Free Rad. Res. Comms., Vol. 9, No. 3-6, pp. 259-266
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ESR SPIN TRAPPING AND CYTOTOXICITY INVESTIGATIONS OF FRESHLY FRACTURED QUARTZ: MECHANISM OF ACUTE SILICOSIS

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Electron spin resonance (ESR) measurements show that grinding of quartz particles in air produces silicon-based (Si· and SiO·) radicals which decay with aging in air. ESR spin trapping measurements provide evidence for the generation of hydroxyl and possibly superoxide radicals from a suspension of fresh quartz particles. The hydroxyl radical generation potential of the fresh quartz particles decreases on storing in ambient air and on the addition of catalase, superoxide dismutase, desferroxamine, or DMSO. Silica-induced lipid peroxidation also decreases on storing the fresh particles in ambient air. These findings suggest that oxygenated radicals play a role in the biochemical mechanism of pneumoconiosis in general and acute silicosis in particular.

KEY WORDS: ESR, spin trapping, free radicals, quartz, cytotoxicity.

INTRODUCTION

This communication summarizes our electron spin resonance (ESR) investigation of the formation of oxygenated radicals by freshly crushed quartz particles in a cell-free aqueous medium, and its potential role in the biochemical mechanism of acute silicosis. This study was undertaken because the mechanisms by which the quartz particles exert their toxic action on cells and the process(es) by which these actions progress to fibrogenesis are still not well understood.[1-3] It is generally believed, however, that the action of quartz particles on the cell membrane is the starting point of the silicotic process.[1,4] Since the first step must involve surface reactions, characterization of the chemical structure of the surfaace of quartz particles has been the subject of several recent studies.[5-7] For example, we reported that mechanical crushing of quartz under normal atmosphere generates silicon-based (Si· and SiO·) radicals which decay with time, and that these radicals are associated with a higher cytotoxicity of fresh quartz dust as compared to that of an aged dust from the same stock.[6,8] Independently Fubini and coworkers[7] have also reported on the formation of Si· and SiO··-type of radicals from quartz particles crushed uneder atmospheric conditions. Gulumian and van Wyk[9] have reported that quartz particles react with hydrogen

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peroxide (H_2O_2) to generate hydroxyl (·OH) radicals. These authors[9] suggested that this process might contribute to quartz's pathogenicity but the mechanism of the ·OH radical formation was not clarified. Earlier, Gabor and Anca[10] had suggested that the cytotoxicity of quartz particles might be associated with the generation of some factor or factors possessing the properties of a free radical and hence capable of promoting the peroxidative chain cleavage of polyunsaturated fatty acid moieties of the phospholipids in the cell membrane. More recently, Weitzman and Graceffa[11] reported that asbestos is able to catalyze the generation of ·OH radicals from H_2O_2. Their later work[12] indicated that lipid peroxidation might be one of the mechanisms of tissue injury by asbestos.

It is also known that ·OH radicals are capable of causing peroxidation by abstracting hydrogen atoms from cell-membrane lipids and initiating lipid peroxidation in lysosomal membrane.[13] Thus the ·OH radical (and also other oxygenated species such as O_2^-, $^1O_2$, and H_2O_2) might be expected to play a role in the quartz toxicity. Earlier studies[14] of the aqueous chemistry of quartz suspensions have reported detection of H_2O_2, implicating the formation of oxygenated radicals as transient species. This observation provided further impetus for the present undertaking.

MATERIALS AND METHODS

Crystalline silica with particle sizes ranging from 0.2 to 2.5 mm was obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, University Park, PA. Particles in the range of smaller than 25 microns were produced by hand grinding in air, with an agate mortar and pestle because of the structural similarity of agate to that of quartz. Also, a mixed particle size rather than a specific range, was employed, to roughly approximate the random particle-size distribution in the mining atmosphere.

ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER 200D ESR spectrometer, employing a self-tracking NMR gaussmeter and a microwave frequency counter. An ASPECT 2000 computer was used for data acquisition, analysis, and spectral simulations to obtain the splitting constants. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used as a spin trap for detecting the hydroxyl and superoxide radicals. The DMPO was purchased from Aldrich and used without further purification, because very weak or no spin adduct signal was obtained from the purchased sample when used by itself. Superoxide dismutase (SOD), linoleic acid (*cis*-9-*cis*-12-octadecadienoic acid) and catalase were obtained from Sigma. All other chemicals were purchased from Fisher or Aldrich.

Peroxidation of the polyunsaturated lipid linoleic acid by freshly ground or aged silica was monitored using a fluorescence method[15] with minor modifications. The reaction mixture in a total volume of 0.5 ml contained freshly ground or aged silica (2.5 mg/ml) and 20 ml of 0.52 mM linoleic acid emulsion in 95% ethanol in HEPES buffer (pH 7.4) without 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 of 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,-tetra methoxypropane to obtain a calibration curve, which was used for calculating the amounts of malondialdehyde produced.

RESULTS AND DISCUSSION

Figure 1 shows some typical ESR spectra of fresh quartz particles. The measured g-values are $g_{\parallel} = 2.0017$ and $g_{\perp} = 2.0007$ as indicated. Such ESR spectra from quartz are characteristic of silicon-based radicals (Si·, SiO·).[6,16] It was found that these radicals decay in air with a half-life of about 30 hours. We stress, however, that the decay followed "first order" kinetics only very approximately and that about 20% of the radicals were detectable even after four weeks of storage in air. It was also noted that the spectral resolution and lineshape depend somewhat on the grinding time and procedure as well as on temperature. Further studies are under progress to understand the details of these observations.

For the detection of short-lived, oxygenated, free radicals, the ESR spin trapping measurements were carried out. Figure 2 shows some typical results of ESR spin trapping measurements. A 0.1 M aqueous solution of the spin trap DMPO with as received (unground) quartz particles did not give a detectable ESR spectrum (Figure 2a). When the quartz particles were crushed in a 0.1 M DMPO aqueous solution or when fresh quartz particles were mixed with 0.1 M DMPO aqueous solution, an ESR spectrum, consisting of a 1:2:2:1 quartet pattern with splittings of $a_N = a_H = 14.9$ G, was observed at $g = 2.0059$ (Figure 2b), which was assigned to the DMPO-OH adduct.[17] As supporting evidence for the ·OH radical formation, ESR spin tapping measurements were made in which 5% ethanol was added as a secondary trap.[17] It has been shown[17] that in presence of ethanol, the intensity of the DMPO-OH spin-adduct signal decreases because ethanol scavenges some of the ·OH radicals to form the

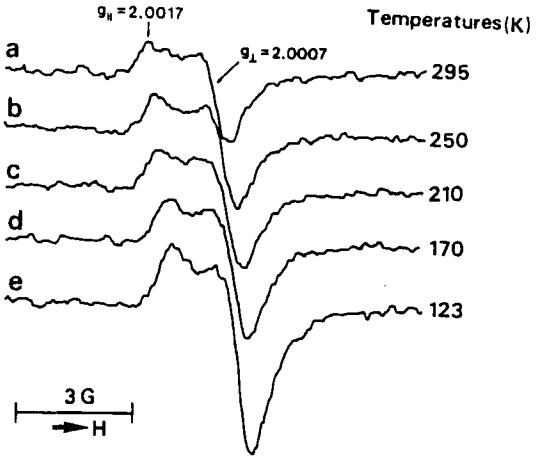

FIGURE 1 Typical ESR spectra from quartz dust freshly produced by an agate ball machine grinder in air. The spectra were recorded at different temperatures as indicated.

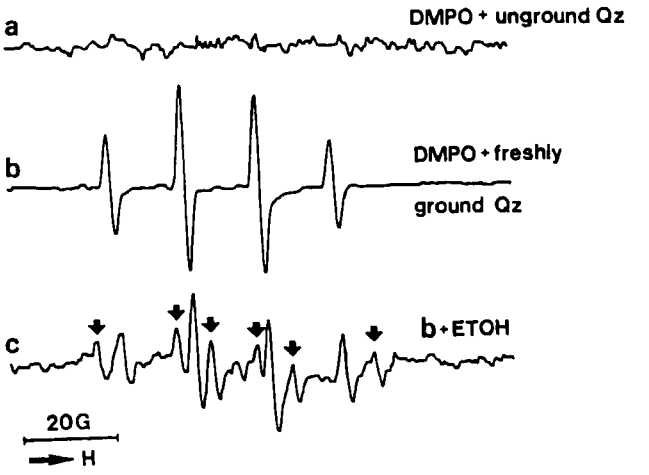

FIGURE 2 ESR spectra recorded 2 minutes after mixing a 100 mM DMPO aqueous solution with (a) unground quartz; (b) freshly ground quartz particles; (c) same as (b) but with 5% ethanol added. The arrows show the signals from the DMPO-ethanolyl radical adduct, attesting to the ·OH radical formation.

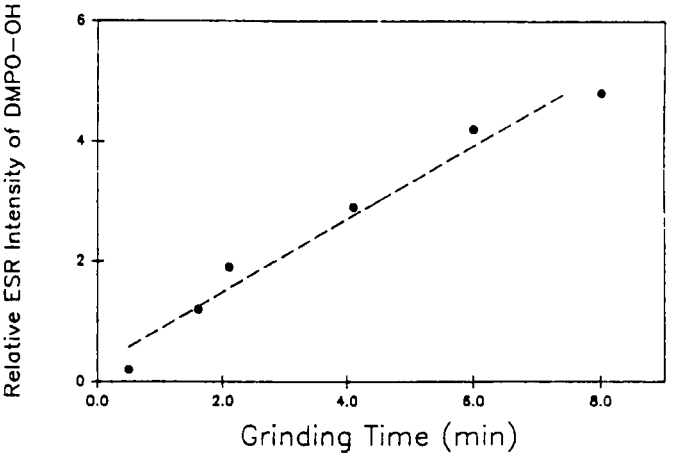

FIGURE 3 Dependence of the ESR intensity of the DMPO-OH adduct (i.e. ·OH radical production) on grinding time (i.e. surface area) of quartz particles.

ethanolyl radicals. The signals from the DMPO-trapped ethanolyl radicals are indicated by arrows in Figure 2b. The measured splitting constants, a_N = 15.8 G and a_H = 22.8 G, for these signals are indeed typical of those of the DMPO–$CHOHCH_3$ adducts,[17] thus supporting the ·OH radical formation in the quartz particle aqueous suspension, without any added H_2O_2.

The concentration of the ·OH radicals, as extimated from the peak-to-peak height of the spin adduct signal, increased with the duration of grinding (Figure 3). This showed that the ·OH radical formation is related to some surface property of fresh

quartz particles.[14] The likely active sites are thought to be the silicon-based radicals,[5-8] since ESR measurements on the same samples showed that the concentration of silicon-based radicals increased with grinding also.

To detect the possible formation of the O_2^- radicals in the fresh quartz particle suspension, SOD (50 μg/ml) and catalase (5000 units/ml) were added individually into the reaction medium. As noted in Figure 4b, SOD reduced the ·OH radical formation to about 65%, indicating that O_2^- radicals might be involved in the mechanism of ·OH radical formation.[11] Catalase, however, completely suppressed the ·OH formation (Figure 4c), indicating that H_2O_2 plays an important role in the ·OH radical formation. The generation and detection of H_2O_2 was reported with wet analytical chemistry, the $KMnO_4$ reduction.[14] Using the same methodology, we did confirm the reducing activity of fresh quartz particle suspension with respect to $KMnO_4$, although the H_2O_2 yield was measured to be an order of a magnitude smaller for our sample. Further experiments showed that DMSO, commonly used radical scavenger,[18] suppresses the ·OH radical formation by more than 70% (Figure 3d). We noted that addition of desferal (1 mM) reduced the ·OH radical formation by more than 80%. Thus the Fenton reaction,

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

seems to play some role in the ·OH radical formation.[19] The Fe^{2+} might be present as a trace impurity or impregnated in the chemical structure of quartz dust.

We also investigated the possible relationship between the ·OH radicals generated by fresh dust particles and the dust's lipid peroxidation potential. Using spin trap ESR, we carried out measurements of the time dependence on the ability of fresh quartz particles to generate ·OH radicals. Parallel measurements were then made on the silica induced lipid peroxidation using linoleic acid as a model lipid. Figure 5A shows time dependence of the ·OH radical production, while Figure 5B shows the time dependence of the lipid peroxidation. It is seen that the ability of fresh ground

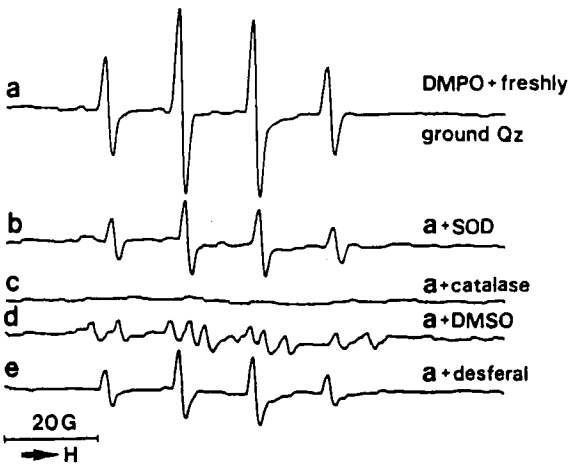

FIGURE 4 ESR spectra recorded 2 minutes after mixing 100 mM DMPO with (a) fresh quartz particles: (b) same as (a) but with SOD (50 μg/ml); (c) same as (a) but with catalase (5000 units/ml); (d) same as (a) but with DMSO (15%); (e) same as (a) but with desferal (1 mM).

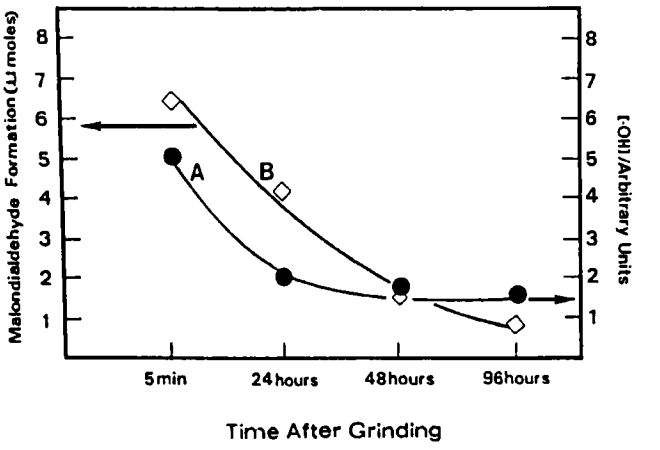

FIGURE 5 Effect of "aging" on the silica's ability to generate hydroxyl radicals, plot A (●) and effect of "aging" of the same sample on the rate of peroxidation of linoleic acid, plot B (◇).

silica to peroxidize a lipid decreases on storage. The time dependence behaviors of the ·OH radicals (Figure 5A) as well as the silica lipid peroxidation potential (Figure 5B) indicates that these radicals might be directly or indirectly involved in the silica induced lipid peroxidation, which, in turn, could result in a progressive degeneration of the membrane structure and eventual loss of the membrane activity.[20]

Based on these results we suggest the following model of the initial events in the reaction of the quartz dust with a cell membrane. The free radicals (Si· and SiO·) on the surface of silica dusts and their associated oxygenated species (·OH, O_2^-, and H_2O_2) are involved in the interaction of cell membrane with quartz dust. The results of this interaction would be the release of reactive oxygenated species (H_2O_2, O_2^-, ·OH, R·, and RO·). These reactive oxygenated species would further react with the cell membrane, leading to additional release of these species and to lipid peroxidation.[21] As to the sites of the reactions between the cell membranes and the quartz dust, we noted that the reaction of quartz particles with H_2O produces silanol (SiOH) groups on the particle surface, as detected by infrared spectroscopy.[22] These silanol groups could form hydrogen bonds with the nitrogen or oxygen atomic sites on the cell membrane. This is supported by the report of a weak (hydrogen) bond formation between a secondary amide (peptide) of proteins and silanol moieties.[23] Such hydrogen bonding could bring the silica surface and cell membrane close enough to provide a favorable environment for the initiation of lipid peroxidation by the solicon-based radicals and their associated oxygenated radicals.

In conclusion, the present ESR spin trapping experiments show that freshly generated, micron-size quartz particles can generate ·OH and, possibly, O_2^- radicals in aqueous media. Moreover, the ability of such particles to generate these oxygenated radicals decreases with the aging of the particles. This result implies that fresh quartz dust should be more fibrogenic than the stale dust.[6,8] Indeed, fresh quartz dust exhibits a greater ability for inducing lipid peroxidation than aged dust. Thus the silicon-based radicals on the quartz particle surface and their associated oxygenated radicals might be involved in the initiation of the lipid peroxidation of the cell membrane which

results in cell death. If further studies confirm these findings, then strategies could be developed for blocking the radical pathways and hence combating silicosis.

Acknowledgements

This research has been supported by the Department of the Interior's Mineral Institute Program administered by the Bureau of Mine through the Generic Technology Center for Respirable Dust under grant G1135142.

References

1. Farber, J.L. How do mineral dusts cause lung injury? *Lab. Invest.* **49,** 379–390, (1983).
2. Reiser, K.M. and Last, J.A. Silicosis and fibrogenesis: fact and artifact. *Toxicology,* **13,** 51–72, (1979).
3. Silicosis and Silicate Disease Committee. Disease associated with exposure to silica and nonfibrous silicate minerals. *Arch. Patho. Lab. Med.,* **112,** 673–720, (1988).
4. Parazzi, E., Secchi, G.C., Pernis, B. and Vigliana, E. Cytotoxic action of silica dusts on macrophages *in vitro. Arch. Environmen. Health,* **17,** 850–859, (1968).
5. Bolis, V., Fubini, B and Venturello, G. Surface characterization of various silica: a tentative correlation between the energies of absorption sites and the different biological activities. *J. Thermal Anal.* **28,** 249–257, (1983).
6. Dalal, N.S., Suryan, M.M., Jafari, B., Shi, X., Vallyathan, V. and Green, F.H.Y. Electron spin resonance detection of reactive free radicals in fresh coal dust and quartz dust and its implications to pneumoconiosis and silicosis. In *Respirable Dust in the Mineral Industries: Health Effects, Characterization, and Control* (eds. R.L. Frantz and R.V. Ramani), P. 25–29. American Conference of Governmental Industrial Hygienists (ACGIH) Publication, ISBN 0-936712-76-7, (1986).
7. Fubini, B., Bolis, V. and Giamello, E. The surface chemistry of crushed quartz dust in relation to its pathogenicity. *Inorganica Chimica Acta,* **138,** 193–197, (1987).
8. Vallyathan, V., Shi, X., Dalal, N.S., Irr, W. and Castranova, V. Generation of free radicals from freshly fractured silica dust: potential role in acute silica-induced lung injury. *Am. Rev. Resp. Dis.,* **138,** 1213–1219, (1988).
9. Gulumian, M. and van Wyk, A. Free radical scavenging properties of polyvinyl-pyridine-N-oxide: a possible mechanism for its action in pneumoconiosis. *La Medicina del Lavoro,* **78,** 124–128, (1987).
10. Gabor, S. and Anca, Z. Effect of silica on lipid peroxidation in the red cells. *Int. Archiv fuer Arbeitsmedizin,* **32,** 553–558, (1974).
11. Weitzman, S.A. and Graceffa, P. Asbestos catalyzes hydroxyl and superoxide radical generation. *Arch. Biochem. Biophy.,* **228,** 373–376, (1984).
12. Weitzman, S.A. and Weitberg, A.B. Asbestos-catalyzed lipid peroxidation and its inhibition by desferrioxiamine. *Biochem. J.,* **225,** 259–262, (1985).
13. Fong, K.L., McCay, P.B., Poyer, J.L., Keel, B.B. and Misra, H. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavine enzyme activity. *J. Biol. Chem.,* **248,** 7792–7797, (1973).
14. Kalbanev, I.V., Berestetskaya, I.V. and Butyagin, P.U. Mechanochemistry of quartz surface. *Kinetika i Kataliz,* **21,** 1154–1158, (1980).
15. Fraga, C.G., Leibovitz, B.Z. and Tappel, A.C. Halogenerated compounds as inducers of lipid peroxidation in tissue slices. *J. Free Rad. Biol.,* **3,** 119–123, (1987).
16. Hochstrasser, G. and Antonini, J.F. Surface states of pristine silica surface. *Surf. Sci.,* **32,** 644–664, (1972).
17. Buettner, G.R. The spin trapping of superoxide and hydroxyl radicals. In *Superoxide Dismutase,* vol. 2 (ed. L.W. Oberly). CRC press, Boca Raton, Florida, pp. 63–81, (1984).
18. Britigan, B.E., Rosen, G.M., Thompson, B.Y., Chai Y. and Cohen, M.S. Do human neutrophils make hydroxyl radicals? *J. Biol. Chem.,* **261,** 17026–17032, (1986).
19. Morehouse, K.M. and Mason, R.P. The transition metal-mediated formation of the hydroxyl free radical during the reduction of molecular oxygen by ferredoxin-ferredoxin: $NADP^+$ oxidoreductase. *J. Biol. Chem.* **263,** 1204–1211, (1988).
20. Girotti, A.M. Mechanism of lipid peroxidation. *J. Free Rad. Biol. Med.,* **1,** 87–95, (1980).
21. Fantone, J.C. and Ward, P.A. Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am. J. Pathol.,* **107,** 397–418 (1982).

22. Tsuchiya, I. Infrared spectroscopic study of hydroxyl groups on silica surface. *J. Phys. Chem.* **86,** 4107–4112, (1982).
23. Summerton, J., Hoenig, S.A., Butler, C. and Chvapil, M. The mechanism of hemolysis by silica and its bearing on silicosis. *Exp. Mol. Pathol.*, **26,** 113–128, (1977).

Accepted by Prof. E.G. Janzen