

Cytotoxicity of yellow sand in lung epithelial cells

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The present study was carried out to observe the cytotoxicity of yellow sand in comparison with silica and titanium dioxide in a rat alveolar type II cell line (RLE-6TN). Yellow sand (China Loess) was obtained from the loess layer in the Gusu Province of China. The mean particle diameter of yellow sand was about 0.003 ± 0.001 mm. Major elements of yellow sand were Si($27.7 \pm 0.6\%$), Al($6.01 \pm 0.17\%$), and Ca($5.83 \pm 0.23\%$) in that order. Silica and yellow sand significantly decreased cell viability and increased $[Ca^{2+}]_i$. All three particles increased the generation of H_2O_2 . TiO_2 did not change Fenton activity, while silica induced a slight increase of Fenton activity. In contrast, yellow sand induced a significant increase of Fenton activity. Silica, yellow sand and TiO_2 induced significant nitrite formations in RLE-6TN cells. Silica showed the highest increase in nitrite formation, while yellow sand induced the least formation of nitrite. Silica and yellow sand increased the release of TNF- α . Based on these results, we suggest that yellow sand can induce cytotoxicity in RLE-6TN cells and reactive oxygen species, Fenton activity and reactive nitrogen species might be involved in this toxicity.

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1. Introduction

The Asian dust storms (called as 'yellow sand') bring a large amount of soil particles from the deserts of Mongolia and China to East Asia and North America. Yellow sand is a dust-laden air mass, and its effect to the atmospheric environment is of large concern in the Asian-Pacific area including Korea, Japan and China. In spring, this phenomenon is very common in this area. Thus, the composition of ion and metal constituents is analysed with continuous sampling of yellow sand. Also, the committee of certified reference material from Asian countries including Korea, Japan, and China tried the collection of the reference material from China Loess. We used this reference material as yellow sand because it is an important core material though its components may be changed by the local condition through the area.

This major atmospheric phenomenon acts as a critical issue because of the seasonal increase of respiratory diseases (Kwon *et al* 2002). Presently, there is no report to explain the mechanism of yellow sand-induced cell injury. Although the exact molecular mechanism by which particles can cause cell injury and death remains unknown; reactive oxygen species (ROS) or reactive nitrogen species (RNS) have been implicated as the major contributor in particle-induced cell injury. It is well known that ROS and/or RNS can damage cells through the peroxidation of cellular lipids, DNA strand breakage, and oxidation of protein eventually leading to cell death.

In an attempt to understand the pathogenesis of yellow sand-induced respiratory tract injury, we compared the effect of yellow sand with that of silica and titanium dioxide (TiO_2) in a rat alveolar type II cell line (RLE-

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Abbreviations used: AEC, Alveolar epithelial cells; AM, alveolar macrophage; RNS, reactive nitrogen species; ROS, reactive oxygen species; TBA, thiobarbituric acid; TiO_2 , titanium dioxide.

6TN) as the target cell. Also, we have focused on the role of ROS and other free radicals in yellow sand-induced cell injury. For this experiment, we used yellow sand (China Loess).

2. Materials and methods

2.1 Particles

2.1a Yellow sand: We used Certified Reference Materials (CRMs) obtained from National Research Centers. In brief, yellow sand (China Loess) was collected in the loess layer (1.8–2.5 m from the surface) in the Gusu Province of China, and the small loess particles obtained after sieving of 100 mesh were blended by a V blender. The particle diameter of yellow sand prepared without grinding was under 10 μm and was irradiated by cobalt 60 for sterilization. The analysed elemental constituents of this material are as follows: Na ($1.33 \pm 0.06\%$), Mg ($1.57 \pm 0.06\%$), Al ($6.01 \pm 0.17\%$), Si ($27.7 \pm 0.6\%$), K ($1.94 \pm 0.10\%$), Ca ($5.83 \pm 0.23\%$), Ti (0.36%), and Fe ($2.94 \pm 0.09\%$).

2.1b Silica and TiO_2 : Silica (α -quartz, Min-U-Sil5) was obtained from US Silica Company (Clarkstown, WV, USA). The Min-U-Sil5 particle had mean particle size of 1.6 μm and 97% less than 5 μm in diameter. α -quartz had a purity of 98.7%. TiO_2 (99% purity) was purchased from Sigma Chemical Co., St. Louis, MO, USA. TiO_2 had particle size of $\sim 1.0 \mu\text{m}$.

2.2 Cell line and culture conditions

The rat alveolar type II cell line, RLE-6TN (ATCC CRL-2300), was maintained in BRFF-RluE (BRFF, SC-10) culture medium. The cells were cultured at 37°C in a humidified incubator with 5% CO_2 . RLE-6TN cells were plated on 24 wells at a density of 2×10^6 and incubated for 24 h. After that, cells were starved for 18 h with 0.1% FBS. After the treatment with particles (100 $\mu\text{g}/\text{cm}^2$) for 18 h, conditioned media were collected for measurement of H_2O_2 and nitrite and for cytokine assay.

2.3 Neutral red assay

The cells were plated at 96-well plate (1×10^4 /well). After the cells were cultured with complete medium for 24 h, they were treated with particles at indicated concentrations. After 24 h incubation, the cells were washed with phosphate-buffered saline. The adherent cells were further incubated with media containing 0.006% neutral red

for 75 min. After washing with PBS, neutral red in the viable cells was eluted into 100 μl of 0.01 N HCl containing 30% ethanol, and absorbance of each well was read at 540 nm with microplate reader (BIO-RAD 550, USA).

2.4 Intracellular calcium level ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ was measured using the method previously described by Yang *et al* (2001). Cover glass was attached to a 1 cm^2 area at the bottom of 35 mm plastic culture dishes. Cells were seeded onto a 22 \times 22 mm cover at a concentration of 10^4 cells/dish. Cells were washed with modified Hanks' solution (pH 7.4) consisting of (in mM): NaCl, 127; MgSO_4 , 0.8; KH_2PO_4 , 0.44; NaHPO_4 , 0.33; MgCl_2 , 1; HEPES, 10; CaCl_2 , 1. Cells were loaded with fura-2/AM (10 μM) for 45 min at 37°C. Fluorescence-loaded cells were washed three times with the same solution to exclude any unloaded fura-2/AM. The fluorescence in RLE-6TN was measured at room temperature using the InCa™ Imaging System (Intracellular Imaging Inc., Cincinnati, Ohio, USA).

2.5 Measurement of H_2O_2

H_2O_2 in culture supernatant was determined as formation of ferric thiocyanate by a method described by Heinzl *et al* (1992). Briefly, 83.3 μl HCl was added to supernatant 166.67 μl , and 50 μl ferrous ammonium sulphate and 20 μl potassium thiocyanate were added to give final concentrations of 3.2 mM and 180 mM, respectively. After 10 min incubation at room temperature, immediate determination of absorbance was done at 492 nm. H_2O_2 concentration was calculated from a H_2O_2 standard curve.

2.6 Evaluation of Fenton activity (measurement of particle activity as a Fenton catalyst)

The capability of a particle to support transitional metal-dependent generation of ROS was measured by an assay that employs deoxyribose as a detector molecule. Based on the degradation of deoxyribose by $\text{OH}\cdot$ and its subsequent reaction with thiobarbituric acid (TBA) to form a coloured product, Fenton activity was evaluated. The reaction tubes contained 1 mM deoxyribose, 1 mM H_2O_2 , 1 mM ascorbate, and particles (100 $\mu\text{g}/\text{ml}$). After 30 min incubation at 37°C, the tubes were centrifuged at 4,000 rpm for 10 min. An aliquot of supernatant (1 ml) was mixed with 1 ml of 1% TBA and 1 ml of 2.8% trichloroacetic acid. The mixture was incubated for 10 min at 100°C followed by cooling to room temperature. The coloured product was measured at 532 nm (Kim *et al* 2000).

2.7 Nitrite determination

Supernatant (100 μ l) was transferred onto a 96-well plate. After the addition of 100 μ l Griess reagent (1 : 1, v/v, N-1-naphthylethylenediamine 0.1% in H₂O, sulfanilamide 1% in 5% H₃PO₄) to each well, absorbance at 550 nm was measured using a microplate reader (BIO-RAD 550, USA). NO₂ concentration was calculated from a NaNO₂ standard curve.

2.8 Cytokine analysis

In supernatant, TNF- α was detected by an enzyme-linked immunosorbent assay (ELISA) method using a goat anti-human TNF- α primary antibody and a peroxidase-conjugated anti-goat secondary antibody (Santa Cruz Inc, Santa Cruz, USA). For the measurement of PDGF-AA, a rabbit anti-human PDGF-AA IgG and a peroxidase-linked goat anti-rabbit IgG (Santa Cruz Inc, Santa Cruz, USA) were used (Kim *et al* 2000).

2.9 Statistical analysis

All values were represented as mean \pm SE. Statistical significance was determined by Student's *t*-test for two-group comparison. A *P*-value less than 0.05% was considered as statistically significant.

3. Results

3.1 Viability

To characterize particle-induced cytotoxicity, cell viability was observed in particle-stimulated RLE-6TN cells using the neutral red assay. At the concentration of 100 μ g/cm², silica and yellow sand significantly decreased cell viability while TiO₂ slightly decreased cell viability. At the concentration of 1 mg/cm², all the particles decreased cell viability. Among these, silica showed the strongest toxic effect on RLE-6TN cells while TiO₂ had the least toxic effect (figure 1).

3.2 Effect of particles on [Ca²⁺]_i

In RLE-6TN cells, yellow sand at the concentration of 5 mg/cm² increased [Ca²⁺]_i within 1 min and its peak level was observed within several minutes. Also, silica (5 mg/cm²) increased [Ca²⁺]_i of RLE-6TN cells. In contrast to these particles, TiO₂ did not change [Ca²⁺]_i (figure 2).

3.3 Generation of H₂O₂

We measured the level of H₂O₂ in particle-stimulated RLE-6TN cells. All three particles increased the generation of H₂O₂. There is no clear difference between the potency of these particles (figure 3).

3.4 Effect of particles on Fenton activity

In RLE-6TN cell, TiO₂ did not change Fenton activity and silica induced a slight increase of Fenton activity. In contrast, yellow sand induced a significant increase of Fenton activity. Yellow sand had a stronger Fenton activity than silica (figure 4).

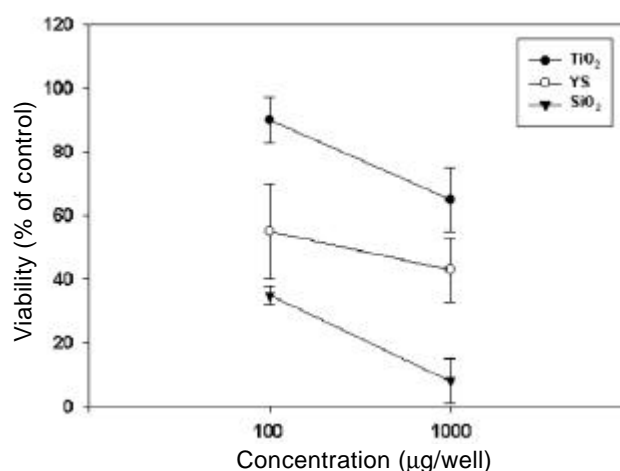


Figure 1. Effect of particles at different concentrations on the viability of RLE-6TN cells. After 24 h incubation, viability was determined using the neutral red assay. Values were represented by mean \pm SE of 6 experiments.

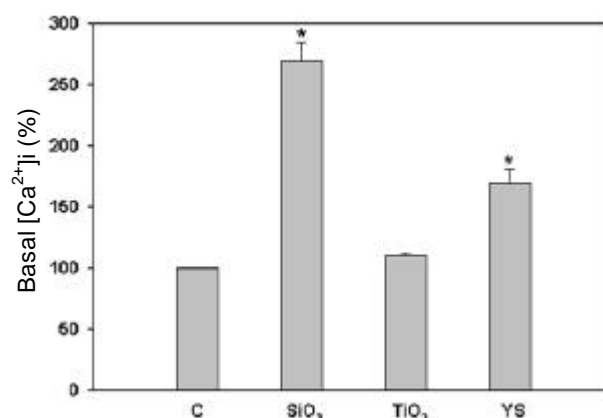


Figure 2. Effect of particles on [Ca²⁺]_i in RLE-6TN cells. Cells were loaded with fura-2/AM (10 μ M) for 45 min at 37°C. C, Control; SiO₂, silica; TiO₂, titanium dioxide; YS, yellow sand. Values were represented by mean \pm SE of 6 experiments. *, Significantly different from control (*P* < 0.05).

3.5 Generation of RNS

Silica, yellow sand and TiO_2 induced significant nitrite formations in RLE-6TN cells. Silica showed the highest increase in nitrite formation, while yellow sand induced the least formation of nitrite (figure 5).

3.6 Effect of particles on TNF- α production

TNF- α production was increased in silica-treated cells and yellow sand-treated cells. In contrast, TiO_2 did not induce significant TNF- α production (figure 6).

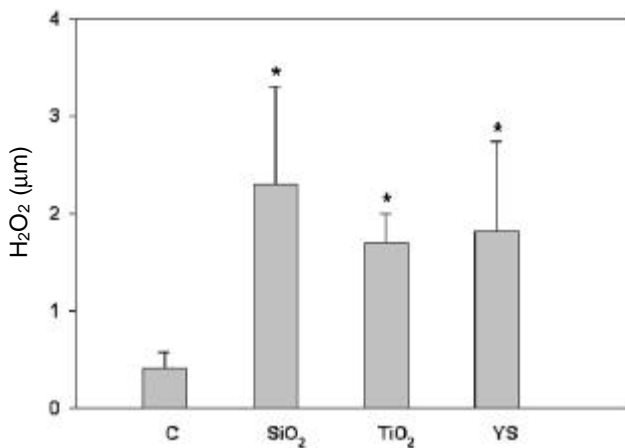


Figure 3. Effect of particles on generation of H_2O_2 in RLE-6TN cells. After the treatment of particles ($100 \mu\text{g}/\text{cm}^2$) for 18 h, conditioned media were collected for measurement of H_2O_2 . Values were represented by mean \pm SE of 6 experiments. *, Significantly different from control ($P < 0.05$).

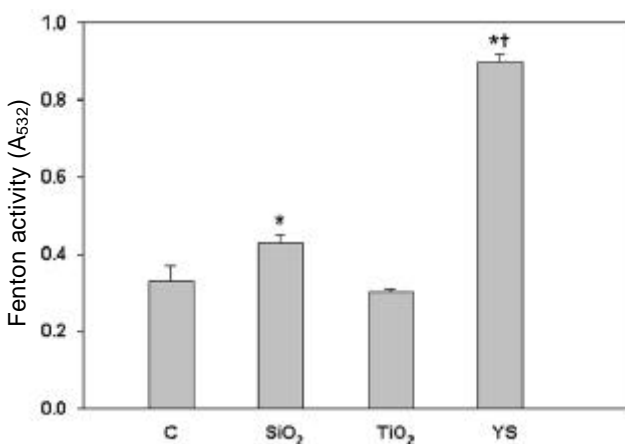


Figure 4. Effect of particles ($100 \mu\text{g}/\text{cm}^2$) on Fenton activity. Values were represented by mean \pm SE of 6 experiments. *, Significantly different from control ($P < 0.05$). †, Significantly different from silica ($P < 0.05$).

4. Discussion

There is a clear association between Asian dust events and enhancement of mortality. During a dust event, a 1.7% increase in the risk of death from all causes was observed. Specifically, the relationship between the Asian dust events and deaths from cardiovascular and respiratory causes was strong. This means that people with advanced cardiovascular and respiratory disease may be susceptible to the Asian dust events (Kwon *et al* 2002). Such an epidemiological report always requires

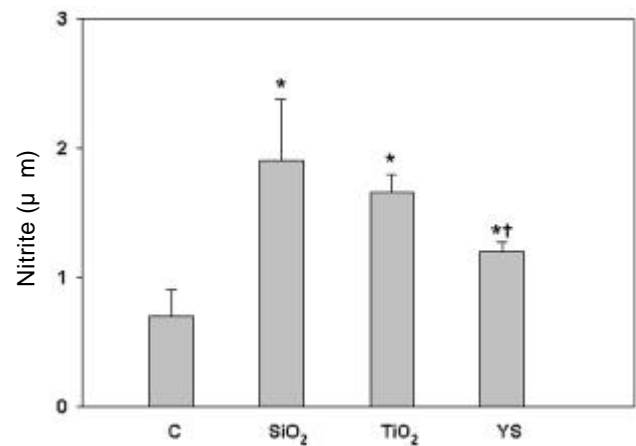


Figure 5. Effect of particles on generation of RNS in RLE-6TN cells. After the treatment of particles ($100 \mu\text{g}/\text{cm}^2$) for 18 h, conditioned media were collected for measurement of nitrite. Values were represented by mean \pm SE of 6 experiments. *, Significantly different from control ($P < 0.05$). †, Significantly different from silica ($P < 0.05$).

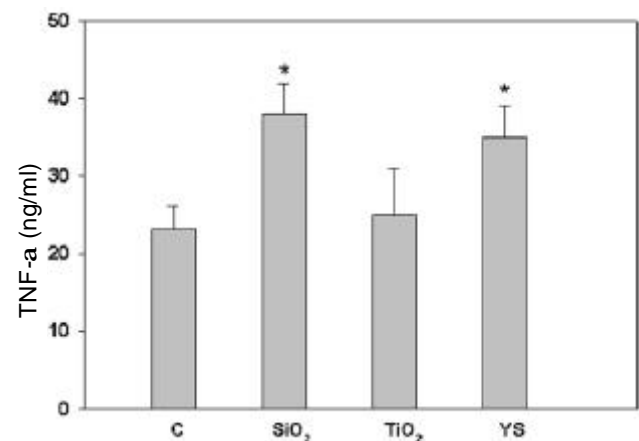


Figure 6. Effect of particles on TNF- α production in RLE-6TN cells. After the treatment of particles ($100 \mu\text{g}/\text{cm}^2$) for 18 h, conditioned media were collected for cytokine assay. Values were represented by mean \pm SE of 6 experiments. *, Significantly different from control ($P < 0.05$).

the evaluation of biological plausibility to explain this phenomenon.

In this experiment, we tried to elucidate the pathogenesis of yellow sand-induced cytotoxicity. Firstly, we observed the effect of yellow sand on cell viability. Silica is a well-known toxic particle while TiO₂ is a relatively inert particle. Thus, we compared the effects of three particles, silica, TiO₂ and yellow sand on cell viability. Cell viability in yellow sand-stimulated cells was higher than that in silica-stimulated cells, and lower than that in TiO₂-stimulated cells. Effects of particles on intracellular calcium level were very similar to cell viability. While TiO₂ did not change [Ca²⁺]_i, silica induced an increase in free [Ca²⁺]_i. Yellow sand increased [Ca²⁺]_i, but the response was weaker than silica. These results suggest that yellow sand exhibits a clear cytotoxicity, and its potency is lower than silica.

ROS are produced by particle-stimulated alveolar macrophages (AM). ROS react as strong mediators in cytotoxicity, carcinogenesis and release of cytokines. Besides AM, fibroblast and bronchial epithelial cells too can generate ROS by stimulation of particles (Kim *et al* 2002; Aljandali *et al* 2001). Asbestos causes apoptosis in alveolar epithelial cells (AEC) including WI-26 (human type I-like cells), A549 (human type II-like cells) and rat alveolar type II cells. Asbestos-induced apoptosis and caspase 3 activation are attenuated by iron chelators or hydroxyl radical scavengers, suggesting a role of iron-catalyzed ROS in asbestos-induced cytotoxicity (Aljandali *et al* 2001).

In this experiment, all particles induced the generation of H₂O₂ without clear difference in potency. In contrast, Fenton activity showed the clear difference between particles. Yellow sand showed high Fenton activity. Also, nitrite was measured as an indicator of acute lung cell injury. Nitric oxide (NO) plays an important role in cytotoxicity through its own action or synergism with ROS. All particles could induce nitrite formation in RLE-6TN cells.

ROS has been reported to play an important role in signal transduction pathways. Low levels of ROS can induce the synthesis of proteins associated with pathophysiological responses. ROS activates protein phosphorylation and NF- κ B nuclear transcription factor binding

affinity. In alveolar type II cells, silica induces the increased expression of TNF- α . This TNF- α -induced generation of ROS leads to the activation of genes for the monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 (Barrett *et al* 1999). Our result showed that silica and yellow sand increased the production of TNF- α in RLE-6TN cells. Thus, we assume that TNF- α to be secreted in particle-stimulated AEC might be involved in the generation of ROS which perhaps play an important role in signal transduction pathway including secretion of cytokines.

From these observations, we suggest that yellow sand can induce cytotoxicity in RLE-6TN cells; and that ROS, Fenton activity and RNS might be involved in this cytotoxicity. Moreover, additional research is necessary to examine a causal relationship between exposure to yellow sand and pulmonary injury.

Acknowledgements

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