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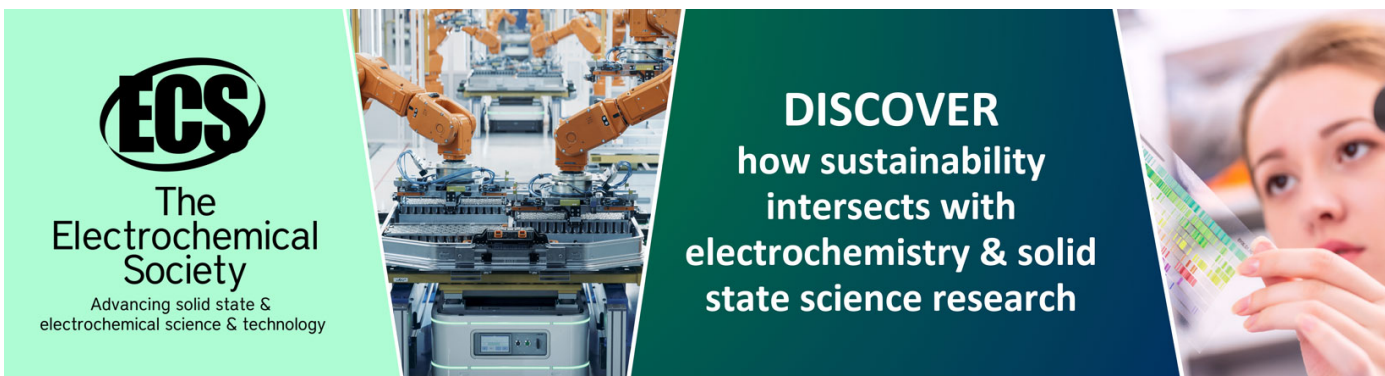
Quartz-Containing Ceramic Dusts: *In vitro* screening of the cytotoxic, genotoxic and pro-inflammatory potential of 5 factory samples

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Quartz-Containing Ceramic Dusts: *In Vitro* Screening of the Cytotoxic, Genotoxic and Pro-Inflammatory Potential of 5 Factory Samples

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Abstract. Inhalation of some respirable crystalline silica (MMAD \leq approx. 4 μ m) leads to inflammatory and malignant diseases. Comprehensive physicochemical/biological data and suitable *in vitro/in vivo* methods may distinguish between more or less harmful quartz-varieties. Within the European Collective Research Project SILICERAM an *in vitro* screening battery was established to evaluate cytotoxicity (LDH-release, MTT-assay), genotoxicity (Comet-assay) and pro-inflammatory potential (PGE₂-liberation, TNF- α mRNA expression) of 5 respirable quartz-containing dusts from ceramic plants: brickwork (BR: 7.8% quartz), tableware granulate/cast (TG/TC: 5.8%/3.1%), tiles (TI: 8.1%), refractory (RF: 3.7%). DQ12 (87% α -quartz) and Al₂O₃ were used as particulate positive and negative controls, respectively. Primary rat alveolar macrophages and the macrophage cell line NR8383 served as model systems. Aluminium lactate was used as inhibitor of biologically active silica, enabling differentiation of silica- and non-specific toxicity. At 200 μ g/cm² (2h) the dusts did not alter significantly LDH-release (except TC), whereas the MTT-assay demonstrated the mainly quartz-independent rank order: DQ12>RF>TG>TI>BR>TC>Al₂O₃. DNA-damage was maximal for BR and TI followed by DQ12>TG>TC>RF>Al₂O₃. All dusts induced PGE₂-liberation (DQ12>BR>TC>TG>TI>RF>Al₂O₃) at 50 μ g/cm² (4h), but TNF- α mRNA (10 μ g/cm², 24h) was only increased by DQ12, TG (quartz-dependently), and TC. In conclusion, these *in vitro* tests were an adequate approach to screen the toxic potential of quartz-containing ceramic dusts, but the quartz-content was too low to differentiate the various quartz-varieties.

1. Introduction

Due to its low solubility and its reactive surface, respirable crystalline silica (RCS) may exert harmful effects on biological targets such as DNA, membranes, and proteins. Inhalation of RCS may thus lead to adverse health effects in the lung, e.g. inflammation, silicosis, and cancer. The debate on adverse effects of RCS and subsequent air limits at workplaces was reinforced by the decision of the International Agency on Research in Cancer (IARC) in 1997 to classify some exposures to RCS as category 1 carcinogen. At present, the different EU states have their own occupational limits for RCS and setting of common RCS exposure limits on a mg/m³ level is difficult, because lung-reactivity of RCS-containing dusts may differ substantially depending on quartz polymorph and industrial sector.

Considering the ubiquitous occurrence, the diverse use of quartz and the complex characteristics of its toxicity, comprehensive physicochemical/biological data sets and suitable *in vitro/in vivo* methods are strongly needed to allow differentiation of various quartz varieties and different quartz processing routes. For that reason, an *in vitro* screening battery was established and used in the European Collective Research Project SILICERAM to evaluate the cytotoxic, genotoxic, and pro-inflammatory potential of RCS-containing ceramic dusts from traditional subsectors of the ceramic industry: brickwork, tableware, tiles, and refractory ware.

2. Materials and Methods

2.1. Test and reference materials

For incubations, test and reference particles were dosed on a mass basis ($\mu\text{g}/\text{cm}^2$) and not on RCS basis due to their unexpectedly low RCS content. Particles were suspended in culture medium without fetal calf serum (FCS) and sonicated for 10 min in a water bath.

2.1.1. *Ceramic dusts.* With 2 high-volume samplers, developed within the SILICERAM project, 5 RCS-containing ceramic dusts were collected in different countries from various industrial sectors or manufacturing plants: brickwork (BR: 7.8% quartz, weight-% by XRD), tableware granulate (TG: 5.8%), tiles (TI: 8.1%), tableware cast (TC: 3.1%), and refractory ware (RF: 3.7%) with geometric mean diameters (weighted by mass) of 5.17, 5.97, 2.14, 4.36, and 7.70 μm , respectively.

2.1.2. *Reference samples.* Quartz DQ12 (mid-size, Dörentrup, Bergbauforschung, Essen, Germany, 87% α -quartz/13% amorphous silica) was used as positive, Al_2O_3 (active, neutral type 507C, Aldrich, Taufkirchen Germany) as particulate negative control with geometric means weighted by mass of 3.01 and 4.23 μm , respectively.

2.2. Cells Systems

Primary rat alveolar macrophages (PAM) isolated from adult Wistar rats and the rat alveolar macrophage cell line NR8383 were used as test systems. PAM were pre-cultured for 24 h in DMEM with GlutaMAXTM, 4.5 g/l D-glucose, 110 mg/l sodium pyruvate, 10% FCS and antibiotics. They were plated at a density of 1.05×10^5 cells/ cm^2 on 24-well plates with a hydrophobic culture surface (Comet-assay, LDH-liberation) or on conventional 4-well plates (PGE₂-release). NR8383 cells were cultured in DMEM supplemented with 200 mM L-glutamine, 15% FCS and antibiotics and were split every 3-4 days. They exhibited 50% adherent and 50% floating morphology. Both cell types were cultured and incubated at 37°C in a humidified atmosphere of 5% CO₂ / 95% air using an incubator.

2.3. Lactate dehydrogenase (LDH)-release

To determine cell viability/membrane integrity of dust-treated PAM, lactate dehydrogenase (LDH)-activity was measured in the incubation supernatant of the Comet-assay. LDH-activity was evaluated according to the manufacturer's protocol using the "Cytotoxicity Detection Kit (LDH)" from Roche Diagnostics (Mannheim, Germany) after 2 h of incubation with 200 $\mu\text{g}/\text{cm}^2$ of the different dusts \pm 10 μM aluminium lactate (AL, to quench quartz-specific biological effects).

2.4. MTT-assay

The metabolic activity/viability of dust-treated NR8383 cells was investigated using the MTT-assay adapted from [1]. In brief, NR8383 cells were seeded in 96-well plates (1×10^5 cells per well) and pre-cultured overnight. After incubation for 2 h with 50 - 200 $\mu\text{g}/\text{cm}^2$ of the different particles (\pm 10 μM AL) cells were washed and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-solution was added. After incubation for 3 h at 37°C the MTT-solution was discarded and the blue MTT-reduction product formazan was dissolved in DMSO. Formazan was determined spectrophoto-

metrically by measuring absorbance at 540 nm, using a microplate reader. The results were expressed as percentage of viability, namely the ratio of absorbance of exposed to unexposed cells x 100.

2.5. Comet-assay

To determine the DNA-damaging potential of the ceramic dust samples, the alkaline *in vitro* Comet-assay (pH>13) according to [2] and [3] with minor modifications was used. Pre-cultured PAM were incubated for 2 h with 200 $\mu\text{g}/\text{cm}^2$ of the dust samples ($\pm 10 \mu\text{M AL}$). Cells were then detached by placing on ice for 10 min. All of the following steps were performed under red light to avoid unspecific DNA-damage. After centrifugation cells were resuspended in pre-heated low-melting agarose (LMA) and applied to pre-coated slides. After gelation, a second layer of LMA was applied and slides were finally immersed in lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 8 g/l NaOH, 1 % Triton-X100, 10% DMSO) and stored overnight at 4°C. They were then placed on a cooled electrophoresis platform and covered with pre-cooled electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13). After DNA-unwinding (20 min) and electrophoresis (20 min at 24V/300 mA) slides were neutralized and stained with ethidium bromide. The tail moment of the nuclei was analyzed using a fluorescence microscope and the Comet Assay III software from Perceptive Instruments (Haverhill, UK). One hundred nuclei were scored per slide.

2.6. Prostaglandin E₂ (PGE₂)-liberation

To assess the pro-inflammatory potential/phagocytic stress induced by the ceramic dusts, PGE₂ was measured in the incubation supernatant of PAM, incubated for 4 h with 50 $\mu\text{g}/\text{cm}^2$ of the ceramic dust or the reference samples. PGE₂ was determined by a highly sensitive and specific competitive enzyme immunoassay using a monoclonal antibody from clone E2R1 [4]. The detection limit was 3 pg/ml of a 96-well plate. Results were calculated from the standard curve by cubic spline interpolation.

2.7. TNF- α mRNA expression

To assess TNF- α mRNA expression, NR8383 cells were cultured for 24 h (10⁶ cells/ml) with 10 $\mu\text{g}/\text{cm}^2$ of the ceramic dusts or the reference particles ($\pm 10 \mu\text{M AL}$). RNA was extracted, using the RNeasy Mini Kit (QIAGEN). Reverse-transcription was performed using TNF- α - and glyceraldehyde-3-phosphate (GAPDH)-specific primer [5] and *Moloney* murine leukaemia virus reverse-transcriptase, leading to amplicon lengths of 175 and 168 bp, respectively. Resulting cDNA was amplified with Taq polymerase and 10 x Long ExpandTM Buffer from Roche (Meylan, France). Amplification reaction consisted of melting (3 min, 94°C), followed by 30 cycles of melting (30 sec, 94°C), annealing (30 sec, 58°C), and extension (2 min, 72°C), and a final extension step (7 min, 72°C). Amplification products were run on 1% agarose gels with Tris·borate/EDTA-buffer (pH 8.0), stained with ethidium bromide, and visualized by UV-transillumination. Relative TNF- α mRNA expression was expressed as the ratio of fluorescence intensities of TNF- α \square and GAPDH (house-keeping gene) bands.

3. Results

The *in vitro* screening battery was initially optimized concerning suitability, sensitivity, concentrations, and incubation times. Optimized protocols were finally used for the main experiments, because of limited amounts of the 5 factory dusts. For the MTT-assay, however, 3 concentrations were used to evaluate concentration-dependency (see table 1). DQ12 and Al₂O₃ were defined as appropriate particulate positive and negative controls (table 1 and figures 1-4). As expected, AL efficiently inhibited the DQ12-induced biological effects (table 1 and figures 2-4). By testing the factory samples (200 $\mu\text{g}/\text{cm}^2$, 2 h) in PAM, all dusts, except DQ12 and TC, mediated only moderate, mostly not quartz-specific, increases in LDH-levels, as compared to Al₂O₃ (figure 1). At 200 $\mu\text{g}/\text{cm}^2$ (2 h), the MTT-assay with NR8383 cells demonstrated the mainly quartz-independent rank order of toxicity: DQ12 > RF > TG > TI \geq BR > TC (table 1). For DQ12, BR, TG, and TC but not for TI and RF decrease in cell viability was concentration-dependent. The marked, concentration-independent effect of RF was most likely of artificial nature due to the dark color of the dusts, thus interfering with photometric formazan

determination. In the Comet-assay with primary rat alveolar macrophages (200 µg/cm², 2 h), BR and TI exhibited the highest DNA-damaging potential, followed by DQ12, TG, TC, RF, and Al₂O₃ (figure 2). About 50 % of the TG-, BR- and TI-mediated effects were quartz-related (as determined by AL-treatment), whereas TC and RF showed values of 20 % and 40 %, respectively. In pre-experiments with BR and TG clear concentration-dependent increase in DNA-damage could be detected (data not shown). All factory dusts induced higher PGE₂-liberation in primary rat alveolar macrophages than Al₂O₃ as particulate negative control (DQ12 > BR > TC > TG > TI > RF; figure 3). Relative TNF-α mRNA expression was significantly increased in NR8383 cells only by DQ12 and TG, both quartz-related, and by TC, not quartz-related, as compared to the vehicle control (figure 4).

Table 1. Results of the MTT-assay with NR8383 cells. Data represent relative means ± standard deviations of 3 independent experiments with 5 replicates per experiment. Significantly different from the corresponding vehicle control: # $P \leq 0.05$, ### $P \leq 0.001$, Student's *t*-test for unpaired values.

Treatment	50 µg/cm ²		100 µg/cm ²		200 µg/cm ²	
	without AL	with AL	without AL	with AL	without AL	with AL
Vehicle control	100 ± 5	100 ± 7	100 ± 5	100 ± 9	100 ± 6	100 ± 3
Al ₂ O ₃	98 ± 4	112 ± 4	95 ± 8	105 ± 7	99 ± 2	99 ± 3
DQ12	86 ± 6	113 ± 7	58 ± 5 [#]	100 ± 7	29 ± 5 ^{###}	95 ± 9
Brickwork (BR)	94 ± 7	97 ± 5	70 ± 9 [#]	91 ± 5	71 ± 6 [#]	75 ± 9
Tableware (TG)	81 ± 9	88 ± 4	64 ± 1 [#]	73 ± 3	57 ± 7 [#]	52 ± 8
Tiles (TI)	71 ± 9 [#]	72 ± 8	66 ± 8 [#]	68 ± 6	73 ± 7 [#]	74 ± 9
Tableware (TC)	93 ± 1	95 ± 11	89 ± 1	80 ± 1	79 ± 8 [#]	71 ± 7
Refractory (RF)	54 ± 12 [#]	57 ± 11	50 ± 11 [#]	53 ± 7	49 ± 5 [#]	61 ± 8

AL = 10 µM aluminium lactate

4. Discussion

All 5 ceramic dust samples mediated more or less cytotoxic, genotoxic and pro-inflammatory effects, with different dusts in each case to exhibit the highest potential. As judged by the experiments with AL there seemed to be no clear correlation between the different biological effects and the quartz content (see 2.1.1 and 2.1.2) of the dust samples. For example, the tile sample demonstrated an even higher clastogenic potential than the positive control DQ12, although the tile sample contained only 8.1 % versus 87.0 % quartz, respectively. Interestingly, in all other assays the toxic potential of the tile sample was rather low, indicating that there were special dust components within the sample with high DNA-damaging potential. In addition, except for the positive control DQ12, cytotoxicity (metabolic capacity and membrane integrity) of the factory samples was nearly RCS-independent, because there was no significant effect of parallel AL treatment. This lack in clear correlation in the *in vitro* tests indicated that other dusts components e.g. special metal compounds, like iron (substantial FeO₂ content in some of the samples), may also account for adverse effects and that dust components like clay were perhaps able to quench the biologic activity of RCS in the ceramic dust samples.

In conclusion, the established *in vitro* battery has been found to be an adequate approach to screen and differentiate the multifactorial toxic potential of quartz-containing ceramic dusts. However, in the present study, the quartz-content of the ceramic dusts was too low to clearly identify and differentiate quartz activities in the various dust samples. To really approach this goal dusts with higher RCS content or the pure quartz varieties used for production in ceramic plants should be compared.

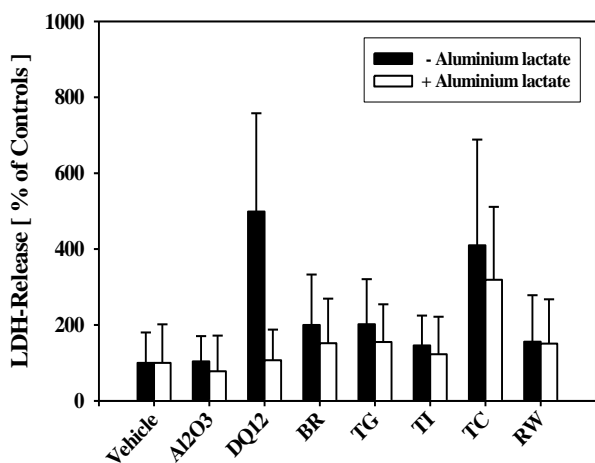


Figure 1. LDH-release. PAM were incubated for 2h with or without $200\mu\text{g}/\text{cm}^2$ of the dusts $\pm 10\mu\text{M}$ AL and LDH-activity was measured in the culture supernatants. Data represent means \pm SD of 3 independent experiments.

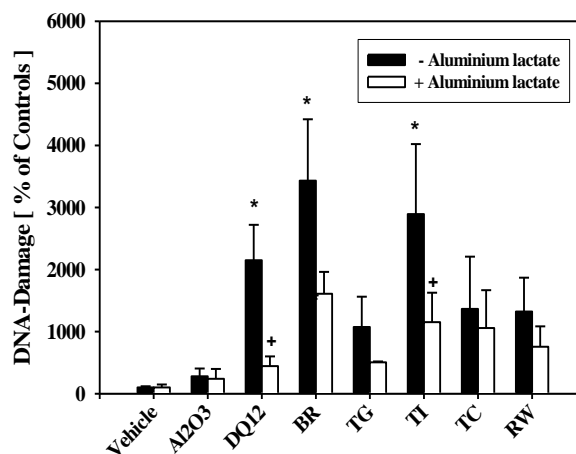


Figure 2. Comet-assay. PAM were incubated for 2h $\pm 200\mu\text{g}/\text{cm}^2$ of the dusts $\pm 10\mu\text{M}$ AL and DNA-damage was measured. Data represent means \pm SD of 3 independent experiments. Significantly different from the vehicle control: * $P \leq 0.05$; from the respective sample without AL: + $P \leq 0.05$, Student's *t*-test for paired values.

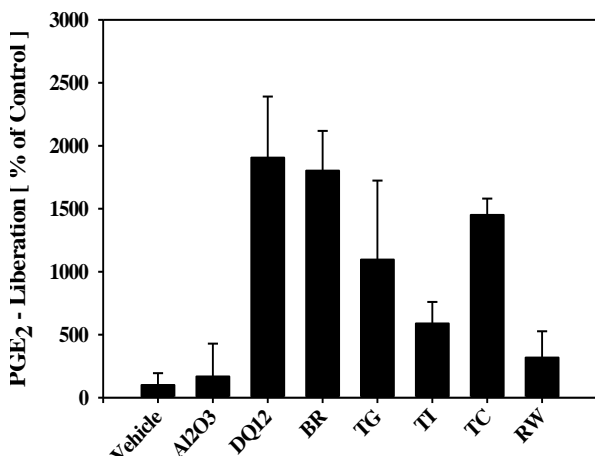


Figure 3. PGE₂-liberation. PAM were incubated for 4h with or without $50\mu\text{g}/\text{cm}^2$ of the test and reference dusts and PGE₂-liberation was evaluated by a competitive enzyme immunoassay. Data represent means \pm SD of 2 independent experiments each performed in duplicate.

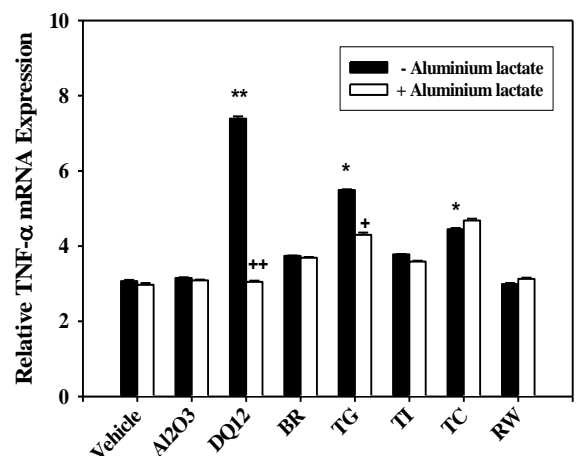


Figure 4. TNF- α mRNA expression. NR8383 were incubated for 24h $\pm 10\mu\text{g}/\text{cm}^2$ of the dusts $\pm 10\mu\text{M}$ AL and TNF- α mRNA expression was determined by PCR. Data represent means \pm SD of 3 independent experiments each performed in triplicate. Significantly different from the vehicle control: * $P \leq 0.05$, ** $P \leq 0.01$; from the respective sample without AL: + $P \leq 0.05$, ++ $P \leq 0.05$; Student's *t*-test for unpaired values.

Acknowledgment

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