

Cytotoxicity of Quartz and Montmorillonite in Human Lung Epithelial Cells (A549)

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Abstract In this study, A549 cell viability, extracellular activities of lactate dehydrogenase (LDH), and tumor necrosis factor (TNF)- α and interleukin (IL)-6 levels were investigated after incubation with quartz (KWC-Q4 and KWC-Q3), Nano-SiO₂, and KWC-M; the micronucleus test and comet assay were carried out to evaluate the genotoxicity. The results showed there were significant differences in the cell death rate and extracellular LDH activities compared with the control group, and showed a good linear relationship in certain concentration range. All mineral particles tested can induce the increase of TNF- α after incubation with mineral powders at 200 μ g/mL for merely 3 h and also induce significant increase of IL-6 for 24 h; the results indicated that inflammatory reaction can be triggered by the exposure of KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M. The results of micronucleus test showed FMN (Frequency of micronucleus number) listed as Nano-SiO₂>KWC-Q3>KWC-Q4. There was no significant FMN increase of KWC-M compared with the control group, which maybe resulted from its high cell mortality at low concentration. The comet assay confirmed the genotoxicity of all tested samples, and the DNA damage: KWC-M>Nano-SiO₂>KWC-Q4>KWC-Q3.

Keywords Quartz • Montmorillonite • A549 • Cytotoxicity

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1 Introduction

More evidences concerning mortality, increased asthma morbidity, and other respiratory disorders with increased concentrations of airborne fine particulate matters whose aerodynamic diameter was less than $2.5\ \mu\text{m}$ have attracted the public attention. Experiments *in vitro* and *in vivo* all have revealed that the exposure can result in pulmonary inflammation, airway hyperreactivity, cell impairment, and epithelial permeability increase. For their small diameter, they can be transported deeply to the alveolar in the lungs, even involved in the blood circulation [1], and there is no effective clearance mechanism. The relationship between them is obviously.

As for the airborne particles, researches can be classified into three kinds: (1) the whole particle, it may attach some organics or adsorb heavy metals around the nucleus; (2) leachate of airborne particles, it often contains the soluble components of the particle itself as well as the attachments on its surface; (3) particles after leaching, it is always called as a bare nucleus. It is revealed that different fractions of particle stir the toxic behaviors to different extent, but the mechanisms are still uncertain [2, 3]. Although it has been ignored for a long time, as one important part of airborne particles, mineral particle counted for a half of the total mass [4]. According to the phase analysis of particles captured during the sandstorm, silicate minerals counted for 60 % approximately. In the normal daily life, clay and diagenetic minerals also play a vital role in the air. Researches focus on the safety assessment of airborne particles, especially the particles attached with organics and heavy metals, and there are some pathogenesis mechanisms being developed. However, the complex composition of particles limits the real reasons that stir the blasting fuse. In this chapter, we focused on the natural mine particles (including silica and montmorillonite) and exposed lung epithelial cells to particles of different size fractions ($<5\ \mu\text{m}$ and $<2.5\ \mu\text{m}$). The particles were analyzed for size, mineral phase, and element composition. The experiment aimed to investigate the cytotoxic and genotoxic potential of natural mine particles to lung epithelial cells and to analyze how the various physicochemical properties were related to chemokine release. The study also aimed to examine whether information on physicochemical characteristics could be used to predict the biological effects of stone quarry particles.

2 Materials and Methods

2.1 Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), RPMI 1640 medium with L-glutamine, penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Hyclone, American. LDH

Detection Kit, TNF- α Detection Kit, and IL-6 Detection Kit were bought from Nanjing Jiancheng Bioengineering Institute, Nanjing city, China. All other reagents and solvents used were of analytical grade. All solutions were prepared with deionized water from a Milli-Q purification system.

2.2 Cell Culture and Treatment

A549 cell line was obtained from the experimental center of the Affiliated Hospital of Luzhou Medical School which was purchased from the American Tissue Type Culture Collection (ATCC, Rockville, MD, USA). It is one of typical epithelial cells of lung adenocarcinoma and is considered as a good model to study the interaction between allogenic material and the respiratory system because of its typical characteristics of II type alveolar epithelial cells in the cultivating process. Besides as a mechanical barrier, lung epithelial cells also act as the first line of breathing defense system and contain important pathogen recognition system of transmembrane and intracellular pathogens. This research aims at elaborating the toxicity effect of A549 cells incubated with different size quartz powder and nature montmorillonite mineral powder (Table 1).

Cells were maintained in RPMI1640 medium containing 10 % fetal calf serum (FBS) and 1 % penicillin/streptomycin at 37 °C with 5 % of CO₂ prior to exposure. The cell line was split with 0.25 % trypsin and the medium was changed the day after.

2.3 Particle Preparation and Analysis Method

Silica rock was obtained from Erlangmiao quarry in Jiangyou, Sichuan. Rocks were firstly crushed into fragments by horizontal planetary ball mill and then milled with deionized water in the ball mill. Particles were grinded through 200 mesh sieve after dehydration in the air dry oven, finally. Because Quartz milling has different median diameters at different times, the powders' size distributions within the

Table 1 The experiment design

| Test item | Concentration ($\mu\text{g/mL}$) | Time (h) |
|--------------------------|------------------------------------|-------------|
| MTT-cell viability | 0, 50, 100, 200, 400, 800 | 24 |
| LDH-cell membrane damage | 0, 50, 200, 800 | 24 |
| TNF- α | 0, 50, 200, 800 | 3, 6, 8, 24 |
| IL-6 | 0, 50, 200, 800 | 24 |
| Comet assay | 0, 50, 100, 200, 400 | 24 |
| Micronucleus test | 0, 50, 100, 200, 400 | 24 |

<5 μm and <2.5 μm fractions were labeled as KWC-Q3 and KWC-Q4, respectively.

Montmorillonite was obtained from Altai, Xinjiang, which appeared light yellow powder. It was first soaked in distilled water for 3 days before removing the floating and suspending debris and then stirred for 3 days with the pulp density of 2 wv%. Granularity of the particle suspension was determined by Laser Particle Analyzer after removing the top supernatant liquid and stood out for 24 h. The suspension was then dehydrated in the air dry oven and grinded through 200 mesh sieve oven finally. The particle size distribution of purified montmorillonite within 10 μm was labeled as KWC-M.

All the dried mineral powders were dispersed in the cultural medium and sonicated for 30 min prior to size distribution analysis by Laser Particle Analyzer again. Besides, mineral phases were determined using X-ray diffraction (XRD) analysis (X'pert PRO PHILIPPOS); semiquantitative element was analyzed by X-ray fluorescence (XRF, Magix, PANalytical); particle surface morphology character was measured by scanning electron microscope (SEM, TM-1000, HITACHI).

2.4 MTT Proliferation Assay

Cell viability was measured by the MTT assay, performed according to kit manual. Briefly, 20 μL of MTT solution (5 mg/mL) per well was incubated with cells (about 2.0×10^5 cells) for further 4 h at 37 $^{\circ}\text{C}$, 5 % CO_2 after the incubation experiment already described above. Then the wells were drained, cells were washed with PBS, and 200 μL of DMSO was added to dissolve the intracellular crystalline formazan product for 30 min at room temperature. The optic density at 490 nm was read spectrophotometrically using a Microplate Reader (Multiskan GO, THERMO) immediately. The results were expressed as a percentage of the absorbance of control cells, called relative proliferation rate (ω), following the equation:

$$\omega \times 100\% = \frac{\text{OD}_{\text{expos.c}} - \text{OD}_{\text{cont.m}}}{\text{OD}_{\text{cont.c}}} \times 100\% \quad (1)$$

where $\text{OD}_{\text{expos.c}}$ stands for the well optic density of cells incubating with different mineral samples, $\text{OD}_{\text{cont.m}}$ for the well optic density of mineral suspensions without cells, and $\text{OD}_{\text{cont.c}}$ for the cells of negative group without exposing to the mineral suspensions or others.

2.5 LDH Activity Assay and Cytokines Release

Cells were incubated with different concentrations (50, 200, and 800 $\mu\text{g/mL}$) of quartz and montmorillonite in six-well plates at 37 $^{\circ}\text{C}$ for 24 h. Subsequently,

culture media were collected and centrifuged in two steps (first at 250 g aiming to remove cells and then at 2,500 g to remove the remaining particles). The final supernatants were stored at -80°C . LDH, IL-6, and TNF- α levels were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' guides. Absorbance was measured and quantified by the Microplate Reader.

2.6 *Micronucleus Assay*

To assess the chromosomal damage of particles to A549 cells, the micronucleus (MN) assay was performed as follows. Briefly, cells were seeded in a 12-well chamber slide at a density of 1×10^5 cells/well and grown for 24 h. Cells were treated with 0, 50, 100, 200, and 400 $\mu\text{g}/\text{mL}$ powder suspensions with 2 v/v% FBS in RPMI1640 for 24 h. Cyclophosphamide (CP, 300 $\mu\text{g}/\text{mL}$, 24 h, Sigma) was used as a positive control. Each sample was run in duplicates. After incubation with different mineral suspensions at 37°C or 24 h, medium was removed and cells were fixed with 1 mL stationary liquid (with methanol and formaldehyde volume ratio is 3:1, precooled at -20°C until staining and evaluation) for 30 min. To stain the cells, stationary liquid was aspirated and cells were stained for 10 min with Wright-Giemsa stain (Baso Diagnostics Inc.) after drying in room temperature and washed twice with phosphate buffer for 3 min. Finally, cells were analyzed by the oil lens of microscope (CX21, Olympus), 1×10^3 cells/slide. Results were shown as the frequency of micronucleated cells (FMN) per slides.

$$\text{FMN} = \frac{N_{\text{MN}}}{\text{TNCC}} \times 1000\% \quad (2)$$

where NMN stands for the number of micronucleated cells in one slide and TNCC for the total number of the cells in the slides.

2.7 *Comet Assay*

A549 cells were seeded in 12-well plates at 5×10^5 cells/mL 1 day before treatment. Cells were exposed to 50, 100, 200, and 400 $\mu\text{g}/\text{mL}$ of particles with 2 v/v% FBS in RPMI1640 for 24 h. After the exposure, the media were removed; cells were washed once by PBS gently and harvested using PBS with the cell concentration at 4×10^5 . 30 μL of the cell suspension which was mixed with 70 μL 0.5 v/v% low-melting agarose (Roth, Karlsruhe) warmed in 37°C before. 90 μL agarose cell solution was applied to microscope slides, covered with coverslip, and fixed 10 min in refrigerator at 4°C . 90 μL agarose (Roth, Karlsruhe) was added on the surface of the first agarose, fixed 10 min in refrigerator at 4°C again, and then covered with coverslip. The cell coverslip was removed before putting the slides

into lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-1 HCl, 1 % Na-Sarcosinate, pH 10; 1 vv% Triton-X-100, and 10 vv% DMSO were added before use) for 2.5 h. Slides were then put into a horizontal electrophoresis tray containing the alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA) precooled at 4 °C allowing the unwinding of the DNA for 20 min. Electrophoresis (25 V, 300 mA) was performed for 30 min. Slides were neutralized in Tris-HCl buffer (0.4 M Tris-HCl, pH 7.5), rinsed in H₂O, dehydrated in 99 % ethanol, and dried overnight. DNA was stained with fluorochrome (20 µg/mL) and comets were analyzed by using a Leica ×2500 microscope with a blind camera. 50 comets chosen randomly were analyzed per slide by the software “CASPER.”

2.8 *Statistic Analysis*

All experiments were repeated three times. The data were presented as the means ± standard deviation (S.D.) of three independent experiments. They were analyzed by using the software of statistical package for the social sciences (SPSS) Version 11.0. The statistical difference between groups was determined with a one-way ANOVA. The minimum level of significance was considered to be $p < 0.05$.

3 Results

3.1 *Mineral Analysis*

Mineral samples used in this study were analyzed by XRD, XRF, and SEM, and the results showed that the SiO₂ content of quartz samples (KWC-Q4 and KWC-Q3) was above 97 %, and the main mineral phase was quartz. The content of SiO₂ in Nano-SiO₂ was 97 % of amorphous structure. The purity of montmorillonite was 96 % by semiquantitative analysis. The SEM images of particle samples were listed in Fig. 1.

3.2 *Cell Viability*

As illustrated in Fig. 2, exposure to 50, 100, 200, 400, and 800 µg/mL quartz, Nano-SiO₂, and montmorillonite for 24 h all induced a statistical significant decrease in the number of viable A549 cells compared with the control group. The figure showed that KWC-Q4 and KWC-Q3 samples caused a concentration-dependent decrease in cell viability. As to the particles in different size range, there was

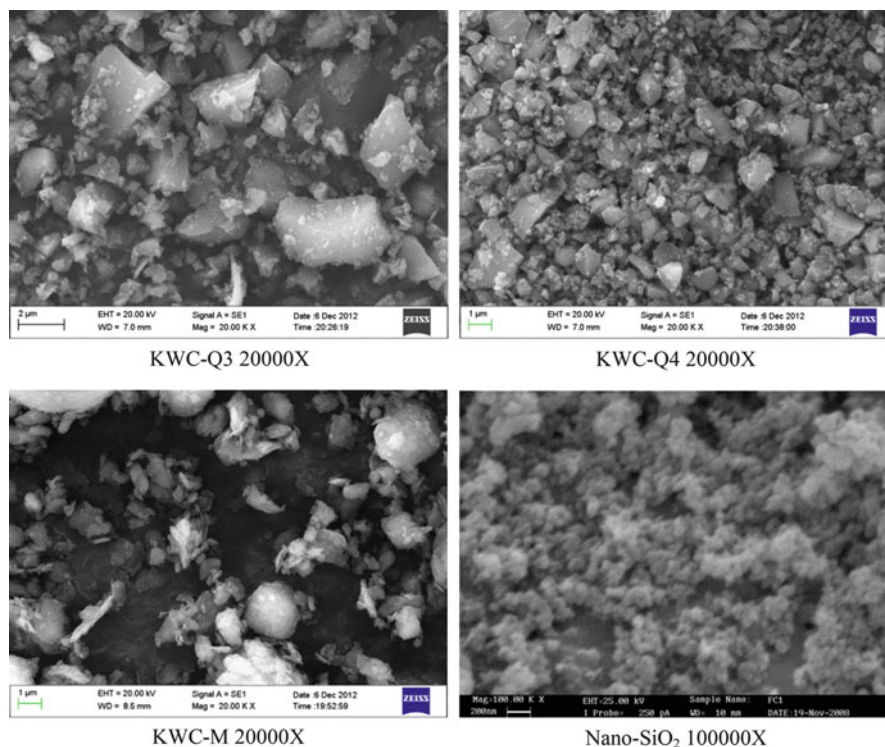


Fig. 1 SEM of mineral samples

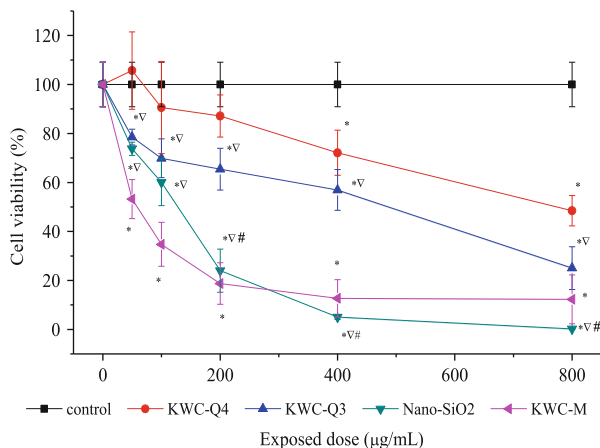
significant difference between KWC-Q4 and KWC-Q3. From low concentration to high concentration, the cell toxic effect induced by KWC-Q3 was significantly higher than KWC-Q4.

The cell viability of Nano-SiO₂ group and KWC-M decreased sharply as the increase of concentration. The dose–effect curve of Nano-SiO₂ from 50 µg/mL to 200 µg/mL was $y = -0.252x + 93.753$, with a correlation coefficient of 0.8695, while decreased to $(5.05 \pm 1.01)\%$, as the powder concentration was 400 µg/mL. KWC-M has the same cell toxic tendency as Nano-SiO₂; the cell viability of KWC-M was $(53.22 \pm 7.94)\%$ at the powder concentration of 50 µg/mL and decreased to $(18.74 \pm 8.46)\%$ as the concentration was 200 µg/mL.

3.3 Effects on Plasma Membrane Permeability

As exposed to mineral powder, there was a continuous interaction between the cell membrane and the mineral surface, resulting in the increase of intracellular LDH enzyme leakage in the medium which reflects the damage of the membrane-

Fig. 2 Cell viability of A549 after incubation with different doses of mineral suspensions for 24 h. Note: Asterisk, triangle, hash $p \leq 0.05$, as compared to the control, the 50 and 200 $\mu\text{g}/\text{mL}$ exposure group of the same kind of powder, $n = 3$

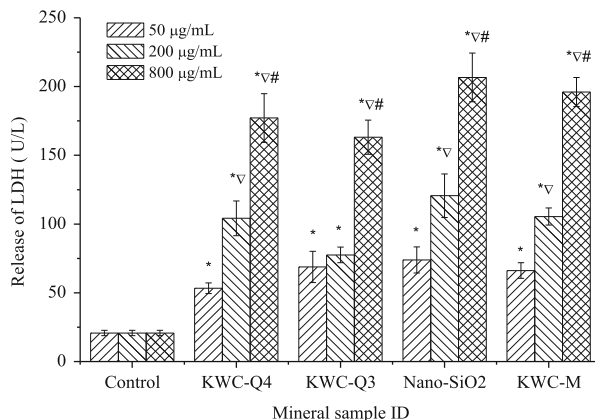


damaged extent. In this study, extracellular LDH was used as an indicator of the cell membrane integrity. Figure 3 shows that all kinds of powders lead to significant increase of extracellular LDH activity compared with the control group. The leakage of A549 cell LDH indicated a dose–effect relationship after exposure to different concentrations. Low dose (50 $\mu\text{g}/\text{mL}$) powder suspension can lead to significant increase of LDH activity, which was two to three times higher than the control. KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M all resulted in significant increase of the activity of LDH at the exposure concentration of 800 $\mu\text{g}/\text{mL}$. LDH activity was increased by approximately 754.4 % and 686.9 %, 896.6 %, and 845.6 %, respectively, as compared with controls. Besides, there was a good linear relationship between LDH release and cell viability. The increase of extracellular LDH indexed the membrane permeability change and the membrane damage.

3.4 Cytokine Release

The content of extracellular TNF- α of A549 after incubation with powder for 8 h was shown in Fig. 4. The content of TNF- α of control group was at (30.69 ± 1.00) ng/L, while the contents of TNF- α of all exposure groups were approximately at 30 ng/L, with no significant difference with the control, or between the two concentrations of the same kinds of samples. Therefore, the TNF- α content of A549 cell incubation with powder suspensions for 3 h, 6 h, and 24 h at 200 $\mu\text{g}/\text{mL}$ was measured. Figure 5 shows the change of TNF- α in the A549 cell medium after exposure to the mineral particle suspensions for 3 h, 6 h, and 24 h. The TNF- α content of control group was at (26.48 ± 1.08) ng/L for 3 h and increased to (31.39 ± 1.31) ng/L 3 h later but had no significant difference among the contents of TNF- α for 24 h, 6 h, and 8 h. The contents of extracellular TNF- α of all samples suspensions were triggered and increased for 3 h, compared with the control group,

Fig. 3 Extracellular LDH activities of A549 cells after incubation with KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M. Note: Asterisk, triangle, hash $p \leq 0.05$, as compared to the control, the 50 and 200 $\mu\text{g}/\text{mL}$ exposure group of the same kind of powder, $n = 3$



but the difference disappeared when the time was stretched to 6 h and maintained stability for 8 h or long. Experiments showed no visible time or dose–effect relationship.

The secretion of IL-6 of A549 cells exposed to different powder suspensions was shown in Fig. 6. Results showed the addition of quartz, Nano-SiO₂, and montmorillonite powder caused the IL-6 secretion of A549 cells to increase significantly compared with the control group. The content of extracellular IL-6 was 1.61 and 1.40 times of the control group, when the exposure concentrations of montmorillonite and Nano-SiO₂ were 50 $\mu\text{g}/\text{mL}$. However, the levels of IL-6 show a declining trend when the exposure concentration continued to increase, which may result from their high and instant toxic effect on cells. The secretion of IL-6 of A549 exposed to KWC-Q4 and KWC-Q3 of the relative low concentration was increased with the concentration. But there was no significant difference of the IL-6 secretion between the neighboring concentrations of all mineral powders. From the figure, KWC-M has a great influence on the secretion of IL-6 of all the mineral suspensions at low exposure dose (50 $\mu\text{g}/\text{mL}$). KWC-Q4 was the only sample that showed a dose–effect relationship in the test concentration range. The experiment indicates that all samples tested can promote the release of IL-6 and trigger the inflammation reaction of A549 cells.

3.5 Micronucleated Cell Test

The cells' micronucleus rate exposed to different mineral suspensions for 24 h was shown in Fig. 7. The micronucleus rate of negative control group cells (–) cultivated with culture solution only was $(5.667 \pm 1.528)\%$ at 24 h; with 300 $\mu\text{g}/\text{mL}$ of cyclophosphamide (CP) as a positive control, the micronucleus rate was $(21.667 \pm 1.528)\%$. All the micronucleus rates of mineral exposure groups had significant difference compared with negative control group, except for

Fig. 4 Change of TNF- α in the A549 cell medium supernatant after incubation with mineral suspensions for 8 h. Note: *Triangle, asterisk, hash* $p \leq 0.05$, as compared with control, 50 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$ of same mineral sample, $n = 3$

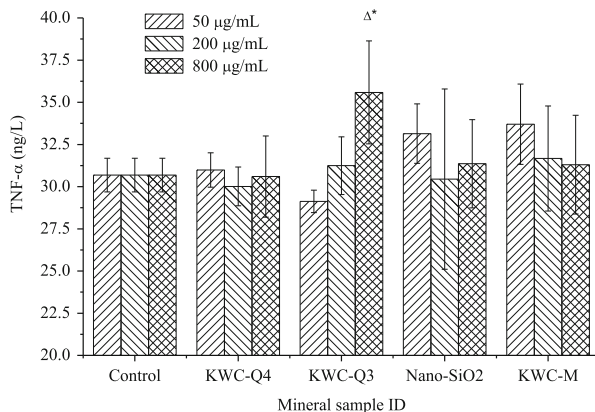


Fig. 5 Concentration change of TNF- α in A549 cell culture medium supernatant after incubation with mineral suspensions at 200 $\mu\text{g/mL}$. Note: *Triangle, asterisk, hash* $p \leq 0.05$, as compared with control, 3 h, 6 h exposure group of the same mineral sample, $n = 3$

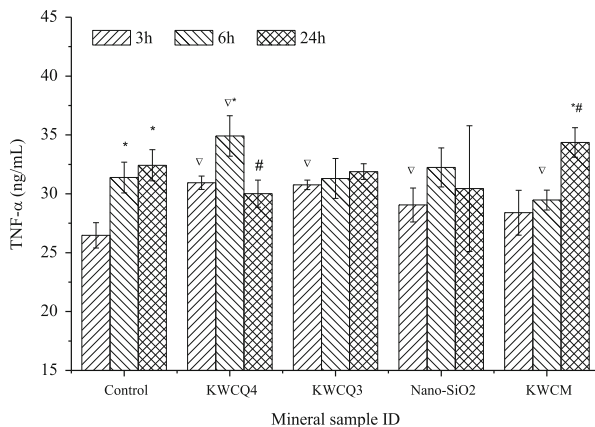


Fig. 6 Change of IL-6 in the A549 cell medium after incubation with mineral particle suspensions. Note: *Triangle, asterisk, circle* $p \leq 0.05$, as compared with negative control group, KWC-Q4, KWC-Q3 exposed group, respectively, $n = 3$

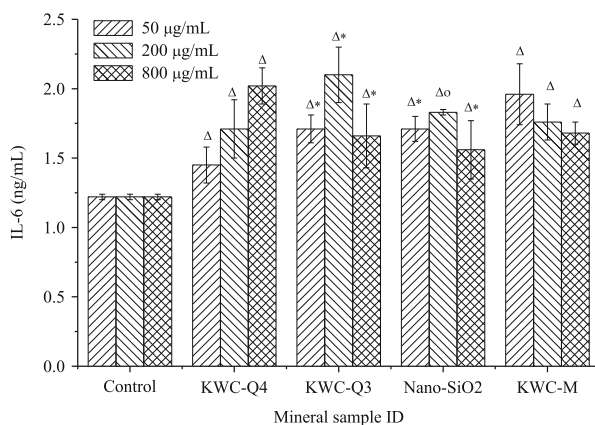
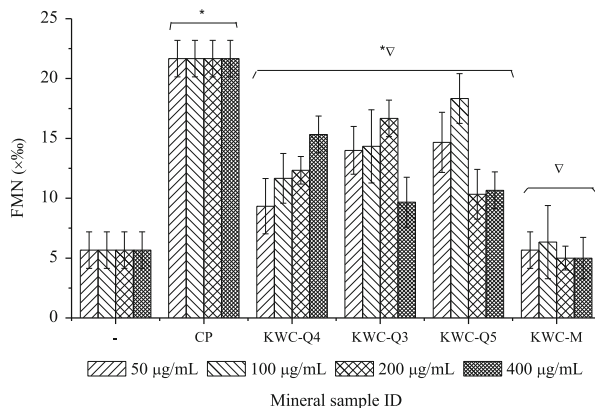


Fig. 7 Micronucleus test of A549 cells after incubation with KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M for 24 h. Note: Asterisk, triangle $p \leq 0.05$, as compared with negative control (-) and positive control (CP) respectively, $n = 3$



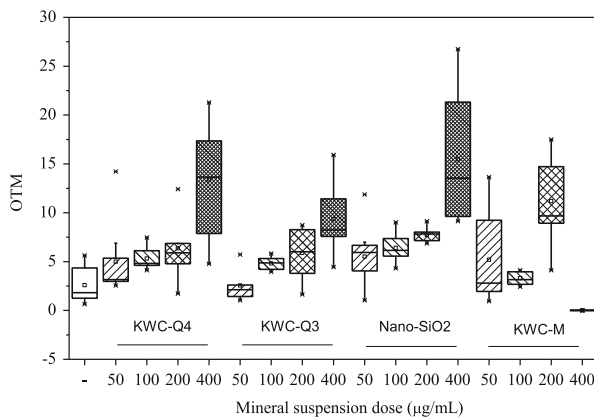
montmorillonite. Cells' micronucleus rate of low concentration groups (50 µg/mL and 100 µg/mL) is as follows: Nano-SiO₂>KWC-Q3>KWC-Q4. KWC-Q3 and KWC-Q4 mineral suspensions caused an increasing trend of cell micronucleus rate with the concentration when the exposure concentration was lower than 200 µg/mL. While the addition of montmorillonite led to higher cell mortality by lessening cell adhesion quickly even at a low concentration of 50 µg/mL, it was meaningless to measure the cell micronucleus rate when the cell mortality was 50 % approximately. The micronucleus test results showed the quartz samples, the Nano-SiO₂, can lead to the increase of cell micronucleus rate, namely the mineral can cause damage to cell's chromosomes, and lead to chromosome aberration; A549 cell micronucleus rate shows a tendency to rise with the increase of exposure concentration, within the condition of low cell mortality.

3.6 Comet Assay

Comet assay is a sensitive test to detect the genotoxicity of allogenic materials. In this chapter, Olive Tail Moment (OTM) was taken as the main indicators analyzed by the CASP software.

The cells of negative control group have no obvious DNA migration, cells appeared bright round head with regular shape, and distinguished difference in background. Cells incubated with different mineral samples exhibited a dose-dependent increase in DNA migration with their tails stretched and rounded out and heads smaller and brighter. The cells became badminton shaped as the DNA was damaged to a great extent. Comet cells OTM is a comprehensive index of cell DNA damage. The box plot of A549 cells comet OTM after incubation with KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M powders for 24 h was shown in Fig. 8. The negative control group cell OTM was 2.589 ± 1.706 . Quartz, Nano-SiO₂, and montmorillonite powder produced significant difference compared with

Fig. 8 Box plot of A549 cells comet OTM after incubation with KWC-Q4, KWC-Q3, Nano-SiO₂, and KWC-M powders for 24 h



control group as the exposure dose was more than 100 µg/mL and appeared an increase trend with the concentration, but no significant difference between the neighboring concentration groups.

4 Discussion

This study focuses on the cytotoxicity of quartz with two different particle sizes, Nano-SiO₂, and montmorillonite. Its results showed the toxic effect of Nano-SiO₂ on A549 cells was obviously higher than that of KWC-Q3 and KWC-Q4, which was in accordance with results of Donaldson [5]. The toxic effect of nanoparticles were significantly higher than that of micron particles, especially when the particle size was smaller than 100 nm, for the biological activity of nanoparticles increased sharply with the decrease of the particle size, which can cause more damage to the lung tissue. For different size range of quartz particles, the toxicity of KWC-Q3 on A549 was more larger than that of KWC-Q4. The toxicity of montmorillonite sample was almost same to Nano-SiO₂ and led to a higher mortality at low dosage. All the test samples showed the cell viability was inversely related to the concentration of treatment in cells.

LDH was a cytoplasm enzyme and leaked into the culture medium with the permeability increasing or the damage of cell membrane. So the extracellular LDH was often analyzed as an index of the membrane integrity [6]. Results showed the extracellular LDH concentration: Nano-SiO₂>KWC-Q3>KWC-Q4 with the same amount of exposure; the leakage of LDH increased with the exposure dosage; there appeared a concentration-dependent relationship which is consistent with the relationship between mortality rate and the exposure dosage.

IL-6 and TNF-α were the main cytokines that mediated the inflammatory reaction, cells will synthesize and secrete the cytokines by the real time of being stimulated by factors which were identified as the ideal indicators to evaluate the

ability of materials to inducing inflammatory response [2, 7]. As one typical II alveolar epithelial cell, A549 cells had been analyzed as the test body, and exhibits a dose-dependent relationship between the inflammatory cytokines secretion and the exposure dosage in Zhang's study [8, 9]. In this experiment, all test samples stimulated the secretion of IL-6, but not all of them exhibited a dose-dependent relationship in the test concentration range. The mineral samples caused the increase of TNF- α in culture medium for 3 h compared with the control group, but the difference disappeared when the exposure time extended to 8 or 24 h. The experiments testified the ability of mineral samples to cause the secretion of inflammatory cytokines and then triggered the inflammatory action. The micronucleus assay and the comet assay also devedined the genotoxicity of quartz, Nano-SiO₂, and montmorillonite.

The montmorillonite sample in the experiment showed a different cytotoxic effect compared with the others' results, which may link with its strong adsorption to promote the interaction with cell membrane and accelerate the cell membrane damage, or the micronanoquartz particles mixed in the suspension. The mechanism of why the montmorillonite produced a lager effect to the A549 cells and the interaction with cells need further study.

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