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ORGAN TOXICITY AND MECHANISMS

**Stefan Geh · Raif Yücel · Rodger Duffin
Catrin Albrecht · Paul J. A. Borm
Lorenz Armbruster · Monika Raulf-Heimsoth
Thomas Brüning · Eik Hoffmann
Albert W. Rettenmeier · Elke Dopp**

Cellular uptake and cytotoxic potential of respirable bentonite particles with different quartz contents and chemical modifications in human lung fibroblasts

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Abstract Considering the biological reactivity of pure quartz in lung cells, there is a strong interest to clarify the cellular effects of respirable siliceous dusts, like bentonites. In the present study, we investigated the cellular uptake and the cytotoxic potential of bentonite particles (Ø < 10 μm) with an α-quartz content of up to 6% and different chemical modifications (activation: alkaline, acidic, organic) in human lung fibroblasts (IMR90). Additionally, the ability of the particles to induce apoptosis in IMR90-cells and the hemolytic activity was tested. All bentonite samples were tested for endotoxins with the in vitro-Pyrogen test and were found to be negative. Cellular uptake of particles by IMR90-cells was studied by transmission electron microscopy (TEM). Cytotoxicity was analyzed in IMR90-cells by determination of viable cells using flow cytometry and by measuring of the cell respiratory activity. Induced apoptotic cells were detected by AnnexinV/Propidiumiodide-staining and gel electrophoresis. Our results demonstrate that activated bentonite particles are better taken up by IMR90-cells than untreated (native) bentonite particles. Also, activated bentonite particles with a quartz content of 5–6% were more cytotoxic than untreated bentonites or bentonites with a quartz content lower than 4%. The bentonite samples induced necrotic as well as apoptotic cell death. In general, bentonites showed a high membrane-damaging potential shown as hemolytic activity in human erythrocytes. We conclude that cellular effects of bentonite particles in human lung cells are enhanced after chemical treatment of the particles. The cytotoxic potential of the different bentonites is primarily characterized by a strong lysis of the cell membrane.

Keywords Bentonite · Quartz · Cellular uptake · Cytotoxicity · Apoptosis · Human lung fibroblasts

S. Geh · A. W. Rettenmeier · E. Dopp (✉)
Institute of Hygiene and Occupational Medicine,
University Hospital, Hufelandstrasse 55,
45122 Essen, Germany
E-mail: elke.dopp@uni-essen.de
Tel.: +49-201-7234578
Fax: +49-201-7234546

R. Yücel
Institute of Cell Biology (Cancer Research),
University Hospital, Essen, Germany

R. Duffin · C. Albrecht · P. J. A. Borm
Institute of Environmental Health Research (IUF),
Düsseldorf, Germany

R. Duffin
ELEGI COLT Lab, University of Edinburgh,
Scotland, UK

L. Armbruster
Verein für Technische Sicherheit und Umweltschutz e.V.,
Gotha, Germany

M. Raulf-Heimsoth · T. Brüning
Research Institute for Occupational Medicine
of the Institutions for Statutory Accident
Insurance and Prevention (BGFA),
Bochum, Germany

E. Hoffmann
Institute of Biology,
Department of Cell Biology and Biosystems
Technology,
University of Rostock, Rostock, Germany

Introduction

The US Department of Health and Human Services in the National Toxicology Program (NTP), the International Agency for Research on Cancer (IARC) and the German Research Foundation (DFG) have classified crystalline silica as a human carcinogen in 1997 on the basis of numerous epidemiological studies and in vivo experiments in rats (US Department of Health and

Human Services 2001; IARC 1997; DFG 1999). Several studies have shown that the occupational exposure to crystalline silica is associated with silicosis and other malignancies in workers (McDonald et al. 2005). The biological activity of quartz particles is highly variable (Donaldson and Borm 1998; Fubini et al. 2001; Schins et al. 2002) and the proposed carcinogenicity of crystalline silica is considered to depend on "inherent characteristics of the material" (IARC 1997). In consideration of these findings, there is a strong interest to clarify the cellular effects of respirable siliceous dusts, like bentonites.

Bentonite is a clay mineral with montmorillonite as its main component. The free silica content of bentonite clays ranges from less than 1% to more than 20%. It also contains mica, feldspar and other minerals in different proportions. Due to its high adsorptive and swelling properties, the use of bentonites is widely spread. Applications can be found in the chemical, pharmaceutical and food industry, cosmetic products (filling material), civil and environmental engineering (sealing material), agriculture (soil conditioner, animal feeding) and metallurgy. Humans are exposed to bentonite particles through both occupational and environmental pathways.

Numerous human pathological observations described severe pulmonary fibroses after occupational exposure of workers to bentonite dust (Vigliani 1954; Rombola and Guardascione 1955; Bassermann 1966; Phibbs et al. 1971; Gibbs und Pooley 1994). Several in vivo and in vitro studies demonstrate direct correlations between fibrogenic and cytotoxic effects in rats and peritoneal rat macrophages and quartz content (Adamis und Timár 1976, 1978; Adamis et al. 1986; Mikhailova-Docheva et al. 1986). Despite of their silicogenic properties, the cytotoxic, apoptosis-inducing and hemolytic potential of bentonite particles have not been investigated thus far.

In the present study, we assessed the cellular uptake and the cytotoxic potential of respirable bentonite particles of varying quartz content and activations (alkaline, acidic, organic) at a range of concentrations and exposure times in a human lung fibroblasts cell line (IMR90). IMR90 cells are human lung fibroblasts and were chosen for the investigations because of their untransformed primary properties. The red blood cell hemolysis assay gave additional information about the cytotoxic effects of bentonites to cellular membranes. Additionally, we analyzed the ability of bentonites to induce apoptosis in IMR90-cells.

Methods

Cell culture

Human lung fibroblasts (IMR90) were purchased from ATCC, USA (No.: CCL-136). Cells were cultivated in MEM + Earls' salts with 10% FCS, 1% L-Glutamin, 1% sodium-pyruvate, 0.1% nonessential amino acids (NAA) and antibiotics (100 U/ml Penicillin and 100 µg/ml Streptomycin) at 37°C and 5% CO_2. All supplements were purchased from CC-Pro, Germany.

Particles

The bentonite samples were provided by the Süd-Chemie AG Moosburg, Germany. Particles were industrially treated with Na_2CO_3 (3–5% bentonite sample 1403, 5% bentonite sample 1400, 3% bentonite sample 1401), with HCl (samples 1402 and 1405), and with distearyl-dimethylamonium-chloride (organic treatment, sample 0015) (Table 1). The content of transition metals in the used bentonite samples is also given in Table 1. The chemical modification of the native bentonite particles causes an intercrystalline exchange of ions like Na^+ or Ca^{2+}. More than 90% of the particles had a diameter <10 µm, about 50% were smaller than 2.5 µm. The quartz content of the particles varied from 0.5 to 6%. Gypsum ($CaSO_4$) and quartz particles (DQ12) were used as negative and positive control particles, respectively. All mineral dust samples were suspended in isotonic NaCl solution, homogenized and sterilized by ultra sonification (Brandson Sonifier 250-D, USA).

Table 1 Applied bentonites with different activation, contents of quartz and transition metals

Mineral	Activation	Quartz content (%)	Content of all transition metals (%)	Iron content (%)	Other transition metals* (%)	pH
0015	Organic: distearyl-dimethylammonium-chloride	1–2 (Cristobalite)	1.75	1.7	0.05	6.9
1403	Alkaline: Na_2CO_3(3–5%)	0.5	2.39	2.2	0.19	10.4
1400	Alkaline: Na_2CO_3(5%)	5–6	3.90	3.7	0.20	10.1
1405	acidic: HCl	1	0.79	0.64	0.15	7.9
1402	acidic: HCl	4–5	2.06	1.8	0.26	4.6
1404	Native (untreated)	1	1.22	1.1	0.12	8.3
1408	Native (untreated)	5–6	4.01	3.8	0.21	8.4
Quartz (DQ-12)	–	95				7.4
Gypsum	–	0				7.5

*Cr, Ni, Cu, Mn, Co, V, La, Sc

Pyrogen test

Incubation of human whole blood samples were performed according to the protocol of the in vitro pyrogen test (IPT) (Hartung et al. 2002) using either fresh or cryopreserved blood. Briefly, particles were suspended in aqua bidest (Sigma-Aldrich, Germany) in a concentration of 5 mg/ml and centrifuged at 2,000 *g* for 8 min. Human blood was added to RPMI 1640 culture media (Biochrom, Germany) at a dilution of 1:5 in polypropylene reaction tubes (Eppendorf, Germany). Samples were prepared by adding 100 µl particle supernatants to 900 µl blood suspension. The tubes were gently shaken and incubated overnight (16–24 h) at 37°C. For a positive control, lipopolysaccharide (LPS) from *Escherichia coli* were used as endotoxine stimuli in concentrations from 0.02 to 20.83 EU/ml. HBSS, diluted 1:1 in pyrogen free aqua bidest, was used as a negative control. After centrifugation, the cell-free supernatants were frozen or assayed immediately by determination of IL-6 with ELISA (R &D Systems, Germany), at 450 nm absorbance.

Transmission electron microscopy (TEM)

Monolayer cultures grown in 35 mm culture dishes until 85% confluence were washed twice with serum free medium and treated with bentonite particles (10 µg/cm^2, 24 h). At the end of each incubation, the monolayers were washed three times with serum free medium and fixed with 2.5% glutaldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C. After postfixation in 2% OsO_4 in 0.1 sodium cacodylate buffer for 1 h at 4°C, the samples were stained en bloc with 1.5% uranylacetate dihydrate and phosphotungstic acid, dehydrated in ethanol series and embedded in epoxy resin, Epon (Serva, Germany).

The measurement of the particle size was performed by TEM (Philips STEM CM12, Netherlands) in combination with a digital imaging system (SIS, Germany) at a magnification of 15,000×. The morphology of the cells, cellular uptake and intracellular distribution of the particles were investigated using ultra thin sections (50 nm) placed on 150 mesh grids, stained with uranylacetate and lead citrate and examined by TEM. For mineral analyses, the system was coupled with an energy dispersive X-ray detector (EDX).

Cytotoxicity

Flow cytometry

IMR90 cells were seeded at a density of 2×10^4 cells/cm^2 in each well of a 6-well plate (Greiner Germany). At a maximum of 80% confluence, cells were treated with bentonite, quartz and gypsum particles at concentrations between 1 and 100 µg/cm^2 for 24 h. Cell viability was evaluated immediately after the exposure by determination of the number of residual cells. Therefore, cells were harvested by trypsin treatment (Sigma, Germany) and diluted in 500 µl phosphate buffered saline (PBS). The cell suspensions were diluted again in 9.5 ml Isoton. The counting procedure was carried out using a Coulter Counter (Beckman-Coulter Z2, USA) on a sample volume of 500 µl. The samples were prepared at a 1:20 dilution. Single measurements were repeated immediately. The cell viability is expressed as percentage of surviving cells compared to the number of untreated cells. All experiments were performed in triplicate.

AlamarBlue assay

The cell viability after particle treatment was determined by examining the mitochondrial reduction activity (cell respiration). The same number of IMR90-cells used for flow cytometry assay were treated with bentonite particles at concentrations between 1 and 50 µg/cm^2 for 20 h. AlamarBlue indicator solution was then added to the cultures at a 1/10 dilution in culture media. Samples were incubated for additional 4 h and supernatants were then analyzed by fluorescence- and UV-spectroscopy. The results were analyzed by comparing the treated values to the untreated control values.

Hemolysis assay

The hemolytic potential of the different bentonite samples was measured based on the method of Harington et al. (1971). Erythrocytes, red blood cells (RBC), were obtained from fresh blood samples of healthy human donors. RBC were washed four times by suspending in saline before centrifugation at 1,200 *g*. The final suspension consisted of 4% RBC suspended in saline. Particles were dispersed in saline to a final concentration between 1.2 and 5 mg/ml. The particle suspension (150 µl) was mixed with 75 µl of RBC suspension and incubated for 10 min at room temperature, with gentle shaking on an orbital plate shaker. After incubation the samples were centrifuged at 717 *g* for 5 min and the amount of hemoglobin released into the supernatant was determined spectrometrically at a wavelength of 540 nm.

Detection of apoptosis

DNA-fragmentation

DNA fragmentation as an indicator of induced apoptosis was analyzed using agarose gel electrophoresis. IMR90-cells (1×10^6 cells) were harvested 72 h after exposure to the bentonite and control particles (concentration 20 µg/cm^2). Mitomycin C (1 µg/ml) was used as positive control. For DNA isolation, a commercial DNA kit (QIAamp, Qiagen, Germany) containing 20 µl protease and chaotropic buffer solution was utilized.

After sodium acetate/ethanol precipitation, the purified DNA (8 µg/lane in a total volume of 25 µl) was separated by a 2% LE-agarose gel (Cambrex, USA), stained with ethidium bromide and visualized under UV light.

Flow cytometry (FACS)

Induced apoptotic cells were quantitatively analyzed by FACS using AnnexinV-FITC/PI staining. Following 72 h exposure to the bentonite particles, 7×10^5 IMR90-cells were harvested (concentration: 20 µg/cm^2). Cells were stained by treating with 5 µl PI solution (250 µg/ml, Immunotech, France) and 6 µl Annexin-V-FITC (Immunotech, France) for 30 min at 4°C in the dark. Finally, 1×10^4 cells were analyzed using a FACS Calibur (Becton Dickinson, USA) and CELL-Quest software.

Statistical analysis

Student's *t* test (2-tailed) was used for comparison of the uptake- and the FACS-results with the untreated control in each set of experiments.

Results

Pyrogen test

The presence of pyrogenic substances like endotoxins can influence the cellular effects of particles or fibers in vitro. By determination of IL-6 in human whole blood we have shown that all particle samples had no relevant endotoxins doses. The amounts of released IL-6 were far below the lowest endotoxin standard of 0.02 EU/ml (data not shown).

Cellular uptake

The number of particles taken up by IMR90-cells ranged from 1.3×10^{-3} to 4.6×0^{-3} particles per µm^2 (concentration: 10 µg/cm^2, exposure time: 24 h). Twenty TEM sections were evaluated, each with an average area of 8,000 µm^2 in each case. The comparison of the obtained data shows clearly that the cellular uptake of alkaline-activated bentonite (1400) was 3.5-fold higher than the uptake of untreated bentonite (1408) (Fig. 1). The phagocytosed particles were solely located in the cytoplasm. No cell organelles, e.g. the nucleus or mitochondria, were affected (Fig. 2). Only the montmorillonite crystal was found intracellularly by EDX analysis (Fig. 3). Minor components of bentonite dust (e.g. quartz, mica) could not be detected inside the cells. Figure 4 shows the size distribution of the phagocytosed montmorillonite particles. A maximum can be found at a diameter of 0.4–1.6 µm. The uptake of untreated bentonite samples was less selective, regarding the particle size (Fig. 4).

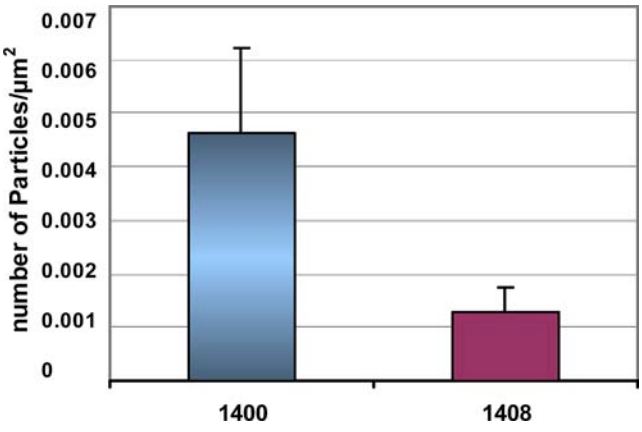

Fig. 1 Cellular uptake, represented by average number of particles/µm^2 (± SD), of an alkaline (1400) and a native (1408) bentonite sample in IMR90-cells. Particles in 20 fields with an average area of 8,000 µm^2 were counted under TEM in each case (concentration: 10 µg/cm^2 exposure time: 24 h)

Fig. 2 Encapsulated particle of montmorillonite in an IMR90-cell (*arrow*). No cell organelles were affected **a** mitochondria, **b** endoplasmatic reticulum (ER); TEM 15,000× (concentration: 10 µg/cm^2 exposure time: 24 h)

Cytotoxicity

IMR90-cells were exposed to the different benonite samples (Table 1) for 24 h at concentrations ranging from 1 to 50 µg/cm^2. The cytotoxicity analysis using flow cytometry indicates that activated bentonites with higher quartz contents (4–6%) had stronger cytotoxic effects than untreated bentonites or bentonites with a lower quartz content (0.5–1%) (Fig. 5, Table 2). The effects of the activated particles with a high quartz content were even stronger than those of DQ12-quartz. The highest cytotoxicity could be observed at 50 µg/cm^2,

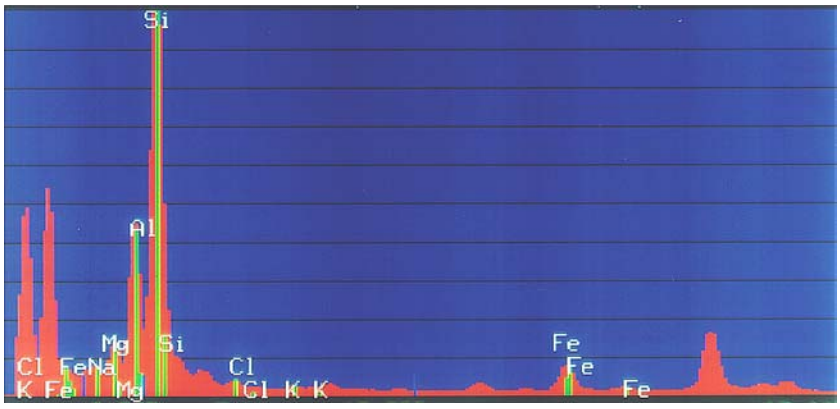

Fig. 3 Energy dispersive X-ray detector analysis of intracellular located bentonite particles. The predominate pattern of silicon and aluminium reveals the montmorillonite crystal (Sample 1400)

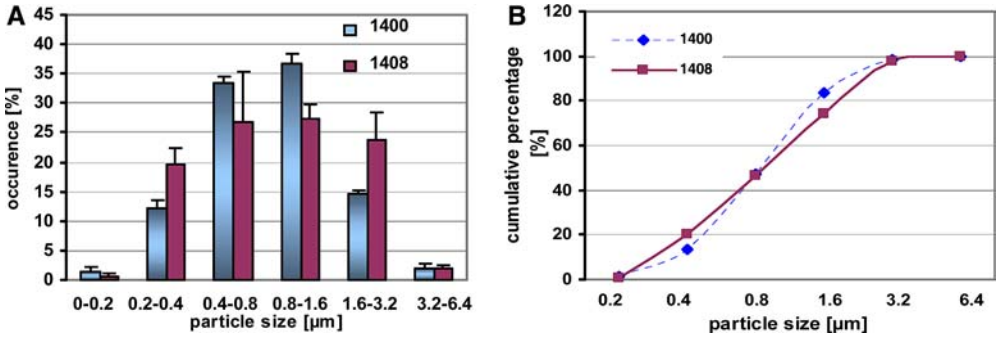

Fig. 4 Distribution of bentonite particles in dependence on the particle size in IMR90-cells: **a** single frequencies (±SD) of occurrence, **b** cumulative percentage

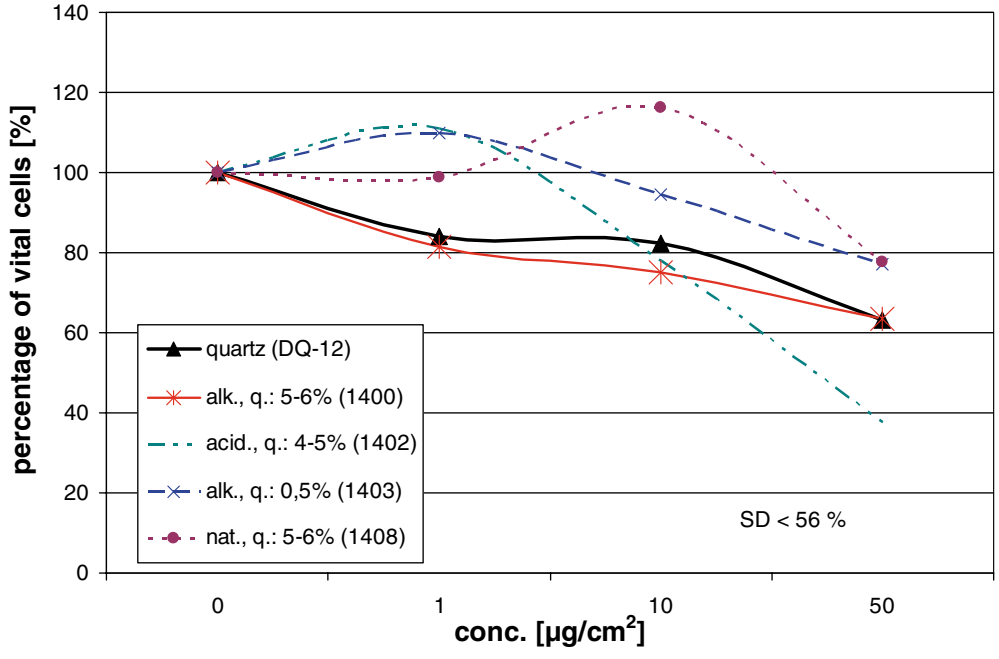

Fig. 5 Cytotoxicity of different bentonite particles in IMR90-cells in relation to quartz, measured by Coulter Counter, exposure time 24 h

but not all bentonites showed a strict dose-dependency. Most bentonite-treated cultures tend to enhance cell proliferation at low concentrations (1–10 µg/cm^2) (Fig. 5, Table 3).

Additionally, we examined the cytotoxic potential of the dust samples by the mitochondrial respiratory activity using the AlamarBlue-assay. The results showed consistently cytotoxic effects in the concentration range tested also by flow cytometry (Fig. 6).

Measurement of hemolysis in RBC (direct membrane damage) was used to investigate the surface reactivity of treated and untreated bentonite particles. Bentonites showed a high dose-dependent hemolytic potential at concentrations from 1.25 to 5 mg/ml. At the highest

Table 2 Results of cytotoxicity analysis (% of viable cells ± SD) after treatment of IMR90-cells with the bentonites 0015, 1404 and 1405 in relation to DQ-12 quartz

Percentage of vital cells		Concentration (µg/cm^2)		
Dust	Comment	1	10	50
DQ-12	Quartz	84 ± 22	82 ± 27	63 ± 25
0015	Org.. Cristobalite: 1%	106 ± 60	69 ± 12	49 ± 2
1405	acidic. q.: 1%	116 ± 7	125 ± 20	93 ± 56
1404	native. q.: 1%	119 ± 30	129 ± 34	78 ± 12

Measurements were carried out by Flow cytometry (exposure time: 24 h, concentrations: 1, 10 and 50 µg/cm^2) *q* Quartz content

Table 3 Percentage (± SD) of red blood cell hemolysis of different bentonite, asbestos, gypsum, and quartz suspensions

Mineral	Concentration (mg/ml)		
	1.25	2.5	5
Gypsum	–	–	0.5 ± 0.0
Chrysotil	68.0 ± 1.8	88.6 ± 2.4	97.5 ± 2.8
0015	7.6 ± 0.1	14.0 ± 0.4	20.1 ± 0.3
1400 (q: 6%)	73.2 ± 1.7	91.6 ± 1.3	95.0 ± 2.9
1402 (q: 5%)	23.1 ± 0.9	44.1 ± 1.1	35.0 ± 1.3
1404 (q: 1%)	72.3 ± 1.9	92.5 ± 2.7	94.6 ± 2.6
1405 (q: 1%)	64.7 ± 1.4	89.4 ± 2.2	89.5 ± 3.9
1408 (q: 6%)	43.8 ± 0.8	80.8 ± 2.9	87.7 ± 2.0
DQ12	15.0 ± 1.9	27.7 ± 0.9	40.0 ± 1.9

Content of quartz in bentonite specimens is given in brackets. Most of all benonites showed high hemolytic activity

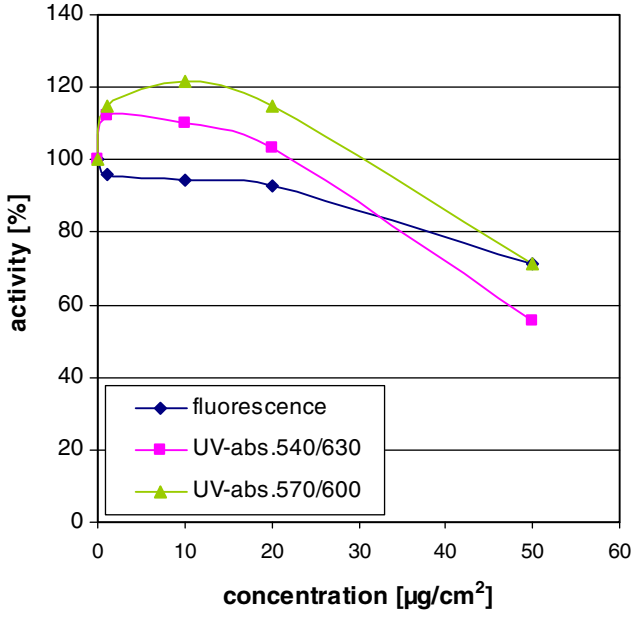

Fig. 6 Results of the cytotoxicity testing of the bentonite 1403 using the AlamarBlue-assay. The measurements are based on the mitochondrial respiratory activity and showed consistently cytotoxic effects in IMR90-cells at the tested concentrations

tested concentration of 5 mg/ml, 2/3 of all tested bentonite samples caused lysis of red blood cells in a range of 88% to 95%. These effects were independent upon the activation and the quartz content. In comparison to other mineral dusts (DQ12, chrysotile), only chrysotile showed similar results. Induction of hemolysis by bentonites were up to 2.1-fold higher than by quartz and 190-fold higher compared to gypsum. Only the organic treated bentonite sample 0015 caused hemolysis below 20%.

Apoptosis

The different cell stainings (Annexin/ FITC, Propidium iodide) led to the differentiation of the cells according to early apoptotic cells (AnnexinV-positive, PI-negative), late apototic/necrotic cells (AnnexinV-positive, PI-positive), and necrotic cells (AnnexinV-negative, PI-positive). After exposure of IMR90-cells to the different bentonites (20 µg/cm^2, 72 h), we observed a slight increase in the number of AnnexinV-positive cells (sample 1405 is shown in Fig. 7). The percentage of apoptotic cells was maximally 1.8-fold increased after exposure to sample 1404 compared to the untreated negative control (Fig 8). This increase is statistically not significant. There was no dependency upon the activation and the quartz content. Also, IMR90-cells exposed to quartz particles (DQ12) did not show typical apoptotic features.

The percentage of late apoptotic/necrotic cells was significantly increased (3.5-fold compared to the untreated control, $P \leq 0.01$) after exposure to the bentonite sample 0015 (Fig. 8). Alkaline or acidic activation of bentonite dust as well as the content of quartz had no influence on the induction of apoptosis in IMR90-cells.

By analyzing the necrotic cell population (AnnexinV-negative, PI-positive), a 3.1-fold increase of necrotic cells (not significant) was measured after exposure of IMR90-cells to the acidic, high quartz containing sample 1402 (Fig. 8).

Additionally, we analyzed the internucleosomal fragmentation of DNA, which is a further characteristic

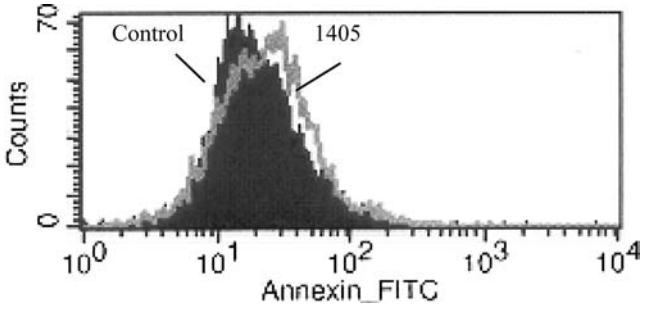

Fig. 7 Induction of apoptosis: FACS histogram of an AnnexinV-stained cell population after bentonite treatment (sample 1405) compared to the untreated control

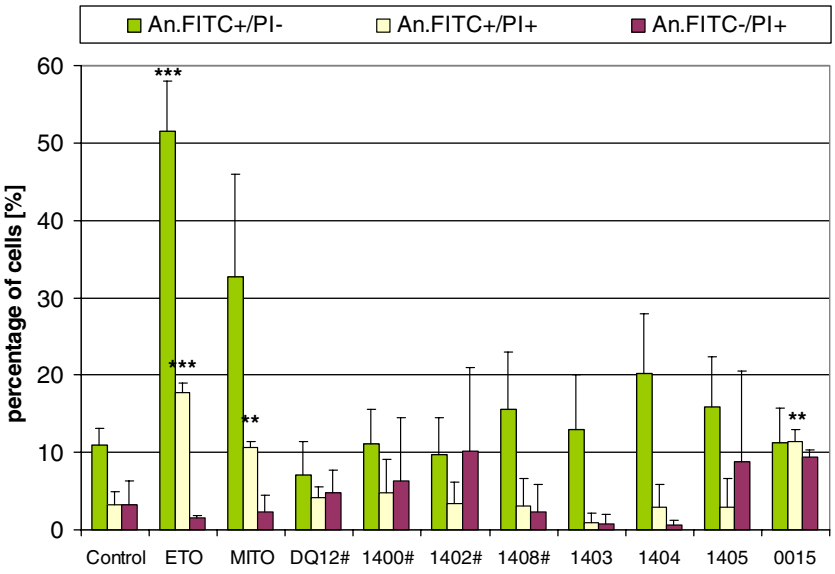

Fig. 8 Percentage (± SD) of AnnexinV-positive, PI/AnnexinV-positive and PI-positive IMR90-cells after 72 h exposure to bentonite and quartz (DQ-12) particles (20 µg/cm^2). Positive controls: ETO = 500 µM etoposide, MITO = 1 µg/ml Mitomycin C, ***$P < 0{,}001$, **$P < 0.01$, #content of quartz more than 4%

feature of apoptosis. IMR90-cells were exposed to DQ12 and bentonite samples for 72 h at a concentration of 20 µg/cm^2. The typical DNA ladder could be seen after application of the bentonite samples 1404 and 1408 (Fig. 9). DNA from untreated cells did not show this typical pattern.

Discussion

The purpose of this study was to investigate the cellular uptake and the cytotoxic potential of respirable bentonite particles in human lung fibroblasts (IMR90) in relation to the quartz content and the activation (alkaline, acidic and organic) of the bentonite samples. IMR90-cells have a normal cell cycle and are therefore an adequate model for primary lung tissue.

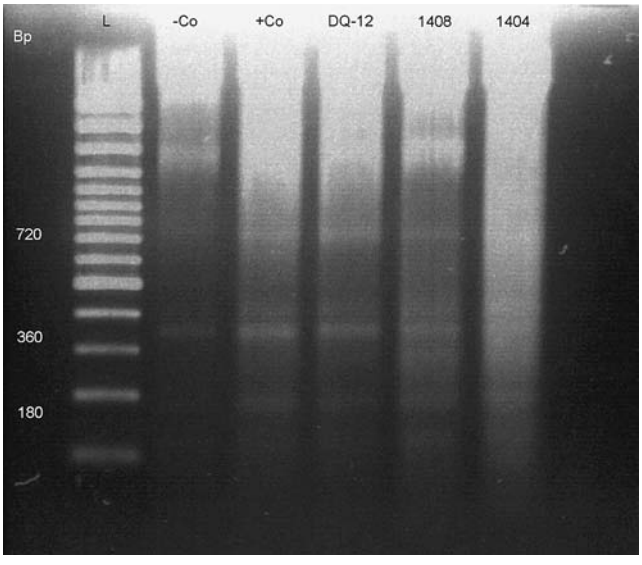

Fig. 9 Agarose gel electrophoresis of DNA from IMR90 cells exposed to bentonite and quartz (20 µg/cm^2, 72 h); *L* DNA Ladder, *−Co* negative control, *+Co* positive control (1 µg/ml Mitomycin C)

The present data demonstrate that the cellular uptake as well as the cytotoxicity of the bentonite particles varies in dependence upon the content of quartz and the activation of the bentonite sample. Adamis and Timár (1978) reported that the cytotoxicity of bentonites as measured by 2,3,5-triphenyltetrazoliumchlorid (TTC) reduction in peritoneal rat macrophages is correlated to the quartz content of the sample. In our study, the cytotoxicity was dependent upon the activation of the dust in combination with an elevated quartz content (≥4%). Cytotoxicity of activated bentonites with a quartz content >4% was stronger than those of pure quartz particles. However, the cytotoxic as well as the hemolytic properties of the DQ12-quartz sample used in the own experiments was relatively low. This might be explained by the age of the sample. Fubini et al. (1995) have shown that "fresh" quartz samples are more reactive than "aged" material. Also, Cakmak et al. (2004) have shown large differences in cytotoxic properties of different quartz samples. The relatively low hemolytic activity of the DQ12-sample suggests a role for cation exchange at the particle surface (Stone et al. 2004).

A high quartz content of our used bentonite samples was mostly related to a high content of transition metals (mainly iron) (positive correlation, Fig. 10). This elevated iron content and not the quartz content might be responsible for the observed increased cytotoxic effects of some bentonite samples. Preliminary data from our laboratory have also shown that the acellular production of OH-radicals is more than twofold increased for the bentonite sample 1400 compared to DQ12-quartz (data not shown). The metal-generated OH-production might potentiate the toxicity of the activated bentonites.

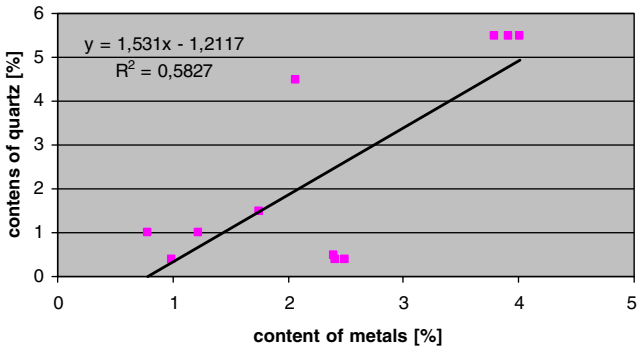

Fig. 10 Calculation of correlation between the content of quartz and the content of transition metals in the used bentonite samples

It can be presumed that the activation of bentonite particles enhances their cation exchange and adsorptive capacities by inter-crystalline ion exchange. Therefore, the membrane-damaging properties of activated bentonite particles can be higher than that of quartz particles. These findings are in agreement with those of Manyai et al. (1969), who tested the hemolytic activity of bentonites and different clay minerals in comparison to quartz. Murphy et al. (1993) reported about a comparatively high cytotoxic potential of aluminium silicates in human umbilical, vein endothelial, neuroblastoma and oligodendroglial cells. The cytotoxicity was measured by the application of trypan blue exclusion test, lactate dehydrogenase assay and by analysis of fatty acid levels. The order of tested particles with cytotoxic properties is: montmorillonite > bentonite = kaolin > > erionite (Murphy et al. 1993).

The mechanisms of cellular toxicity of aluminium silicate clays are poorly understood. Several hypotheses are based on the unique charge and hydrogen bounding properties of the clays (Davies and Preece 1983; Davies et al. 1984; Oscarson et al. 1986; Woodworth et al. 1982). Probably, bentonites are able to lyse liposomes because of direct charge interactions between the particle and the cellular organelles (Woodworth et al. 1982).

An important factor in the pathogenesis of diseases is the process of apoptosis (Thompson 1995). Several studies have described the induction of apoptosis in human and rodent alveolar macrophages after treatment with quartz dust (Iyer and Holian 1997; Thibodeau et al. 2003). Leigh et al. (1997) detected a dose-dependent apoptosis induction in rat alveolar and granulomatous cells after instillation of quartz. Apoptosis in alveolar macrophages can lead to a persistent release of cytokines. The results of the present study clearly show that bentonites as well as quartz particles do not induce a strong increase of apoptosis in human lung fibroblasts. The application of etoposide as positive control gave evidence that apoptosis can be induced in IMR90-cells. Bentonite particles induced both necrotic and apoptotic cell death in IMR90-cells. Similar results were reported by Gao et al. (2001). These authors have tested quartz and kaolin for apoptosis induction in rat alveolar macrophages and found apoptotic as well as necrotic cell death. No data are available in the literature about effects of quartz or quartz-containing particles on induction of apoptosis in fibroblasts.

Compared to other studies in the literature, relatively low particle concentrations (up to 50 μg/cm^2) were used in the own experiments. Bentonite and quartz concentrations >50 μg/cm^2 were not tested for cytotoxicity, because these (uptake, apoptosis) and further (genotoxicity) experiments were/are carried out at non-toxic particle concentrations (1–20 μg/cm^2). Real exposure conditions according to the allowed maximum work place concentration (MAK-value for inhalable dust: 4 mg/m^2; MAK- und BAT-Werte-Liste der DFG, 2004) are even >tenfold lower (0.03–0.1 μg/cm^2) than the chosen particle concentrations in the experiments (calculation of dust concentration in the lung according to the lung surface, inhaled dust concentration per day and lung volume per day). Further in vivo experiments are necessary to investigate the cellular effects of bentonite dust on lung cells and macrophages at low doses, which are comparable to real exposure conditions.

Altogether, we conclude from our in vitro experiments that the cytotoxic potential of bentonite particles is mainly caused by a lysis of the cell membrane. This effect can be enhanced by activation of the bentonite dust. This observed cytotoxic effect leads to a slightly more pronounced necrotic than apoptotic cell death.

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