Generation of Oxygen Radicals by Minerals and Its Correlation to Cytotoxicity

Val Vallyathan

Pathology Section, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia

Occupational exposure to mineral dust causes pneumoconiosis and other diseases. A cytotoxicity assay to predict the potential of minerals to cause disease would be of great value as a prevention strategy. This study compares the ability of several minerals to generate the more potent oxidizing agent, hydroxyl radical (•OH), and their cytotoxicity and lipid peroxidation potentials. Crystalline silica, the most potent cytotoxic and pathogenic mineral studied, showed the least ability to generate •OH radicals while inducing the maximal lipid peroxidation. Coal mine dust, showing the maximal ability to generate •OH radicals, was the least cytotoxic in bioassays of toxicity and induction of lipid peroxidation. Based on these results, it would appear that the ability of minerals to induce lipid peroxidation provides a better correlation with known cytotoxicity and pathogenicity of minerals than does their ability to generate oxygen radicals. — Environ Health Perspect 102(Suppl 10):111–115 (1994)

Key words: oxygen radicals, cytotoxicity, pneumoconiosis, hemolysis, enzymes, surface iron, lipid peroxidation

Introduction

Inhalation of inorganic minerals and coal mine dust can produce pneumoconiosis, a lung disease characterized by an initial pulmonary inflammatory response in the lung associated with an increase in cell-mediated secretions of cytokines, growth factors, lysosomal enzymes, hydrogen peroxide, and superoxide anion (*1–4*). Several studies suggest that many of these factors work together to cause cell injury and the progression of the disease process (*3,4*). The possible mechanisms leading to this fibrotic lung disease are under intense investigation. To this end, we have devoted considerable effort during the last several years toward understanding the surface properties and chemical structure of minerals and their relation to oxygen radical generation and related cytotoxicity (*5–7*).

Several recent studies indicate that fracturing of crystalline silica, which may occur in occupations such as sandblasting, tunneling, rock drilling, or silica flour mill operations, may result in the generation of silicon oxygen radicals on the cleavage planes of silica and that freshly fractured silica can react with aqueous medium to generate hydroxyl (•OH) radicals (*5–7*). Studies have shown that freshly fractured silica is cytotoxic, induces the release of cytosolic enzymes from alveolar macrophages, and enhances the generation of •OH radicals during phagocytosis (*5,8–10*). In addition to crystalline silica, our earlier studies also suggest that freshly fractured coal exhibits surface reactivity not found in aged coal (*11,12*). The concentration of surface-based radicals in freshly fractured silica and coal decreases with aging in air.

The objective of the present study is to evaluate the potential role of several occupational mineral dusts in the generation of •OH radicals from hydrogen peroxide (H_2O_2) and to correlate *in vitro* cytotoxicity and lipid peroxidation potential with oxygen radical generation. In an attempt to generate the oxygen radical and measure the radical efficiently, we used H_2O_2 and dust mixtures in the presence of an •OH radical trap and electron spin resonance (ESR) as a direct spectroscopic technique for measuring the •OH radical. The •OH radical generated from the H_2O_2 dust mixtures forms a stable radical adduct DMPO-OH with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) with a characteristic 1:2:2:1 hyperfine quartet signal. The signal peak heights of this stable adduct provide a quantitative measure of the radical generated. To correlate the oxygen radical-generating potential with cytotoxicity, we measured red blood cell hemolysis, lactate dehydrogenase (LDH), β-*N*-acetylglucosaminidase (β-NAG) and β-glucuronidase (β-GLUC) release from alveolar macrophages (AM) exposed to dusts. The potential to induce lipid peroxidation as a measure of free radical-induced toxicity was determined using a noncellular *in vitro* assay.

Materials and Methods

Crystalline silica (min-U-sil) was obtained from Pennsylvania Sand and Glass Corp. (Pittsburgh, PA). All other minerals were obtained from primary milling and mining facilities in the United States. Bituminous coal mine dust was obtained from Blacksville, WV, coal mines, which represented the Pittsburgh coal seam. All the minerals were classified to < 5 μm size with the aid of an Accucut Particle Classifier (Donaldson-Majal Division, St. Paul, MN). Scanning electron microscopy (SEM) analysis with the aid of an automated image analysis system (LeMont), and X-ray spectrometric analysis on samples prepared on Nucleopore filters were made at ×1000 magnification to determine the elemental composition and particle diameter. Surface area measurements were made by the nitrogen adsorption technique according to the method of Brunauer et al. (*13*).

Electron Spin Resonance Measurements

Electronic spin resonance (ESR) measurements were made with the aid of a spin trap DMPO to monitor the generation of •OH radical from all the minerals. The reaction mixtures in a final volume of 1 ml contained 10 mg dust, 100 μl 1 M DMPO, and 100 μl 0.1 M H_2O_2. The reaction was initiated in a 5-ml plastic syringe by the addition of H_2O_2, mixed well in a vortex for 10 sec and filtered through a 0.45-μm nylon Acrodisc filter attached to the syringe. For the ESR measurements, 250-μl flat quartz cells were used as a sample container and measurements made on a Varian E109 ESR operating at x-band (~9.4 GHz)

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Address correspondence to Val Vallyathan, Pathology Section, NIOSH, 1095 Willowdale Road, MS 211, Morgantown, WV 26505. Telephone (304) 291-4582. Fax (304) 284-5467.

frequency. A microwave power of 50 mW and modulation amplitude of 2 G were found to be adequate for the optimal development of signal peak heights without compromising resolution of the spectrum.

Cytotoxicity Measurements

Cytotoxic potential of all the minerals was measured by determining cellular membrane damage, i.e., hemolysis of red blood cells, and release of cytosolic enzyme LDH from AM, as well as the potential of the dusts to induce the selective release of lysosomal enzymes β-GLUC and β-NAG. In addition, the potential to induce lipid peroxidation by all the dusts was measured as an index of free radical-induced cellular damage.

Hemolysis

Hemolytic potential of the minerals was measured based on the method by Harrington et al. (*14*) using a 2% suspension of sheep erythrocytes for incubation with the dusts (1 mg/ml) for 1 hr at 37°C. The amount of hemoglobin released was measured in the supernatant after centrifugation by monitoring the absorbance at 540 nm. Percentage of hemoglobin released as a result of dust interaction was calculated as the ratio of absorbance value obtained from cells lysed with Triton X-100.

Isolation of Alveolar Macrophages

For the enzyme release studies, AM were harvested from pathogen-free Sprague-Dawley rats by pulmonary lavage using calcium and magnesium-free Hank's balanced salt solution. Alveolar macrophages were centrifuged at 500*g* for 5 min at 4°C and resuspended in HEPES-buffered medium containing 5 mM glucose. Trypan blue dye exclusion tests and microscopic estimates were made to determine the cell viability and differential cell populations (*15*). Viable AM were found to be the predominant cell type in all the lavages.

Enzyme Measurements

Alveolar macrophages (2×10^6/ml) were incubated with (1 mg/ml) and without dusts for 2 hr in a shaking water bath at 37°C. Incubation was terminated by rapid cooling and centrifugation. The supernatant was separated and used in all the three enzyme assays. LDH was estimated in a total reaction mixture of 3 ml containing phosphate buffer, pH 7.4, 100 µl enzyme supernatant, 0.07 mg/ml NADPH, and 100 µl of 0.0007 M sodium pyruvate (*16*). The reaction was initiated by the addition of sodium pyruvate and LDH activity was calculated as the percent of enzyme released by comparing with the total enzyme released from cells lysed with Triton X-100. β-NAG was assayed according to the method of Lockhard and Kennedy (*17*) and β-GLUC was assayed according to the method of Sellinger et al. (*18*). These lysosomal enzymes were also expressed as the percent of enzyme released as a result of dust interaction by comparing with the total enzyme present in the cells without dust treatment.

Table 1. Physical and chemical characteristics of minerals.

Mineral/coal	MMAD,[a] µm	Surface/area, m^2/g	% < 1µm	% Si[b]	Surface iron, mg/100 mg
Coal	1.78	7.4	18	2.3	0.119
Feldspar	2.21	2.2	7	15.2	0.097
Kaolin	0.75	12.5	29	0	0.029
Bentonite	0.85	9.8	18	0.8	0.062
Talc	1.67	9.3	19	3.7	0.314
Silica	3.5	4.7	11	99	0.028

[a]MMAD, mass median aerodynamic diameter. [b]Percent concentration of silica by number as determined by X-ray spectrometric analysis of 1000 or more particles.

Surface Iron Measurements

Surface iron was measured using a spectrophotometric method according to Roth et al. (*19*). Minerals (10 mg) were treated with 5.5 ml 0.3 M sodium citrate, 1 M sodium bicarbonate, and 100 mg sodium dithionate and heated for 30 min at 80°C. Mixtures were centrifuged and supernatant diluted to 100 ml. Aliquots of the supernatant were treated with 1 ml 10% hydroxylamine hydrochloride and 2 ml 0.5% *O*-phenanthroline for 5 min, diluted to 100 ml, and absorbance read at 508 nm. Iron standards were treated similarly and graphed for the conversion of surface iron present in mineral samples.

Lipid Peroxidation

Lipid peroxidation potential by all minerals was monitored by measuring the malondialdehyde (MDA) generated during the incubation of dust with linoleic acid for 1 hr in a buffered medium without the addition of any promoters. The reaction was terminated by the addition of 0.3 ml 5 N HCl and 0.625 ml 40% trichloroacetic acid (*20*). After adding and mixing 0.625 ml 2.0% thiobarbuturic acid to the reaction mixture, the tubes were heated in a water bath at 95°C for 20 min. The thiobarbuturic acid reactive substance developed a color which was measured at 535 nm after cooling and centrifugation for 10 min at 600*g*. Malondialdehyde produced was calculated from a standard graph.

Results

Physical and Chemical Characteristics of Dusts

The major physical and chemical characteristics of the dusts used in this study are presented in Table 1. The mass median aerodynamic diameter (MMAD) of all the dusts was < 5 µm. The particle diameter never exceeded 7 µm in dust samples and less than 2% of the dust exceeded the limit of 5 µm. However, several dusts showed large proportions of particles in the lower size range of less than 1 µm. These particle size differences are well reflected in the surface area measurements showing a wide variability between dusts (Table 1).

•OH Radical Generation

All the minerals reacted with H_2O_2 in the presence of DMPO and produced DMPO-OH radical adducts showing typical 1:2:2:1 quartet signals. All the ESR signals were identical and showed only major differences in peak intensities. Figure 1 shows the typical ESR spectra obtained from crystalline silica (min-U-sil), talc, bentonite, and coal. The spectra shown for crystalline silica (Figure 1*a*), talc (Figure 1*b*), and bentonite (Figure 1*c*) are presented 10 times bigger than the coal spectra (Figure 1*d*). Crystalline silica generated the least amount, whereas coal generated the maximal amount of •OH radicals. Controls with mixtures of DMPO and dusts and DMPO with H_2O_2 showed barely detectable signals. The splitting constants of hyperfine couplings were characteristic of •OH radical adducts of DMPO. To confirm the •OH radical generation, we added a competitive •OH radical scavenger, ethanol, in increasing concentrations to the reaction mixture. The addition of 33% ethanol in the mixtures with dusts and 0.1 M DMPO and 10 mM H_2O_2 resulted in a six-line spectrum characteristic of DMPO ethanolyl radical adducts. •OH radical scavengers such as catalase and metal chelators such as deferoxamine, inhibited approximately 90% of the

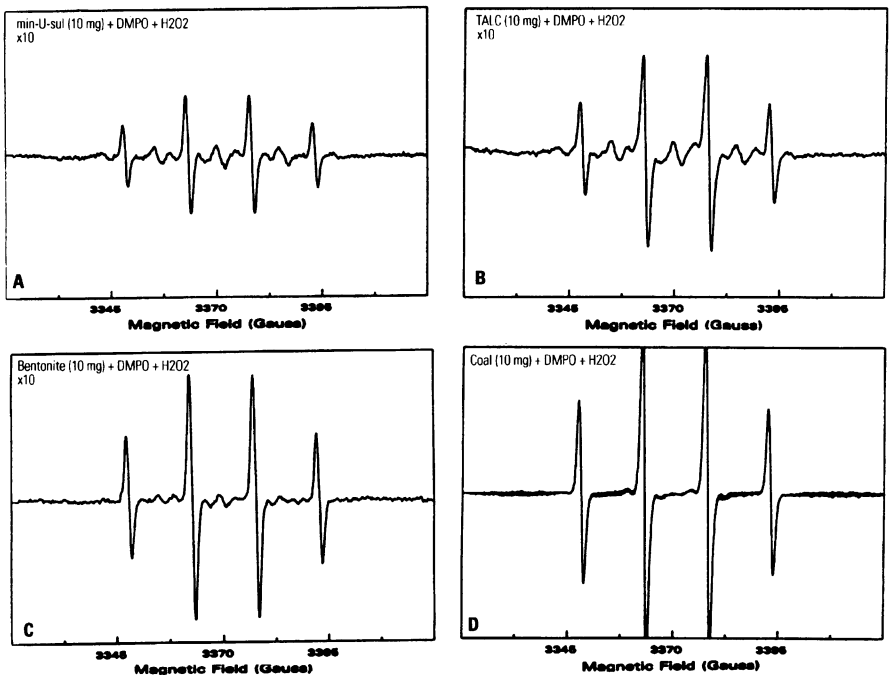

Figure 1. Typical ESR spectra obtained from (*A*)10 mg crystalline silica; (*B*), 10 mg talc; (*C*), 10 mg bentonite; (*D*), and 10 mg coal; with 10 mM H_2O_2 in the presence of 0.1 M DMPO. Note the gain used for the 10-mg coal sample, which produced a signal 18-fold stronger than the bentonite generating the maximal •OH radical generation on a equal mass basis.

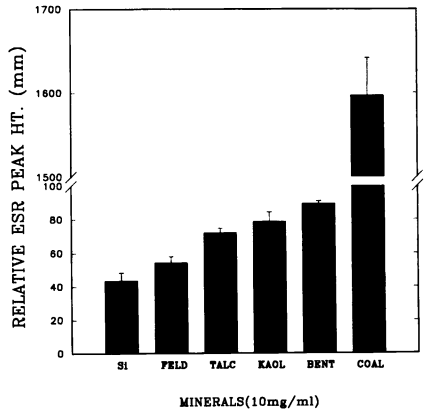

Figure 2. Bar graph illustrating the relative intensities of DMPO-OH adduct signals generated by reacting 10 mg/ml minerals with 10 mM H_2O_2 in the presence of 100 mM DMPO as a spin trap. Note that the coal generated signals 18-fold greater than the bentonite.

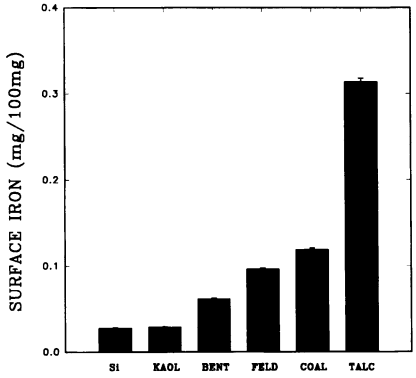

Figure 3. Surface iron concentration in the minerals.

•OH radical generation by dusts from H_2O_2.

Figure 2 illustrates the relative potential of all minerals in equal mass concentration (10 mg/ml) in the generation of •OH radicals from 10 mM H_2O_2 in the presence of 100 mM DMPO. It is evident from the data that coal mine dust generated the strongest •OH radical signal, while crystalline silica generated the weakest •OH radical signal. Surface iron concentrations of coal mine dust and crystalline silica showed a direct correlation to the •OH radical generation (Figure 3). However, talc, with the highest concentration of surface iron, exhibited a relatively weak ESR signal compared to coal (Figure 2).

Cytotoxicity Studies

The hemolytic potential of all the minerals in equal mass concentration (1 mg/ml) is shown in Figure 4. Bentonite, kaolin, and crystalline silica showed the greatest hemolytic activity, whereas feldspar and coal were the least hemolytic. Release of cytosolic enzyme, LDH, and selective release of lysosomal enzymes, β-GLUC and β-NAG, were maximal for kaolin, crystalline silica, and talc, and least for bentonite and coal in equal mass concentrations (Figure 5). On the other hand, the combined cytotoxicity index of all three enzymes and hemolysis showed kaolin, feldspar, and crystalline silica most cytotoxic and coal and talc least cytotoxic in equal mass concentrations (Figure 6).

Figure 7 illustrates data on the lipid peroxidation potential of the different minerals in equal mass concentrations. The data illustrate that feldspar, crystalline silica, and talc induce the greatest lipid peroxidation, and kaolin, coal, and bentonite induce the least lipid peroxidation. It is interesting to note that coal and talc with greater concentrations of surface iron, and

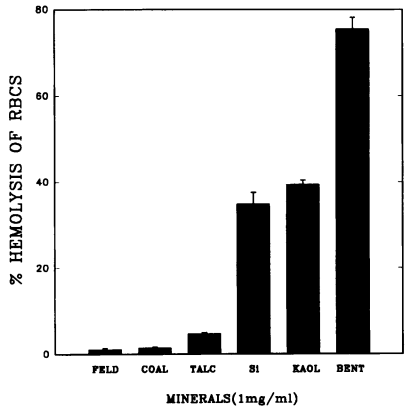

Figure 4. Bar graph showing the percent hemolysis potential of minerals on an equal mass basis (1 mg/ml). Note that bentonite and kaolin are the most cytotoxic and feldspar and coal are the least cytotoxic minerals.

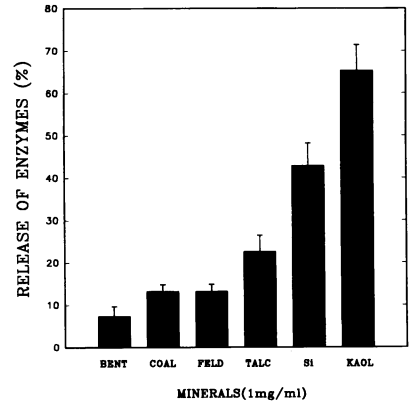

Figure 5. Bar graph illustrating the relative release of enzymes (LDH, β-GLUC, β-NAG) from alveolar macrophages on an equal mass basis (1 mg/ml). In this combined cytotoxicity index of enzymes, bentonite is the least cytotoxic and kaolin and silica are the most cytotoxic minerals.

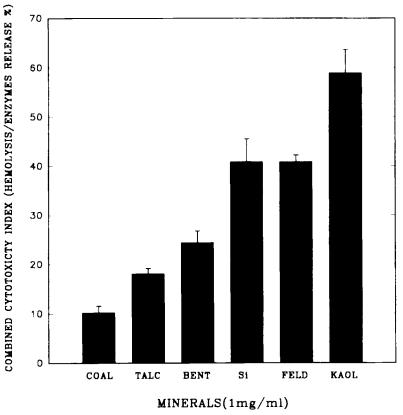

Figure 6. Bar graph representation of the combined toxicity index of hemolysis and LDH, β-GLUC, and β-NAG release on an equal mass basis.

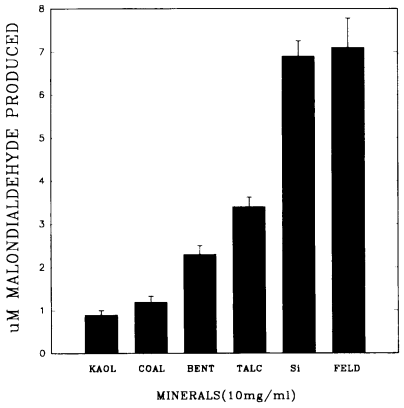

Figure 7. Lipid peroxidation potential of the minerals.

coal and bentonite with the greatest •OH radical generation potential, were minimally effective in inducing lipid peroxidation.

Figure 8 presents the combined cytotoxicity index based on equal surface area (0.1 m^2) of the minerals. The combined cytotoxicity index was calculated by adding all the three enzyme data (% released) and hemolysis percent. The data was then normalized for coal with a cytotoxicity index of 1. Coal was found to be least cytotoxic

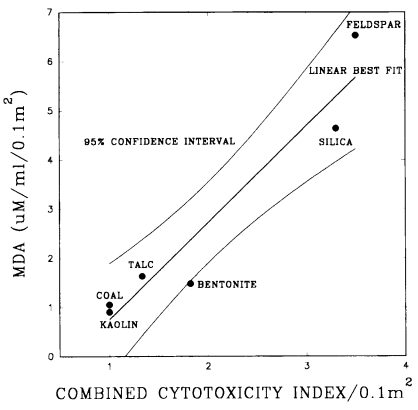

Figure 8. Comparison of the relative cytotoxicity index of minerals (hemolysis and enzyme release) on an equal surface area basis (0.1 m^2) vs lipid peroxidation. The data indicate a good correlation between the cytotoxicity index and MDA produced.

in the combined cytotoxicity on an equal mass basis. Similarly, the lipid-peroxidation data on an equal surface area basis were also normalized to 1 for coal. The data indicate a good positive correlation between cytotoxicity index and lipid peroxidation.

Discussion

In this study we evaluated the ability of several common occupational minerals to generate oxygen radicals from H_2O_2. The •OH radical generation potential of the dusts was then compared with the cellular toxicity and potential to induce lipid peroxidation. This investigation is important because several studies in recent years have implicated iron on the minerals as the causative agent inducing the generation of oxygen radicals leading to lipid peroxidation, cellular injury, and the disease process (*21*). The data presented here demonstrates that the concentration of surface iron in minerals is directly related to the potential of the dust to generate •OH radicals. However, •OH radical generation by these higher iron-containing minerals does not appear to be directly related to the cytotoxicity of minerals or to their potential to induce lipid peroxidation. In contrast, lipid peroxidation potential shows a better correlation with the known pathogenicity of dusts.

Among the six minerals studied, crystalline silica (the most potent toxic and pathogenic mineral) showed the least ability to produce •OH radicals from H_2O_2. This inability of •OH radical generation by crystalline silica correlated well with the surface iron concentration, while in cytotoxicity measurements, silica was not the most cytotoxic compared to feldspar, bentonite, and kaolin (Figures 3–5). However, when lipid peroxidation (a specific irreversible cell membrane injury induced by •OH radicals) was measured, crystalline silica induced the maximal lipid peroxidation. The significance of this lipid peroxidation potential parallels the cytotoxicity and pathogenicity of silica.

On the other hand, coal mine dust, which showed the maximal ability to generate •OH radicals with a corresponding high concentration of surface iron, is generally considered least cytotoxic and pathogenic in the absence of crystalline silica and other minerals. This correlates with the data presented here on the ability of coal to induce toxicity and lipid peroxidation. Therefore, the potential to produce the •OH radical alone may not be directly related to the ability of minerals to induce damage. Other factors and events involved in cytotoxicity, coupled with •OH radical generation, are likely to be important in inducing the cell injury by "lipid peroxidation."

Conclusion

Based on the current evidence, it appears that neither *in vitro* toxicity studies nor the ability of the minerals to generate the more potent oxidizing agents, such as •OH from H_2O_2, can directly predict the potential for toxicity and resultant pulmonary fibrosis. There appears to be a better correlation between the ability of the minerals to induce lipid peroxidation and known toxicity and fibrogenicity in humans.

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