 24°C for 30 min and observed using fluorescent microscopy. The fluorescence intensity of hemocytes was quantified by ImageJ analysis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ as compared with the control.

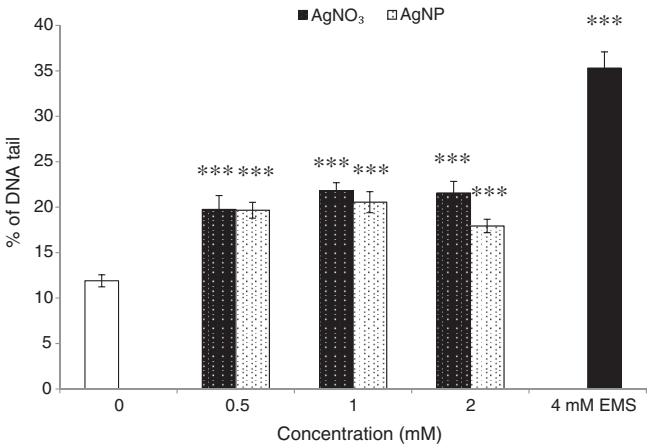


Fig. 5. Percentage of DNA in the comet tail, induced after larvae exposure to AgNPs and silver nitrate. Three replicates were carried out and 100 randomly selected cells per treatment were scored per each treatment. Ethyl methane sulfonate (EMS) (4 mM) was used as positive control. $***P < 0.001$.

exposed to silver nitrate what would agree with the oxidative stress and genotoxic effects detected. The lack of effects of *Hsp70* and *Duox* after AgNPs exposure would agree with the low toxicity shown by this compound; nevertheless, changes in the levels of expression of *Hsp70* were observed after exposure to silver nitrate as indication of a higher toxic action. In summary, although a general pattern is observed for both compounds, the effects observed after silver nitrate exposure tend to be higher.

To explain how AgNPs exert their effects, we determined the release of ions in a due time experiment lasting for 96 hr and the obtained results are indicated in Figure 7. As observed, AgNPs show some instability in dispersion and around 12% was dissolved into its ionic form after 24 hr. No relevant changes were detected from that point on. Accordingly, some of the reported effects would be due to the Ag ions released.

DISCUSSION

Our studies in Drosophila confirm that AgNPs are able to cross the intestinal barrier of the larvae inducing oxidative stress and genotoxicity in hemocytes present in hemolymph. In addition, the observed changes in the expression of *Sod2* and *p53* genes give support to the results obtained in this *in vivo* model.

It should be emphasized that in spite of the wide spread use of AgNPs, few *in vivo* studies have evaluated its harmful effects following ingestion. Oral consumption of water

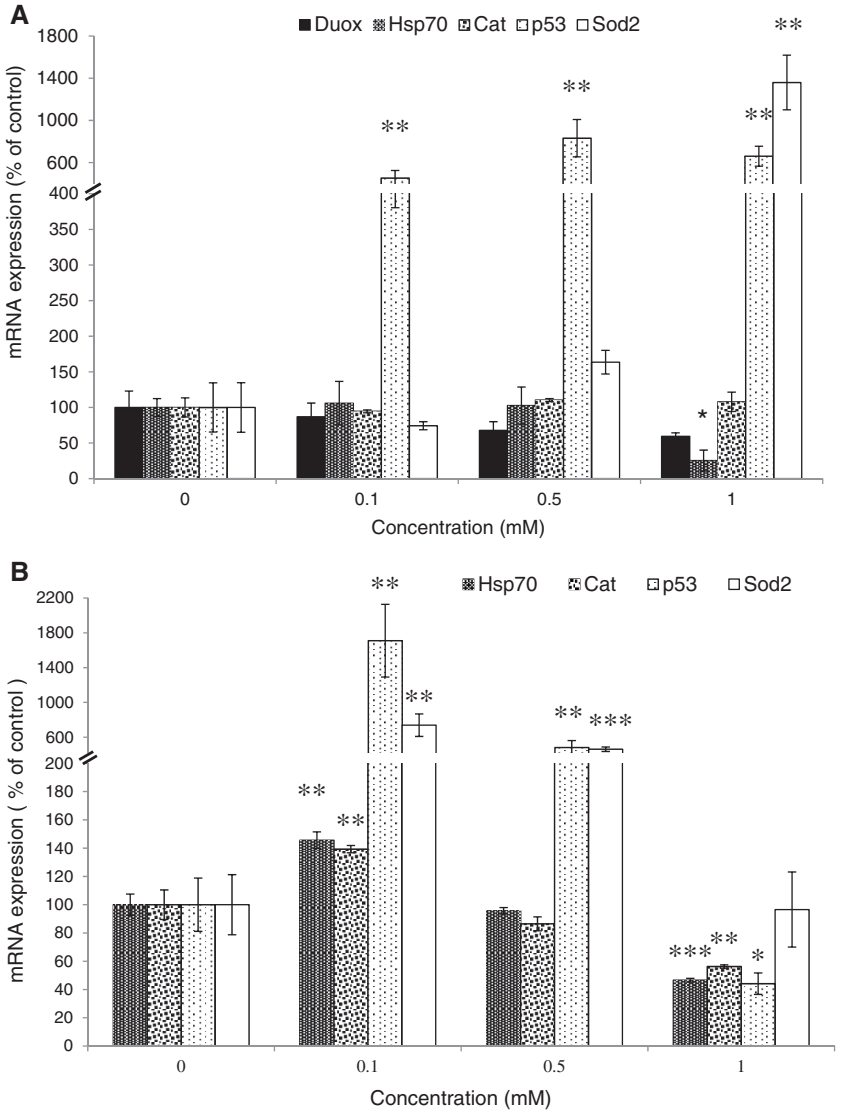


Fig. 6. Expression of *p53, Cat, Sod2, Hsp70*, and *Duox* genes in 96 hr larvae exposed to different doses of AgNPs (**A**) and silver nitrate (**B**). The expressions were normalized using *β-actin* and are presented compared to control values. Data represent the mean ± standard error of the mean of three independent experiments. $*P < 0.05$, $**P < 0.01$.

soluble dispersions of AgNPs can occur from numerous sources (Vance et al. 2015). Therefore, consumers are very likely exposed orally to AgNPs, either intentionally or accidentally (Gaillet and Rouanet 2015). In this context, our results in an *in vivo* model like Drosophila are useful for a general comprehension of the risk associated with the oral ingestion of AgNPs.

Interestingly, the observed effects were not directly related with the toxicity associated to AgNPs exposure. As observed, doses up to 1 mM were not able to induce changes in the egg-to-adult survival but the dose of 0.5 mM was able to induce significant increases in both the intracellular levels of ROS and the levels of DNA breaks. In spite of this lack of toxicity, phenotypical changes in the offspring were observed, as the appearance of a yellow-like phenotype. This cuticle depigmentation has already been reported in previous studies (Panacek et al. 2011; Avalos et al. 2015; Phatak et al. 2016) and it is associated with the internal presence of silver, irrespective of its form. It has been proposed that this effect is due to the interaction of silver with precursor metabolites of the melanization pathway. Nevertheless, it seems that this is an indirect effect due to decreased activity of copper (Cu)-dependent enzymes, such as tyrosinase and Cu-Zn Sod. Despite the constant level of Cu present in tissues, consumption of AgNPs might produce sequestration of Cu ions creating a condition that resembles Cu starvation. This hypothesis was reaffirmed by observing that extra addition of Cu in

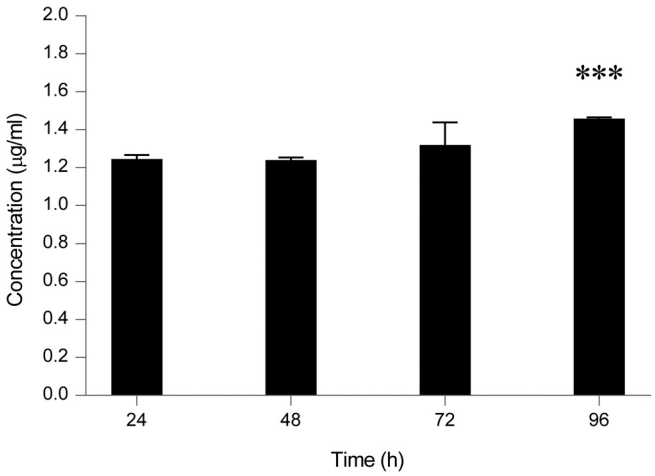


Fig. 7. Dissolution of AgNPs to their ionic forms in Milli-Q water, evaluated at different time intervals (24, 48, 72, and 96 hr) using ICP-MS. The initial concentration was 10 µg/mL. The reported data were determined by averaging three independent experimental values. ***$P < 0.001$.

the diet restores normal pigmentation (Armstrong et al. 2013).

Different NPs are characterized by its ability to produce increased ROS levels (Fu et al. 2014), including AgNPs (Flores-López et al. 2018). Overproduction of ROS can induce oxidative stress, resulting in cells failing to maintain normal physiological redox-regulated functions (Meng et al. 2009). This oxidative stress stage can suppose the induction of DNA-strand breaks leading to genotoxic effects (Kermanizadeh et al. 2015). In our case, AgNPs were able to increase the levels of intracellular ROS in Drosophila. This mechanism has also been observed in mice (Shrivastava et al. 2016), as well as in other *in vivo* models like zebrafish (Krishnaraj et al. 2016), and the nematode *Caenorhabditis elegans* (Chatterjee et al. 2014). Although no previous studies have directly measured the induction of oxidative stress in Drosophila, Posgai et al. (2011) were able to determine indirectly its role in parameters such development, mating success, and survivorship. In their study, these effects were partially or fully reversible by vitamin C. Vitamin C also rescued cuticle and pigmentation defects in AgNPs fed flies. As a whole these results indicate that AgNPs ingestion produce oxidative stress, and suggest that antioxidants can act as a potential remediation for AgNPs toxicity. The role of AgNPs on oxidative stress was confirmed by the significant overexpression of *Sod2* in treated larvae of Drosophila. *Sod2* overexpression following AgNPs exposure was reported in different *in vivo* studies like in *C. elegans* (Roh et al. 2012) and in the Cape River crab *Potamonautes perlatus* (Walters et al. 2016). In addition, different *in vitro* studies like in human immortalized keratinocytes (HaCaT) cells have shown the ability of AgNPs exposure to enhancing *Sod2/Cat/Gpx* activity (Tyagi et al. 2016). *Cat* expression showed relevant changes only after silver nitrate exposure, with different patterns according exposure: over-expression at low dose and downregulation at the highest tested dose. This response is quite similar to the response observed in Drosophila after CuONPs exposure (Alaraby et al. 2016b). A similar pattern was observed for the *Hsp70* gene, measuring general stress response, pointing out the higher toxic effects of silver nitrate.

As previously indicated, oxidative stress is associated with DNA damage induction. Among the different ways used to measure DNA damage, the comet assay offers many advantages. This assay measures primary DNA damage at the level of single cells. Although the standard assay detects mainly DNA strand breaks, slight modifications allow the detection of more specific types of DNA damage, as oxidative damage or DNA repair (Karlsson et al. 2015).

Only four *in vivo* studies have been reported until now using the comet assay to determine the genotoxicity of AgNPs. Although in rats no genotoxicity was observed when using bone marrow cells (Dobrzyńska et al. 2014), positive findings were reported in mice in liver (Li et al. 2014), and in testis and lung (Asare et al. 2016). In addition, increases in DNA damage were also observed in plants like *Allium cepa* and *Nicotiana tabacum* (Ghosh et al. 2012). Although no previous data were reported in Drosophila using the comet assay, two studies used the wing-spot assay to detect genotoxicity. This assay detects both somatic mutation and/or somatic recombination in the wing blade cells. Although small but significant increases in the frequency of total spots were observed in one of the studies (Demir et al. 2011), no variation was observed in the other one (Avalos et al. 2015). This would point out the high sensitivity of the comet assay with regard to other assays like the wing-spot test. It should be remembered that while the comet assay measure primary DNA damage, the wing-spot assay measures fixed DNA damage. In addition, while the comet assay was applied to hemocytes, the wing-spot assay was applied to the cells of the wings imaginal disks.

Genotoxic effects were also indicated by the overexpression of the *p53* gene. These results agree with those previously reported in Drosophila by Ahamed et al. (2010). Authors found overexpression of both the cell cycle checkpoint p53 and in the cell signaling protein p38, which are involved in the DNA damage repair pathway. These results confirm those previously reported by the same authors in two types of mammalian cells, like mouse embryonic stem cells and mouse embryonic fibroblasts (Ahamed et al. 2008). Interestingly, these effects were even more severe in coated than in uncoated AgNPs.

In our study, we have simultaneously used AgNPs and silver nitrate. This permits to identify whether the associated effects are due to the NPs themselves or to the Ag ions released. As an overall our results show that the effects of silver nitrate are always more marked than those produced by AgNPs. This would mean that the detrimental observed effects are mainly due to the intracellular presence of Ag ions. In fact, we have observed a significant release of Ag

ions by AgNPs, reinforcing the proposal of Stensberg et al. (2011). Interestingly, a recent study using 5 and 50 nm citrate coated AgNPs reported a noninduction of genotoxicity (Lebedová et al. 2018), which emphasizes silver ions release as the underlying mechanism of AgNPs genotoxicity. According to that, a Trojan-horse-effect is accepted to explain the genotoxic effects of AgNPs, where their uptake would be followed by a release of silver ions.

Our conclusion is that AgNPs exposure is able to produce genotoxic effects in an *in vivo* model like Drosophila, *via* the induction of oxidative stress. In parallel, changes in the expression of genes involved in oxidative stress and genotoxicity pathways were also overexpressed. Interestingly, these genotoxic effects were reported at lower doses than those necessaries to induce toxic effects, like those affecting egg-to-adult survival. In addition, the intercomparison between AgNPs and silver nitrate would reinforce the potential role of released ions as source of the observed detrimental effects. Finally, our results confirm the useful role of Drosophila when the potential health effects of nanoparticles are evaluated.

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AUTHOR CONTRIBUTION

RM and AH planned the experiments. MA and SR carried out the experimental part of the work as well as the carried out the statistical analysis and prepared figures and tables. RM and AH wrote the final manuscript.

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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