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KEYWORDS

Quartz hazard, carbon dust, free radical, cytotoxicity, inflammation, oxidative stress

ABSTRACT

In order to evaluate the effect of carbonaceous materials on the pathogenic activity of quartz dusts, mixtures of carbon soot (1% and 10%) and quartz (Min-U-Sil) have been prepared, then milled so to attain an intimate association of carbon and the quartz surface. Both cellular and cell-free tests show that carbon associated to quartz completely inhibits the typical free radical generation of quartz dusts (through Fenton activity and homolytic cleavage of a C-H bond) and suppresses the oxidative stress and inflammation induced by quartz alone on MH-S murine macrophage cells (lipid peroxidation, nitric oxide release, tumour necrosis factor-α synthesis). Cytotoxic response to quartz is also largely reduced. An extremely pure quartz milled with 10% of soot showed inactivating effects on the adverse reactions to quartz similar to Min-U-Sil quartz. None of these effects takes place when the same experiments are carried out with mechanically mixed samples, which suggests that carbon acts not just as a radical quencher, but because of its association to the quartz surface.

INTRODUCTION

Silicosis – the most ancient occupational disease – is related to exposure to quartz, or other crystalline silica polymorphs. Exposure to silica may also be associated with some autoimmune diseases.[1-3] Quartz and cristobalite dusts have been classified as human carcinogens by IARC (International Agency for Research on Cancer) in 1997 (Monograph 68)[2] and recently re-confirmed in class 1 in Monograph 100C.[3] In 1997 the final evaluation included a preamble indicating that "carcinogenicity was not found in all industrial circumstances, but might be dependent on inherent characteristics of the crystalline silica dust or on external factors affecting its biological activity or distribution of its polymorphs". This statement is absent in monograph 100C, but the so called "variability of quartz hazard"[4,5] is much stressed through the whole "mechanisms" section.

Related studies revealed that such variability could be explained by the differences among various crystalline silica sources, caused by the fragmentation path and the association of different kinds of impurities with the quartz particle.[3]

Interestingly, in the same Monograph n° 68 coal dust was classified in class 3 (inadequate evidence of carcinogenicity to humans) in spite of the well known presence of relatively high levels of quartz particles in coal, usually ranging from 0.5 to 8 % of the dust. Coal dust is in fact a complex mixture containing different minerals including quartz, the overall composition depending on origin, particle size and coal seam. One would thus expect that any quartz-containing dust should be considered carcinogenic, unless the presence of carbon modifies the surface properties of the associated quartz particles, hence their pathogenicity.

No lung cancer has been found among miners nor evidenced in animal studies. The effect of coal mine dust on health includes simple pneumoconiosis, emphysema and accelerated loss of lung functions. Quartz increases the fibrogenic potential of coal dusts but no correlation was ever observed between the quartz content of coal mine dusts and their cytotoxicity. Old studies compared the fibrogenic potential of quartz-containing coal dust with the same amount of mere quartz dust, and it resulted that quartz alone was more active than that same quartz amount in coal

dust.[5-8] A loss of biological activity was also found when quartz was ground with coal dust[5,9] which suggests that the low toxicity of quartz-containing coal dust might be due to impurities, such as carbon or other mineral phases at the quartz surface.[9]

In spite of occasional suggestions of a possible protecting role of carbon when in intimate contact with quartz as in coal mine dusts,[16] firm evidence about this has not been provided, nor are available data on the amount of carbon dust required for such effect.

This is the aim of the present study. To this purpose a model system was built, by mixing a well characterized commercial carbon soot[17] with a commercial quartz dust of well known fibrogenicity (Min-U-Sil 5 quartz).

Two different loadings of carbon soot (1 and 10% in weight, respectively) were considered. The mixtures of quartz and soot were then milled in an agate jar to promote intimate contact between the components.

Following a multidisciplinary protocol which proved successful in previous studies on silica, we have associated cell-free and cellular tests.[18,19] On the one hand, some surface properties (charge, surface radicals and free radical generation) known to be involved in the toxicity of crystalline silica were evaluated on carbon-loaded samples and compared with that of the pristine quartz dust. On the other hand, the potential of carbon-loaded quartz to induce harmful effects was then investigated in an alveolar murine macrophage (MH-S) cell line by measuring cytotoxicity, induction of inflammatory responses and oxidative stress.

These latter results were then compared to the effects produced by a mechanical non-milled mixture of the same composition. To evaluate whether the obtained results can be extrapolated to other quartz samples, some of the above tests have been performed on two other samples consisting in milled mixtures of soot and, respectively: i) a very pure quartz[19] (known to be active only in the Fenton reaction); and: ii) a powdered refractory brick from metallurgic industry featuring several components, including quartz, so to cover a range from the purest to the very contaminated quartz dust.

MATERIALS AND METHODS

Materials.

The commercial quartz dust Min-U-Sil 5[2] was purchased from US Silica Company (U.S. Silica, Berkeley Spring plant; lot number 15062696).

Pure quartz (Qz-p) was obtained by grinding in a ball mill (agate jar, Retsch MM200) a very pure natural quartz crystal from Madagascar for 12 h at 27 Hz.

An industrial silica dust (SiO_2 50%. Al_2O_3 43%, Fe_2O_3 1.4%) used for the preparation of refractory bricks was kindly supplied by a metallurgic local industry.

Carbon soot from Sigma-Aldrich s.r.l (St. Louis, MO; lot number 390127-25G) was obtained by resistive heating of graphite. An exhaustive physico-chemical characterization of this carbon dust has been performed by Lopez-Fonseca in 2007.[17] Briefly this material is made up of spherical carbon particles of about 20 nm, with a carbon content of 97.4 wt.%, a hydrogen content of 0.4 wt.%, a nitrogen content of 0.7 wt.%, an oxygen content of 0.8 wt.%, and a sulphur content of 0.8 wt.%. Moreover, the sample contained approximately 10 wt.% of adsorbed hydrocarbons, and the BET surface area is approximately 80 $m^2\ g^{-1}$.

Chemical Reagents.

All reagents were from Sigma-Aldrich, when not otherwise indicated.

5,5'-dimethyl-1-pyrroline-1-oxide (DMPO) was from Alexis Biochemicals (Plymouth Meeting, PA). To minimize contamination by degradation products, DMPO was purified by passing through charcoal then stored in the dark at 4°C.

Preparation of carbon-loaded quartz dusts.

Milling was performed in a ball mill (agate jar, Retsch MM200) for 1 h at 27 Hz. Two different loadings of carbon soot - low (1% wt) and high (10% wt) - were employed. Carbon-loaded quartz are hereafter referred to as QzC1 and QzC10, respectively.

All unloaded quartz samples have been subjected to the same milling process used for mixtures. Moreover, mechanical mixtures of carbon soot (1% and 10%) and Min-U-Sil quartz are referred to respectively as QzC1-mix and QzC10-mix.

Surface area measurements.

The surface area of the dusts was measured by means of the BET method based on N_2 adsorption using a Micromeritics ASAP 2020 apparatus (Norcross, GA). Samples were degassed for several hours prior to N_2 analysis, which was carried out at liquid nitrogen temperature (77K). Surface area measurements have been repeated twice.

Particle morphology

The samples were examined by means of scanning electron microscopy (SEM) in the secondary electron imaging mode (Stereo Scan 420 Leica) at two different magnifications.

ζ-potential measurements.

The ζ-potential of the quartz dusts was evaluated by means of electrophoretic light scattering (ELS) (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.), a technique in which the velocity of particles in an oscillating electric field - which is proportional to their ζ-potential - is measured by light scattering.

Quartz specimens were suspended (6 mg/10 ml) in ultrapure water (MilliQ, Billerica, MA) and sonicated for 2 minutes in ice, to avoid any excessive heating of samples, with a probe sonicator (100 W, 20 kHz, Sonoplus, Bandelin, Berlin, Germany). The ζ-potential was measured after adjusting the pH step by step by addition of either 0.1 M NaOH or 0.1 M HCl.

Free Radical Detection.

Free radical generation was monitored by EPR spectroscopy using DMPO as trapping agent, following a well established procedure.[15,20] EPR spectra were recorded at room temperature on a Miniscope 100 X band EPR spectrometer (Magnettech, Berlin, Germany) at a microwave power level of 10 mW, scan range of 120 G, and modulation amplitude of 1 G. The number of radicals

released is proportional to the intensity of the EPR signal after double integration. Kinetics of free radical yield was followed for at least one hour. Each test was repeated three times.

***$HO^{\bullet}$ radical release**:* 75 mg of each dust was suspended in 500 µL of buffered solution (0.5 M potassium phosphate buffer, pH 7.4) and 250 µL of 0.15 M DMPO. The reaction was started by adding hydrogen peroxide (500 µL of 0.20 M solution in distilled water) to the quartz particle suspension and the radical yield was progressively measured in an aliquot of 50 µL of the suspension.

$COO^{\bullet-}$ radical release: 75 mg of each dust was suspended in 250 µL of 0.15 M DMPO. The reaction was initiated by adding formate ion (250 µL of 1.0 M HCOONa solution in 0.5 M potassium phosphate buffer, pH 7.4) as target molecule. Carboxyl radical yield was measured as previously indicated for hydroxyl radicals.

Detection of surface radicals and paramagnetic centres on the particles.

The EPR spectra of the pristine and mixed dusts were recorded in vacuum at 77K on a Bruker EMX spectrometer operating in the X-band mode (9.5 GHz) following a technique reported in previous crystalline silica studies.[21] The spectra were recorded with the following instrument setting: scan range, 400 G; receiver gain, $1*10^4$; microwave power, 10 mW; modulation amplitude, 1G; scan time, 80 s. Three scans were usually performed.

Cells.

Murine alveolar macrophages (MH-S, a continuous cell line derived from Balb/cJ mice) were provided by Istituto Zooprofilattico Sperimentale "Bruno Ubertini" (Brescia, Italy). Cells were cultured in Petri dishes in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% FBS up to 90% confluence, then incubated in the same culture medium for 24 h, in the absence or presence of silica before the assays. The protein content of cell monolayers, cell suspensions and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). Each result obtained from cell cultures, suspensions, or lysates was related to the amount of cellular proteins in the same sample.

Measurement of extracellular LDH activity.

The cytotoxic effect of silica was measured as leakage of lactate dehydrogenase (LDH) activity into the extracellular medium as previously described,[22] with a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT). Extracellular LDH activity (LDH out) was calculated as a percentage of the total (intracellular + extracellular) LDH activity (LDH tot) in the dish.

Measurement of thiobarbituric acid-reactive substances (TBARS).

TBARS assay, used as a screening method for lipid peroxidation, was performed after a 24 h-incubation using a Synergy HT microplate reader as previously described.[22] TBARS values were expressed as nmol/mg cellular proteins.

Measurement of nitric oxide (NO) synthesis.

After a 24 h-incubation of cells (cultured in 35 mm diameter Petri dishes) in the absence or presence of silica particles, the extracellular medium was tested for the content of nitrite, which is a stable derivative of NO, using the Griess method as previously described.[23] A blank was prepared with RPMI medium and its absorbance was subtracted from the one measured in the samples; absorbance values were corrected for the content of cell proteins and results were expressed as nmol/mg cellular proteins. In order to exclude that our results could be influenced by silica or carbon contamination by endotoxins (such as bacterial lipopolysaccharide), which are potent activators of inducible NOS, we have performed in parallel experiments for nitrite accumulation also in the presence of polymyxin B (10 μg/ml), which is able to bind endotoxin, neutralizing its toxic effects. Nitrite levels were not modified in the presence of polymyxin B (data not shown), so we can affirm that our silica samples, loaded or not with carbon, were free of contaminants, and NO was significantly generated as a result of MH-S cells exposure to silica particles.

Measurement of tumour necrosis factor-α (TNF-α) production.

After a 24 h-incubation of cells (cultured in 35 mm diameter Petri dishes) in the absence or presence of silica particles, the extracellular medium was collected and centrifuged at 13000 x *g* for 2 h. The concentration of the cytokine was determined in the supernatant by using the conventional ELISA kit from Bender MedSystems (Wien, Austria), following the manufacturer's instructions.

Absorbance was measured at 450 nm with a Synergy HT microplate reader. The cytokine amount, corrected for the content of cell proteins, was expressed as percentage increase of TNF-α versus the respective control incubated without silica (assumed as 100%).

Statistical analysis.

All data in text and figures are provided as means ± SEM. Results were analyzed by a one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test (software: SPSS 19.0 for Windows, SPSS Inc., Chicago, IL). $p < 0.05$ was considered significant.

RESULTS

Preparation of carbon-loaded quartz dusts.

The presence of carbon converts the colour of the pristine quartz dust (Figure 1) from white to grey (1%) and to dark black (10%), respectively. Upon milling, carbon-loaded quartz (part E and F) turned slightly darker than the simple mixture of the two components (part C and D), may be because of the intimate contact of carbon with the quartz particle surface documented in the following.

Surface Area.

The original quartz dusts and carbon soot largely differ in specific surface area (Table 1). Low carbon loading (1%) did not significantly change the surface area by respect to the pristine quartz. The surface area increased in the sample QzC10 reaching a value only slightly lower than what expected for a simple mixture.

Particle Morphology.

A morphological characterization (not reported) of the unloaded and carbon-loaded quartz has been performed by means of SEM. No visible modifications in the particle morphology following milling have been observed. The particle size remains in the same range of the pristine quartz, the only difference being a lower number of small particles adhering to bigger ones, as typically observed with quartz dusts obtained by grinding. This suggests the building of a thin carbon layer on quartz preventing electrostatic interaction among the particles.

Surface charge.

Figure 2 shows the ζ-potential values as a function of pH in the 0.5-9.0 pH range for quartz, carbon soot and the two carbon-loaded quartz dusts QzC1 and QzC10.

Quartz exhibits a negative surface charge over the entire pH range never attaining the point of zero charge, as reported in the literature.[24] The shape of the ζ-potential curve is different from that of amorphous silicas[25,26] and pure crystalline silica,[27] likely because of metal contaminants in the pristine quartz. Carbon soot exhibits a negative surface charge only above pH 3, while from pH 0 to

3 is positively charged, being less acidic than quartz.[28] Carbon-loaded quartz dusts are negatively charged over the whole pH range, similarly to pristine quartz. However a shift toward less negative potential values – i.e. toward the carbon soot curve - is observable in the presence of 10% of carbon soot in the whole curve - except at very acidic pH - while no significant shift was observed in the low carbon-loaded quartz.

Generation of oxygen and carbon-centred free radicals.

All samples were tested for their potential to generate either $HO^{\bullet}$ radicals from hydrogen peroxide or carbon-centred radicals, following the homolytic cleavage of a hydrogen-carbon bond in the formate ions, as done in previous studies with quartz dusts.[29]

Figure 3 shows EPR/spin trapping spectra of the DMPO/$HO^{\bullet}$ and DMPO/$COO^{\bullet}$ adducts obtained after contact of both unloaded and carbon-loaded quartz QzC1 and QzC10 with hydrogen peroxide (part A) or with sodium formate (part B). Min-U-Sil quartz generates both hydroxyl and carboxyl radicals[30] (Fig. 3, curve a) while no radical release is observed for carbon soot (data not reported). The presence of carbon at the surface of quartz completely inhibits both free radicals yield even at the lower loading, as shown by the absence of the DMPO adduct in both free radical generation experiments.

Presence of surface radicals and paramagnetic centres.

Figure 4 shows the EPR spectra of Min-U-Sil, QzC1 and QzC10 recorded under vacuum and at low temperature. Quartz dust (curve a) exhibits a typical spectrum corresponding to the overlapping of Silicon- and Oxygen-centred radicals (Si-O, $Si\text{-}O_2$, $Si\text{-}O_3$, O_2^-) produced by the cleavage of Si-O-Si bonds during the grinding process and subsequent reaction with atmospheric oxygen.[21,31] The paramagnetic centres of $[AlO_4]^0$ and titanium impurities[32] typical of commercial quartz dusts are also visible.

Carbon-loaded quartz dusts (curves b and c) exhibit the typical spectrum of carbon soot (not reported). The intensity of the signal increases from QzC1 to QzC10. In the spectrum of QzC10 two different signals are seen, both centred at g = 2.003, due to two different carbon radical species. One

signal is intense and broad and can be assigned to interacting spins, the other less intense and sharper signal is due to localized paramagnetic species.[33] In both cases the signal of quartz dust is no more visible. Conversely, the typical signal of quartz are still present in the non milled mixture of 1% carbon and quartz (curve d). This is clear evidence of a basic difference in surface and subsurface layers between mixed and milled samples.

Cytotoxicity.

The cytotoxic effects observed after a 24 h-incubation with pristine and carbon-loaded quartz in MH-S cells are reported in figure 5. As already reported[30] the pristine quartz exhibited a concentration-dependent cytotoxicity, measured as leakage of intracellular LDH activity into the extracellular medium. The presence of carbon decreased - but not completely suppressed - the cytotoxicity of quartz nearly at the same extent with QzC1 and QzC10. Indeed, at the highest concentration tested (80 μg/cm^2) both carbon-loaded dusts were still cytotoxic, although at a significantly lower degree than quartz. When carbon soot (1 and 10%) and quartz dusts (40 and 80 μg/cm^2) were simply mixed with a spatula and not milled, the presence of carbon did not exert any protective effect on quartz cytotoxicity (Figure 5, inset). Moreover, carbon soot alone did not show any cytotoxic effect (data not shown).

Cellular oxidative stress.

The oxidative stress caused by the dusts was evaluated through their ability to induce the oxidation of cell membrane lipids (expressed as TBARS generation) (Figure 6), after a 24 h-incubation with MH-S cells. The accumulation of lipid peroxidation markers increased with the concentration of quartz and this increase was significant from 20 to 80 μg/cm.[30] The presence of carbon at the surface of quartz decreased significantly the ability of pristine quartz (at 40 and 80 μg/cm^2) to oxidize membrane lipids. In contrast, simply mixed dusts (40 and 80 μg/cm^2) did not have any inhibitory effect on lipid peroxidation markers accumulation (Figure 6, inset). Moreover, carbon soot alone did not have any oxidative activity as assessed by the TBARS measurement (data not shown).

Induction of inflammation.

The ability of quartz and carbon-loaded quartz to induce an inflammatory response was carried out by measuring in the culture medium of MH-S cells after a 24 h-incubation the extracellular levels of nitrite (a stable derivative of NO in oxygenated systems) and the production of TNF-α. The accumulation of nitrite and TNF-α were significantly increased by pristine quartz (Figures 7 and 8), as previously reported.[30] Both carbon-loaded dusts QzC1 and QzC10 decreased significantly the amount of NO synthesized by the cells when compared to the one produced in the presence of pristine quartz (Figure 7). The TFN-α generation decreased similarly in carbon-loaded dusts if compared to the pristine quartz (Figure 8). In these experiments too, simply mixed dusts (40 and 80 $\mu g/cm^2$) did not have any inhibitory effect on nitrite and TNF-α accumulation (Figures 7 and 8, inset). Moreover, carbon soot alone did not exert any induction of nitrite and TNF-α accumulation as assessed in NO and TNF production measurements (data not shown).

Effect of carbon soot at the surface of other crystalline silica dusts.

Results concerning Qz-p are reported in Figure 9. Part A illustrates the generation of $HO^{\bullet}$ free radicals via Fenton-like reaction. As already reported,[19] such sample is able to generate $HO^{\bullet}$ radicals. Addition of carbon soot at the surface of quartz completely inhibited this ability at both amount of carbon, as reported above for Min-U-Sil dust.

The cytotoxic effects observed after a 24 h-incubation with Qz-p and carbon-loaded quartz in MH-S cells are reported in figure 9. When MH-S cells were incubated with increasing concentrations of Qz-p, obtained by grinding in a ball mill a very pure natural quartz from Madagascar, Qz-p exhibited a significant concentration-dependent cytotoxicity, similarly to Min-U-Sil (Figure 9B). But in this case, the presence of 1% carbon did not modify the cytotoxicity of this pure quartz, whereas the presence of 10% carbon completely suppressed the effect of the corresponding Qz-p (Figure 9B). Moreover, using Qz-p we confirmed the data observed with the higher concentrations of Min-U-Sil on nitrite production. When MH-S cells were incubated with Qz-p at 80 $\mu g/cm^2$, Qz-p significantly increased the nitrite accumulation. Again, the presence of 1%

carbon did not modify this increase, whereas 10% carbon completely suppressed the effect of Qz-p on nitrite accumulation (Figure 9C).

The powdered refractory brick also exhibited a Fenton activity which was suppressed when milled with carbon (not reported for brevity). This specimen both alone and when milled with carbon was non cytotoxic nor inflammogenic on MH-S cells (data not shown for brevity) likely because of the high aluminum content

DISCUSSION

All results here reported point to a beneficial effect of carbon on quartz, when upon milling is somehow spread onto the quartz surface, as already reported for other metal nanoparticles.[34-36] .The effects were the same on QzC1 and QzC10 a suggesting that 1% of carbon is sufficient for the inactivation at the quartz surface of the reactive sites associated to the adverse responses to quartz dusts.

Two different mechanisms may be hypothesized for the inactivation of quartz caused by the associated carbon:

1 carbon hides and/or quenches the active sites at the quartz surface;

2 carbon acts as a scavenger toward free radicals as several other carbon based materials, e.g. carbon black,[37] multi-walled carbon nanotubes,[38] and fullerenes.[39]

Because inactivation takes place only with the milled samples and not with mixtures, we assume that mechanism 1 is the most likely one. Mechanisms 2 could also take place but only with particles close one to the other.

Support to this interpretation comes from the EPR evidence that the surface radicals generated by grinding,[19,21] disappeared in carbon-loaded dusts. Conversely, the mechanical mixture with 1% of carbon soot – where loose contact between the particles takes place - only reduced the intensity of the EPR signal of the surface radicals, which shows that a specific interaction between radicals at the quartz surface and carbon particles (probably through carbon centred radicals) occurred because of milling. Accordingly, the presence of carbon in milled dusts suppresses the potential to generate free radical species (Fenton activity and homolytic rupture of the C-H bond). This confirms that carbon particles hinder the active surface sites, inhibiting a direct contact with target molecules.

Cytotoxicity, oxidative stress and inflammation

The modifications of surface properties by carbon particles completely suppressed the signs of oxidative stress and inflammation induced by pristine quartz to MH-S cells. A decrease of membrane integrity (indicated by the LDH leakage) was still present in the presence of carbon-

loaded dusts at the highest dose, although it was significantly reduced in comparison to pristine quartz. This effect might be ascribed to some mechanical damage to the membrane or other still unknown factors not relatable to oxidative stress or inflammogenicity.

The cytotoxicity of silicas has been generally related either to free radical generation or to the interaction between cell membranes and chemical functionalities at the particle surface.[40] Cell membrane damage could be caused by strong hydrogen bonding of undissociated silanols and charges of the dissociated ones interacting with the membrane components.

Hindering of surface radicals, suppression of free radicals generation and the different topography of silanol groups may all account for the remarkable reduction in cytotoxicity. The mechanism previously hypothesized for quartz toxicity[29,41] involves different interactions between the particle surface and cell components. Modifications as those caused by carbon coverage may cause different cell activation mechanisms, thus leading to a great decrease of oxidative stress and synthesis of pro-inflammatory cytokines such as TNF-α and subsequently to a lower cytotoxicity.

Carbon in coal mine dusts could indeed be responsible for decreasing the pathogenic potential of quartz, as hypothesized time ago by Donaldson and Borm (1998).[4]

In a recent multidisciplinary study[42-45] on industrial quartz dusts two were nearly inactive and two very active both in cellular toxicity and "*in vivo*" inflammogenicity. Inactivity was then mainly ascribed to their aluminium content, much higher in the two "less toxic" samples. Interestingly one of the non toxic samples also contained substantial amounts of carbon impurities which might have contributed to their inertness as in the present case.

The inhibition of cellular responses only occurs when carbon and quartz are in intimate contact as in the milled samples. Several factors may contribute to the absence of inhibition observed in the simply mixed samples. Quartz and carbon particles show different physico-chemical properties (size, surface hydrophilicity, surface charge), that affect agglomeration/aggregation kinetics, cellular uptake and localization within the cell.[46] In the simple mixtures carbon is not firmly associated to the silica particles. As a consequence when in contact with an aqueous environment -

such as biological fluids or culture media - quartz particles may depart from the carbon ones and are likely to be taken up by cells in a different way.

Further investigations are needed to understand better the carbon-silica/cell interaction, to verify: i, if the same protective effects may be obtained with different carbon samples; ii, the amount of carbon required for each kind of silica source; iii, the extent of milling required to "activate" the effect of carbon, also in view of possible uses of carbon-based materials as a mean to inactivate hazardous silica dusts. The present study however clarifies the molecular basis of a remarkable case of "variability of quartz hazard".

FIGURES

Figure captions

Figure 1. Visible aspect of: (A) pristine quartz, (B) carbon soot, (C and D) quartz and carbon (1% and 10%) simply mixed with a spatula and (E and F) carbon-loaded samples obtained by milling, hereafter indicated as QzC1 and QzC10.

Figure 2. ζ-potential of pristine quartz (Qz), low and high carbon-loaded quartz (QzC1, QzC10), and carbon soot (C-soot) as a function of pH. The ζ-potential was measured after adjusting the pH step by step by addition of either 0.1 M NaOH or 0.1 M HCl.

Figure 3. Free radical generation from aqueous suspension of the pristine and carbon-loaded quartz dusts. EPR spectra of DMPO-HO• (A) and DMPO-$COO^{\bullet -}$ (B) adducts of (a) pristine quartz (Qz), (b) low carbon-loaded quartz (QzC1) and (c) high carbon-loaded quartz (QzC10). Hydroxyl radicals were obtained via a Fenton-like reaction, in the presence of H_2O_2, while carboxyl radicals via homolytic cleavage of a C-H bond in the formate ions. All spectra have been collected after 60 min of incubation.

Figure 4. EPR spectra of a) pristine quartz (Qz), b) low carbon-loaded quartz (QzC1), c) high carbon-loaded quartz (QzC10) and d) simply mixed quartz and carbon QzC1 mix recorded in vacuum and at low temperature (77K). The spectrum c was divided ten times while spectra b and d were multiplied ten times in order to fit them into the figure. The spectra were recorded with the following instrument setting: scan range, 400 G; receiver gain, 1*104; microwave power, 10 mW; modulation amplitude, 1G; scan time, 80s; and three scans.

Figure 5. Effect of the various dusts on LDH release into the extracellular medium of MH-S cells. Cells were incubated for 24 h in the absence (0, control) or presence of quartz (Qz), low carbon-loaded quartz (QzC1), and high carbon-loaded quartz (QzC10) at the concentration of 10, 20, 40, 80 $\mu g/cm^2$. After the incubation, LDH activity was measured in duplicate and calculated as described

in the Methods section. Data are presented as means ± SEM (n=5). * control *vs*. all the other experimental conditions: *, $p < 0.05$; **, $p < 0.001$ and ***, $p < 0.0001$. ###, $p < 0.0001$ Qz 80 µg/cm^2 *vs*. QzC1 80 µg/cm^2. °, $p < 0.05$ Qz 40 µg/cm^2 *vs*. QzC10 40 µg/cm^2 and °°°, $p < 0.0001$ Qz 80 µg/cm^2 *vs*. QzC10 80 µg/cm^2. Inset: Effect of silica particles incubated in the absence or presence of 10% (QzC10 mix) or 1% carbon (QzC1 mix) on LDH release into the extracellular medium of MH-S cell cultures.

Figure 6. Effect of the various dusts on thiobarbituric acid-reactive substances (TBARS) production in MH-S cells. Cells were incubated for 24 h in the absence (0, control) or presence of quartz (Qz), low carbon-loaded quartz (QzC1), and high carbon-loaded quartz (QzC10) at the concentration of 10, 20, 40, 80 µg/cm^2. TBARS production was measured as described in the Methods section. Each measurement was performed in duplicate, and data are presented as means ± SEM (n=3). * control *vs*. all the other experimental conditions: *, $p < 0.05$ and ***, $p < 0.0001$. ##, $p < 0.001$ Qz 40 µg/cm^2 *vs*. QzC1 40 µg/cm^2 and ###, $p < 0.0001$ Qz 80 µg/cm^2 *vs*. QzC1 80 µg/cm^2. °°°, $p < 0.0001$ Qz 40 µg/cm^2 *vs*. QzC10 40 µg/cm^2 and Qz 80 µg/cm^2 *vs*. QzC10 80 µg/cm^2. Inset: Effect of silica particles incubated in the absence or presence of 10% (QzC10 mix) or 1% (QzC1 mix) carbon on TBARS production in MH-S cell cultures.

Figure 7. Effect of the various dusts on extracellular levels of nitrite in MH-S cell cultures. Cells were incubated for 24 h in the absence (0, control) or presence of quartz (Qz), low carbon-loaded quartz (QzC1), and high carbon-loaded quartz (QzC10) at the concentration of 10, 20, 40, 80 µg/cm^2. After incubation, nitrite accumulation in the extracellular medium was determined as described in the Methods section. Each measurement was performed in duplicate, and data are presented as means ± SEM (n=3). * control *vs*. all the other experimental conditions: ***, $p < 0.0001$. ###, $p < 0.0001$ Qz 40 µg/cm^2 *vs*. QzC1 40 µg/cm^2 and Qz 80 µg/cm^2 *vs*. QzC1 80 µg/cm^2. °°°, $p < 0.0001$ Qz 40 µg/cm^2 *vs*. QzC10 40 µg/cm^2 and Qz 80 µg/cm^2 *vs*. QzC10 80 µg/cm^2. Inset:

Effect of silica particles incubated in the absence or presence of 10% (QzC10 mix) or 1% (QzC1 mix) carbon on extracellular levels of nitrite in MH-S cell cultures.

Figure 8. Effect of the various dusts on extracellular levels of TNF-α in MH-S cell cultures. Cells were incubated for 24 h in the absence (0, control) or presence of quartz (Qz), low carbon-loaded quartz (QzC1), and high carbon-loaded quartz (QzC10) at the concentration of 10, 20, 40, 80 μg/cm^2. After incubation, TNF-α accumulation in the extracellular medium was determined as described in the Methods section. Results are expressed as percentage increase of TNF-α versus the respective control incubated without silica (assumed as 100%). Each measurement was performed in duplicate, and data are presented as means ± SEM (n = 3). * control vs. all the other experimental conditions: *, p<0.05; ***, p < 0.0001 °°, p < 0.005 Qz 20 μg/cm^2 *vs.* QzC10 20 μg/cm^2; °°°, p < 0.0001 Qz 40 μg/cm^2 *vs.* QzC10 40 μg/cm^2 and Qz 80 μg/cm^2 *vs.* QzC10 80 μg/cm^2. Inset: Effect of silica particles incubated in the absence or presence of 10% (QzC10 mix) or 1% (QzC1 mix) carbon on extracellular levels of TNF-α in MH-S cell cultures.

Figure 9: A) Free radical generation from aqueous suspension of the pristine and carbon-loaded pure quartz dusts. EPR spectra of DMPO-HO• of (a) pristine quartz from Madagascar (Qz-p), (b) low carbon-loaded quartz (Qz-pC1) and (c) high carbon-loaded quartz (Qz-pC10). All spectra have been collected after 60 min of incubation. B) Effect of pure quartz (Qz-p) on LDH release in the extracellular medium of MH-S cells. Cells were incubated for 24 h in the absence (0, control) or presence of Qz-p, low carbon-loaded pure quartz (Qz-pC1), and high carbon-loaded pure quartz (Qz-pC10) at the concentrations of 40 and 80 μg/cm^2. After the incubation, LDH activity was measured in duplicate and calculated as described in the Materials and Methods section. Data are presented as means $\pm$ SEM (n=3). * control *vs*. all the other experimental conditions: *, p < 0.05; and ***, p < 0.0001. °°, p < 0.002 Qz-p 80 μg/cm^2 *vs*. Qz-pC10 80 μg/cm^2. C) Effect of Qz-p on extracellular levels of nitrite in MH-S cell cultures. Cells were incubated for 24 h in the absence (0, control) or presence of Qz-p, low carbon-loaded pure quartz (Qz-pC1), and high carbon-loaded pure

quartz (Qz-pC10) at the concentrations of 40 and 80 µg/cm^2. After incubation, nitrite accumulation in the extracellular medium was determined as described in the Materials and Methods section. Each measurement was performed in duplicate, and data are presented as means ± SEM (n=3). * control *vs*. all the other experimental conditions: ***, $p < 0.0001$. °°, $p < 0.002$ Qz-p 80 µg/cm^2 *vs*. Qz-pC10 80 µg/cm^2.

TABLES.

Table 1. Specific surface.

Samples	Specific Surface Area (BET) m^2/g
Carbon soot	80.0*
Qz	5.2 ±0.7
QzC1	4.9 ± 0.9
QzC10	11.6 ± 0.2

* from ref. 18

Figure 1

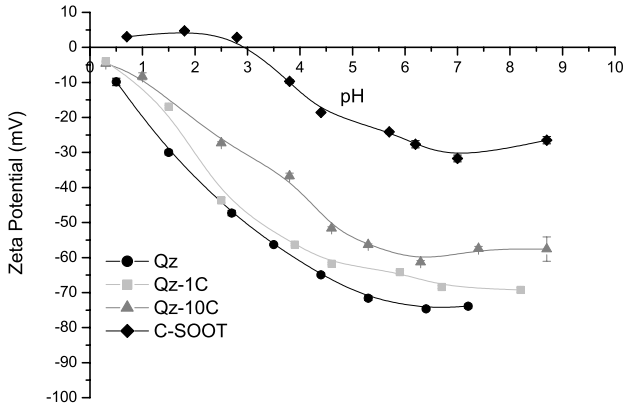

Figure 2

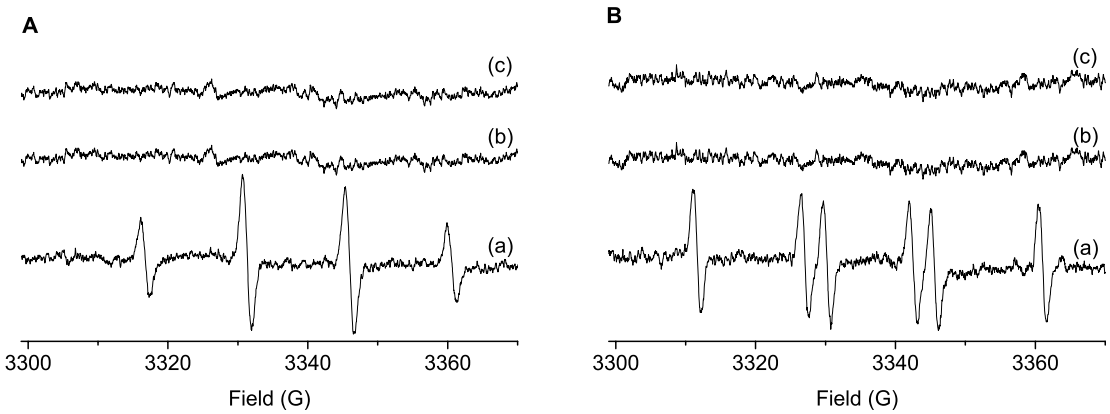
A
(c)
(b)
(a)
3300
3320
3340
3360
Field (G)
B
(c)
(b)
(a)
3300
3320
3340
3360
Field (G)

Figure 3

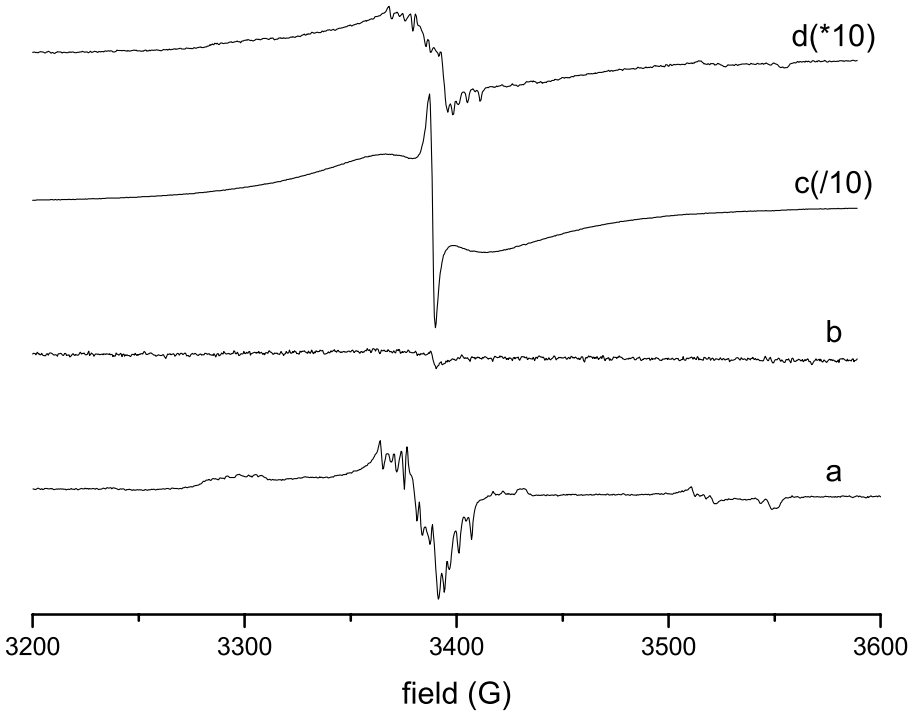

Figure 4

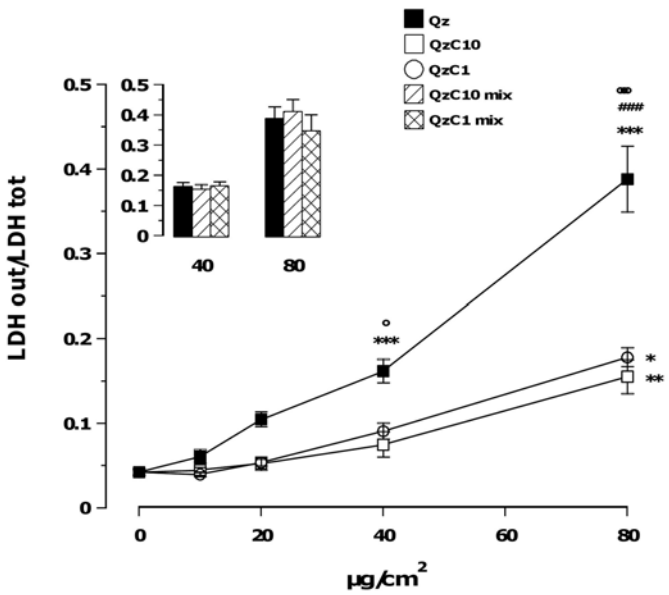
Qz
QzC10
QzC1
QzC10 mix
QzC1 mix
LDH out/LDH tot
µg/cm²
0
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80
40
80
***
°
°°
###
*
**

Figure 5

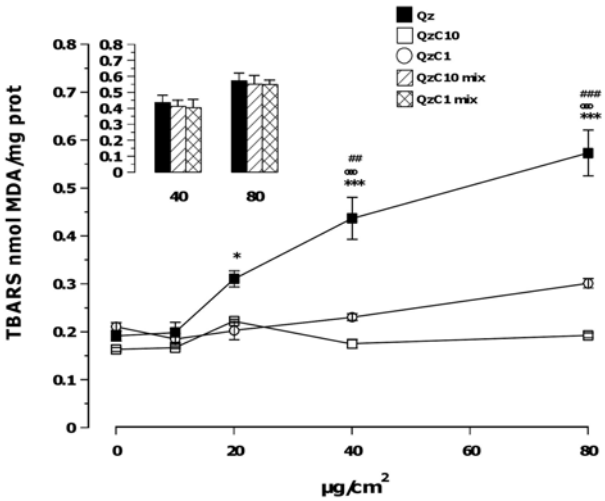
Qz
QzC10
QzC1
QzC10 mix
QzC1 mix
TBARS nmol MDA/mg prot
µg/cm²
40
80
*
##
ααα
***
###
ααα
***

Figure 6

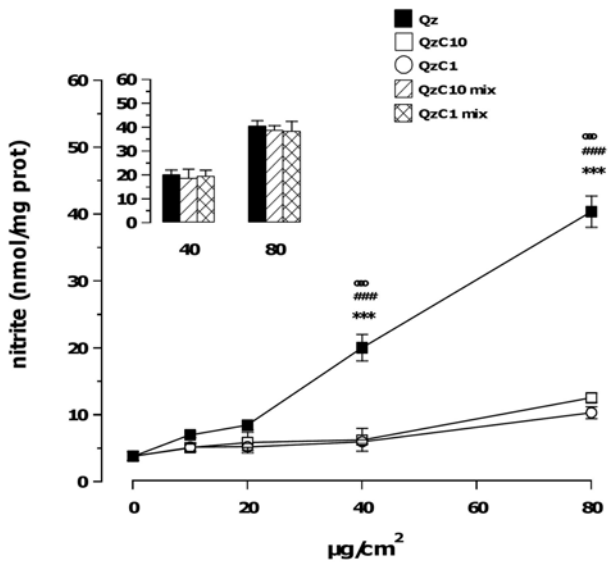
Qz
QzC10
QzC1
QzC10 mix
QzC1 mix
nitrite (nmol/mg prot)
µg/cm2
&&
###
***
0
20
40
60
80

Figure 7

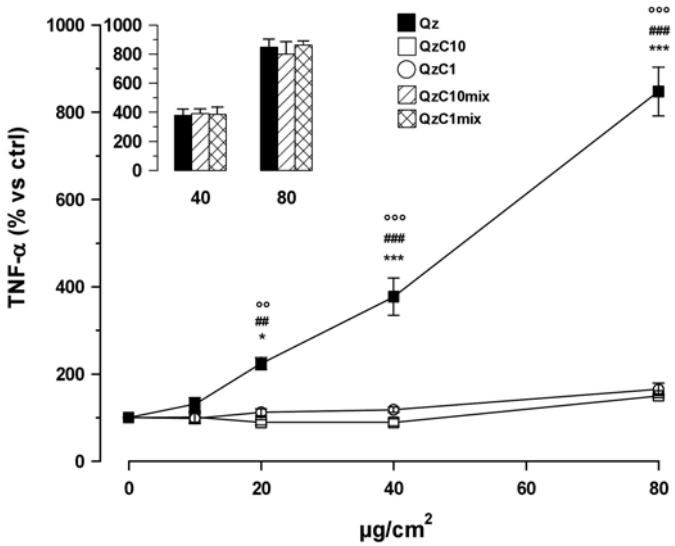
TNF-α (% vs ctrl)
µg/cm2
Qz
QzC10
QzC1
QzC10mix
QzC1mix
°°
##
*
°°°
###
***
°°°
###
***
0
200
400
600
800
1000
0
20
40
60
80
40
80

Figure 8

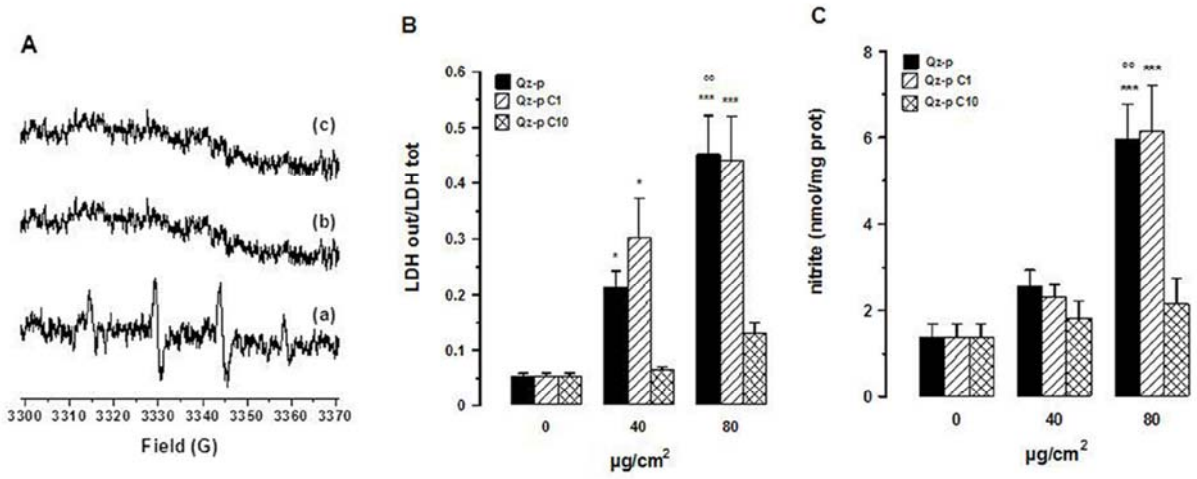
A
(c)
(b)
(a)
3300 3310 3320 3330 3340 3350 3360 3370
Field (G)
B
Qz-p
Qz-p C1
Qz-p C10
LDH out/LDH tot
0.6
0.5
0.4
0.3
0.2
0.1
0
0
40
80
µg/cm2
C
Qz-p
Qz-p C1
Qz-p C10
nitrite (nmol/mg prot)
8
6
4
2
0
0
40
80
µg/cm2

Figure 9

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Author Contributions

‡ M.G., M.T. and S.D. equally contributed to this study.

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ABBREVIATIONS

EPR, Electron Paramagnetic Resonance Spectroscopy; DMPO, 5,5-dimethyl-pirroline-N-oxide; ELS, electrophoretic light scattering; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid-reactive substances; NO, nitric oxide; MH-S, murine alveolar macrophages; IARC, International Agency for Research on Cancer; Qz-p, pure quartz; QzC, carbon-loaded quartz; SEM, scanning electron microscopy; TNF-α, tumour necrosis factor-α.

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