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# Comparing Heat-treated Silica Particle with Silica Particles for the Ability to Induce Superoxide Release from Rat Alveolar Macrophages

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**Abstract**

Crystalline silica can devitrify with the formation of cristobalite and other crystalline silica species when exposed to prolonged high temperatures, which is of potential concern because crystalline silica is classified as carcinogenic. Silica particles activate macrophages to release oxidants, which contribute to inflammation and injury in the lower respiratory tract. Our aim was to compare silica particles with heat-treated silica particles for their ability to induce superoxide release from rat alveolar macrophages. We estimated the ability of four types of silica particle samples and heat-treated silica particles with different number average particle diameter to induce lucigenin-dependent chemiluminescence (CL) from macrophages based on the number of silica particles. A strong positive correlation was observed between particle diameter and the ability to induce CL in both the silica and heat-treated silica samples. Moreover, the ability of heat-treated silica samples to induce CL was approximately 43% of that of the silica samples. These results suggest that heat-treated silica reduces superoxide release from macrophages, and that the heat-treated reduces the biological effects of silica.

**Keywords:** Silica; Quartz; Heat-treated; Superoxide; Reactive oxygen species; Macrophage

## Introduction

In 1987 the International Agency for Research on Cancer classified crystalline silica as a probable carcinogen [1] and in 1997 reclassified it as a Group 1 carcinogen [2]. Alveolar macrophages play a critical role in crystalline silica-induced cytotoxicity and genotoxicity [3,4].

Heating modifies the state of the silica surface and progressively decrease the membranolytic activity of silica dust toward erythrocytes [5-7], by eliminating from the surface the sites responsible for the interaction with cell membrane components. Fubini et al. demonstrated that cytotoxicity of crystalline silica to the rat lung alveolar epithelial cell line was greatly reduced for heat-treated silica at 800°C and eliminated for heat-treated silica at 1300°C [8].

We observed a close positive correlation between silica particle diameters and the ability to induce the release of superoxide from macrophages (in contributing). Therefore, it is possible to compare the ability to induce the release of superoxide with silica and heat-treated silica by the method.

The purpose of this study was to investigate the effect of heat-treated silica particles at 800°C in the ability to induce superoxide release from rat alveolar macrophages. Although the silica particles were heat-treated at 1300°C too, the samples could not be used for the experiment because of aggregating.

## Materials and Methods

### Silica samples

Silica (S-5631, Particle size: 0.5–10 μm) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). We suspended silica particles in distilled water, and four samples (A, B, C and D) of different particle sizes were obtained depending on the sedimentation rate of the suspension. A part of each four sample (HA, HB, HC and HD) was heat-treated by an electric furnace (ks-1502, ADVANTEC, Tokyo, Japan). The electric furnace was programed as follows: 500°C for 1 h, 500°C for 10 min, 800°C for 4 h, 800°C for 1 h, and then turned off. The diameters of particles in the four samples were measured using a laser scattering particle size distribution analyzer (LA-700, Horiba Ltd., Kyoto, Japan). Silica particle numbers per unit weight were counted using a hemocytometer under an optical microscope (Microphot-FX, Nikon corp., Tokyo, Japan) (objective lens: (20; eyepiece: (10; intermediate magnifier: (2; resolving power of lens: $\lambda/2N.A.=0.3667$ μm, where $\lambda=0.55$ μm and numerical aperture (N.A.)=0.75).

All samples were dried and heat-sterilized at 80°C for 48 h and suspended in fetal bovine serum (FBS) at concentrations of 1 mg/ml. The suspensions were incubated for 15 min at 37°C, and spin-washed three times in Hanks' balanced salt solution (HBSS) at 900×*g* for 20 min. Pellets were resuspended at 0.77, 3.08, and 5.38 mg/ml. These suspensions were stored at 4°C.

## Cell isolation

Bronchoalveolar lavage was performed on F344 rats (Japan Slc, Inc., Hamamatu, Japan) to isolate alveolar macrophages for in vitro experiments. Briefly, rats were injected peritoneally with 5% pentobarbital sodium at 25 mg/kg. The lungs were lavaged with cold (4°C) HBSS containing 100 U of penicillin and 100 μg of streptomycin per 1 ml with a 10 ml plastic syringe. This process was repeated until a total of 50 mL lavage fluid was collected. The lavage fluid was centrifuged at 250×g, for 10 min at 4°C. The pellet was resuspended in 5 mL of RPMI-1640 medium (HEPES modification, Sigma Chemical Co.) with 10% FBS and penicillin (100 U/mL) and streptomycin (100 μg/mL). In all experiments, the viability of cells was higher than 95 percent as measured by the trypan blue exclusion test. The suspension stored at 4°C until an assay. All procedures associated with this study were reviewed and approved by the Institutional Animal Care and Use Committee at Osaka Prefectural Institute of Public Health.

## CL measurements

The method for measuring lucigenin-dependent CL from the macrophages exposed to various mineral samples has been described in previous reports [9-12].

The isolated cells ($1.5\times10^5$) were transferred to a luminometer tube containing sample suspension (65 μl), 10% FBS, 0.1 mM lucigenin, and in some experiments 1,000 unit/ml superoxide dismutase (SOD). The final volume of each tube was 1 ml. The light emission of each sample was detected at 15-min intervals using a luminescence reader (ALOKA BLR-201, Mitaka, Tokyo, Japan). The CL response of all samples, including the negative control (no particle), was measured at constant rotation every 15 min using a stock suspension of cells. We performed all reactions at 37°C in RPMI 1640, and each measurement three times.

## Statistical analysis

We estimated the ability to induce CL per sample particle for exclusion of sample dose effects, as described previously [10,11]. Briefly, we plotted the relationships between the administered numbers of samples and CL response. A slope (i.e., $\beta_1$) of linear regression of the administered numbers of samples and CL was estimated as the ability to induce CL per sample. As $\beta_1$ was a constant, it was possible to universally compare with each sample and to examine relationship between the ability to induce CL and sample geometries. In this study, we examined the relationship between $\beta_1$ and number average particle diameter of samples by linear regression in two groups of silica samples and heat-treated silica samples. Moreover, we compared the slopes (i.e., $\beta_2$) of linear regression of two groups to examine the reducibility of heat-treatment.

# Results

## Silica size per unit weight

Table 1 shows the results of silica samples from the laser scattering particle size distribution analyzer, and the results of particle number per unit weight of silica samples using a hemocytometer and an optical microscope. Each diameter of heat-treated silica was considerably similar with that of silica. We used the number average particle diameter as the particle size.

Time course of the ability to induce CL per dust particle ($\beta_1$)

We calculated $\beta_1$ to compare the CL response of each sample at a value not related to the number of sample particles. Table 2 shows $\beta_1$ and $r^2$ values of the regression line with CL and number of sample particles. All samples dose-dependently induced a CL response. Each response was almost completely inhibited by SOD, which is a superoxide scavenger (data not shown). Figure 1 shows a representative relationship between the number of sample particles and CL at 45 min. The ability to induce CL of heat-treated silica samples was reduced than silica samples.

| Sample | A | HA | B | HB | C | HC | D | HD |
|---|---|---|---|---|---|---|---|---|
| median diameter (μm) | 3.79 | 4.99 | 2.89 | 3.08 | 0.99 | 0.83 | 0.68 | 0.58 |
| number average particle diameter (μm) | 5.43 | 5.27 | 4.34 | 4.52 | 2.31 | 2.22 | 1.10 | 1.15 |
| % diameter: 90.0% (μm) | 8.09 | 8.95 | 6.71 | 6.92 | 2.95 | 2.84 | 1.39 | 1.08 |
| number per unit weight (/μg) | 6650 | 6780 | 9255 | 9285 | 55286 | 56143 | 340500 | 412500 |
| ratio of number per unit weight | (1 : 1.020) | | (1 : 1.003) | | (1 : 1.016) | | (1 : 1.211) | |

**Table 1:** Particle diameter and number per unit weight of silica samples.

| Timea | 0 | | 15 | | 30 | | 45 | | 60 | | 75 | | 90 | | 105 | | 120 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\beta_1$[b] | $r^2$[c] | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ | $\beta_1$ | $r^2$ |
| A | −0.6 | 0.59 | 25.2 | 0.73 | 40.5 | 0.99 | 66.2 | 0.95 | 104 | 0.97 | 151.3 | 0.99 | 174.7 | 0.98 | 166.4 | 0.95 | 145.6 | 0.95 |
| HA | −0.3 | 0.40 | 5.5 | 0.49 | 15.9 | 0.85 | 27.6 | 0.98 | 48.3 | 0.99 | 66.4 | 1.00 | 87.0 | 0.98 | 92.1 | 0.99 | 88.9 | 0.97 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B | −0.1 | 0.01 | 18.8 | 0.72 | 35.4 | 1.00 | 59.9 | 1.00 | 101.5 | 1.00 | 139.2 | 0.99 | 148.4 | 0.99 | 139.5 | 0.96 | 118.6 | 0.95 |
| HB | −0.2 | 0.40 | 10.8 | 0.92 | 14.9 | 0.91 | 25.0 | 0.98 | 41.4 | 0.96 | 63.4 | 0.96 | 77.4 | 0.97 | 83.3 | 0.96 | 80.4 | 0.96 |
| C | 0.0 | 0.05 | 1.2 | 0.54 | 4.1 | 0.96 | 9.0 | 0.99 | 15.5 | 0.99 | 22.9 | 0.99 | 25.4 | 0.99 | 23.4 | 0.96 | 19.0 | 0.92 |
| HC | 0.0 | 0.02 | 4.0 | 0.94 | 5.3 | 0.93 | 7.3 | 0.98 | 10.3 | 0.98 | 15.1 | 0.97 | 17.9 | 0.97 | 19.6 | 0.97 | 19.1 | 0.96 |
| D | 0.0 | 0.86 | 0.1 | 0.14 | 1.2 | 0.99 | 1.8 | 0.99 | 2.5 | 1.00 | 3.2 | 0.99 | 3.7 | 0.99 | 3.8 | 0.97 | 3.5 | 0.96 |
| HD | 0.0 | 0.00 | 0.2 | 0.67 | 0.2 | 0.48 | 0.4 | 0.89 | 0.5 | 0.90 | 0.6 | 0.94 | 0.7 | 0.91 | 0.9 | 0.91 | 1.1 | 0.94 |

**Table 2:** Slope ($\beta_1$) and $r^2$of the regression lines for CL and number of silica particles.

[a]Time after administration (min). [b]$\beta_1$ ($\times 10^{-8}$) is the slope of the regression line for the estimated number of silica particles administered and CL response with three concentrations and a duplicate negative control. The CL response is the mean value of the three measurements. [c]Square of the correlation coefficient of the regression line.

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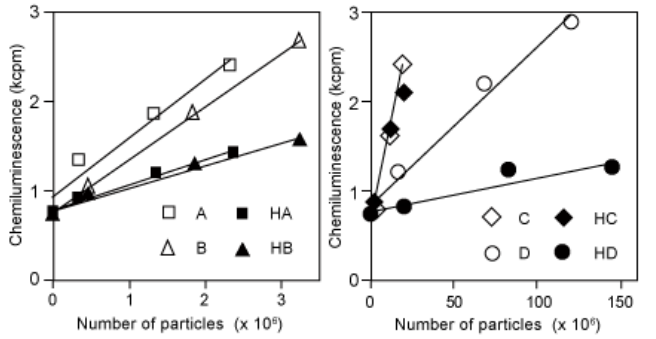


**Figure 1:** The relationship between number of dust particles and CL response at 45 min. The lines represent regression lines. The slope (β1) of the line was taken as a measure of the ability to induce CL per silica particle.

## Relationship between the ability to induce CL per dust particle (β1) and silica diameter

Figure 2 shows a representative relationship between $\beta_1$ and number average particle diameter in the two groups (silica particles and heat-treated silica particles). A close correlation was found between $\beta_1$ and sample particle diameter in both the two groups. $\beta_2$ of regression lines of the heat-treated silica group was weaker than that of silica group.

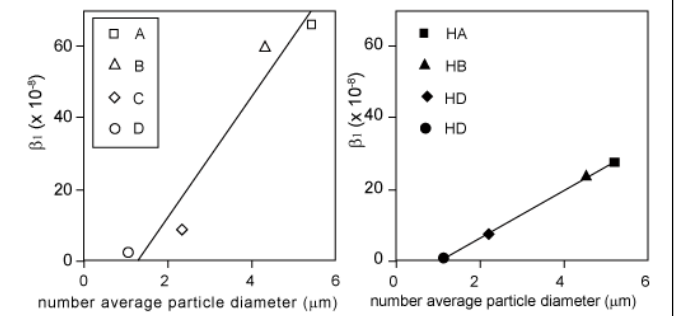


Figure 2: The relationship between particle size (the number average particle diameter) and *β1* in Table 2. The lines represent regression lines. Data at 45 min.

Table 3 presents $\beta_2$ and $r^2$ of the regression lines with particle diameter and $\beta_1$. A close correlation was found between sample particle diameter and $\beta_1$ at each time-point in both the two groups. Silica group was more active than heat-treated silica group. $\beta_2$ of heat-treated silica group was 43 percent of silica group at the average measurement period.

| Time[a] | 15 | | 30 | | 45 | | 60 | | 75 | | 90 | | 105 | | 120 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\beta_2$[b] | $r^2$[c] | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ | $\beta_2$ | $r^2$ |
| Silica[d] | 6.31 | 0.95 | 10.2 | 0.94 | 16.7 | 0.95 | 26.9 | 0.93 | 38.4 | 0.95 | 43.3 | 0.96 | 41.1 | 0.97 | 35.7 | 0.97 |
| HS[e] | 1.75 | 0.59 | 3.92 | 0.99 | 6.89 | 0.99 | 12.1 | 1.00 | 17.2 | 0.98 | 22.1 | 0.99 | 23.5 | 0.99 | 22.6 | 0.99 |
| Ratio[f] | 3.60 | | 2.61 | | 2.43 | | 2.23 | | 2.23 | | 1.96 | | 1.75 | | 1.58 | |

**Table 3:** Slope ($\beta_2$) and $r^2$ of the regression lines for $\beta_1$ in Table 2 and particle size.

[a]Time after administration (min). [b]$\beta_2$ ($\times 10^{-8}$) is the slope of the regression line for β1 in Table 2 and particle size (the number average particle diameter). [c]Square of the correlation coefficient of the regression line for $\beta_1$ in Table 2 and particle size. [d]Group of four silica

samples. eGroup of heat-treated four silica samples (HS). $^{f}\beta_2$ of silic /$\beta_2$ of HS.

## Discussion

We found the ability of heat-treated silica samples to induce CL from rat alveolar macrophages was weaker than that of silica samples. It was possible to universally compare the effect of particle diameter between the two groups when $\beta_2$ (Figure 2 and Table 3) was excluded.

These results suggest that the heat-treated of silica reduce the superoxide release from macrophages. Our results consist with oxidants from macrophages relate to carcinogenicity of silica particles.

Heating modifies the state of the silica surface and progressively decrease the membranolytic activity of silica dust toward erythrocytes [5-7], by eliminating from the surface the sites responsible for the interaction with cell membrane components. Although cytotoxicity of crystalline silica to the rat lung alveolar epithelial cell line was greatly reduced for heat-treated silica at 800°C and eliminated for heat-treated silica at 1300°C, heat-treated at 1300°C did not affect silica micromorphology and crystallinity. However, surface of silica, still showed some hydrophilic patches in heat-treated at 800°C, but heat-treated at 1300°C was fully hydrophobic [8]. Although we heat-treated the four types of silica particles at 800 and 1300°C, the samples of heat-treated silica at 1300°C were not examined by the particle aggregation. It was assumed that the surface of silica was made to change by the heat-treated at 1300°C very much.

We suggested previously that the induction superoxide release from macrophages occurs nonspecifically for various types of mineral fibers depending on fiber length, and that the kinetics of the induction superoxide release from macrophages is similar between silica particles and mineral fibers; moreover, this depends on silica particle size and mineral fiber geometry. However, present results show that the activity of the induction superoxide release from macrophages is different for two types of silica particles, may differ surface character. It seems that the present macrophage activity results are inconsistent with the nonspecific mineral fiber results. We considered to the inconsistency that the fiber length affects to release superoxide from macrophages further than the fiber surface characteristics. In fact, we previously demonstrated that the activity of mineral fiber to induce superoxide release from macrophages was approximately 8.3 times greater than that of silica [12].

In conclusion, our results suggest that the ability of heat-treated silica samples to release superoxide from macrophages reduced to approximately 43 percent of that of silica sample and that heat treatment reduces the biological effects of silica.

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