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Does the crystal habit modulate the genotoxic potential of silica particles? A cytogenetic evaluation in human and murine cell lines

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ABSTRACT

Crystalline silica inhaled from occupational sources has been classified by IARC as carcinogenic to humans; in contrast, for amorphous silica, epidemiological and experimental evidence remains insufficient. The genotoxicity of crystalline silica is still debated because of the inconsistency of experimental results ("variability of silica hazard"), often related to the features of the particle surfaces. We have assessed the role of crystal habit in the genotoxicity of silica powders. Pure quartz (crystalline) and vitreous silica (amorphous), sharing the same surface features, were used in an *in vitro* study with human pulmonary epithelial (A549) and murine macrophage (RAW264.7) cell lines, representative of occupational and environmental exposures. Genotoxicity was evaluated by the comet and micronucleus assays, and cytotoxicity by the trypan blue method. Cells were treated with silica powders for 4 and 24 h. Quartz but not vitreous silica caused cell death and DNA damage in RAW264.7 cells. A549 cells were relatively resistant to both powders. Our results support the view that crystal habit *per se* plays a pivotal role in modulating the biological responses to silica particles.

1. Introduction

Occupational exposure to inhalable crystalline silica has been associated with the development of silicosis, lung cancer, and airway diseases [1,2]. Accordingly, in 1997, IARC classified quartz as "carcinogenic to humans" [3]. The same agency considered both natural and synthetic amorphous silica as not classifiable (group 3), due to insufficient epidemiological evidence and experimental data. Crystalline silica has also been reported as inert, in some *in vitro* studies [4]. This variability of silica toxicity is referred to as "variability of silica hazard" and has been frequently discussed by the scientific community since the IARC classifications were published. Fubini and Hubbard [5] have discussed the toxicological variability of silica in light of two aspects, namely the crucial role played by particle surface and the importance of the source of silica flours. The authors stressed the concept that toxicity is ascribable not only to the various forms of silica (*e.g.*, crystalline, amorphous, natural, synthetic, mineral, biogenic) but also to the surface properties of particles, which are largely influenced by the "history" of the flours. The pivotal role played by surface chemistry in inducing biological effects of quartz has been widely reported [6–9]. Particularly, ROS generation (either on the particle surface or within the cytoplasm of phagocytes) is considered the initial step in the process of inflammation and subsequent fibrosis induced by crystalline silica [10,5]. Although several research groups have demonstrated genotoxic properties of quartz *per se*, in the absence of inflammatory cells [7], the secondary inflammation is generally regarded as the driving force for the carcinogenicity of crystalline silica [1]. However, some evidence suggests that crystal habit might not be a prerequisite for silica particle toxicity: silicosis and lung cancer have also been found among workers exposed to vitreous (amorphous) silica [11–13]. This hypothesis was further stressed by Ghiazza et al. [14], who found that vitreous silica and pure quartz share some effects on cultured macrophages, including the activation of nitric oxide synthase and tumor necrosis factor-α production. Based on the relationship between ROS generation and DNA damage [14–15], loss of DNA integrity has been suggested to be a useful biomarker for silica exposure [16].

We have examined the cytotoxic and genotoxic potential of two well-characterized "silica models", crystalline pure quartz (PQ) and amorphous vitreous silica (VS), sharing surface features and differing only in crystal habit, to investigate the importance of particle habit in triggering adverse biological responses. In *vitro* exposure of a pulmonary epithelial cell line, A549, was carried out because this

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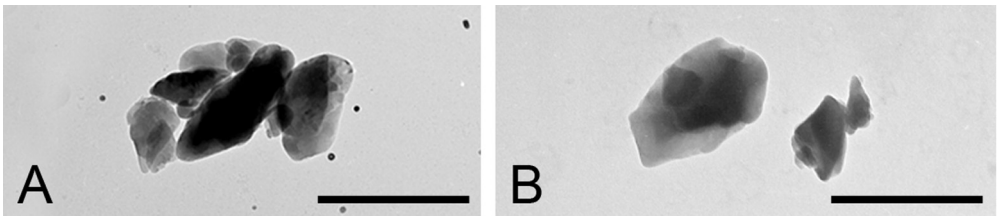

Fig. 1. Transmission electron micrograph of pure quartz (A) and vitreous silica (B), exhibiting similar morphology (scale bar = 500 nm).

cell line is a model for quartz-induced lung carcinogenesis [17]. The mouse alveolar macrophage (RAW264.7) cell line was also selected for the study because of the critical role played by macrophages in silicosis [10]. Genotoxicity was evaluated by the comet assay and the micronucleus (MN) test. The trypan blue exclusion technique was used to assess cytotoxicity. Five doses (0, 5, 10, 20, 40 and 80 μg/cm^2) of flours were tested at two different exposure times (4 and 24 h).

2. Materials and methods

2.1. Particle preparation

The silica powders used in the present investigation were prepared and provided by Ghiazza et al. [14]. Pure quartz (PQ) flour was obtained by grinding (in a ball mill/ agate jar) a very pure natural crystal from Madagascar for 12 h (Fig. 1A). Vitreous silica (VS) (Fig. 1B) was obtained by grinding very pure silica glass (Suprasil for optical applications) for 3 h. These procedures guaranteed that the particles had the same size frequency distribution and surface micromorphology, close to those typical of commercial quartz dusts [8].

2.2. Particle characterization

Particle characterization was performed in the framework of the collaborative project "Mechanisms responsible for cytotoxicity and genotoxicity of silica nanoparticles and nanometric fibrous silicates having strictly controlled size, structure and composition" (PRIN 2007498XRF) by X-ray diffraction (XRD), BET method, electron paramagnetic resonance spectroscopy (EPR), and scanning electron microscopy (SEM), and was previously published by Ghiazza et al. [14]. Surface areas (m^2/g) were 4.1 ± 0.011 (VS) and 5.0 ± 0.015 (PQ). SEM analysis showed, for both VS and PQ, 80% of particles ranging from 0.5–5 μm, with indented irregular surface topology. XRD confirmed the nature of the particles as amorphous (VS) and crystalline (PQ). X-ray photoelectron spectroscopy (XPS) analysis confirmed that no surface impurities were acquired during grinding of the materials.

2.3. Cell lines and exposure

Before the experiments, the flours were baked (220 °C, 3 h) to eliminate possible lipopolysaccharide contamination. A highest (80 μg/cm^2) dose stock suspension was obtained by dispersing the particles in complete cell culture medium. Stock suspension was freshly prepared and sonicated in a Bransonic Ultrasound bath for 30 min at 37 °C (35 kHz) to reduce aggregation, immediately before exposure; the other doses were obtained by dilution in the treatment wells. Nominal doses were expressed as μg of particles per cm^2 of monolayer.

RAW264.7 and A549 cell lines, provided by Prof. Lucia Migliore (University of Pisa), were selected as representative models of occupational and environmental exposures (Gonzalez et al., 2010; 19 Guidi et al., 2013). RAW264.7 were [18,19] cultured in minimal essential medium (MEM) supplemented with 10%, 1% pen/strep and 1% L-glutamine. A549 was cultured in F12 supplemented with 10% FBS, 1% pen/strep. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2/95% air. RAW264.7 cells at confluence were detached using a cell scraper, seeded into 6-well plates at 200,000 cells/well, and allowed to attach for 24 h. Confluent A549 cells were detached with trypsin 2.5% for 10 min at 37 °C, seeded into 24-well plates at 50,000 cells/well, and allowed to attach for 24 h. Semi-confluent cell lines were treated with 0, 5, 10, 20, 40 or 80 μg/cm^2 PQ and VS.

The comet assay was used to evaluate genotoxicity after 4 and 24 h of exposure to silica particles. Methylmethanesulphonate (MMS), CAS [66-27-3] 0.5 mM, was used as positive control. The micronucleus test was used to evaluate chromosomal damage after 48 h exposure. Mitomycin C (MMC), CAS [50-07-7], 0.1 μg/ml, was used as positive control. Two samples for each experimental point were set up; three independent experiments were carried out for each treatment.

2.4. Cell viability

Before and at the end of the treatments, aliquots of both exposed and control cells were used for assessment of cell viability by the trypan blue exclusion technique. Trypan blue solution (0.4%; Sigma–Aldrich) was mixed with the cell pellet. Successively, the mixture was smeared on a Bürker chamber, kept for 5 min, and scored for white (live) and blue (dead) cells.

2.5. Comet assay

RAW264.7 and A549 cells were seeded and treated for DNA damage evaluation as previously described [19]. The single-cell gel electrophoresis (or comet) assay was performed according to Singh and coauthors [20], with slight modifications. Briefly, cell suspensions were embedded in agarose, spread onto microscope slides, lysed (NaCl 2.5 M, Na_2EDTA 100 mM, Trizma base 10 mM, 10% dimethylsulphoxide, 1% Triton X-100; pH 10) and kept for at least 1 h at +4 °C in the dark. Successively, slides were treated 20 min with alkali (NaOH 300 mM, Na_2EDTA 1 mM, pH > 13) and electrophoresed for 20 min at 25 V and 300 mA; field strength 0.76 V/cm.

After the run, the slides were neutralized with Tris–HCl (0.4 M, pH 7.5), stained with ethidium bromide, and observed under a fluorescence microscope (400×). To evaluate the feasibility of RAW264.7 and A549 cells for DNA strand breakage analysis by the comet assay, MMS was used as a positive control. The percentage of DNA migrated towards the anode was quantified by an image analyser (Kinetic Imaging Ltd., Komet, Version 5). For each experiment (three independent experiment were performed), two slides were used *per* treatment and for each slide 25 cells were randomly analysed. DNA damage was evaluated by the percentage of DNA in the tail (%tail DNA), which is considered the most informative and reliable parameter [21–23].