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In vivo genotoxic effects of four different nano-sizes forms of silica nanoparticles in *Drosophila melanogaster*

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HIGHLIGHTS

- Genotoxicity of amorphous silica (SAS) nanoparticles has been shown in *Drosophila*.
- Positive effects in the comet assay (with and without enzymes) were obtained.
- Oxidative DNA-damage induction was inversely associated to SAS size.
- No somatic and recombination mutations were obtained in the wing-spot test.
- No genotoxic effects were obtained with microparticulated silica dioxide.

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ABSTRACT

Although the use of synthetic amorphous silica (SAS) is steady increasing, scarce information exists on its potential health risk. In particular few and conflictive data exist on its genotoxicity. To fill in this gap we have used *Drosophila melanogaster* as *in vivo* model test organism to detect the genotoxic activity of different SAS with different primary sizes (6, 15, 30 and 55 nm). The wing-spot assay and the comet assay in larvae haemocytes were used, and the obtained results were compared with those obtained with the microparticulated form (silicon dioxide). All compounds were administered to third instar larvae at concentrations ranging from 0.1 to 10 mM. No significant increases in the frequencies of mutant spots were observed in the wing-spot assay with any of the tested compounds. On the other hand, significant dose-dependent increases in the levels of primary DNA damage, measured by the comet assay, were observed for all the SAS evaluated but mainly when high doses (5 and 10 mM) were used. These *in vivo* results contribute to increase the database dealing with the potential genotoxic risk associated to SAS nanoparticles exposure.

1. Introduction

Nanotechnology industry is rapidly growing, due to the novel physicochemical properties of nanomaterials that are defined by their small size, with at least one dimension less than 100 nm. This supposes that such nanomaterials are increasingly spread into the environment and, in this way, human exposure certainly occurs. It is assumed that the important biological reactivity of nanometals may also imply an increased toxicity, both systemic and specifically on the genetic material. For such reasons, nanotoxicology and nanogenotoxicology are extending as a novel field, looking for the potential toxic and genotoxic risk of nanomaterials as well as for their mechanisms of action [1–6].

Synthetic amorphous silica (SAS) nanoparticles are used as a food additive in many processed foods, as well as in pharmaceutical drug tablets, glass, electronics, and as hydrophobic anticancer drug [7]. With respect to SAS toxicity it is assumed that it is mediated by inflammatory and oxidative stress mechanisms, as it has been shown in both *in vivo* and *in vitro* [8–11], from the genotoxic point of view the obtained results are contradictory. When testing for primary DNA damage positive effects in the comet assay were obtained in human lung alveolar epithelial cells [12] and in human umbilical vein endothelial cells [13] but not effects were observed in mouse fibroblasts [14]. Similarly, in the micronucleus assay although positive effects were observed in mouse fibroblasts [15], negative results were also reported in Balb/3T3 mouse fibroblasts [16]. With respect to *in vivo* approaches, studies using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* were unable to demonstrate the induction of genetic damage by SAS exposure using the comet assay. The only one study carried out with *Drosophila melanogaster* showed that larval exposure to SAS supposes its internalization through intestine track producing cellular stress and apoptosis in midgut cells [17].

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Due to this lack of confirmatory results on the genotoxic potential of SAS exposure we have plan to use *Drosophila* to get further inside on its genotoxic risk. It must be pointed out that *Drosophila* is one of the most genetically and experimentally accessible model organisms used in biology. It must also be stressed that about 75% of human disease genes have related sequences in *Drosophila*, suggesting that it can serve as an effective model to study the function of a wide array of genes involved in human disease [18,19]. In addition to the technical advantages of using somatic cells as a target, such as scoring the effects on the exposed individuals without waiting for the two subsequent generations, somatic mutation inductions are directly linked with cancer processes, which suppose a relevant role on human health [20]. Thus, *Drosophila* is considered a very potent *in vivo* tool to detect the potential damaging effect of new environmental contaminants.

The advantages of *Drosophila* to detect potential genotoxicants have already been used to determine the potential risk of nanomaterials. In fact, this *in vivo* model has already been used to evaluate the internalization of nanoparticles, as well as its cell uptake and tissue distribution [21,22]. In addition *Drosophila* has also been used to determine the potential genotoxic harmful effects of different nanomaterials [4,23–26].

In this context, in the present study we have use *Drosophila* to study the potential genotoxicity of four different SAS characterized by their different sizes. SAS was administered to larvae and the targeted cells were those from the wing imaginal disk and haemocytes.

2. Experimental

2.1. Drosophila strains

For the *Drosophila* wing-spot test two *D. melanogaster* strains were used: the *multiple wing hairs* strain with the genetic constitution *mwh/mwh* and the *flare-3* strain with the genetic constitution of $flr^3/In\,(3LR)\,TM3,\,Bd^s$. More detailed information on genetic markers and descriptions of the phenotypes is obtained in Lindsley and Zimm [27]. The studies were carried at the Akdeniz University and both strains were kindly provided by Prof. R. Marcos (Universitat Autònoma de Barcelona, Spain). The wild-type strain Oregon R^+, proficient for all types of repair, was used for the comet assay. These strains were cultured in bottles with standard *Drosophila* medium, at a temperature of $25 \pm 1^\circ C$ and a relative humidity of ~60%.

2.2. Chemicals

Low melting-point agarose (LMA), normal melting-point agarose (NMA), trisma base, ethidium bromide (EtBr), fluorescein diacetate (FDA), *N*-lauroylsarcosine sodium salt solution, endonuclease III (endo III), formamidopyrimidine DNA glycosilase (FPG), EDTA disodium salt dehydrate, phosphate-buffered saline solution without Ca^{+2}, Mg^{+2} (PBS), HEPES, potassium chloride (KCl), bovine serum albumin (BSA), triton X-100, sodium chloride (NaCl), sodium hydroxide (NaOH) and ethyl methanesulfonate (EMS) were obtained from Sigma Chemical Co. (St. Louis, MO).

Four different synthetic amorphous silica (SAS) nanoparticles (LEVASIL®-types) with sizes 6, 15, 30 and 45 nm were obtained from H.C. Starck GmbH, Engineered Material Solutions (Goslar/Germany). The microparticulated form of silicon dioxide (SiO_2, CAS No: 7631-86-9) was provided by Sigma–Aldrich.

2.3. Nanoparticles characterization

According to the manufacturer the physical characteristics of the different nanosized SAS are: for 6 nm, density (1.1 g/cm^3), surface area (min. 450 m^2/g); for 15 nm, density (1.205 g/cm^3), surface area (200 m^2/g); for 30 nm, density (1.343 g/cm^3), surface area (100 m^2/g) and for 55 nm, density (1.39 g/cm^3) and surface area (50 m^2/g). We further characterized the selected SAS by using transmission electron microscopy (TEM), dynamic light scat-tering (DLS) and laser Doppler velocimetry (LDV) methodologies. TEM methodologies were carried on a JEOL JEM-2011 instrument to determine size and morphology. DLS and LDV were performed on a Malvern Zetasizer Nano-ZS zen3600 instrument for the characterization of hydrodynamic size and zeta potential, for these measures SAS were dispersed in a 5% solution prepared with distilled water. For dispersion, SAS were subjected to ultrasonication (S-250D, Branson Sonifier) at 20 kHz for 16 min in an ice-cooled bath.

2.4. Drosophila wing-spot test protocol

To carry out the wing-spot assay virgin flr^3 females were mated to *mwh* males, as previously described [4]. Eggs from this cross were collected during 8-h periods in culture bottles containing standard food medium. The resulting 3-day-old larvae were then transferred to plastic vials with 4.5 g of *Drosophila* instant medium (Carolina Biological Supply Co., Burlington, NC) prepared with 9 mL of non-toxic concentrations of the four selected SAS (0.1, 1, 5 and 10 mM). Distilled water was used as negative controls and 1 mM EMS as positive control. For each treatment five plastic vials were used and 25 larvae per vial were included. Two replicated were done by experiment. Larvae were fed on this medium until pupation and the emerged adults were conunted and stored in plastic vials with 70% ethanol. After that he wings of the emerged adults were removed, mounted and scored for the presence of mutant clones. In each experiment we scored 80 wings (40 individuals). Wings were carefully removed from adults and mounted in Faure's solution on microscope slides and scored at 400× magnification for the presence of spots. Single and large *mwh* or flr^3 spots, as well as twin spots were recorded as previously reported [24,25].

2.5. Haemocytes collection and comet assay protocol

Larval haemocytes were collected according to Irving et al. [28] and Carmona et al. [29,30] with minor modifications. Third instar larvae were extracted from the culture medium, washed, sterilized with ~5% sodium hypochlorite and dried with filter paper. To collect the haemolymph and circulating haemocytes, the cuticle of each larva was disrupted using two fine forceps, avoiding damage to internal organs. A total of 40–60 larvae per treatment were used. The comet assay was conducted as previously described by Singh et al. [31], with minor modifications. Cell samples (~40,000 cells in 20 μL) were carefully resuspended in 140 μL of 0.75% LMA prepared in PBS. The cells and agarose were gently mixed by repeated pipetting, and layered onto microscope slides pre-coated with 1% NMA (dried for 25 min). The slides were immediately covered with cover slips and kept on ice for 5 min to solidify the agarose. After solidification, the cover slips were removed and 80 μL of molten 0.75% LMA prepared in PBS was spread on the slides. The slides were again covered with cover slips and kept on ice for 5 min. Then, the cover slips were removed and the slides were immersed in cold, freshly made lysis solution for 2 h at 4 °C in a dark chamber. To avoid additional DNA damage, the next steps were performed under dim light. Slides were 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 25 V (1 V/cm) and 300 mA. After electrophoresis, slides were neutralized with three washes of 5 min in fresh chilled with 400 mM Tris buffer (pH 7.5). The slides were stained with 50 μl of ethidium bromide (EtBr) solution (60 μg/mL) for 10 min and covered with a cover slip. For the visualizing of DNA damage, slides were examined at 400× magnification using a fluorescence microscope (Nikon Eclipse E200) connected to a CCD camera and an image analysis system (Comet assay IV version 4.11, Kinetic Imaging, UK). Randomly selected 100 cells (50 cells on each one of the two replicate slides) were analyzed per treatment. 4 mM EMS was used as positive control in the comet assay. The percentage of DNA in tail was the parameter used as a measure of DNA damage induction.

To determine the induction of oxidized bases the comet assay was complemented with the use of FPG and endo III enzymes