

Generation of Oxygen Radicals and Mechanisms of Injury Prevention

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Exposure to crystalline silica can result in damage to the lung parenchyma and scarring that can lead to fibrosis. Pulmonary damage may be the direct consequence of toxic interaction between quartz particles and cell membranes, or it may be due to silica-induced production of oxidant species by pulmonary phagocytes, that in turn overwhelms pulmonary antioxidant systems and causes lung injury. Data indicate that grinding or fracturing quartz particles breaks Si-O bonds and generates $\cdot\text{Si}$ and $\text{Si-O}\cdot$ radicals on the surface of the cleavage planes. Upon contact with water, these silica-based radicals can generate hydroxyl radicals ($\cdot\text{OH}$). These surface radicals decay as fractured silica dust is aged. Freshly fractured quartz is significantly more potent than aged silica in directly causing lipid peroxidation, membrane damage, and cell death. Furthermore, freshly ground silica is a more potent stimulant of alveolar macrophages than aged silica. This silica-induced activation results in the production of superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide ($\text{NO}\cdot$), and other oxidant species that can damage lung cells. Tetrandrine, an herbal medicine that exhibits antifibrotic activity in rat models of silicosis, effectively blocks the ability of quartz to stimulate oxidant release from pulmonary phagocytes. — *Environ Health Perspect* 102(Suppl 10):65-68 (1994)

Key words: silicosis, silica radicals, alveolar macrophages, oxygen radicals, nitric oxide, fibrosis, tetrandrine

Introduction

Occupational exposure to crystalline silica can result in the development of pulmonary fibrosis. Acute silicosis can develop rapidly (1-3 years) after inhalation of relatively high levels of silica dust. This acute disease is associated with dyspnea, fatigue, cough, and weight loss, and is characterized histologically by alveolar proteinosis and diffuse fibrosis (1). Chronic silicosis can develop over a period of 20 to 40 years and may progress from simple silicosis with few symptoms to progressive massive fibrosis where restrictive lung impairment is evident. Histologically chronic silicosis is characterized as nodular fibrosis with collagen arranged in a unique spiral pattern (2,3).

Cellular injury and tissue damage are believed to be important steps in the development of silicosis (4). Damage to the lung parenchyma may result from the direct toxicity of silica or indirectly from silica-

induced activation of pulmonary phagocytes to produce oxidant species that in turn injure lung cells (5).

Several theories have been advanced to explain the direct cytotoxicity of crystalline silica. The first is that silanol groups (SiOH) can form hydrogen bonds with the oxygen or nitrogen groups in biologic membranes causing loss of membrane integrity and cell death (6). A second theory is that the negative surface charge associated with SiO^- groups is critical to cytotoxicity (7). A third theory is that radical sites are present on the surface of silica that may contribute to its ability to injure lung tissue (8).

Crystalline silica may also damage the lung indirectly by stimulating the production of phagocyte-derived oxygen metabolites. Such oxidants include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot\text{OH}$) (9). Indeed, O_2^- , H_2O_2 , and $\cdot\text{OH}$ have been associated with membrane damage and cell lysis (10,11), and can damage tight junctions between epithelial cells in culture (12). In addition to reactive oxygen metabolites, recent evidence indicates that activated macrophages can generate nitric oxide $\text{NO}\cdot$ (13). Once formed, $\text{NO}\cdot$ can react with O_2^- to produce a potent oxidant, peroxytrite (14).

The objective of this review is to summarize data, collected in my laboratory in collaboration with several scientists, that supports the theory that silica can directly

generate radicals as well as stimulate radical production in alveolar macrophages.

Results and Discussion

Occupations such as sandblasting, silica flour milling, rock drilling, and tunneling are associated with relatively high risks of pulmonary fibrosis. Common to these operations is crushing, grinding, or fracturing silica particles and generating fresh surfaces. While fracturing the crystalline lattice of quartz, Si-O bonds would break and surface radicals form. This theory can be tested by grinding silica and measuring radical species using an electron spin resonance (ESR) spectrometer (15). Data indicate that surface radicals are generated during the fracturing of quartz. A typical ESR signal for a dry sample of freshly ground silica is given in Figure 1A. This signal, centered around $g = 2.0015$, is characteristic of $\cdot\text{Si}$ or $\text{Si-O}\cdot$ radicals. The intensity of this signal (peak to peak) is proportional to the number of surface radicals and decays with aging (Figure 1B). The half-life for the decay of these surface radicals in air is approximately 30 hr.

These silicon-based surface radicals can react in aqueous solution to generate oxygenated free radicals. Such radical production can be monitored by ESR using DMPO (5,5-dimethyl-1-pyrroline-1-oxide) as a spin trap (16). As shown in Figure 2A, an ESR spectrum centered around $g = 2.0059$ and exhibiting a 1:2:2:1 quartet pattern is obtained that is characteristic of a DMPO-OH adduct. Indeed, ethanol, an

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•OH radical scavenger, decreases this quartet pattern. It is likely that the Fenton reaction is involved in the generation of •OH radicals, since both the H₂O₂ scavenger (catalase) and the Fe²⁺ chelator (desferal) decrease the •OH signal (Figure 2C, E). The ability of ground silica to generate •OH in aqueous solution decays with aging, exhibiting a half-life of approximately 20 hr.

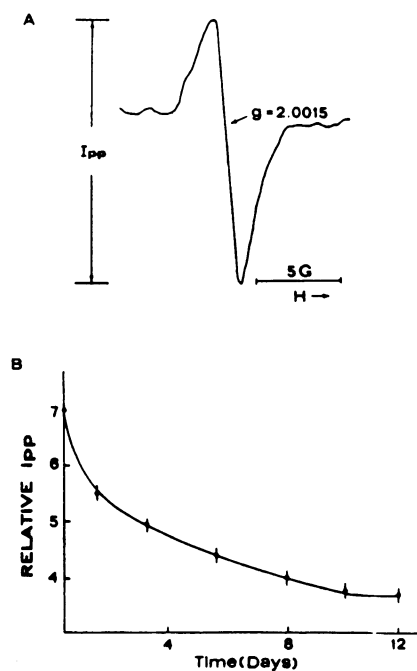


Figure 1. (A) ESR signal generated from a dry sample of crystalline silica immediately after grinding in air for 30 min. (B) Decay of silicon-based radical concentration as a function of aging in air. Data modified from Vallyathan et al. (17).

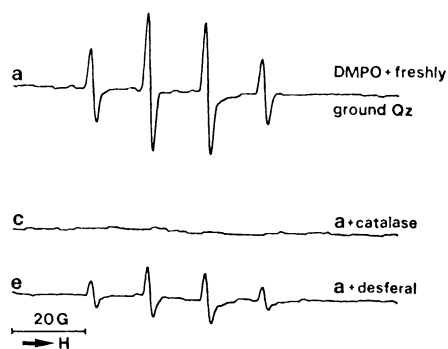


Figure 2. ESR signal obtained from freshly ground silica suspended in aqueous solution containing 100 mM DMPO (A). DMPO-OH spectrum of fresh silica suspended in the presence of catalase (C) or desferal (E). Data are modified from Vallyathan et al. (17).

Table 1. Direct toxicity of fresh versus aged silica.^a

Parameter	Aged Si	Fresh Si
Hemolysis ^b	2 ± 1%	39 ± 1%
Membrane leakiness ^c	15 ± 2%	58 ± 8%
Lipid peroxidation ^d	1.5 ± 0.4 μmol mal	7.5 ± 0.6 μmol mal

^aValues are means ± SE of three or more experiments. Data modified from Vallyathan et al. (17,18). ^bPercent hemolysis of a 2% suspension of red blood cells exposed to 1 mg/ml of fresh or aged (2 days) silica for 1 hr at 37°C. ^cMembrane integrity of alveolar macrophages exposed to 15 mg/ml fresh or aged (14 days) silica for 30 min at 37°C measured by trypan blue dye exclusion. ^dMalondialdehyde production of linoleic acid exposed to 5 mg/ml fresh or aged (4 days) silica for 1 hr at 37°C.

Grinding of crystalline silica is not only associated with the generation of radicals but also results in an increase in the direct toxicity of silica *in vitro* (17,18). As shown in Table 1, freshly ground silica is approximately 19 times more lytic to red blood cells than ground dust tested after a 2-day aging period, is approximately four times more potent in causing loss of membrane integrity in alveolar macrophages than silica aged for 14 days, and induces five times more lipid peroxidation than quartz aged for 4 days. Figure 3 shows that there is a direct correlation between radicals generated by ground silica and its ability to oxidize lipids *in vitro*.

To summarize, the direct toxicity of crystalline silica is due in part to radicals generated on the cleavage planes of fractured dust. The enhanced cytotoxicity of fresh versus aged silica may explain in part the relatively high incidence of silicosis in sandblasters, rock drillers, and silica millers.

Evidence exists that silica can activate alveolar macrophages to generate oxidants, and thus indirectly cause lung injury. *In vitro* exposure of alveolar macrophages to silica increases oxygen metabolism by approximately 3-fold and stimulates chemiluminescence approximately 12-fold above control (19). At least part of this activation is associated with silica-induced enhancement of O₂⁻ and H₂O₂ production (Figure 4). Freshly ground silica is a more potent stimulant of alveolar macrophages than aged silica (18). Indeed, freshly fractured silica causes approximately three times more H₂O₂ production and approximately 14 times more chemiluminescence from alveolar macrophages than ground silica, which was aged for 14 days prior to use (Figure 5).

Silica-induced potentiation of alveolar macrophages can be demonstrated *in vivo*. Figure 6 shows that alveolar macrophages

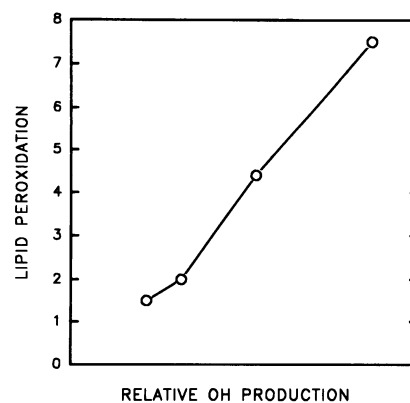


Figure 3. Relationship between the ability of ground silica to generate •OH and to cause lipid peroxidation of linoleic acid. Data were obtained using silica aged for 0, 1, 2, or 4 days after grinding. Data modified from Vallyathan et al. (17).

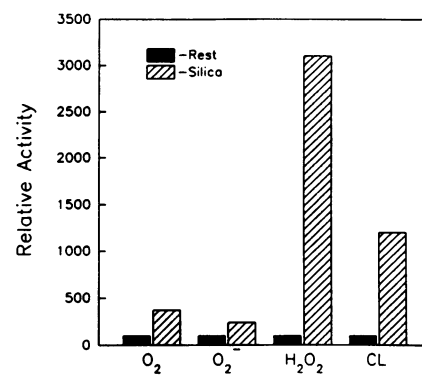


Figure 4. Effect of *in vitro* exposure of silica on alveolar macrophages. Cells were exposed at 37°C to 2 mg/ml silica for oxygen consumption (O₂) and superoxide release (O₂⁻), to 1 mg/ml silica for hydrogen peroxide (H₂O₂), and to 0.5 mg/ml for chemiluminescence (CL). Data given as percentage of the resting (unexposed) levels and are modified from Castranova et al. (19).

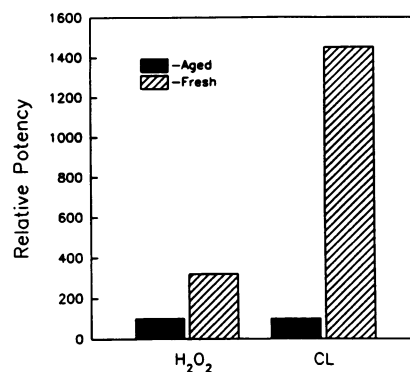


Figure 5. Effect of freshly ground silica vs silica that was stored for 14 days after grinding on alveolar macrophages. Hydrogen peroxide (H₂O₂) and chemiluminescence (CL) are given a percentage of the resting (unexposed) levels and are modified from Vallyathan et al. (18).

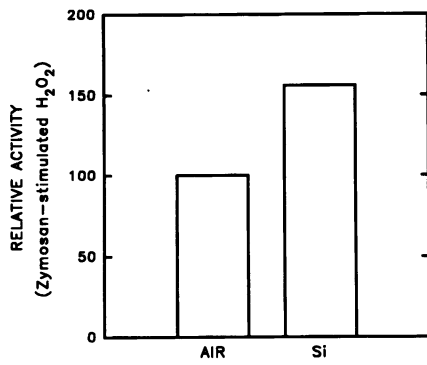


Figure 6. Effect of inhalation of silica (117 mg/m³ for 6 hr) on H₂O₂ production by alveolar macrophages in response to zymosan (2 mg/ml). Macrophages were harvested by bronchoalveolar lavage 4 days postexposure. Data are percentages of the control (filter air) values and are modified from Castranova et al. (20).

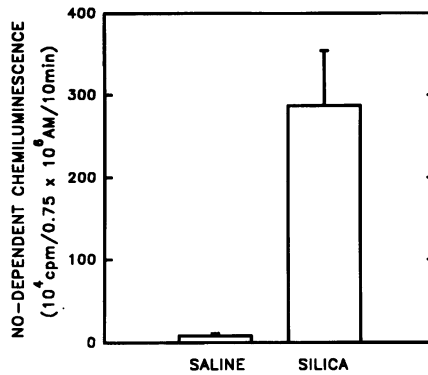


Figure 8. Effect of *in vivo* silica exposure on NO-dependent zymosan-stimulated chemiluminescence from alveolar macrophages. Macrophages were harvested 1 day after intratracheal instillation of silica (10 mg/g bw). NO-dependent CL was calculated as CL in the presence of 2 mg/ml unopsonized zymosan minus CL in the presence of zymosan plus 1 mM L-NAME (an inhibitor of NO synthase). Data are means ± SE of four experiments and are modified from Blackford et al. (21).

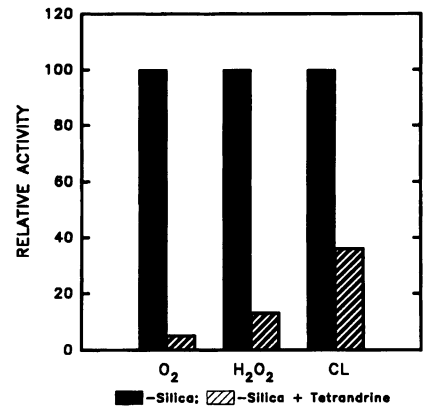


Figure 9. Effect of tetrandrine *in vitro* on silica-induced activation of alveolar macrophages. For oxygen consumption (O₂), cells were exposed at 37°C to 1.7 mg/ml silica in the absence or presence of tetrandrine (75 μM). For hydrogen peroxide release, cells were exposed at 37°C to 1 mg/ml silica in the absence or presence of tetrandrine (27 μM). For chemiluminescence, cells were exposed at 37°C to 0.5 mg/ml silica in the absence or presence of 40 μM tetrandrine. Data modified from Castranova et al. (19).

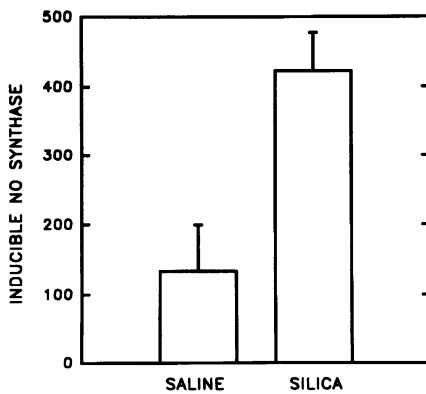


Figure 7. Effect of *in vivo* silica exposure on mRNA levels of the inducible form of nitric oxide synthase in pulmonary phagocytes. Bronchoalveolar lavage cells were harvested 1 day after intratracheal instillation of silica (10 mg/g bw). Cellular mRNA was evaluated for the inducible form of nitric oxide synthase with a ³²P-labeled probe. Data are means ± SE of four experiments and are modified from Blackford et al. (21).

harvested from rats exposed to silica are primed and produce 56% more H₂O₂ upon stimulation with unopsonized zymosan particles (20). Note that although cells harvested from silica-exposed rats are macrophages and granulocytes, unopsonized zymosan does not activate granulocytes. *In vivo* exposure to silica also induces nitric oxide synthase in pulmonary phagocytes and increases NO-dependent chemiluminescence generated by zymosan-stimulated macrophages (21). Figure 7 shows that mRNA for inducible nitric oxide synthase is increased approximately 3-fold in pulmonary phagocytes harvested 1 day after intratracheal instillation of silica. This NO-synthase is primed to produce approxi-

mately 37 times more NO-dependent chemiluminescence upon *in vitro* stimulation with unopsonized zymosan (Figure 8).

The data above suggest that silica activates and primes alveolar macrophages to generate reactive oxidants that can damage lung tissue. In this indirect manner, silica can express additional cytotoxicity. Freshly fractured silica is a more potent stimulant of phagocytes than aged silica, and thus, may exhibit enhanced fibrogenic potential.

Tetrandrine is a Chinese herbal medicine derived from *Stephania tetrandra*. This bisbenzylisoquinoline alkaloid has a molecular weight of 622.73 and an empirical formula of C₃₈H₄₂O₆N₂. Its structure is characterized by methoxy groups at C7 and C12, uncharged nitrogens at N2 and N2', and two 17-carbon ring members connected by a double oxygen bridge between C8-C7' and C11-C12'. Tetrandrine has been reported to decrease several silica-induced pulmonary responses in a rat model, such as collagen and hydroxyproline accumulation, lung weight gain, and nodule production (22-25).

Data from my laboratory indicate that tetrandrine effectively inhibits the stimulation of alveolar macrophages *in vitro* (19). Tetrandrine decreases silica-induced oxygen consumption by 95%, inhibits quartz-stimulated H₂O₂ production by 87%, and depresses silica-dependent chemiluminescence by 64% (Figure 9). ID₅₀ values for this inhibition by tetrandrine are in the range of 30 μM. Tetrandrine (administered orally, 33 μg/g bw daily) is also effective *in vivo*, i.e., decreasing potentiation of H₂O₂

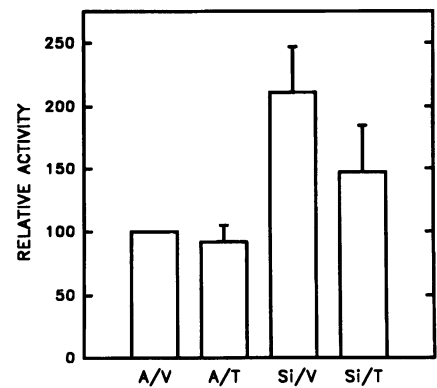


Figure 10. Priming of zymosan-stimulated H₂O₂ production in alveolar macrophages harvested from silica-exposed rats: effect of oral tetrandrine treatment. Rat were intratracheally instilled with 40 mg silica and orally treated for 30 days with tetrandrine (33 μg/g bw) or vehicle (H₂O). After 30 days, alveolar macrophages were harvested and H₂O₂ release measured at 37°C in response to unopsonized zymosan stimulation (2 mg/ml). Data are means ± SE of three experiments and are modified from Castranova et al. (20).

production in alveolar macrophages harvested 30 days after an intratracheal instillation of 40 mg silica (20). Figure 10 shows that tetrandrine decreases the *in vivo* priming of alveolar macrophages by 30%.

The above data indicate that tetrandrine is effective in blocking silica-induced activation of alveolar macrophages *in vitro* and silica-dependent priming of the phagocytes *in vivo*. These inhibitory effects are obtained at doses of tetrandrine that do not compromise the viability of these phago-

cytes. Therefore, tetrandrine may express antifibrotic activity in part by decreasing the production of damaging oxidant species from alveolar macrophages.

In conclusion, data presented in this review support the hypothesis that oxidant

injury plays a role in the pathogenesis of silicosis. These reactive oxygen species can be derived from two sources: direct $\cdot\text{OH}$ generation from freshly fractured silica, and O_2^- , H_2O_2 , and $\text{NO}\cdot$ produced from silica-exposed alveolar macrophages. In addition,

information not presented in this review indicates that oxygen species from recruited blood leukocytes also play a role in the development of silica-induced lung injury (5).

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