DO SILICON-OXYGEN RADICALS PLAY A ROLE IN THE QUARTZ-INDUCED HEMOLYSIS AND FIBROGENICITY?

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INTRODUCTION

In an earlier communication[1] from our laboratory it was reported that mechanical crushing of coal and quartz under normal air atmosphere generates of free radicals on the particle surfaces, and that these radicals decay with time, hence pointing to a higher toxicity of fresh dusts in relationship to pneumoconiosis and silicosis. More recently Fubini et al.[2] have also reported the detection by electron spin resonance (ESR) of the formation of SiO_2^- and Si_2^--type of radicals from quartz particles crushed under atmospheric conditions. In agreement with earlier ESR studies on single crystals of quartz crushed under high vacuum ($\sim 10^{-10}$ torr)[3] and subsequent exposure to air,[3] and to other gases,[4] these radicals were identified[2] as being formed by the homolytic cleavage of the Si-O-Si bonds and the reactions of the $Si^•$ and $SiO^•$ radical with atmosphere. Fubini et al.[2] also suggested that these radicals might be involved in the mechanism of the fibrotic action by silica, either by transforming the particle surface into a selective oxidating agent or as an initiator of a sequence of reactions leading to fibrosis. Earlier Gabor and Anca[5] had reported that lipid peroxidation caused by free radicals on the silica surface might be involved in the red blood cell membrane damage. Thus far, however, no parallel cytotoxicity, fibrogenicity, and free radical studies on a given quartz dust sample have been reported, except for some earlier work from our laboratory.[1,6,7] We now present more recent results obtained from parallel cytotoxicity, fibrogenicity, and free radical measurements on a freshly made quartz dust. The dust's free radical content was measured using ESR spectroscopy while its cytotoxicity potential was estimated via hemolysis. Hemolysis was employed as the toxicity test because it is a widely used method for estimating the potential of a dust for disrupting the cell membrane.[8] The fibrotic potential was followed by measuring the dust-induced lipid peroxidation, using linoleic acid as a model lipid. As discussed below, the results obtained suggest new clues to the mechanism of the quartz-related cytotoxicity and fibrogenicity.

MATERIALS AND METHODS

Reagents

Crystalline quartz particles with a size range of 0.2–2.5 mm were obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, University Park, Pennsylvania. These particles were crushed in air to obtain quartz dust samples with particle sizes smaller than 20 microns. We chose to work with a dust with mixed particle sizes, rather than a specific range, as an effort to simulate the mining atmosphere. An agate mortar-pestle arrangement was used for the crushing and grinding because of the close similarity of the structure of agate to that of quartz. Diethylenetriaminepentaacetic acid (DETAPAC) were purchased from Sigma. All other chemicals were purchased from Fisher or Aldrich.

Hemolysis Experiments

Hemolytic activity of silica was measured, following an establishsd procedure,[9] as the amount of hemoglobin released from a 2% suspension of sheep erythrocytes after incubation with 10 mg of silica dust for one hour at 37°C. The hemoglobin release was estimatsd via the absorbance at 540 nm using a Gioford spectrophotometer. The procedure was calibrated by substituting the silica dust by a phosphate buffer solution as a negative control (background) and 0.5% Triton-X-100 as a positive control (100% hemolysis). The percentage of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = (I_{silica} - I_{neg}) / (I_{pos} - I_{neg})$$

where I_{silica} is ths absorbance after incubation with the silica dust, while I_{neg} and I_{pos} are those with buffer only and 0.5% Triton-X-100, respectively.

Lipid Peroxidation Measurements

Peroxidation of the polyunsaturated lipid linoleic acid (cis-9-cis-12-octadecadienoic acid) by freshly ground or aged silica was monitored using a fluorescence method[10] with minor modifications. The reaction mixture in a total volume of 0.5 ml contained freshly ground or aged silica and 20 µl of 0.52 mM linoleic acid emulsion in 95% ethanol in HEPES buffer (pH 7.4) with calcium and glucose. The mixture was heated for one hour in a shaking water bath at 37°C. This procedure was followed by the addition and mixing of 0.5 ml of 3% sodium dodecyl sulfate and then of 2.0 ml 0.1 N HCl, 0.3 ml 10% phosphotungstic acid and 1.0 ml 0.7% 2-thiobarbituric acid. The mixture was then heated for 30 min at 95–100°C and the reactive substance formed was extracted with 5 ml 1-butanol after cooling. The extraction was then centrifuged at 3000 rpm for one minute and the fluorescence of the butanol layer was measured using a

515 nm excitation and 555 nm emission, with a Perkin-Elmer fluorospectrophotometer (Model MPG-36). Malondialdehyde standards were prepared from 1,1,3,3,-tetramethoxypropane to obtain a calibration curve, which was used for calculating the amounts of malondialdehyde produced.

ESR Measurements

ESR spectroscopy was used for identifying the crushing-induced silicon-oxygen radicals, and to follow their concentration as described elsewhere.[1,6,7] The ESR measurements were made with a Bruker ER 200D spectrometer operating at X-band (~9.5 GHz) frequencies, and 100 kHz magnetic field modulation. The magnetic field was calibrated with a self-tracking NMR gaussmeter (Bruker, model ERO35M). The microwave frequency was measured with a Hewlett-Packard, Model 5340A, digital frequency counter. All ESR measurements were made at room temperature.

RESULTS AND DISCUSSION

Figure 1 shows a typical, room temperature, ESR spectrum of freshly ground quartz particles. The spectrum is not identical but similar to those reported earlier for the measurements made at room temperature and ambient air environment.[1,2] Here we focused on the major species, characterized by g = 2.0015, and assigned to a combination of silicon-oxygen radicals.[1,2] To correlate the radical content with hemolysis, it was necsssary to control the radical concentration. The first method used for this was thermal annealing. Thus the free radical concentration was measured via ESR (at room temperature) after thermal annealing from 50° to 800°C for 30 minutes at each temperature. Figure 2 shows the change in the radical concentration on thermal treatment (Plot A) and the corresponding hemolysis msasurements (Plot B). The data for the samples heated above 300°C show that while the free radical content decreases sharply with the heat treatment above 300°C, the hemolytic potential remains virtually unchanged for heating up to 550°C, and starts to decrease on further heating only. It, thus, follows that there is little, if any, direct correlation between the concentration of the free radicals and the hemolytic potential of the dust samples.

Second, measurements of both the radical concentration and the hemolytic potential were made at several time intervals after the dust preparation. Figure 3 shows the time dependence of the free radical concentration on storing the dust in air after grinding (Plot A) and the hemolysis induced by the same sample (Plot B). It is seen that while the radical concentration decreases with a half-life of about one and a half day, in agreement with our earlier studies,[1,5,7] the hemolytic potential does not change noticeably over at least two weeks, again showing that the grinding-induced radicals on the quartz particles do not play any direct role in the mechanism of ths hemolysis by quartz particles.

As the third method for controlling the radical concentration, some freshly ground quartz particles were boiled in a phosphate buffer for about 30 minutes. ESR measurements on thess samples showed that their radical concentration decreased to about 10%, while their hemolytic activity decreased to almost zero. In order to find if this decrease was related to the silicon-oxygen radicals, experiments were

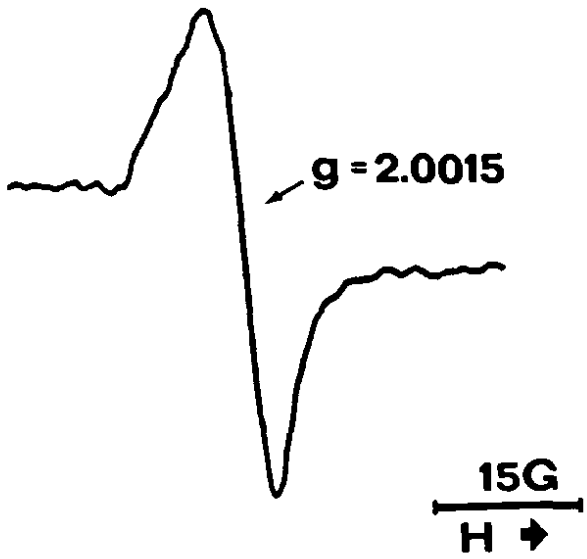

Figure 1. A typical, room temperature, ESR spectrum of freshly ground quartz particles.

conducted as an attempt to restore the hemolytic activity. It was found that while an exposure to a phosphate buffer or a $KMnO_4$ solution (a strong oxidant) did not restore the hemolytic activity, the addition of DETAPAC, a strong metal chelate, to the incubation medium restored the activity to about 60%. Thus, ths boiling-induced reduction in the silica's hemolytic potential cannot be attributed to the loss of the radicals on boiling, since it is unlikely that the addition of DETAPAC could restore the silicon-oxygen radicals. These results seem to indicate that the attachment of metal ions to the particle surfaces causes the loss in the hemolytic activity by quenching certain reactive (surface) sites. This conclusion is not unprecedented since earlier hemolysis studies[8] have shown that the presence of metal ions such as Al^{3+} causes a significant decrease in the quartz dust's hemolytic potential. We indeed confirmed that addition of Al^{3+}, Cu^{2+}, or Fe^{2+} ions, at about 1.0 mM concentration, to the incubation medium results in the loss of the hemolytic activity. The new result obtained here is that the subsequent addition of DETAPAC restores it, implying that the metal ions were only loosely bonded to the silica surface.

The above results are consistent with an earlier suggestion[8] that surface silanol (SiOH) groups play a key role in the mechanism of hemolysis by quartz particles. Metal ions are expected to be bonded via the surface silanol (SiOH) groups by replacing the H^+ ions, thus reducing the number of silanol groups responsible for red blood cell membrane damage.[8] Infrared studies on heated silica-gel[11] and silica surfaces[12] demonstrated that silanol groups are formed on the silica surface, and that these moieties are annealed only if silica is heated to higher than 700°C.[11,13]. Since the present work shows that the hemolytic activity of silica decreases markedly on heating to 700°C (Figure 2), the role of the silanol groups in the hemolysis by silica seems fairly well establishsd. This finding is consistent with an earlier

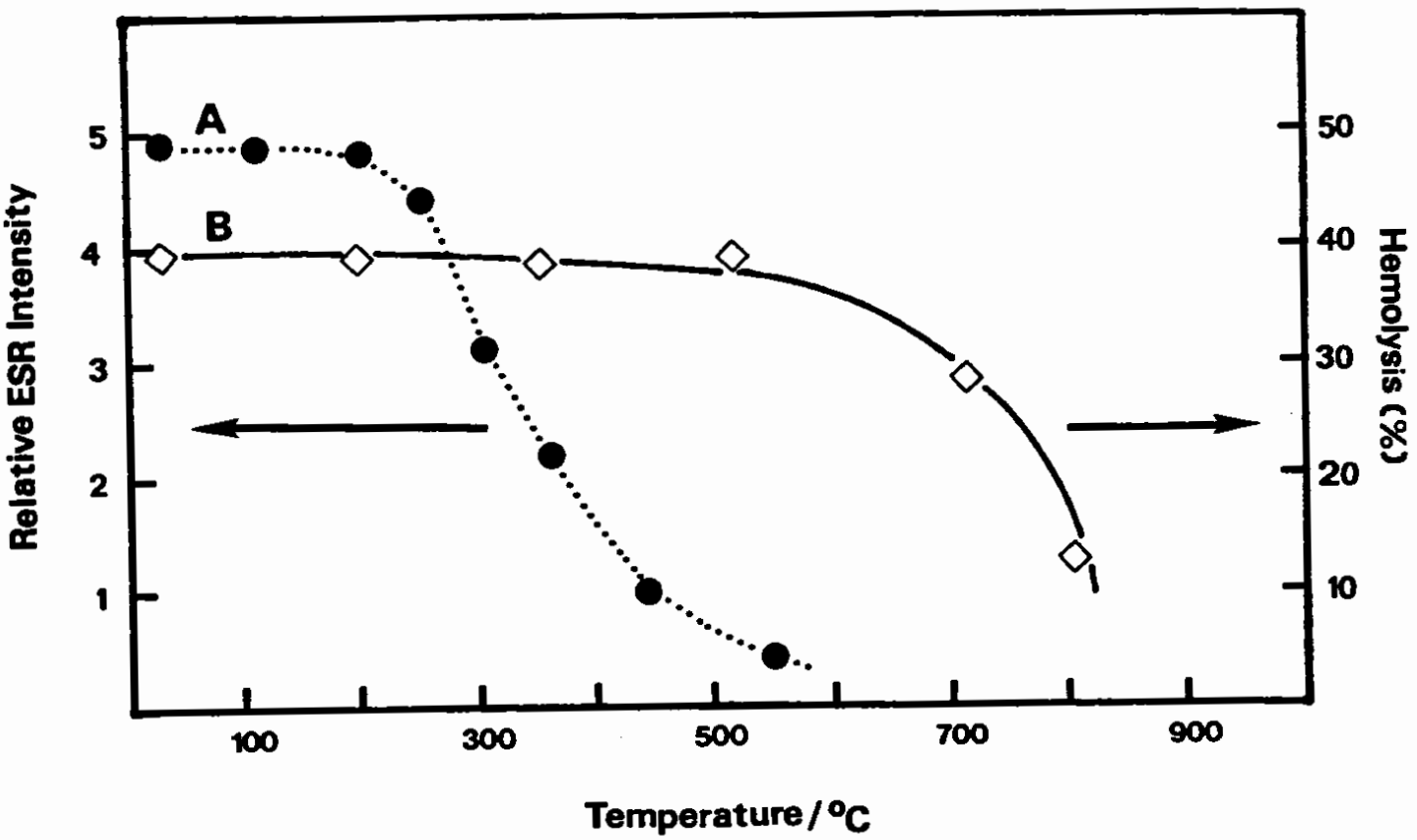

Figure 2. The effect of heating on the ESR intensity of the grinding-induced silicon-oxygen radicals, plot A(•), and silica-induced hemolysis by the same samples, plot B (◇).

report[8] of the reduction in the silica toxicity by Al^{3+} and polyvinyl-pyridine-N-oxide (PVPNO).

For obtaining further clues to the mechanism of silica's fibrogenicity, we investigated the possible relationship between silicon-oxygen radicals on fresh dust particles and the dust's lipid peroxidation potential by parallel measurements of the time dependence of radical content by ESR and of the silica-induced lipid peroxidation using linoleic acid as a model lipid.[10] Figure 4 shows the time dependence of the lipid peroxidation. It is seen that the ability of freshly ground silica to peroxidize a lipid decreases on storage, since the rate of silica-induced lipid peroxidation declined markedly over the first 48 hours after grinding and remained fairly constant thereafter. The similarity of the time dependence of the lipid peroxidation (Figure 4) with the decay behavior of the silicon-oxygen radicals (Figure 3, plot A) indicates that these radicals might be directly or indirectly involved in ths silica-induced lipid peroxidation, which may result in a progressive degeneration of the membrane structure and eventual loss of membrane activity[14]

In conclusion, this work shows that the fracture-induced silicon-oxygen radicals are not directly involved in the mechanism of the erythrocyte hemolysis by quartz. This is consistant with earlier reports which suggest that dust-induced hemolysis and lipid peroxidation proceed via independent mechanisms.[15,16] Thus the hypothesis[5] that lipid peroxidation caused by free radicals on the silica surface might be directly involved in the erythrocyte membrane damage does not seem likely. However, these radicals might be directly or indirectly involved in an oxidative-type chain reaction leading to macrophage membrane perturbation through lipid peroxidation and eventual fibrosis as noted earlier.[2,6,7] It is interesting to note that fibrotic action, as a result of failed phagocytosis, was suggestsd to be due to the perturbation of macrophage membrane and the consequent release of a macrophage fibrotic factor.[17] Recent ESR studies have shown that silica particles release •OH radicals in the presence of exogenous H_2O_2[18] and even without it,[7] and that the amount of the •OH radicals formed decreases with the "aging" of the quartz dust.[6,7]. Thus it is suggested that the •OH radical related mechanism of fibrogenesis by silica might be a fruitful new approach to understanding the pathogenesis of the silica-induced lung injury.

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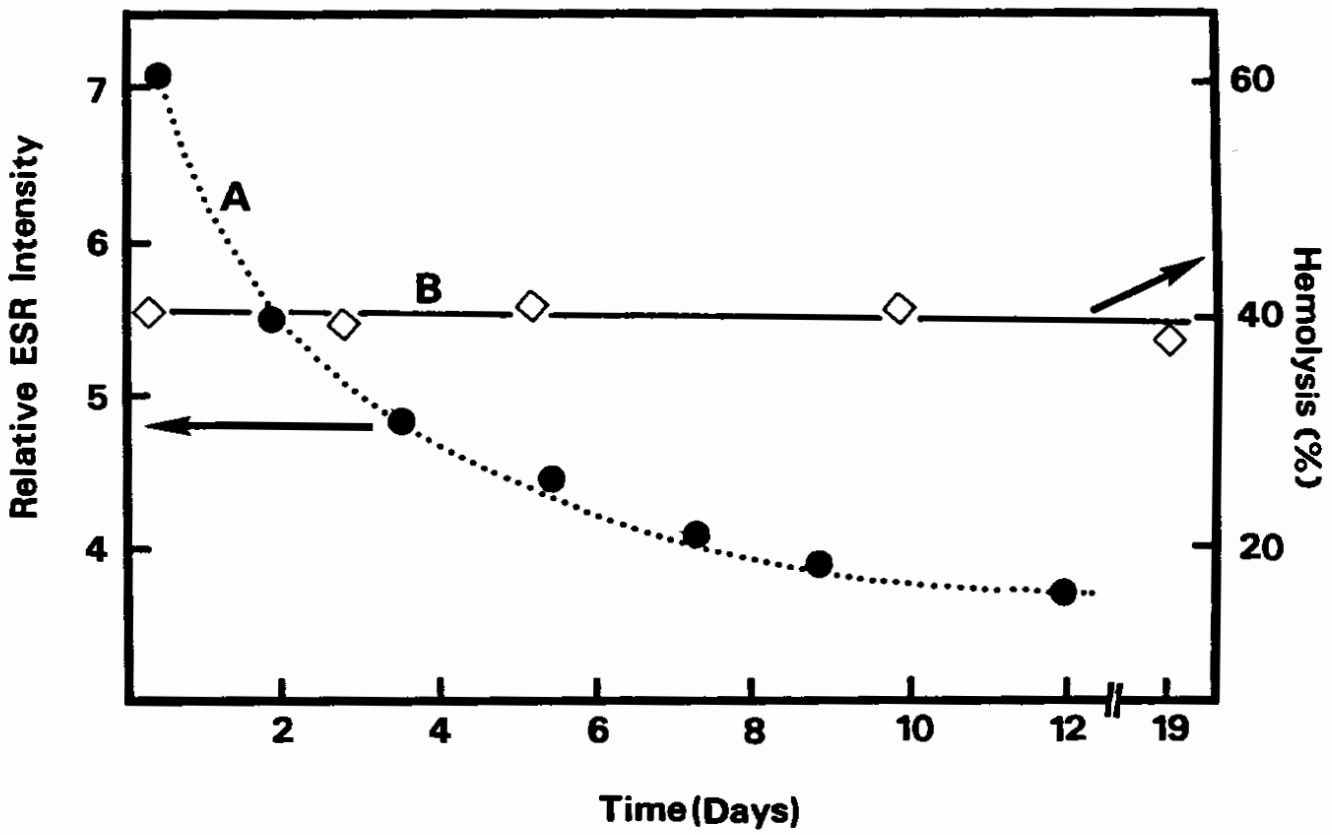

Figure 3. Time dependence of the ESR intensity of the silicon-oxygen radicals, plot A (•), on storing in air, and the hemolysis, plot B (◇), by the same sample.

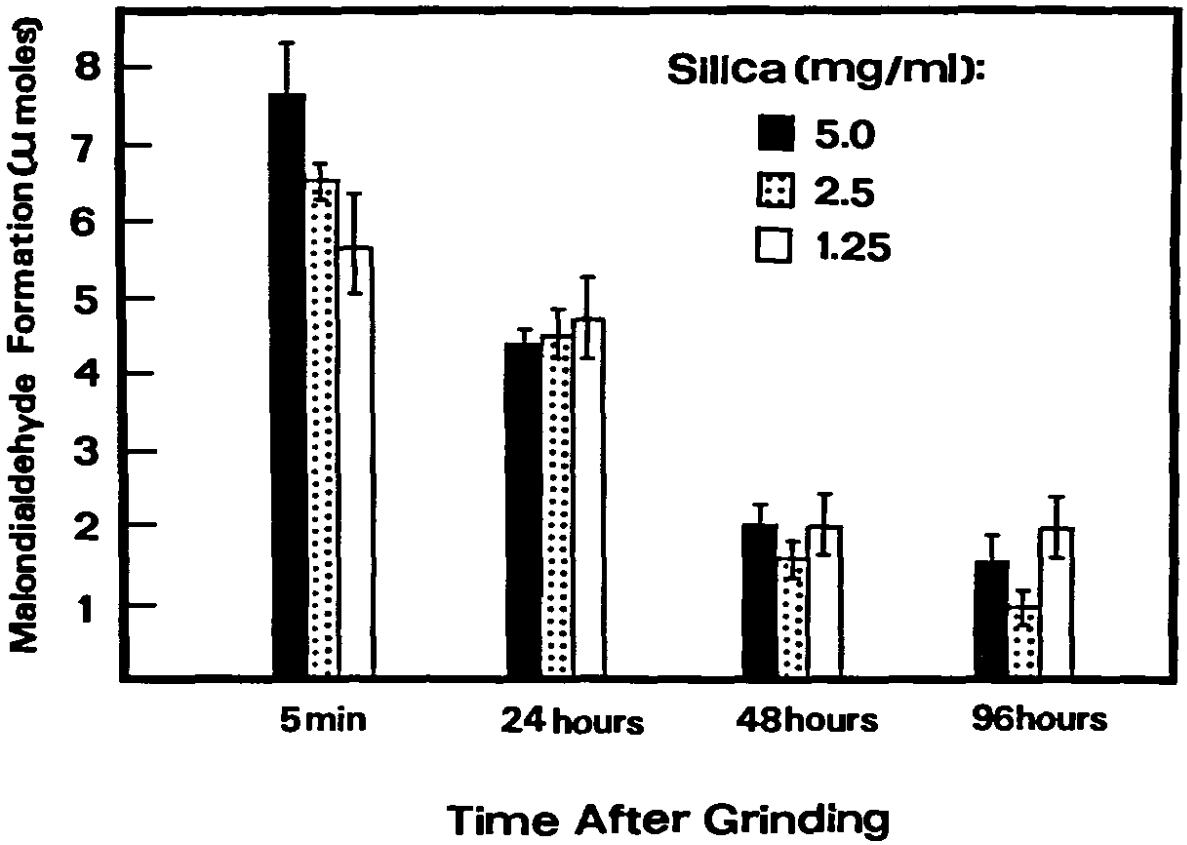

Figure 4. Effect of "aging" of the quartz dust on the rate of peroxidation of linoleic acid.

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