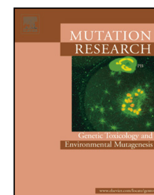
Mutation Research 791 (2015) 1–11

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres



Genotoxicity of copper oxide nanoparticles in *Drosophila melanogaster*

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A R T I C L E I N F O

Article history:
Received 9 May 2015
Received in revised form 19 June 2015
Accepted 21 July 2015
Available online 23 July 2015

Keywords:
Comet assay
DNA damage
Hemocytes
Malondialdehyde
Mitotic recombination
Wing-spot test

A B S T R A C T

Copper oxide nanoparticles (CuONPs) are used as semiconductors, catalysts, gas sensors, and antimicrobial agents. We have used the comet and wing-spot assays in *Drosophila melanogaster* to assess the genotoxicity of CuONPs and ionic copper ($CuSO_4$). Lipid peroxidation analysis was also performed (Thiobarbituric Acid Assay, TBARS). In larval hemocytes, both CuONPs and $CuSO_4$ caused significant dose-dependent increases in DNA damage (comet assay). In the wing-spot assay, an increase in the frequency of mutant spots was observed in the wings of the adults; CuONPs were more effective than was $CuSO_4$. Both agents induced TBARS; again, CuONPs were more active than was $CuSO_4$. The results indicate that CuONPs are genotoxic in *Drosophila*, and these effects may be mediated by oxidative stress. Most of the effects appear to be related to the presence of copper ions.

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1. Introduction

The physico-chemical properties of nanoparticles (NPs), such as nanoscale size and large surface area, are attractive for technological and industrial applications. However, the same properties may contribute to health risks; the toxicological properties of NPs may differ from those of soluble forms of the same elements. Extremely small NPs may reach the nucleus by penetration via nuclear pores or during mitosis and may interact directly with DNA organized in chromatin or chromosomes, causing genetic damage [1–3]. However, to induce genotoxicity, NPs do not necessarily need to be in contact with DNA. For example, NPs may perturb proteins involved in DNA replication or mitotic division, and may also generate ROS that can damage DNA [3].

Copper oxide nanoparticles (CuONPs) are used in applications such as electric conductors, catalysts, gas sensors, and antimicrobial agents [4,5]. Environmental and human exposures are to be expected. The potential genotoxic risk of CuONPs has been studied, especially *in vivo* [6–8]. This nanomaterial may induce genotoxic effects in different cells and organism models, both *in vitro* and *in vivo* [9,10].

The available data indicate that CuONPs are mutagenic and cause DNA strand breaks in bacteria such as *Escherichia coli* and *Salmonella typhimurium* [5,11]. *In vitro* studies indicate that CuONPs can induce DNA damage, DNA strand breaks and chromosome alterations in human lung epithelial, skin, peripheral blood and cancer cell lines [12–17]. In addition, DNA fragmentation, DNA methylation, and chromosome damage have also been reported in murine cell lines exposed to CuONPs [17,18].

Some *in vivo* studies reported an increase in the frequency of micronucleus formation in peripheral blood cells of mice treated acutely with CuONPs [19]. Increased neoplastic lesions in F334 male rats exposed to CuONPs by intratracheal instillation have also been reported [20]. Other *in vivo* genotoxic effects, such as point mutations, DNA alterations, and DNA strand breaks have been reported in terrestrial plants [21,22] and marine molluscs [23].

Recent studies have shown that CuONPs genotoxicity may be mediated by oxidative stress, as evidenced by an increase of reactive oxygen species (ROS) production, oxidative DNA damage, and depletion of antioxidant defence in bacteria [24] and cultured human cells [13]. However, few data on oxidative stress-

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http://dx.doi.org/10.1016/j.mrgentox.2015.07.006
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related DNA damage of CuONPs in experimental animals have been reported [19].

The fruit fly *Drosophila melanogaster* has been used to detect genotoxicity and mutagenicity of several metal and metal-oxide NPs [25–31]. This system offers physiological and genetic advantages for the study of acute and chronic effects and underlying mechanisms of action [28,32]. We have assessed DNA damage (comet assay), mutagenic and recombinogenic activities (wing-spot assay), and levels of oxidative stress (TBARS assay) associated with exposure of *D. melanogaster* to CuONPs.

2. Materials and methods

2.1. Strains

The following mutant *Drosophila* strains were used for the wing-spot test: the *multiple wing hairs* strain with genetic constitution *y; mwh j*; and the *flare-3* strain with constitution $flr^3/ln(3LR)\,TM3, Bd^s$. The *multiple wing hairs marker* (*mwh*, 3–0.3) is a completely recessive homozygous viable mutation, which is kept in homozygous condition. It produces multiple trichomes per cell instead of the normally unique trichome in the wing cells. The *flare-3* marker (flr^3, 3–38.8) is a recessive mutation that affects the shape of wing hairs, producing malformed wing hairs that have a flare shape. Given their zygotic lethality, *flare* alleles have to be kept in stocks over balancer chromosomes carrying multiple inversions and a dominant marker that is a lethal homozygous ($TM3, Bd^s$). More detailed information on genetic markers and descriptions of the phenotypes is given by Lindsley and Zimm [33]. In this study, the flr^3 strain was also used for comet assay experiments with larval hemocytes. This strain was chosen based on the low background level of DNA damage in blood cells from untreated third instar larvae. Both strains were cultured in glass bottles with standard medium for *Drosophila* (*i.e.*, agar, corn flour, and yeast) at $25 \pm 1\,^{\circ}$C and relative humidity ~60%.

2.2. Chemicals

Copper oxide NPs (CuONPs, <50 nm average particle size, surface area 29 m^2/g, 100% purity, CAS 1317-38-0, Ref. No. 544869), ethyl methane sulphonate (EMS, 100% purity, CAS 62-50-0), phenylthiourea (PTU), N-lauroylsarcosine sodium salt, Triton X-100, Trizma base, sodium hydroxide, sodium chloride, and ethylene diamine tetra acetic acid (EDTA) disodium salt were obtained from Sigma-Aldrich (St Louis, MO, USA). Low-melting-point agarose (LMA), and normal-melting-point agarose (NMA), phosphate-buffered saline (PBS), and 4,6-diamidine-2-phenylindole (DAPI) were from Life Technologies Corporation (Carlsbad, CA, USA); copper sulphate ($CuSO_4 \times 5H_2O$, 100% purity, CAS 7758-99-8, Ref. No. 102790), potassium chloride (KCl), trichloacetic acid (TCA) and 2-thiobarbituric acid (TBA) were from Merck Company (Darmstadt, Germany).

2.3. CuONPs preparation

Various concentrations of CuONPs were prepared with distilled water. Dispersion was carried out by sonication in an ultrasonic bath (Elmasonic S, 37 kHz) for 30 min at room temperature. $CuSO_4$ was used to compare the genotoxicity of ionic and nanoparticulated forms. This compound was prepared with distilled water and diluted by magnetic stirring for 10 min at room temperature. Distilled water was used as negative control; the mutagenic agent EMS was used as positive control in each experiment carried out with both the wing-spot and comet assays.

2.4. NPs characterisation

To confirm the physical characteristics of CuONPs, the following techniques were performed: Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV). TEM was carried out with a JEOLJEM- 2011 instrument to determine the size of CuONPs in dry form. DLS and LDV were performed with a Malvern Zetasizer Nano-ZS zen3600 instrument to measure the hydrodynamic diameter and zeta potential in aqueous suspension, respectively. For TEM analyses CuONPs were measured at 2.56 mg/mL. For DLS and LDV techniques, CuONPs samples were measured at 10 μg/mL.

2.5. Lipid peroxidation assay

Third instar larvae of *Drosophila* (72 ± 4 h old) were treated with three different concentrations of CuONPs or $CuSO_4$. Control larvae received untreated instant medium for *Drosophila* rehydrated with distilled water. Larvae were exposed to the compounds for approximately 24 h. The lipid peroxidation (Thiobarbituric Acid Reactive Substances, TBARS) assay was performed according to the modified method of Tironi et al. [35]. A sample of larvae (approximately 0.5 g) was used in each exposure and control group. Three replications and one independent experiment were assessed. Control or CuONPs-treated larvae were homogenised with 0.5% w/v TCA. The homogenates were maintained for 30 min on ice and then were filtered. A mixture (0.5 mL filtered tissue homogenate + 0.5 mL 0.5% w/v TBA solution) was incubated for 30 min at 70 °C. The absorbance was measured at 532 nm using a Thermo Scientific SpectronicGenesys 10 UV–vis Scanning spectrophotometer (WI, USA). TBARS levels were converted to malondialdehyde (MDA) and expressed as mg MDA/kg sample, using molar extinction coefficient = $1.56 \times 10^5\,M^{-1}$.

2.6. Comet assay

An *in vivo* comet assay with hemocytes (blood cells) of *D. melanogaster* larvae was performed to detect DNA damage [36,37]. This assay has been shown to be highly sensitive, allowing for the detection of low levels of several kinds of DNA damage, such as double- and single-strand DNA breaks, alkali-labile sites, and incomplete repair sites [38].

Third instar (72 ± 4 h old) larvae were placed in plastic vials containing *Drosophila* instant medium (4.5 g) prepared with solutions (10 mL) of various non-toxic concentrations of CuONPs (0.24, 0.48, and 0.95 mg/mL) or $CuSO_4$ (0.25, 0.50, and 0.75 mg/mL).

Hemocytes from *Drosophila* hemolymph were collected according to Marcos and Carmona [37]. Briefly, the hemolymph and circulating hemocytes were collected directly into cold PBS solution (one drop) containing 0.07% PTU and separated in a 1.5 mL microcentrifuge tube. Pooled hemolymph was centrifuged (300 g, 10 min), the supernatant was discarded, and the pellet was resuspended in cold PBS (20 μL).

The comet assay was performed as described by Singh et al. [39] with minor modifications. Cell samples (~40,000 cells, 20 μL) were carefully resuspended in 75 μL 0.75% LMA and layered onto microscope slides pre-coated with 1% NMA (dried at room temperature). Two gels were mounted in each slide and covered with a coverslip. Immediately after agarose solidification (for 10 min at 4 °C), the coverslips were removed and the slides were immersed in cold fresh lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100 and 1% *N*-lauroylsarcosine, pH 10) for 2 h at 4 °C in a dark chamber. To prevent additional DNA damage, the following steps were performed under dim light: the slides were placed for 25 min in a horizontal gel electrophoresis tank filled with cold electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13) to

allow DNA unwinding. Electrophoresis was carried out in the same buffer for 20 min at 25 V and 300 mA (0.96 V/cm). Unwinding and electrophoresis processes were done at 4 °C. After electrophoresis, slides were neutralised with two washes of 0.4 mM Tris (pH 7.5) for 5 min each. The slides were stained with 20 μL DAPI (1 μg/mL) per gel. The images were examined at 400× magnification with a Nikon Eclipse E200 fluorescence microscope coupled with a CMOS digital camera. One hundred randomly selected cells (50 cells on each one of the two replicate slides) were analysed per treatment. The percentage of DNA in the tail (% DNA tail) was used to measure DNA damage, since this is the most widely used and recommended parameter for comet data analysis [40]. The % DNA tail was computed using Comet Score 1.5 Image Analysis System (TriTek Corp., Virginia, USA).

2.7. Wing-spot test

The wing-spot test was used as a short test system based on the loss of heterozygosity (LOH) in normal genes, and the corresponding expression of recessive markers, called *multiple wing hairs* (*mwh*) and *flare-3* (*flr*^{3}), in the wing blades of adult flies [42]. Thus, the induced genotoxic effects are microscopically observed as an increase in the frequency of mutant clones cells (*mwh* or *flr*^{3} phenotype) in wing slide preparations. This assay can detect a wide range of mutational events, such as point mutations, deletions, certain types of chromosome aberrations (non-disjunction), and mitotic recombination [42].

Virgin females of the *flr*^{3} strain were mated to *mwh* males as previously described [37]. Eggs from this cross were collected during 8 h periods in culture bottles containing the standard medium. The resulting 3-day-old larvae (third instar larvae) were then placed in plastic vials containing 4.5 g *Drosophila* instant medium (Carolina Biological Supply, Burlington, NC) prepared with 10 mL solutions of various non-toxic concentrations of CuONPs (0.24, 0.48, and 0.95 mg/mL) and $CuSO_4$ (0.25, 0.50, and 0.75 mg/mL). Larvae were fed in this medium until pupation. The surviving adults were collected and stored in 70% ethanol. Afterwards, their wings were removed with fine tweezers and mounted in Faure's solution on microscope slides. The wings were scored at 400 × magnification for the presence of small single spots, large single spots, and twin spots. Single *flr*^{3} spots were also scored, but these were included in the total mutant spots, as has usually been done in previous works [26,31,42]. In each series, 80 wings were scored (from 40 individuals). Scoring of flies and data evaluation were conducted following the standard procedures for the wing-spot test, as used in recent investigations [45].

2.8. Statistical analysis

ANOVA was used to analyse differences in MDA levels with different treatments and compounds. The Tuckey *post-hoc* test was performed to compare negative controls *versus* different treatments of CuONPs and $CuSO_4$. Results were considered statistically significant at $P \leq 0.05$. All data was presented as arithmetic mean ± standard deviation (SD).

For the comet assay, a generalised linear model (GLM) was used to analyse differences in% DNA tail. The conservative Scheffe *post-hoc* test was performed to compare negative controls *versus* different treatments. The GLM is analogous to traditional ANOVA, but it allows the use of nonparametric and heteroscedastic data, which was our case [41]. Before analysis with GLM, the homogeneity of variance and normality assumption of data was tested with the Bartlett and Kolmogorov–Smirnov tests, respectively. Results were considered statistically significant at $P \leq 0.05$. All data was presented as arithmetic mean ± standard error, and 95% confidence intervals were constructed.

For the wing-spot assay, the conditional binomial test was applied to assess differences between the frequencies of each type of spot in treated and concurrent negative control with significant levels $\alpha = \beta = 0.05$ [46]. The multiple-decision procedure was used to judge the overall response of an agent as positive, negative, or inconclusive [47]. The treatment was considered as positive if the frequency of mutant clones in the treated series was at least m (multiplication factor) × greater than in the control series. Since small single spots and total spots have a comparatively high spontaneous frequency, m was fixed at a value of 2 (testing for a doubling of the spontaneous frequency). For large single spots and twin spots, which have a low spontaneous frequency, $m = 5$ was used. The frequency of clone formation was calculated, without size correction, by dividing the number of *mwh* clones per wing by 24,400, which is the approximate number of cells inspected in one wing [48]. For mitotic recombination induction, the *Z*-test with binomial proportions was applied to assess differences between the percentages of recombination events induced by controls and treated series with CuONPs and $CuSO_4$ compounds.

3. Results

3.1. Physical characterisation of CuONPs

TEM was used to characterise aerodynamic size of CuONPs. NPs displayed spherical shapes and showed low levels of agglomeration (Fig. 1A–C). The size of the CuONPs agglomerates ranged from 6.21–81.45 nm diameter, and the average (±SD) diameter was 29.84 ± 15.28 nm (Fig. 1D). TEM images and analyses of representative NPs ($n = 100$) indicated that size was not different from the manufacturer's indications (less than 50 nm).

DLS and LDV techniques were used to measure hydrodynamic diameter and zeta potential of CuONPs, respectively. The diameter average in water suspension was different and higher than TEM analyses, reaching the mean (±SD) value of 263.83 ± 17.92 nm (Fig. 2A). Finally, the average (±SD) of zeta potential was −24.40 ± −1.28 for CuONPs (Fig. 2B), indicating a good stability and dispersion of this nano-compound in aqueous solution for feeding *D. melanogaster* larvae.

3.2. Toxicity of CuONPs and $CuSO_4$ in Drosophila

The concentrations of CuONPs and $CuSO_4$ used in the genotoxicity experiments with *D. melanogaster* strains were selected according to previous toxicity and viability studies carried out in the laboratory. Initially, the doses administered ranged from 0.24–7.5 mg/mL, and within this dose range, elevated toxicity was observed for both compounds, reflected as a reduced percentage of larvae developing into imago and a significant delay in the time required for the larvae to develop into adult stage. In general, in all the preliminary toxicity experiments, $CuSO_4$ was more toxic than CuONPs. Hence, while for CuONPs a suitable larval viability (>80%) was reached at 0.95 mg/mL, $CuSO_4$ could not be evaluated at concentration >0.75 mg/mL. Choice of concentrations was based on reduction in the percentage of developing treated larvae (a clear indication that the compounds affected the larvae) and the number of emerging larvae and adults after treatment (high enough to perform genotoxicity experiments with the wing-spot and comet tests) [37].

3.3. Lipid peroxidation induced by CuONPs

Third instar *Drosophila* larvae were exposed to CuONPs and $CuSO_4$ by feeding for 24 ± 4 h at the same concentrations used for the comet and wing-spot tests. After the treatments, larval tissues of *D. melanogaster* were used to evaluate the MDA marker for

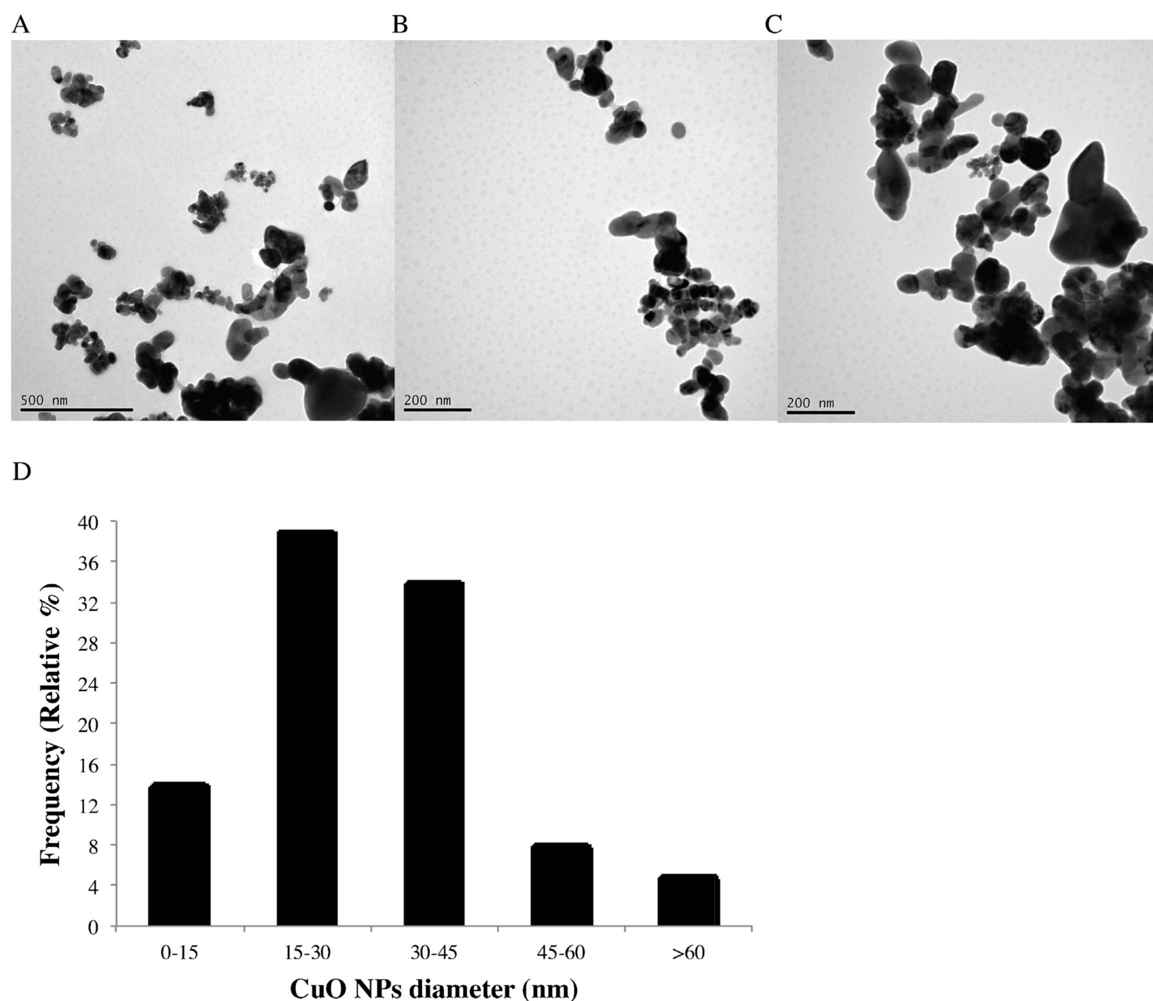

Fig. 1. Physical characterisation of CuONPs with TEM. (A) is a random field view of NPs (scale bar representing 500 nm). (B) and (C) are TEM images with high magnification (bar scales representing 200 nm). (D) is a histogram showing size distribution of CuONPs.

oxidative stress. MDA values after CuONPs treatments increased significantly at all concentrations in comparison with the negative control ($P \leq 0.05$) and this increase in MDA values was dose-dependent (Fig. 3A). Similarly, $CuSO_4$ showed a significant increase in MDA levels ($P \leq 0.05$) in treated larvae, with a linear dose-response relationship (Fig. 3B).

3.4. DNA damage assessed with the comet assay

The comet assay results are summarized in Fig. 4(A and B). Both compounds were administered by feeding to third instar larvae. Afterwards, the hemolymph was extracted from the larvae, and circulating hemocytes were isolated by centrifugation for comet test experiments. Both CuONPs and $CuSO_4$ induced significant DNA damage in larval hemocytes, compared with negative controls. The negative and positive control values for % tail DNA agreed with the background range observed in recent studies [49–51].

3.5. Genotoxicity: wing-spot test

The data from the wing-spot test are summarized in Tables 1 and 2. Table 1 shows the results obtained with the transheterozygous flies (*mwh/flr*^{3}), while Table 2 present the data from the balanced heterozygous flies (*mwh/TM3/Bd*^{s}). The results with *mwh/flr*^{3} flies indicate that CuONPs induce significant increases in the frequency of total mutant spots, mainly inducing small single *mwh* spots, as compared with the negative control. Nevertheless, the data obtained with *mwh/TM3/Bd*^{s} flies indicate inconclusive results at the same concentration of CuONPs. It should be noted that balanced heterozygous flies have abolished somatic recombination; therefore, the recombination events induced by CuONPs could be quantified contrasting mutant spots scored for both transheterozygous and balanced heterozygous flies. Thus, the results obtained from this analysis indicate that CuONPs can induce significant mitotic recombination events (between 8% and 20%), suggesting that the genotoxicity observed for CuONPs was mainly promoted by mutation events (~80%) (Fig. 5A).

Tables 3 and 4 summarize the results obtained with $CuSO_4$ treatments of *mwh/flr*^{3} and *mwh/TM3/Bd*^{s} flies, respectively. The results with *mwh/flr*^{3} flies indicate that $CuSO_4$ can induce significant increases in the frequency of total mutant spots (mainly inducing small single *mwh* spots), while the results from *mwh/TM3/Bd*^{s} flies showed were inconclusive. However, $CuSO_4$ does not induce significant mitotic recombination events (<8%) in comparison with CuONPs (Fig. 5B).

In this study, the negative control frequencies observed (0.39–0.46) were in accordance with the normal background range observed in the laboratory, and are not significantly different from previous results [52,53]. The positive controls carried out with 0.12 mg/mL EMS showed a clear response, and the mutant spot frequencies also agreed with previous and recent studies [26,54,55].

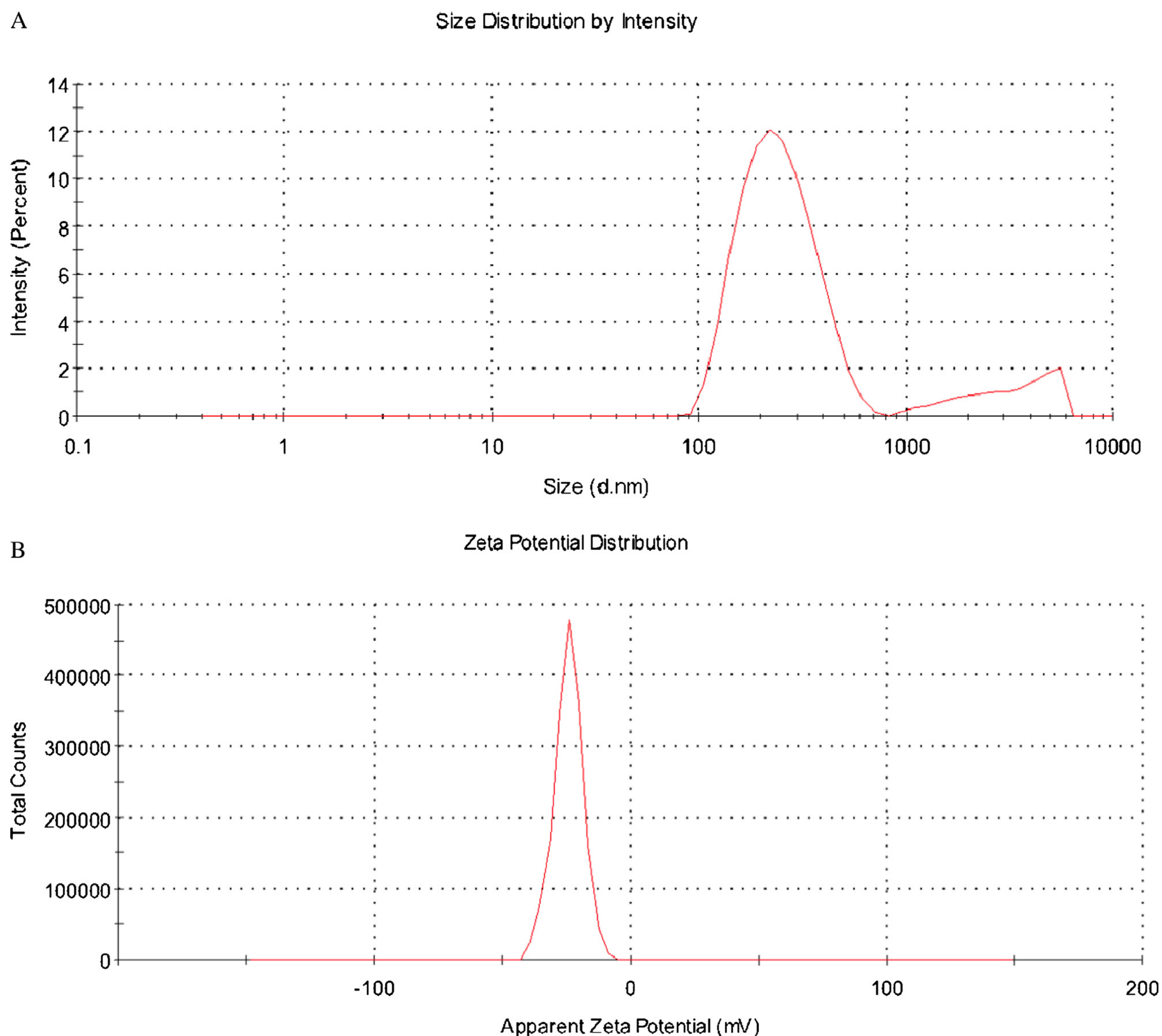

Fig. 2. Hydrodynamic characterisation of CuONPs. (A) and (B) are hydrodynamic size and Zeta Potential measured with DLS and LDV techniques, respectively.

Table 1
Genotoxicity data obtained from the *Drosophila* wing-spot test experiments with copper oxide nanoparticles (CuONPs). Results from *mwh/flr*^{3} wings.

Compound, concentration (mg/mL)	Small single spots (1–2 cells) ($m=2$)			Large single spots (>2 cells) ($m=5$)			Twin spots ($m=5$)			Total spots ($m=2$)			Frequency of clone formation per 10^5 cells
	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
CuONPs													
Control	26	0.35		2	0.03		1	0.01		32	0.40		1.60
0.24	33	0.41	–	1	0.01	–	2	0.03	–	38	0.48	–	1.90
0.48	38	0.48	i	0	0.00	–	1	0.01	i	45	0.56	i	2.30
0.95	48	0.60	+	2	0.03	i	1	0.01	i	51	0.64	+	2.60
EMS													
0.12	195	2.44	+	59	0.74	+	35	0.44	+	328	4.10	+	16.80

No: number of spots, Fr: frequency, D: statistical diagnosis, +: positive, –: negative, i: inconclusive, m: multiplication factor, probability levels, $\alpha=\beta=0.05$, 80 wings were analysed for each concentration (40 individuals).

4. Discussion

CuONPs are used as catalysts, semiconductors, gas sensors, and in the manufacture of textile and medical accessories with antimicrobial properties [4,5,56]. Relevant human and environmental exposures to these NPs are expected and genotoxic risk must be studied.

In general, few studies on the potential genetic damage of CuONPs using whole organisms are available in the literature. To fill this gap, we have assessed the genotoxicity and oxidative stress in *D. melanogaster*.

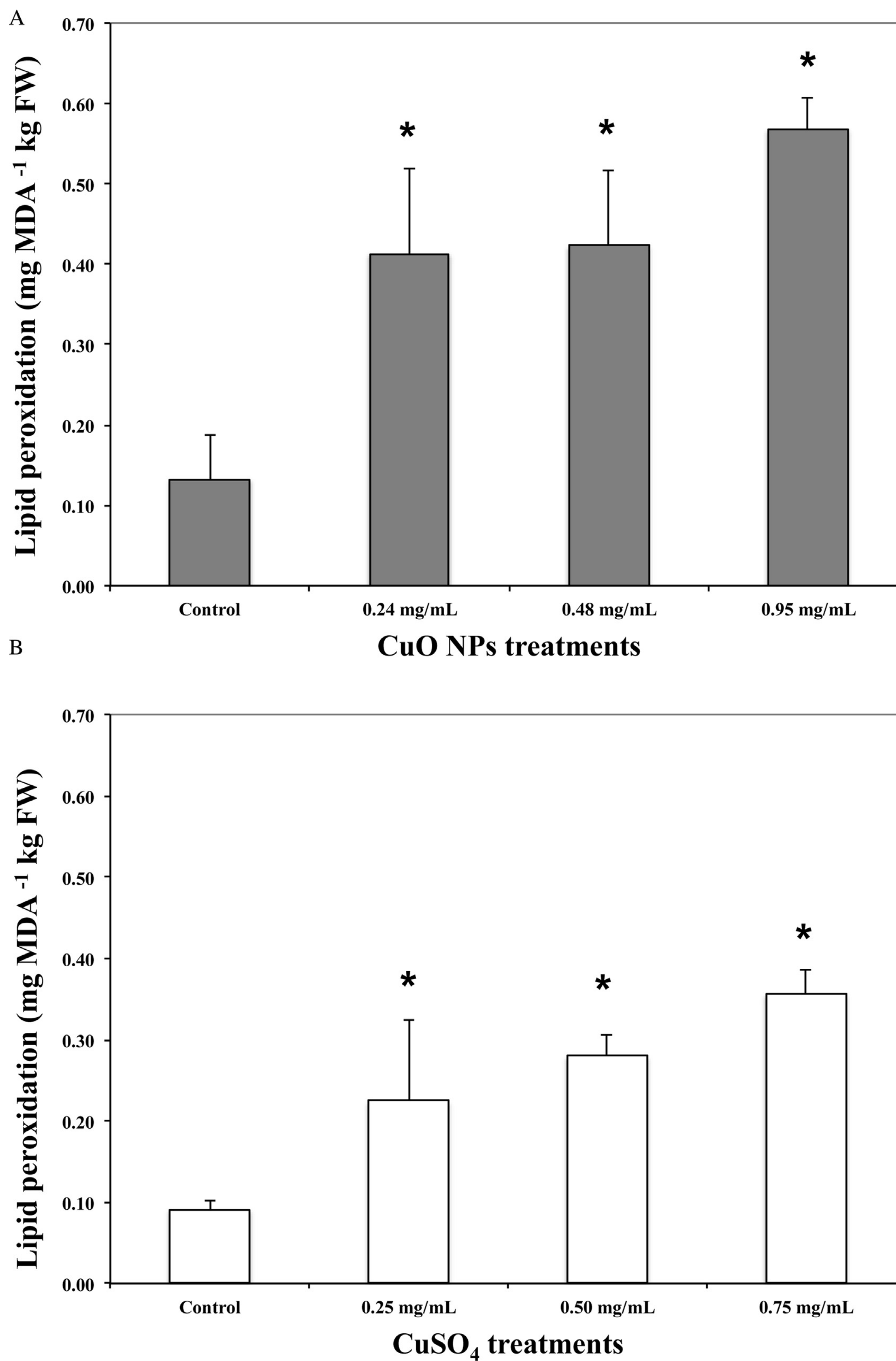

Fig. 3. Lipid peroxidation, measured as TBARS (Thiobarbituric Acid Reactive Substances) accumulation in *D. melanogaster* treated with different concentrations of CuONPs (A) and $CuSO_4$ (B). Bars show MDA average (mg/kg FW) and error bars show standard deviation ($n = 3$). Asterisks indicate significant differences ($P \leq 0.05$) between the controls and different concentrations of CuONPs and $CuSO_4$.

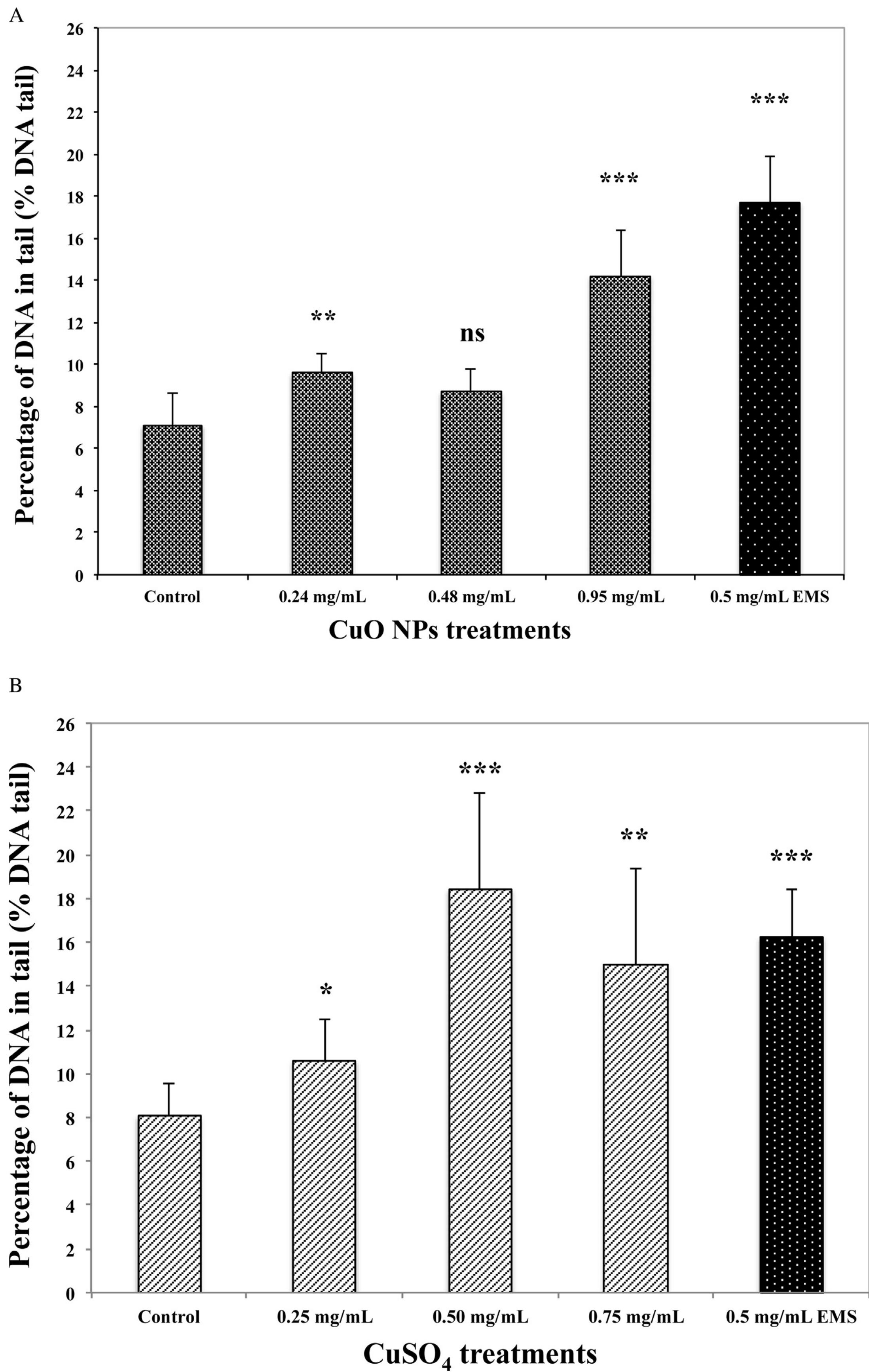

Fig. 4. Primary DNA damage measured by the *in vivo* comet assay in hemocytes from *D. melanogaster* larvae treated with CuONPs (A) and $CuSO_4$ (B). Distilled water was used as negative controls and ethyl methane sulphonate (EMS) as positive control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ^{ns}no significant *vs.* negative control.

Table 2
Genotoxicity data obtained from the *Drosophila* wing-spot test experiments with copper oxide nanoparticles (CuONPs). Results from *mwh/TM3/Bd^s* wings.

Compound, concentration (mg/mL)	Small single spots (1–2 cells) (m = 2)			Large single spots (>2 cells) (m = 5)			Twin spots (m = 5)			Total spots (m = 2)			Frequency of clone formation per 10^5 cells
	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
CuONPs													
Control	31	0.39		0	0.00					31	0.39		1.59
0.24	31	0.39	–	2	0.03					33	0.41	–	1.68
0.48	36	0.45	–	0	0.00					36	0.45	–	1.84
0.95	42	0.53	i	0	0.00					42	0.53	i	2.17
EMS													
0.12	119	1.49	+	20	0.25	+				139	1.74	+	7.13

No: number of spots, Fr: frequency, D: statistical diagnosis, +: positive, –: negative, i: inconclusive, m: multiplication factor, probability levels, α = β = 0.05, 80 wings were analysed for each concentration (40 individuals).

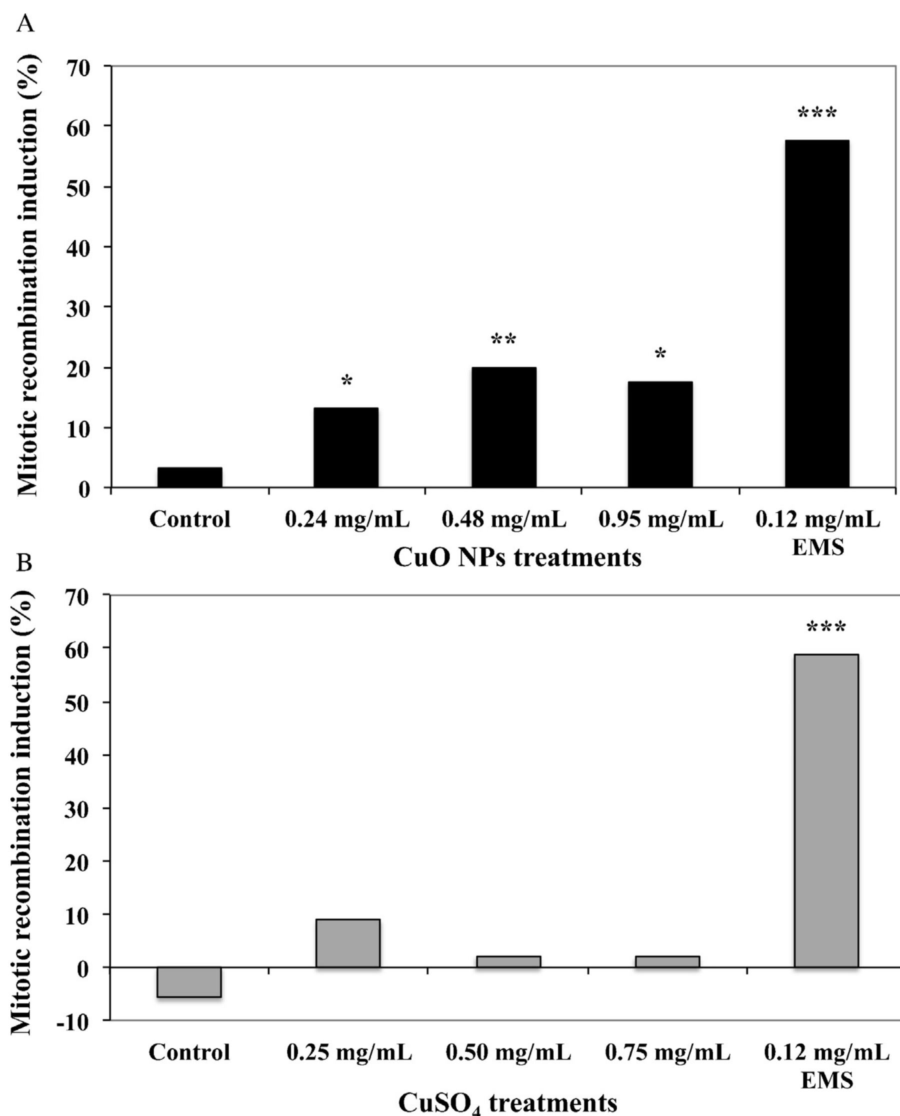

Fig. 5. Mitotic recombination induced by CuONPs (A) and $CuSO_4$ (B) in the wing-spot test of *D. melanogaster*. Ethyl methane sulphonate (EMS) was used as a positive recombinogenic agent in this study. *P < 0.01, **P < 0.001, and ***P < 0.0001 *vs.* negative control.

The physical characterization of CuONPs showed a primary particle size average of ∼30 nm diameter measured with TEM, in agreement with the size data given by the manufacturer with the same microscopy methods. According to the DLS results, the hydrodynamic size of CuONPs was larger than TEM analyses, with an average of ∼264 nm, suggesting an agglomeration tendency of these particles in aqueous suspension. These results were similar to other studies showing CuONPs sizes >200 nm in aqueous media and <50 nm in the dry state, by TEM analysis [24,57]. These differences are commonly explained by the tendency of NP to agglomerate in aqueous medium [58,59]. Although agglomeration of NPs may reduce the surface area reactivity and consequently reduce

Table 3
Wing-spot test data after copper sulphate ($CuSO_4$) treatments. Results obtained from *mwh/flr*^{3} wings.

Compound, concentration (mg/mL)	Small single spots (1–2 cells) ($m=2$)			Large single spots (>2 cells) ($m=5$)			Twin spots ($m=5$)			Total spots ($m=2$)			Frequency of clone formation per 10^5 cells
	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
$CuSO_4$													
Control	32	0.40		2	0.03		1	0.01		35	0.44		1.80
0.25	39	0.49	–	4	0.05	i	2	0.03	–	45	0.56	–	2.29
0.50	45	0.56	i	1	0.01	–	1	0.01	–	47	0.59	–	2.41
0.75	46	0.58	i	5	0.06	i	1	0.01	–	52	0.65	+	2.66
EMS													
0.12	246	3.08	+	57	0.71	+	47	0.59	+	371	4.64	+	19.01

No: number of spots, Fr: frequency, D: statistical diagnosis, +: positive, –: negative, i: inconclusive, m: multiplication factor, probability levels, $\alpha=\beta=0.05$, 80 wings were analysed for each concentration (40 individuals).

Table 4
Wing-spot test data after copper sulphate (CuSO4) treatments. Results obtained from *mwh/TM3/Bds* wings

Compound, concentration (mg/mL)	Small single spots (1–2 cells) ($m=2$)			Large single spots (>2 cells) ($m=5$)			Twin spots ($m=5$)			Total spots ($m=2$)			Frequency of clone formation per 10^5 cells
	No	Fr	D	No	Fr	D	No	Fr	D	No	Fr	D	
$CuSO_4$													
Control	37	0.46		0	0.00					37	0.46		1.88
0.25	41	0.51	–	0	0.00					41	0.51	–	2.09
0.50	46	0.58	–	0	0.00					46	0.58	–	2.37
0.75	49	0.61	–	2	0.03	i				51	0.64	i	2.62
EMS													
0.12	142	1.78	+	11	0.14	+				153	1.91	+	7.82

No: number of spots, Fr: frequency, D: statistical diagnosis, +: positive, –: negative, i: inconclusive, m: multiplication factor, probability levels, $\alpha=\beta=0.05$, 80 wings were analysed for each concentration (40 individuals).

toxic effects [60], our research indicates that CuONPs can induce deleterious biological effects in *Drosophila*. The zeta potential of CuONPs measured with the LDV technique reached an average of ~ -25 in water suspension. This measure indicates moderate colloidal stability of CuONPs in liquid suspension (*i.e.*, resistance to agglomeration in water), indicating suitable exposure conditions for nanogenotoxicity assays with *D. melanogaster*.

Oxidative stress has been proposed as one of the main mechanisms involved in toxicity and genotoxicity of nanoscale particles [9]. NPs can generate ROS in the cells that can potentially cause indirect oxidative damage to DNA through free radical attack and/or by interaction with MDA, which is one of the low-molecular-weight end-products formed via the decomposition of certain primary and secondary lipid peroxidation products [13,61]. In this study, we have found that although CuONPs can induce lipid peroxidation, this effect was also observed after exposure to the ionic $CuSO_4$, but at lower level. Thus, the induction of oxidative stress via lipid peroxidation is not an intrinsic characteristic effect of CuONPs, but of copper compounds. These results are in agreement with those of other authors, where CuONPs were found to generate oxidative stress leading to oxidative DNA lesions and genotoxicity in bacteria, plants, and mice [19,22,24].

The results obtained with the *in vivo* comet assay in hemocytes of *D. melanogaster* showed that CuONPs treatments induced DNA strand breaks in circulating hemocytes (equivalent to mammalian blood cells). Similar results have been reported in hemocytes of marine mussels *Mytilus galloprovincialis* [23] and in cultured human lung cells [12,14], indicating that these NPs induce primary DNA damage in different kind of cells both *in vitro* and *in vivo*.

The genotoxicity assessment of CuONPs with the wing spot test indicated significant genotoxic effects, where ~80% were induced by mutagenic activity, and ~20% were promoted by recombination events in somatic cells of *D. melanogaster*. In this context, it should be noted that the quantification of the recombinogenic activity of a compound is of primary importance for genotoxicity screening, because aberrant recombination events are commonly associated with carcinogenesis [44].

The above result agrees with recent *in vivo* studies where CuONPs produced chromosome damage and oxidative stress in mice exposed via intraperitoneal injection [19]. DNA alterations and mutations have also been observed in terrestrial plants [21,22]. Thus, the data available support the idea that CuONPs can cause DNA damage.

In the present study, both CuONPs and $CuSO_4$ showed similar genotoxic effects in *D. melanogaster*, indicating that the genotoxicity of CuONPs is induced not only by NPs *per se*, but also possibly by the release of Cu ions. Nevertheless, the source and mechanisms of genotoxicity in our results are interesting, in term of health risks, because $CuSO_4$ is a well-known toxic metal for living organisms [62,63] and it induces DNA damage and mutations in plants, invertebrates, and mammals [34,64–66].

In conclusion, CuONPs can induce DNA strand breaks detected by the comet assay in hemocytes of *D. melanogaster*, and this genetic damage could result in somatic mutation and recombination events, as evidenced in the wing-spot test. The observed genotoxic effects in the *D. melanogaster* model can be explained partially by the oxidative stress induced by CuONPs exposure. This study demonstrates the value of *D. melanogaster* in nanogenotoxicity studies, in order to assess genetic damage and related mechanisms of metal-oxide NPs.

Conflict of interest

There is no conflict of interest.

Acknowledgments

The authors thank the financial support given by FONDECYT-CONICYT 11110181 project, Dirección General de Investigación y

Postgrado, Universidad Católica de Temuco, DGIP UCT CD 2010-01 project, and MECESUP UCT 0804 project. We are grateful to B. Escobar and J. Scherpenisse for collaboration and technical assistance in the laboratory, and also to M.J. Rivadeneira and M.M. Carmona for revising the English version of the manuscript.

References

[1] N. Singh, B. Manshian, G.J. Jenkins, S.M. Griffiths, P.M. Williams, T.G. Maffeis, C.J. Wright, S.H. Doak, NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials, Biomaterials 30 (2009) 3891–3914.

[2] L.-C. Cheng, X. Jiang, J. Wang, C. Chen, R.-S. Liu, Nano–bio effects: interaction of nanomaterials with cells, Nanoscale 5 (2013) 3547–3569.

[3] Z. Magdolenova, A. Collins, A. Kumar, A. Dhawan, V. Stone, M. Dusinska, Mechanisms of genotoxicity. A review of *in vitro* and *in vivo* studies with engineered nanoparticles, Nanotoxicology 8 (2014) 233–278.

[4] M.J. Hajipour, K.M. Fromm, A.A. Ashkarran, D.J. de Aberasturi, I.R. de Larramendi, T. Rojo, V. Serpooshan, W.J. Parak, M. Mahmoudi, Antibacterial properties of nanoparticles, Trends Biotechnol. 30 (2012) 499–511.

[5] O. Bondarenko, K. Juganson, A. Ivask, K. Kasemets, M. Mortimer, A. Kahru, Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review, Arch. Toxicol. 87 (2013) 1181–1200.

[6] A. Kumar, A. Dhawan, Genotoxic and carcinogenic potential of engineered nanoparticles: an update, Arch. Toxicol. 87 (2013) 1883–1900.

[7] R. Magaye, J. Zhao, L. Bowman, M. Ding, Genotoxicity and carcinogenicity of cobalt-, nickel-and copper-based nanoparticles, Exp. Ther. Med. 4 (2012) 551–561.

[8] K. Klien, J. Godnić-Cvar, Genotoxicity of metal nanoparticles: focus on *in vivo* studies, Arch. Ind. Hyg. Toxicol. 63 (2012) 133–145.

[9] H. Wang, F. Wu, W. Meng, J.C. White, P.A. Holden, B. Xing, Engineered nanoparticles may induce genotoxicity, Environ. Sci. Technol. 47 (2013) 13212–13214.

[10] S. Chibber, S.A. Ansari, R. Satar, New vision to CuO, ZnO, and TiO2 nanoparticles: their outcome and effects, J. Nanopart. Res. 15 (2013) 1–13.

[11] X. Pan, J.E. Redding, P.A. Wiley, L. Wen, J.S. McConnell, B. Zhang, Mutagenicity evaluation of metal oxide nanoparticles by the bacterial reverse mutation assay, Chemosphere 79 (2010) 113–116.

[12] H.L. Karlsson, P. Cronholm, J. Gustafsson, L. Moller, Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes, Chem. Res. Toxicol. 21 (2008) 1726–1732.

[13] M. Ahamed, M.A. Siddiqui, M.J. Akhtar, I. Ahmad, A.B. Pant, H.A. Alhadlaq, Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells, Biochem. Biophys. Res. Commun. 396 (2010) 578–583.

[14] K. Midander, P. Cronholm, H.L. Karlsson, K. Elihn, L. Möller, C. Leygraf, I.O. Wallinder, Surface characteristics, copper release, and toxicity of nano-and micrometer-sized copper and copper (II) oxide particles: a cross-disciplinary study, Small 5 (2009) 389–399.

[15] S. Alarifi, D. Ali, A. Verma, S. Alakhtani, B.A. Ali, Cytotoxicity and genotoxicity of copper oxide nanoparticles in human skin keratinocytes cells, Int. J. Toxicol. 32 (2013) 296–307.

[16] A. Semisch, J. Ohle, B. Witt, A. Hartwig, Cytotoxicity and genotoxicity of nano-and microparticulate copper oxide: role of solubility and intracellular bioavailability, Part. Fibre Toxicol. 11 (2014) 10.

[17] S. Di Bucchianico, M.R. Fabbrizi, S.K. Misra, E. Valsami-Jones, D. Berhanu, P. Reip, E. Bergamaschi, L. Migliore, Multiple cytotoxic and genotoxic effects induced *in vitro* by differently shaped copper oxide nanomaterials, Mutagenesis 28 (2013) 287–299.

[18] F. Perreault, S.P. Melegari, C.H. da Costa, A.L.O.F. d. Rossetto, R. Popovic, W.G. Matias, Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures, Sci. Total Environ. 441 (2012) 117–124.

[19] M.-F. Song, Y.-S. Li, H. Kasai, K. Kawai, Metal nanoparticle-induced micronuclei and oxidative DNA damage in mice, J. Clin. Biochem. Nutr. 50 (2012) 211.

[20] M. Yokohira, N. Hashimoto, K. Yamakawa, S. Suzuki, K. Saoo, T. Kuno, K. Imaida, Lung carcinogenic bioassay of CuO and TiO2 nanoparticles with intratracheal instillation using F344 male rats, J. Toxicol. Pathol 22 (2009) 71–78.

[21] S. Lee, H. Chung, S. Kim, I. Lee, The genotoxic effect of ZnO and CuO nanoparticles on early growth of buckwheat, Fagopyrum esculentum, Water Air Soil Pollut. 224 (2013) 1–11.

[22] D.H. Atha, H. Wang, E.J. Petersen, D. Cleveland, R.D. Holbrook, P. Jaruga, M. Dizdaroglu, B. Xing, B.C. Nelson, Copper oxide nanoparticle mediated DNA damage in terrestrial plant models, Environ. Sci. Technol. 46 (2012) 1819–1827.

[23] T. Gomes, O. Araújo, R. Pereira, A.C. Almeida, A. Cravo, M.J. Bebianno, Genotoxicity of copper oxide and silver nanoparticles in the mussel Mytilus galloprovincialis, Mar. Environ. Res. 84 (2013) 51–59.

[24] O. Bondarenko, A. Ivask, A. Käkinen, A. Kahru, Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action, Environ. Pollut. 169 (2012) 81–89.

[25] G. Vecchio, A. Galeone, V. Brunetti, G. Maiorano, L. Rizzello, S. Sabella, R. Cingolani, P.P. Pompa, Mutagenic effects of gold nanoparticles induce aberrant phenotypes in *Drosophila melanogaster*, Nanomed.: NBM 8 (2012) 1–7.

[26] E. Demir, F. Turna, G. Vales, B. Kaya, A. Creus, R. Marcos, In vivo genotoxicity assessment of titanium, zirconium and aluminium nanoparticles, and their microparticulated forms, in *Drosophila*, Chemosphere 93 (2013) 2304–2310.

[27] G. Vales, E. Demir, B. Kaya, A. Creus, R. Marcos, Genotoxicity of cobalt nanoparticles and ions in *Drosophila*, Nanotoxicology 7 (2013) 462–468.

[28] M. Alaraby, A. Hernández, B. Annangi, E. Demir, J. Bach, L. Rubio, A. Creus, R. Marcos, Antioxidant and antigenotoxic properties of CeO2 NPs and cerium sulphate: studies with *Drosophila melanogaster* as a promising *in vivo* model, Nanotoxicology 0 (2014) 1–11.

[29] E. Demir, G. Vales, B. Kaya, A. Creus, R. Marcos, Genotoxic analysis of silver nanoparticles in *Drosophila*, Nanotoxicology 5 (2011) 417–424.

[30] S. Sabella, V. Brunetti, G. Vecchio, A. Galeone, G. Maiorano, R. Cingolani, P.P. Pompa, Toxicity of citrate-capped AuNPs: an *in vitro* and *in vivo* assessment, J. Nanopart. Res. 13 (2011) 6821–6835.

[31] E.R. Carmona, B. Escobar, G. Vales, R. Marcos, Genotoxic testing of titanium dioxide anatase nanoparticles using the wing-spot test and the comet assay in *Drosophila*, Mutat. Res 778 (2015) 12–21.

[32] G. Vecchio, A fruit fly in the nanoworld: once again *Drosophila* contributes to environment and human health, Nanotoxicology 9 (2015) 135–137.

[33] D.L. Lindsley, G.G. Zimm, The genome of *Drosophila melanogaster*, Elsevier Sci. 1 (1992) 1133.

[34] T. Guecheva, J.A. Henriques, B. Erdtmann, Genotoxic effects of copper sulphate in freshwater planarian in vivo, studied with the single-cell gel test (comet assay), Mutat. Res 497 (2001) 19–27.

[35] V.A. Tironi, M.C. Tomás, M.C. Añón, Lipid and protein deterioration during the chilled storage of minced sea salmon (*Pseudopercis semifasciata*), J. Sci. Food Agric. 87 (2007) 2239–2246.

[36] E.R. Carmona, T.N. Guecheva, A. Creus, R. Marcos, Proposal of an *in vivo* comet assay using haemocytes of *Drosophila melanogaster*, Environ. Mol. Mutagen. 52 (2011) 165–169.

[37] R. Marcos, E.R. Carmona, The wing-spot and the comet tests as useful assays detecting genotoxicity in *Drosophila*, in: A. Dhawan, M. Bajpayee (Eds.), Genotoxicity Assessment, Humana Press, Springer, 2013, pp. 417–427.

[38] R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. Ryu, Y. Sasaki, Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.

[39] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.

[40] T.S. Kumaravel, B. Vilhar, S. Faux, A. Jha, Comet assay measurements: a perspective, Cell Biol. Toxicol. 25 (2009) 53–64.

[41] B.M. Bolker, M.E. Brooks, C.J. Clark, S.W. Geange, J.R. Poulsen, M.H.H. Stevens, J.-S.S. White, Generalized linear mixed models: a practical guide for ecology and evolution, Trends Ecol. Evol. 24 (2009) 127–135.

[42] U. Graf, F. Würgler, A. Katz, H. Frei, H. Juon, C. Hall, P. Kale, Somatic mutation and recombination test in *Drosophila melanogaster*, Environ. Mutagen. 6 (1984) 153–188.

[44] G. Luo, I.M. Santoro, L.D. McDaniel, I. Nishijima, M. Mills, H. Youssoufian, H. Vogel, R.A. Schultz, A. Bradley, Cancer predisposition caused by elevated mitotic recombination in bloom mice, Nat. Genet. 26 (2000) 424–429.

[45] G.A. Arossi, M. Lehmann, R.R. Dihl, M.L. Reguly, H.H.R. De Andrade, Induced DNA damage by dental resin monomers in somatic cells, Basic Clin. Pharmacol. Toxicol. 106 (2010) 124–129.

[46] M.A. Kastenbaum, K.O. Bowman, Tables for determining the statistical significance of mutation frequencies, Mutat. Res. 9 (1970) 527–549.

[47] H. Frei, F.E. Würgler, Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result, Mutat. Res. 203 (1988) 297–308.

[48] A. Alonso-Moraga, U. Graf, Genotoxicity testing of antiparasitic nitrofurans in the *Drosophila* wing somatic mutation and recombination test, Mutagenesis 4 (1989) 105–110.

[49] I. Mukhopadhyay, D.K. Chowdhuri, M. Bajpayee, A. Dhawan, Evaluation of *in vivo* genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline comet assay, Mutagenesis 19 (2004) 85–90.

[50] E. Demir, B. Kaya, Studies on the genotoxic properties of four benzyl derivatives in the *in vivo* comet assay using haemocytes of *Drosophila melanogaster*, Fresen. Environ. Bull. 22 (2013) 1590–1596.

[51] E. Demir, In vivo genotoxicity assessment of diflubenzuron and spinosad in *Drosophila melanogaster* with the comet assay using haemocytes and the smart assay, Fresen. Environ. Bull. 21 (2012) 3894–3900.

[52] E.R. Carmona, A. Creus, R. Marcos, Genotoxicity testing of two lead-compounds in somatic cells of *Drosophila melanogaster*, Mutat. Res. 724 (2011) 35–40.

[53] M. Gürbüzel, H. Uysal, H. Kizilet, Assessment of genotoxic potential of two mycotoxins in the wing spot test of *Drosophila melanogaster*, Toxicol. Ind. Health 31 (2015) 261–267.

[54] A. Karadeniz, B. Kaya, B. Savaş, Ş.F. Topcuoğlu, Effects of two plant growth regulators, indole-3-acetic acid and β-naphthoxyacetic acid, on genotoxicity in *Drosophila* SMART assay and on proliferation and viability of HEK293 cells from the perspective of carcinogenesis, Toxicol. Ind. Health 27 (2011) 840–848.

[55] M. Gürbüzel, I. Çapoglu, H. Kizilet, Z. Halici, F. Özçiçek, L. Demirtas, Genotoxic evaluation of two oral antidiabetic agents in the *Drosophila* wing spot test, Toxicol. Ind. Health 30 (2014) 376–383.

[56] B. Fahmy, S.A. Cormier, Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells, Toxicol. In Vitro 23 (2009) 1365–1371.
[57] H.L. Karlsson, P. Cronholm, Y. Hedberg, M. Tornberg, L. De Battice, S. Svedhem, I.O. Wallinder, Cell membrane damage and protein interaction induced by copper containing nanoparticles – importance of the metal release process, Toxicology 313 (2013) 59–69.
[58] A. Dhawan, V. Sharma, Toxicity assessment of nanomaterials: methods and challenges, Anal. Bioanal. Chem. 398 (2010) 589–605.
[59] V. Sharma, P. Singh, A.K. Pandey, A. Dhawan, Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles, Mutat. Res. 745 (2012) 84–91.
[60] S.P. Melegari, F. Perreault, R.H.R. Costa, R. Popovic, W.G. Matias, Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*, Aquat. Toxicol. 142 (2013) 431–440.
[61] B. Halliwell, S. Chirico, Lipid peroxidation: its mechanism, measurement, and significance, Am. J. Clin. Nutr. 57 (1993) 715S–724S.
[62] D.L. de Romaña, M. Olivares, R. Uauy, M. Araya, Risks and benefits of copper in light of new insights of copper homeostasis, J. Trace Elem. Med. Biol. 25 (2011) 3–13.
[63] M. Trivedi, N. Sangai, A. Renuka, Assessment of toxicity of copper sulphate pentahydrate on oxidative stress indicators on liver of gold fish (*Carassius auratus*), Bull. Environ. Pharmacol. Life Sci. 1 (2012) 52–57.
[64] D. Prá, S.I.R. Franke, R. Giulian, M.L. Yoneama, J.F. Dias, B. Erdtmann, J.A.P. Henriques, Genotoxicity and mutagenicity of iron and copper in mice, Biometals 21 (2008) 289–297.
[65] M. Yıldız, İ.H. Ciğerci, M. Konuk, A.F. Fidan, H. Terzi, Determination of genotoxic effects of copper sulphate and cobalt chloride in *Allium cepa* root cells by chromosome aberration and comet assays, Chemosphere 75 (2009) 934–938.
[66] M. Gürbüzel, H. Kizilet, Evaluation of genotoxicity of two sulphate compounds using the somatic mutation and recombination test in *Drosophila melanogaster*, Fresenius Environ. Bull. 19 (2010).