

Assessing Toxicity of Fine and Nanoparticles: Comparing *In Vitro* Measurements to *In Vivo* Pulmonary Toxicity Profiles

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Previous studies have reported little correlation between the relative toxicity of particle types when comparing lung toxicity rankings following *in vivo* instillation versus *in vitro* cell culture exposures. This study was designed to assess the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of several fine or nanoscale particle types in rats. In the *in vivo* component of the study, rats were exposed by intratracheal instillation to 1 or 5 mg/kg of the following particle types: (1) carbonyl iron (CI), (2) crystalline silica (CS) (Min-U-Sil 5, α -quartz), (3) precipitated amorphous silica (AS), (4) nano-sized zinc oxide (NZO), or (5) fine-sized zinc oxide (FZO). Depending on particle type and solution state, these particles range in size from 90 to 500 nm in size. Following exposures, the lungs of exposed rats were lavaged and inflammation (neutrophil recruitment) and cytotoxicity end points (bronchoalveolar lavage [BAL] fluid lactate dehydrogenase [LDH] values) were measured at 24 h, 1 week, 1 and 3 months postexposure. For the *in vitro* component of the study, three different culture conditions were utilized. Cultures of (1) rat L2 lung epithelial cells, (2) primary alveolar macrophages (AMs) (collected via BAL from unexposed rats), as well as (3) AM—L2 lung epithelial cell cocultures were incubated with the particle types listed above, and the culture fluids were evaluated for cytotoxicity end points (LDH, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan [MTT]) as well as inflammatory cytokines (macrophage inflammatory 2 protein [MIP-2], tumor necrosis factor alpha [TNF- α], and interleukin-6 [IL-6]) at one (i.e., cytokines) or several (cytotoxicity) time periods. Results of *in vivo* pulmonary toxicity studies demonstrated that instilled CI particles produced little toxicity. CS particles produced sustained inflammation and cytotoxicity. AS particles produced reversible and transient inflammatory responses. NZO or FZO particles produced potent but reversible inflammation which was resolved by 1 month postinstillation exposure. Results of *in vitro* pulmonary cytotoxicity studies demonstrated a variety of responses to the different particle types, primarily at high doses. With respect to the LDH results, L2 cells were the most sensitive and exposures to nano- or fine-sized ZnO for 4 or 24 h were more cytotoxic than exposures to CS or AS particles. Macrophages essentially were resistant and epithelial macrophage cocultures generally reflected the epithelial results at 4 and 24 h incubation, but not at 48 h

incubation. MTT results were also interesting but, except for nano- and fine-sized ZnO, did not correlate well with LDH results. Results of *in vitro* pulmonary inflammation studies demonstrated that L2 cells did not produce MIP-2 cytokines, but CS- or AS-exposed AMs and, to a lesser degree, cocultures secreted these chemotactic factors into the culture media. Measurements of TNF- α in the culture media by particle-exposed cells demonstrated little activity. In addition, IL-6 secretion was measured in CS, AS, and nano-sized ZnO-exposed cocultures. When considering the range of toxicity end points to five different particle types, the comparisons of *in vivo* and *in vitro* measurements demonstrated little correlation, particularly when considering many of the variables assessed in this study—such as cell types to be utilized, culture conditions and time course of exposure, as well as measured end points. It seems clear that *in vitro* cellular systems will need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particle types.

Key Words: particle toxicity; nanoparticles; rat; *in vivo*; *in vitro*; pulmonary toxicity; cytotoxicity; ultrafine particles; fine particles.

The successful development of *in vitro* assays as predictive screens for assessing particle toxicity is an important goal during early product development prior to more substantive inhalation toxicity testing. If properly validated, the advantages of these early screening tests would result since they are simpler, faster, and less expensive than their *in vivo* counterparts. Moreover, successful development of *in vitro* toxicity systems should reduce animal use for hazard screening of potential pulmonary toxicants and to evaluate new particulate materials in small quantities. Although implementation of *in vitro* lung toxicity screening systems would be highly beneficial relative to current testing protocols, the accuracy and predictability of these tests with particulates have not been compared to *in vivo* results.

Very few systematic attempts have been made to validate the results from *in vitro* studies to *in vivo* toxicity effects on the same materials. Seagrave and coworkers assessed the pulmonary toxicity of diesel engine particulate extracts using *in vivo*

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assessments followed by *in vitro* screening studies on the same particulate test substances. These authors noted that the rank order of hazard potency from the *in vitro* assays in general did not correspond well to the ranking results from the *in vivo* comparisons of the same samples. In fact, most of the extracts that were determined to be in the least toxic range of tested particulates following assessment using *in vitro* assay methodology were, ironically, the most toxic following particle instillation in to the lungs of rats. The authors concluded that *in vitro* assays may provide cell type-specific mechanistic information, but do not accurately reflect *in vivo* comparative toxicity results (Seagrave *et al.*, 2002, 2005).

A recently published review/commentary focusing on the safe handling of nanotechnology has recommended strategic research strategies to support sustainable nanotechnologies by maximizing benefits and minimizing environmental and health risks (Maynard *et al.*, 2006). This international panel of scientists recommended five grand challenges which were selected to stimulate research and to bring focus to a range of complex multidisciplinary issues. One of the five key grand challenges cited in the Maynard *et al.* (2006) commentary is to develop and validate alternatives to *in vivo* toxicity testing of engineered nanomaterials within the next 5–15 years. Conceptually, there would appear to be several limitations inherent to *in vitro* assay/cell culture systems in simulating the complex biological effects of inhaled or instilled particles in the lungs of experimental animals. These include, but are not limited to, (1) particle dose, (2) selection of cell types for simulating the lung microenvironment (concomitant with the issue of single-cell culture systems vs. coculture systems), (3) characterization and exposure interactions of lung cells with particle types in culture versus lung fluids, (4) time course of effects (acute vs. chronic) *in vivo* versus 1, 4, 24, or 48 h incubation *in vitro*, and (5) appropriate end points for hazard evaluation.

In this study, we have attempted to assess the impact of many of these variables utilized for *in vitro* toxicity studies with the stated goal of developing an *in vitro* culture system that could provide a reasonably accurate, predictive early screen for assessing the pulmonary toxicity of particulate materials. Accordingly, we have utilized an *in vivo* pulmonary bioassay methodology to assess the toxicity of five different intratracheally instilled particles. Each of these particles has been shown to elicit characteristic pulmonary responses following inhalation or instillation exposures. Subsequently, we have conducted systematic studies wherein these same well-characterized particles have been incubated with lung cells *in vitro*, using three different culture conditions, including a range of doses and incubation time periods. For the evaluation of relevant biomarkers, we have selected inflammation and cytotoxicity indices as comparative end points for both *in vitro* and *in vivo* studies. The sustainability of these biomarkers represents important criteria for assessing the toxicity of inhaled particulates.

MATERIALS AND METHODS

Experimental Design

A systematic method has been devised to assess the efficacy and predictability of *in vitro* studies and to compare the results obtained with lung inflammation and cytotoxicity data obtained from *in vivo* pulmonary bioassay studies. Accordingly, we evaluated two pulmonary cell types (immortalized rat lung epithelial cells [L2 cells] and primary rat alveolar macrophages [AMs]) both separately and in an epithelial/macrophage coculture system for implementation of *in vitro* studies with five different particle types. The results were then used to compare with data generated from an *in vivo* pulmonary bioassay studies in exposed rats using the same fine-sized and nano-sized particles. The five particulates that were evaluated were (1) carbonyl iron (CI) particles, (2) crystalline silica (CS) (Min-U-Sil 5, α -quartz) particles, (3) precipitated amorphous silica (AS) particles, (4) nano-sized zinc oxide (NZO) particles, and (5) fine-sized zinc oxide (FZO) particles. Iron, α -quartz, AS, and zinc oxide have unique pulmonary inflammatory profiles. The end points for the *in vivo* studies were bronchoalveolar lavage (BAL) biomarkers of neutrophilic inflammation and cytotoxicity (BAL fluid lactate dehydrogenase). For the *in vitro* toxicity study, cytotoxicity was assessed by measuring for lactate dehydrogenase (LDH) and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) activity in the culture fluid. Inflammatory mediators included measurement of cytokines in the culture fluids—macrophage inflammatory 2 protein (MIP-2), tumor necrosis factor alpha (TNF- α), and interleukin 6 (IL-6). In addition, we have assessed the predictive value of an *in vitro* hemolytic assay as a possible screening tool for gauging *in vivo* pulmonary toxicity effects of particles.

Preparation and Characterization of Particle Suspensions

Particle preparation. CI powder (metallic iron) was purchased from BASF Chemical Company (Wayne, NJ) at > 99.5% purity. CS (Min-U-Sil 5, α -quartz) was purchased from U.S. Silica Co. (Berkeley Springs, WV) at > 99% purity. AS (Zeofree 80) was purchased from J. M. Huber Corporation (Edison, NJ) at 98% purity. Nano-ZnO and fine-ZnO were purchased from Sigma Aldrich (St Louis, MO) at > 99.0% purity. The particle systems used here were produced via a “top down” approach (grinding or milling a bulk material into an ultrafine dust), therefore resulting in a wide distribution of particle sizes, > 20%. Stock suspensions (10 mg/ml) of each particle were prepared in ultrapure Milli-Q water, phosphate-buffered saline (PBS) solution (sterile filtered), and F-12K cell culture media. Each suspension was probe sonicated (Ultrasonic Processor, 50 W/60 Hz) for 30 min prior to exposure and characterization. For *in vivo* studies, each stock solution was subsequently diluted serially to yield concentrations of 1 and 5 mg/kg. For the *in vitro* studies, each stock solution was diluted serially to yield seven concentrations ranging from 0.001 μ g/ml (ppm) to 30 mg/ml (0.052–520 μ g/cm²). Note that we have made calculations that indicate that the 52 and 520 μ g/cm² doses are considered to be overload doses (Faux *et al.*, 2003). This conclusion was based on a calculation to convert μ g/cm² dose units to cm²/cm² units (i.e., μ g/cm² \times surface area of the particle). The various surface areas of the particle types used in this study were the following: CI = 2.5 m²/g, CS = 5.1 m²/g, AS = 77.7 m²/g, NZO = 12.1 m²/g, and FZO = 9.6 m²/g.

Accordingly, using calculations, we converted the following doses at 52 and 520 μ g/cm², respectively, to cm²/cm²: CI = 1.3 and 13 cm²/cm², CS = 2.65 and 26.5 cm²/cm², AS = 40.4 and 404 cm²/cm², NZO = 6.29 and 62.9 cm²/cm², and FZO = 5.0 and 49.9 cm²/cm².

Given that 1–3 cm²/cm² is considered to be an overload dose for low toxicity dusts such as fine-sized TiO₂, all the particle doses equivalent to 52 and 520 μ g/cm² were considered to be overload doses (Faux *et al.*, 2003).

Particle characterization. In both the dry powder form and the suspension, each particle system was analyzed for various physicochemical properties. Surface area was measured by the Brunauer-Emmett-Teller (BET) method; size, size distribution, and surface charge were examined by dynamic

light-scattering (DLS) spectroscopy; and crystallinity was determined by X-ray diffraction (XRD).

BET measurements (Micrometrics ASAP 2405) were taken on each particle sample in its dry state under N_2 adsorption to measure specific surface area (Brunauer *et al.*, 1938).

DLS (Malvern Zetasizer Nano-S, Model Zen1600) was used to determine size, size distribution, and surface charge. DLS measurements were taken on each particle sample in each suspension: ultrapure water, PBS solutions, and F-12K cell culture media (Berne and Pecora, 1975).

XRD (Philips X'PERT automated powder diffractometer, Model 3040) was used to determine purity, crystal structure, and crystallite size of the NZO and FZO particle samples (Otwinski and Minor, 1997).

Pulmonary Toxicity of Particles, In Vivo

Animals. Male Crl:CD (SD)IGS BR rats (Charles River Laboratories, Inc, Raleigh, NC) were approximately 8 weeks old at study start (240–255 g). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee. The animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care as described in Warheit *et al.*, 1997.

Groups of rats (five rats per group per dose per time point) were intratracheally instilled with particle doses of 1 or 5 mg/kg in PBS. Groups of PBS-instilled rats served as controls. BAL fluid from the lungs of PBS and particle-exposed rats were analyzed at 24 h, 1 week, 1 month, and 3 months postinstillation exposure (pe). Specifically, groups of rats were exposed to (1) vehicle control—PBS, (2) negative control—CI, (3) positive control—CS (Min-U-Sil 5), (4) AS, (5) nano-sized ZnO, and (6) fine-sized ZnO particle types.

Pulmonary lavage. The lungs of both control and particle-exposed rats were lavaged with PBS (PBS) solution at 37°C as described previously (Warheit *et al.*, 1991). Methodologies for cell counts, cell differentials, and pulmonary cytotoxicity biomarkers in lavaged fluids were conducted as previously described (Warheit *et al.*, 1991, 1997). Briefly, the first 12 ml of lavaged fluids recovered from the lungs were centrifuged at $700 \times g$, and 2 ml of the supernatant was removed for LDH and total protein.

LDH release. LDH release was measured using Olympus Lactate Dehydrogenase reagents. Data were collected and recorded on an Olympus AU640 Chemistry Analyzer at 340/380 nm (Roe *et al.*, 1972).

Statistical analyses. Each experimental value was compared to the corresponding control value for each time point. One-way ANOVA and Bartlett's tests were calculated for each sampling time. When the *F*-test from ANOVA was significant, the Dunnett's or Dunn's test was used to compare means from the control group and each of the groups exposed to particles. Statistical significance versus PBS control was established as $p < 0.05$. Statistical tests were performed with SAS 9.1 software (SAS Institute Inc, Cary, NC).

Cytotoxicity of Particles, In Vitro

Cells in culture. Three different cell culture systems were used in this study: (1) immortalized rat L2 lung epithelial cells (American Type Culture Collection, Manassas, VA), (2) rat lung AMs, and (3) cocultures of (1) and (2). All cell culture systems were cultured in F-12K medium (Kaighn's modification of Ham's F-12 medium) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (see Fig. 1) (Warheit *et al.*, 1991). All experiments were conducted at a cellular density of 200,000 cells/cm².

Tests for LDH release, mitochondrial activity (MTT), production of inflammatory mediators (MIP-2), cytokine production (TNF- α and IL-6), and hemolytic potential were performed on all three *in vitro* cell culture systems and done in triplicate.

LDH release. Cells were seeded in 24-well plates, exposed to increasing concentrations of particle suspensions (0.0052–520 $\mu\text{g}/\text{cm}^2$, where 52 and 520 $\mu\text{g}/\text{cm}^2$ are particle-overload concentrations), and incubated for four different

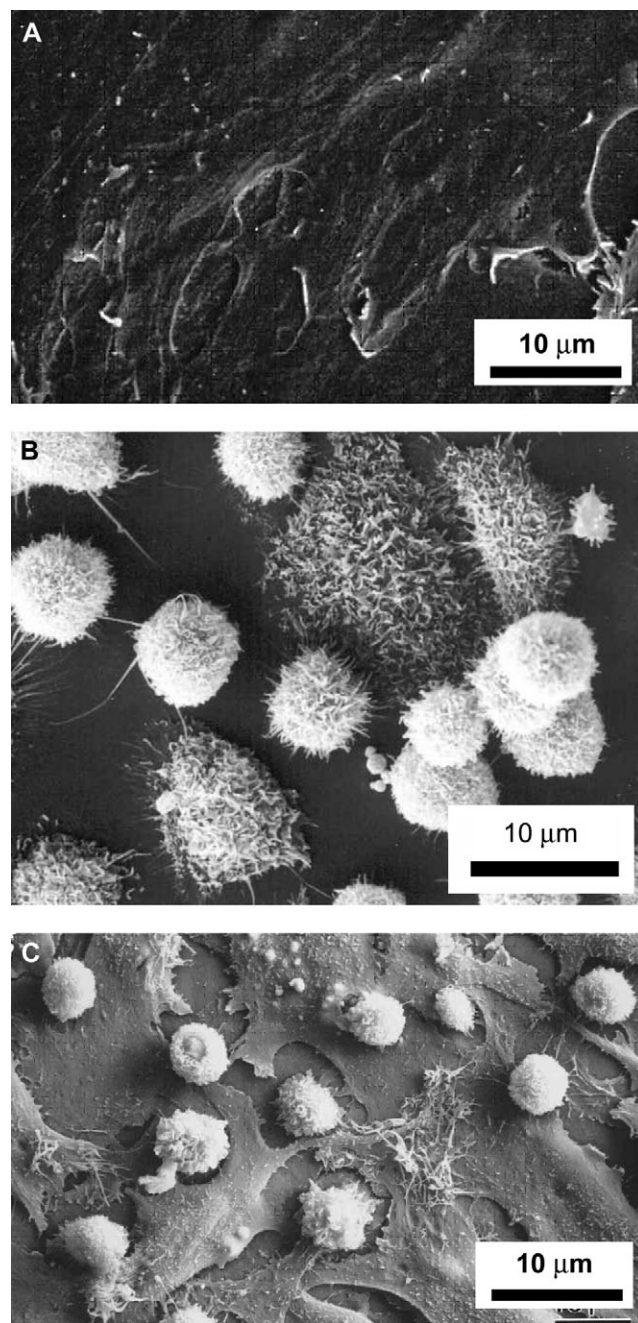


FIG. 1. Scanning electron micrographs of each of the *in vitro* test systems. Scanning electron microscopic morphological demonstrations of each of the *in vitro* test systems. SEM of (A) rat lung epithelial cells, (B) primary AMs (collected via BAL from unexposed rats), and (C) lung epithelial cell/AM cocultures.

time points ranging 1–48 h. After incubation, the plate was centrifuged at 1900 rpm for 4 min. The media were transferred into a fresh 24-well plate and analyzed for LDH release as described earlier in “Materials and Methods.”

Mitochondrial activity. The MTT assay (Sigma Aldrich) was used to evaluate mitochondrial activity (Mossman, 1983). Cells were seeded in 24-well plates and exposed as mentioned before. After incubation, the cell culture

media were aspirated; 150 μ l of MTT (5 mg/ml) was added to each well and incubated for 4 h. Afterwards, 850 μ l of the MTT solubilization solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well. The resulting formazan crystals were solubilized in acidic isopropanol and quantified by measuring absorbance at 570 nm. Data were calibrated to the appropriate calibration curve.

Production of MIP-2. The Rat Macrophage Inflammatory Protein-2 Enzyme Immunometric Assay Kit (Alpco Diagnostics, Salem, NH) was used to evaluate the production of MIP-2. Cells grown in 24-well plates were exposed to varying particle concentrations (0.0052–520 μ g/cm²) (Sherry *et al.*, 1992; Widmer *et al.*, 1993; Wolpe *et al.*, 1989).

Production of TNF- α . The TNF- α Enzyme Immunometric Assay Kit (Assay Designs, Ann Arbor, MI) was used to determine the level of TNF- α in the biological samples. Cells grown in 24-well plates were exposed to the same particle concentrations (0.0052–520 μ g/cm²) (Argilés *et al.*, 1997; Beutler and Cerami, 1998).

Production of IL-6. IL-6 Enzyme Immunometric Assay Kit (Assay Designs) was used to evaluate the production of IL-6 (Carmeliet, 1990; Hirano *et al.*, 1990; Kishimoto, 1989). Cells grown in 24-well plates were exposed to the same particle concentrations as mentioned above (Carmeliet, 1990).

Hemolytic potential of particle suspension. The hemolytic potential of the particles used in these studies was measured using the method of Harington *et al.* (1971). Erythrocytes were obtained from the fresh blood of healthy human donors. The RBCs were washed four times by suspending in PBS solution before centrifugation at 1200 \times g. The final suspension consisted of 4% by volume RBC suspended in PBS. Each particle type was suspended in PBS, as previously described, and diluted in saline to give a stock solution of 30 mg/ml. The dilutions ranged from 15 to 0.059 mg/ml. Each suspension (150 μ l) was

mixed with 75 μ l of erythrocyte suspension and incubated for 30 min at 37°C. After incubation, the samples were centrifuged at 717 \times g for 10 min at room temperature. Each particle suspension was measured against a blank, PBS negative control, and 1% Triton X-100 positive control. The amount of hemoglobin released into the supernatant was determined spectrophotometrically at 540 nm (Razzaboni and Bolsaitis, 1990).

Statistical analyses. Statistical analysis was performed as described earlier, i.e., statistical significance versus a control of μ g/cm² dose was established as $p < 0.05$.

RESULTS

Particle Characterization

We tested three different *in vitro* systems and compared the results to *in vivo* toxicity profiles of test systems exposed to five different particle samples (Fig. 2). The particle types, including CI, CS (Min-U-Sil), AS, NZO, and FZO, were characterized for physicochemical properties in their dry native state, in a water suspension, in PBS solution, and in cell culture media.

Different physicochemical properties were observed among each of the particles used in this study (Figs. 3 and 4). Further, certain properties of each particle varied when suspended in the different solvents. In general, as compared to the particle's dry native state, the mean particle size and percent size distribution were increased when placed in a water suspension. The mean

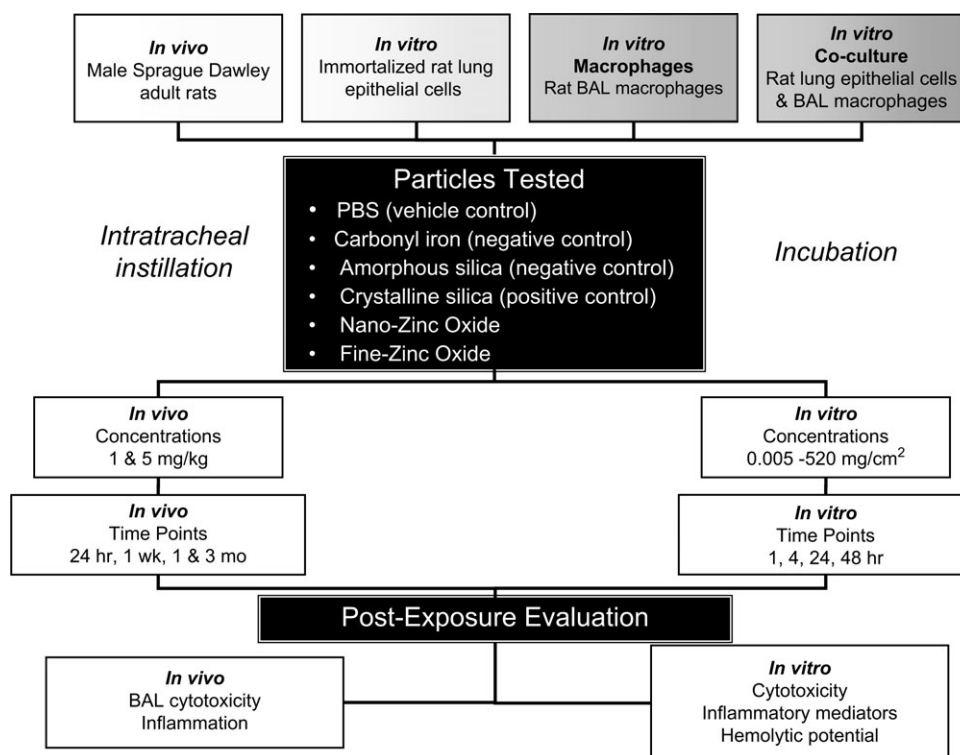
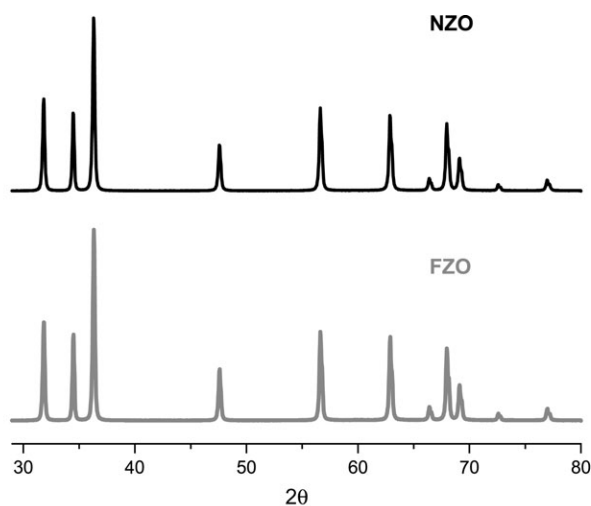


FIG. 2. Experimental design. The design of the *in vivo* and *in vitro* experiments. Test systems were exposed to CI, CS (Min-U-Sil 5), AS, NZO, and FZO particles. For each experiment, both dose-response and time-response relationships were established.



Sample	Abbreviation	Reported primary particle size (nm)	Surface area (m ² /g)	Density (g/ml)	Calculated size in dry state (nm)	Crystallinity
Carbonyl iron	CI	800–3000	2.5	6.7	358	Cubic
Crystalline silica	CS	1600	5.1	2.6	452	α-quartz
Amorphous silica	AS	1000–3000	77.7	2.2	354	Amorphous
Nano-ZnO	NZO	50–70	12.1	5.6	90	Hexagonal
Fine-ZnO	FZO	< 1000	9.6	5.6	111	Hexagonal

FIG. 3. Particle characterization in the dry state. (Top) XRD pattern of both ZnO particle samples (hexagonal crystals). (Bottom) The manufacturers reported primary particle size from the sample specification sheet and the authors' characterization of each particle type including surface area, density, calculated size in dry state, and crystallinity of each particle sample.

particle size and size distribution were increased further in both PBS and F-12K cell culture media suspensions. Aggregation was consistently most pronounced in the F-12K cell culture media. In all particle systems, surface charges were decreased when dispersed in the cell culture media, except for CS (Min-U-Sil) particle types.

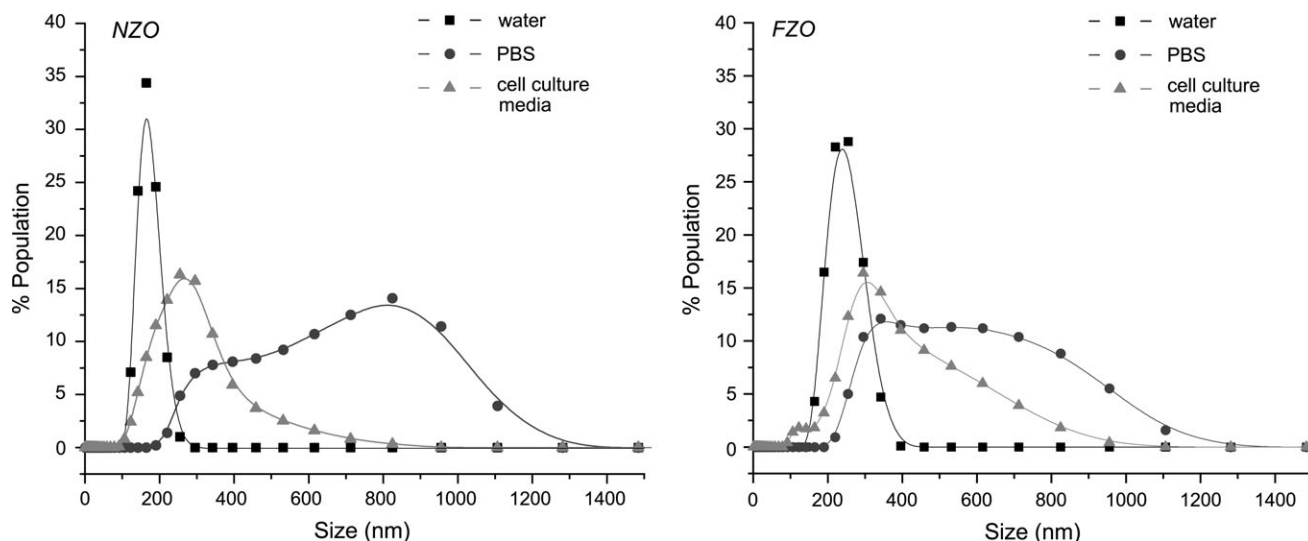
In all cases, the mean particle sizes reported by the manufacturer were significantly different than the actual measured size using BET and XRD in the native dry state and DLS in three different suspensions. From the formula $D = 6/(d \times A)$, where D is particle diameter, d is particle density, and A is measured surface area, the particle's average diameter can be calculated, assuming that the particle is spherical. Similarly, the full width at half maximum peak height of the ZnO signature can be used to estimate crystallite size. Both of the calculations are estimates and neither includes aggregation parameters. For example, in the NZO system, the supplier reported a particle size of 50–70 nm. However, three independent sizing techniques indicated that the NZO particles ranged from 90 to 283 nm, which was similar to the size of the FZO particles. The XRD patterns of both NZO and FZO

particle samples were identical, and DLS sizing results show the little differences in size of the two zinc oxide particle systems suspended in solvents. Three techniques, independent from the manufacturer's reported particle size, indicate that the NZO particles ranged from 90 to 283 nm depending on the particle's state.

***In Vivo* Effects of Instilled Particle Types as Measured by BAL Fluid Total Cell Numbers, LDH, and Inflammatory Responses**

In Vivo BAL Fluid LDH Responses

Exposures to 1 and 5 mg/kg CS particles produced elevated and sustained cytotoxic responses at all measured pe time points (24 h, 1 week, 1 month, and 3 months) (Fig. 5). AS exposures resulted in increased BAL fluid LDH responses at 24 h pe without subsequent significant effects. Exposures to the NZO or FZO particle suspensions produced enhanced cytotoxic responses at the 24-h and 1-week pe time periods but were not different from PBS-instilled controls thereafter. CI



Sample	Solution	Average aggregate size in solution (nm)	% distribution	Surface charge (mV)	Aggregation state
Carbonyl iron	Water	564.5	47.6	-60.52	Mild
	PBS	560.8	80.3	-51.29	Mild
	F-12K media	578.9	86.5	-33.62	Mild
Crystalline silica	Water	534.1	19.8	-61.93	Moderate
	PBS	530.0	66.9	-16	Moderate
	F-12K media	471.5	67.0	-1.1	Moderate
Amorphous silica	Water	213.5	24.4	-48.43	Mild
	PBS	260.7	73.0	-26.96	Mild
	F-12K media	382.2	98.0	-5.26	Moderate
Nano-ZnO	Water	167.5	16.2	-54.51	Moderate
	PBS	313.7	31.3	-28.5	Moderate
	F-12K media	283.0	44.8	-5.7	Severe
Fine-ZnO	Water	242.9	18.8	-55.76	Mild
	PBS	318.6	34.9	-14.9	Moderate
	F-12K media	372.4	45.2	-9.4	Moderate

FIG. 4. Particle characterization in the wet state. (Top) Size distribution of both zinc oxide (ZnO) particle samples. (Bottom) Average aggregate size, percent particle size distribution, surface charge, and aggregation state of each particle sample in ultrapure water, PBS solution, and F-12K cell culture media. Aggregation state was qualified by the time the particles stay in suspension: with 24 h is “severe,” 1–7 days is “moderate,” and more than a week is “mild.”

exposures did not produce any significant effects compared to controls. Further, longer term BAL fluid analyses as well as histopathological evaluations at later postexposure time points (i.e., 1 and 3 months) indicated that the effects associated with CI, AS, NZO, and FZO exposures were transient.

In Vivo BAL Fluid Total Cell Numbers

The numbers of cells recovered by BAL from the lungs of high-dose CS-exposed (5 mg/kg) groups were significantly higher than any of the other groups for all pe time periods

(Fig. 6). The number of cells at the 24-h time point for rats exposed to AS, NZO, and FZO were significantly higher than the other time points. This increase at the 24-h time point is a transient effect due to the instillation procedure and was diminished at the 1-week time point.

In Vivo Pulmonary Inflammation

The numbers of cells recovered by the lavage from the lungs of rats exposed to CS (Min-U-Sil, 5 mg/kg) were substantially higher than any of the other groups for all pe time periods.

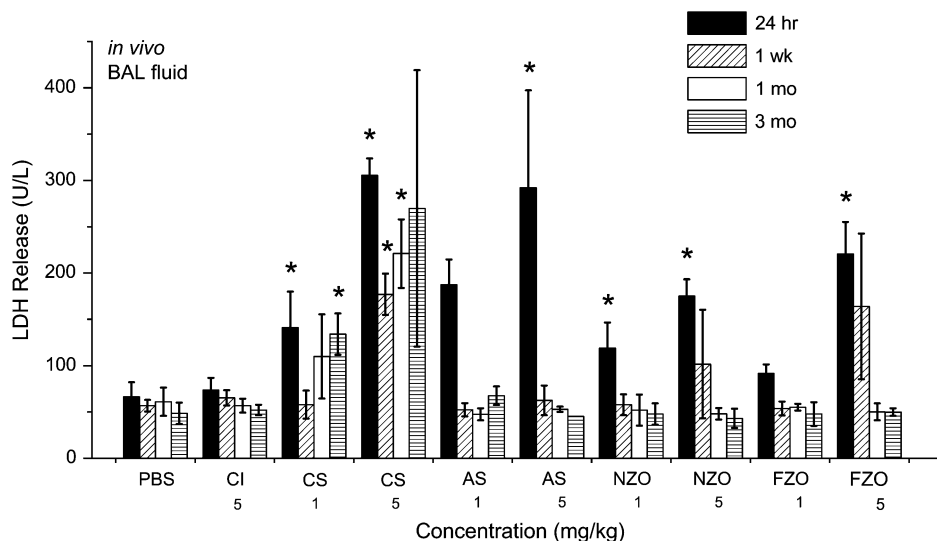


FIG. 5. Dose and time-course relationships of the cytotoxic effects in the *in vivo* test system. BAL fluid LDH values for rats exposed to PBS, CI, CS, AS, NZO, and FZO and corresponding controls at 24 h, 1 week, 1 month, and 3 months postexposure. Values given are means \pm SD. Increases in BAL fluid LDH values were measured in the lungs of high-dose (5 mg/kg) NZO and FZO at 24 h and 1 week postexposure. Exposures to 5 mg/kg CS particles produced a sustained increase in BAL fluid LDH values through the 3-month postexposure period (* $p < 0.05$).

Figure 7 demonstrates pulmonary inflammatory responses as indicated by the percentages of neutrophils present in BAL fluid of groups of PBS or particle-exposed rats. Intratracheal instillation exposures of all particle types produced short-term, pulmonary inflammatory responses, as evidenced by increases in the percentages of BAL-recovered neutrophils relative to PBS-instilled controls, measured at 24 h pe.

Exposures to 5 mg/kg CI or to 1 or 5 mg/kg AS particles produced transient and reversible neutrophilic lung inflammatory responses at 24 h pe. The responses to AS were much more

potent when compared to CI particles. Intratracheal instillation exposures to high-dose NZO (5 mg/kg) or FZO particles produced substantial lung inflammatory responses measured at 24 h pe followed by a minimal, recruitment of neutrophils through 1 week pe (i.e., 15–20% polymorphonuclear leukocytes). These effects were not measured at the 1- and 3-month pe time points, indicating resolution of the inflammatory responses. In contrast, exposures to CS particles (1 and 5 mg/kg) produced persistent pulmonary inflammatory responses, with both doses producing > 25% neutrophils through 3 month postexposure.

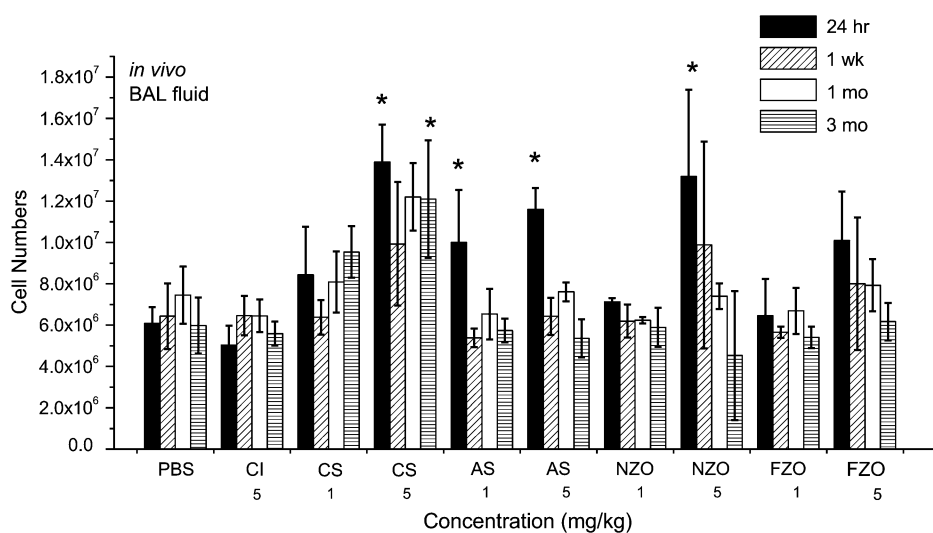


FIG. 6. Dose and time-course relationships of the cell numbers in the *in vivo* test system. Numbers of cells recovered in BAL fluids from rats exposed to PBS, CI, CS, AS, NZO, and FZO and controls at 24 h, 1 week, 1 and 3 months pe. Values given are means \pm SD (* $p < 0.05$).

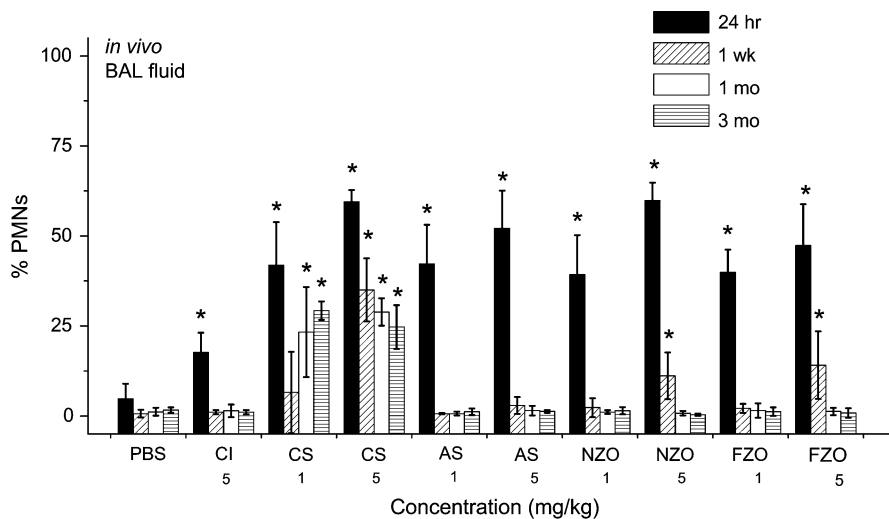


FIG. 7. Dose and time-course relationships of the % polymorphonuclear leukocytes (% PMNs) in the *in vivo* test system. Pulmonary inflammation in rats exposed to PBS, CI, CS, AS, NZO, and FZO and controls as observed by % neutrophils (% PMNs) in BAL fluids at 24 h, 1 week, 1 month, and 3 months postexposure. Values given are means \pm SD (* p < 0.05).

In Vitro Cytotoxicity Studies

LDH Studies (1, 4, 24, and 48 h)

LDH values from various doses of five different particle types at four different time points were compared with corresponding culture media (control) values.

L2 (rat lung epithelial cells) LDH values. After a 1-h incubation period with lung epithelial cells, all the particles produced increased LDH levels in the culture media only at the highest dose: 520 $\mu\text{g}/\text{cm}^2$ (Fig. 8).

After a 4-h incubation period, CI produced significant increases in LDH levels only at 520 $\mu\text{g}/\text{cm}^2$. CS and AS particles produced significant LDH increases at 52 and 520 $\mu\text{g}/\text{cm}^2$. In contrast, NZO or FZO particulates produced increases in LDH levels at five concentrations: 0.052, 0.52, 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$.

After a 24-h incubation period, CI produced no significant increases in LDH values at any doses. CS and AS particles produced significant increases in LDH levels at 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$. In contrast, NZO and FZO particles produced increases in LDH levels at 0.52, 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$.

At 48-h incubation period, exposures to CI, NZO, or FZO particles produced no significant increases in LDH values at any doses. CS and AS particles produced significant increases in LDH levels at 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$.

AMs (rat primary AMs) LDH values. After a 1-h incubation period with rat AMs, none of the particles produced increased LDH levels in the culture media (Fig. 9).

After a 4-h incubation period, CS produced a significant increase in LDH levels versus controls at 520 $\mu\text{g}/\text{cm}^2$. No significant increases in LDH values were measured in any of the other particles at any time periods.

After a 24-h incubation period, CI and CS produced significant increases in LDH levels versus controls at 520 $\mu\text{g}/\text{cm}^2$. No significant increases in LDH values were measured in any of the other particles at any time periods.

After a 48-h incubation period, none of the particles produced increased LDH levels in the culture media at any time periods.

Coculture (epithelial/macrophage coculture) LDH values. After a 1-h incubation period with rat epithelial/macrophage cocultures, none of the particles produced increased LDH levels in the culture media (Fig. 10).

After a 4-h incubation period, CS particles produced increased LDH values only at 520 $\mu\text{g}/\text{cm}^2$. In contrast, NZO and FZO particles produced increases in LDH levels at doses of 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$. No significant increases in LDH values were measured in CI or AS particles at any time periods.

After a 24-h incubation period, exposures to CI or AS particles produced increased LDH values only at 520 $\mu\text{g}/\text{cm}^2$. CS, NZO, and FZO particles produced significant increases in LDH levels at 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$.

At 48 h incubation, exposures to CI, NZO, or FZO produced no significant increases in LDH values. CS and AS particles produced significant increases in LDH levels at 5.2, 52, and 520 $\mu\text{g}/\text{cm}^2$.

To summarize the *in vitro* LDH findings with L2 lung epithelial cells, primary rat AMs or epithelial/macrophage cocultures, the L2, and cocultures appeared to have the greatest sensitivity to CS particles as well as to NZO and FZO particles. The significant effects associated with these particles appeared to be dose related. It is noteworthy that the macrophage cultures appeared to be resistant to the cytotoxic effects of particles in culture, as evidenced by the lack of LDH secretion by these cells.

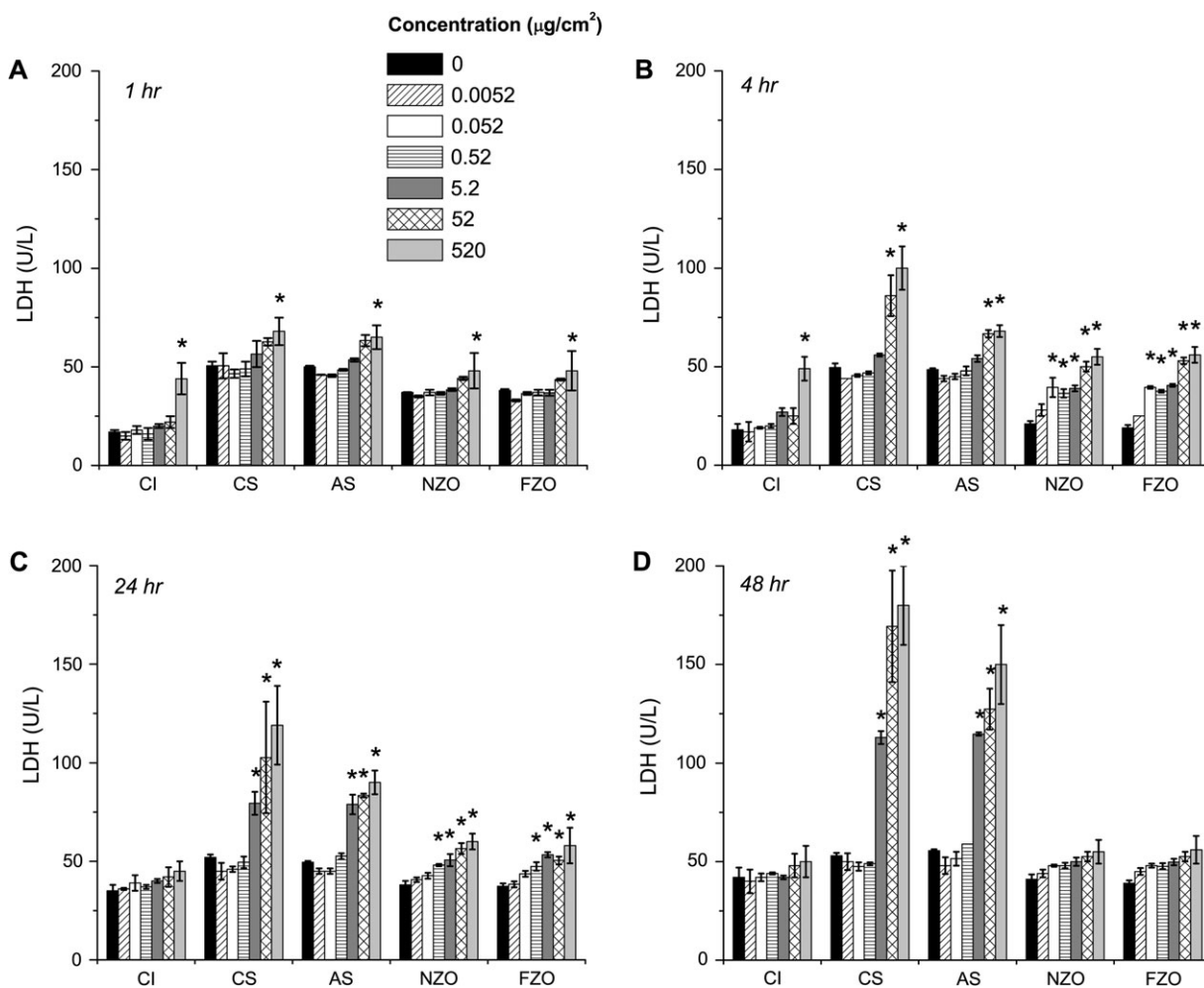


FIG. 8. Dose and time-course relationships of the cytotoxic effects in the *in vitro* L2 test system. The dose and time-response relationships of L2 cells exposed for 1, 4, 24, or 48 h to CI, CS, AS, NZO, and FZO. Cellular response is measured in LDH release (U/L) over varying concentrations ranging from 0.0052 to 520 mg/cm² (**p* < 0.05).

MTT Studies (4 and 24 h)

MTT values from various doses of the five different particle types at two different time points were compared with corresponding culture media (control) values. Data were assessed at the 4- and 24-h time points. An independent experiment confirmed that ZnO has no effect directly on MTT reduction.

L2 (rat lung epithelial cells) MTT values. After a 4-h incubation period, both NZO and FZO particles produced decreases in MTT levels at 5.2 and 52 µg/cm² (Fig. 11). No significant decreases in MTT values were measured in CI, CS, or AS particles at this time period.

After a 24-h incubation period, CI and AS particles produced significant decreases in MTT levels at 5.2 and 52 µg/cm². CS and NZO particles produced significant MTT decreases at 52 µg/cm². FZO particulates produced decreases in MTT levels at doses of 5.2 and 52 µg/cm².

AMs (rat primary AMs) MTT values. After a 4-h incubation period, AS, NZO, and FZO particles produced decreases in cellular MTT levels at doses of 5.2 and 52 µg/cm² (Fig. 11). No significant decreases in MTT values were measured in CI, CS, or AS particles at this time period.

After a 24-h incubation period, AS and NZO particulates produced decreases in cellular MTT levels at 5.2 and 52 µg/cm². FZO particles produced decreased MTT cellular levels at 52 µg/cm². No significant decreases in MTT values were measured in CI or CS particles at this time period.

Coculture (epithelial/macrophage coculture) MTT values. After a 4-h incubation period, FZO particles produced decreases in cellular MTT levels at doses of 5.2 and 52 µg/cm² (Fig. 11). NZO particles produced decreased MTT levels at 52 µg/cm². No significant decreases in MTT values were measured in CI, CS, or AS particles at this time period.

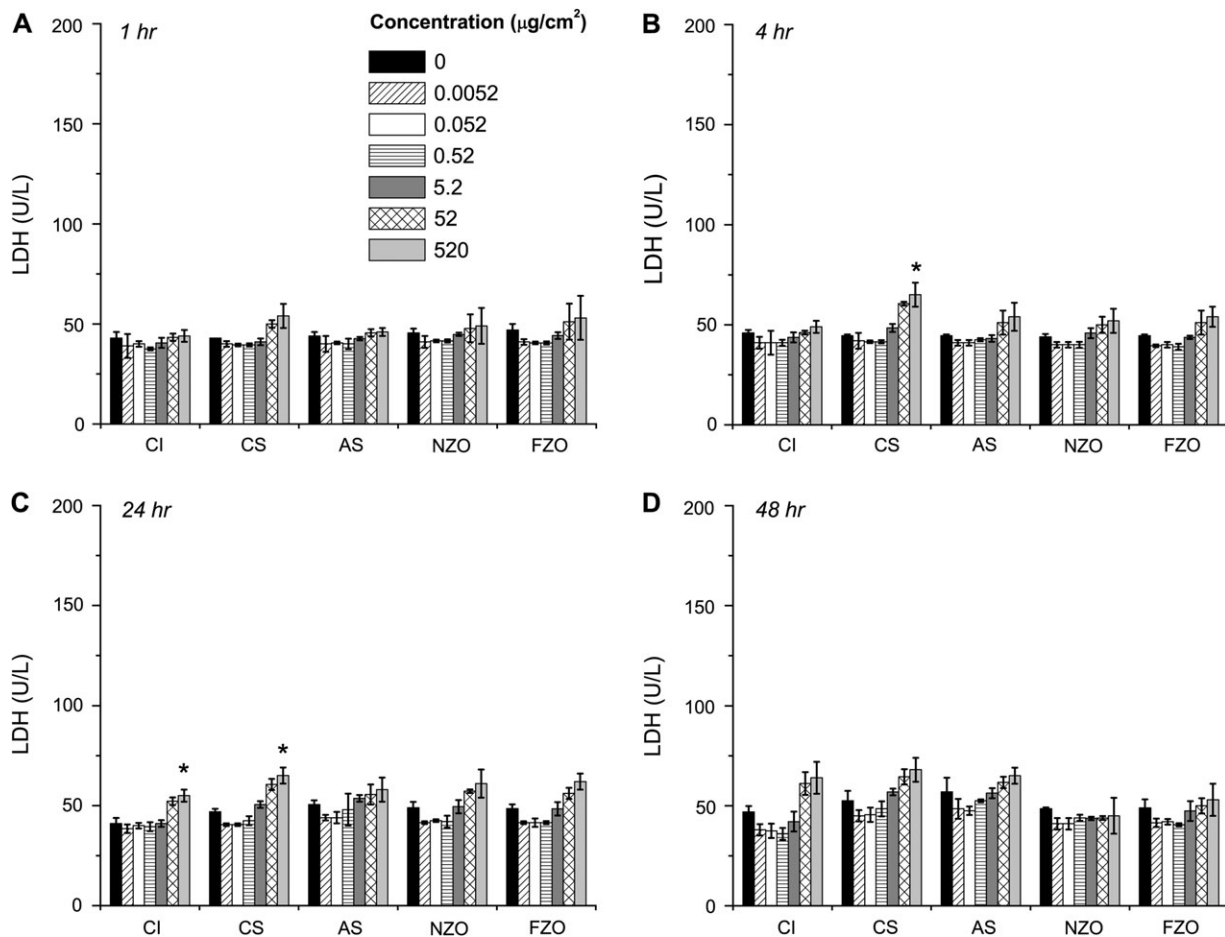


FIG. 9. Dose and time-course relationships of the cytotoxic effects in the *in vitro* macrophage test system. The dose and time-response relationship of the macrophage test system exposed for 1, 4, 24, or 48 h to CI, CS, AS, NZO, and FZO. Cellular response is measured in LDH release (U/L) over varying concentrations ranging from 0.0052 to 5200 µg/cm² (**p* < 0.05).

After a 24-h incubation period, AS, CS, and FZO particulates produced decreases in cellular MTT levels at 5.2 and 52 µg/cm². No significant decreases in MTT values were measured in CI- or NZO-exposed cocultures at this time period.

In Vitro—Inflammatory Mediator Studies

MIP-2 production by cells exposed *in vitro* for 24 h to AS, CS, NZO, or FZO particles is shown in Figure 12. Little or no MIP-2 production was measured in the culture fluids of lung epithelial cells exposed to any of the particle types. Rat AMs exposed to AS or CS particulates, but not to NZO or FZO particles generated MIP-2 in the culture fluid. Similarly, epithelial/macrophage cocultures exposed to AS or CS generated MIP-2 in the culture fluid, although the macrophage cultures appeared to be more efficient when compared to the cocultures.

TNF-α production by cells exposed *in vitro* for 24 h to AS, CS, NZO, or FZO particles is shown in Figure 13. It was interesting to note that *in vitro* exposures of lung epithelial

cells, macrophages, or cocultures to CS particles did not produce any secretions of TNF-α in the cell culture fluid. Cocultures exposed to AS particle types produced TNF-α at doses of 0.52 and 5.2, but the effect did not conform to a dose-response relationship.

IL-6 production by cells exposed *in vitro* for 24 h to AS, CS, NZO, or FZO particles is shown in Figure 14. The results of studies were rather mixed and the relevance of the results was difficult to interpret. In this regard, macrophages and cocultures produced IL-6 following exposures to CS, but only cocultures produced IL-6 in response to AS particulates. NZO and FZO particle exposures produced very little IL-6 and were not dose related.

To summarize the results of *in vitro* cytokine generation studies as a surrogate for *in vivo* inflammation, it is difficult to draw any meaningful conclusions regarding the relevance of measured biomarkers as predictors of *in vivo* inflammatory responses. This is due, in large part, to the finding that each test produced different results. The data suggest that MIP-2 generation and corresponding recruitment of neutrophils by macrophages may be operative in the lungs of AS- or CS-exposed rats.

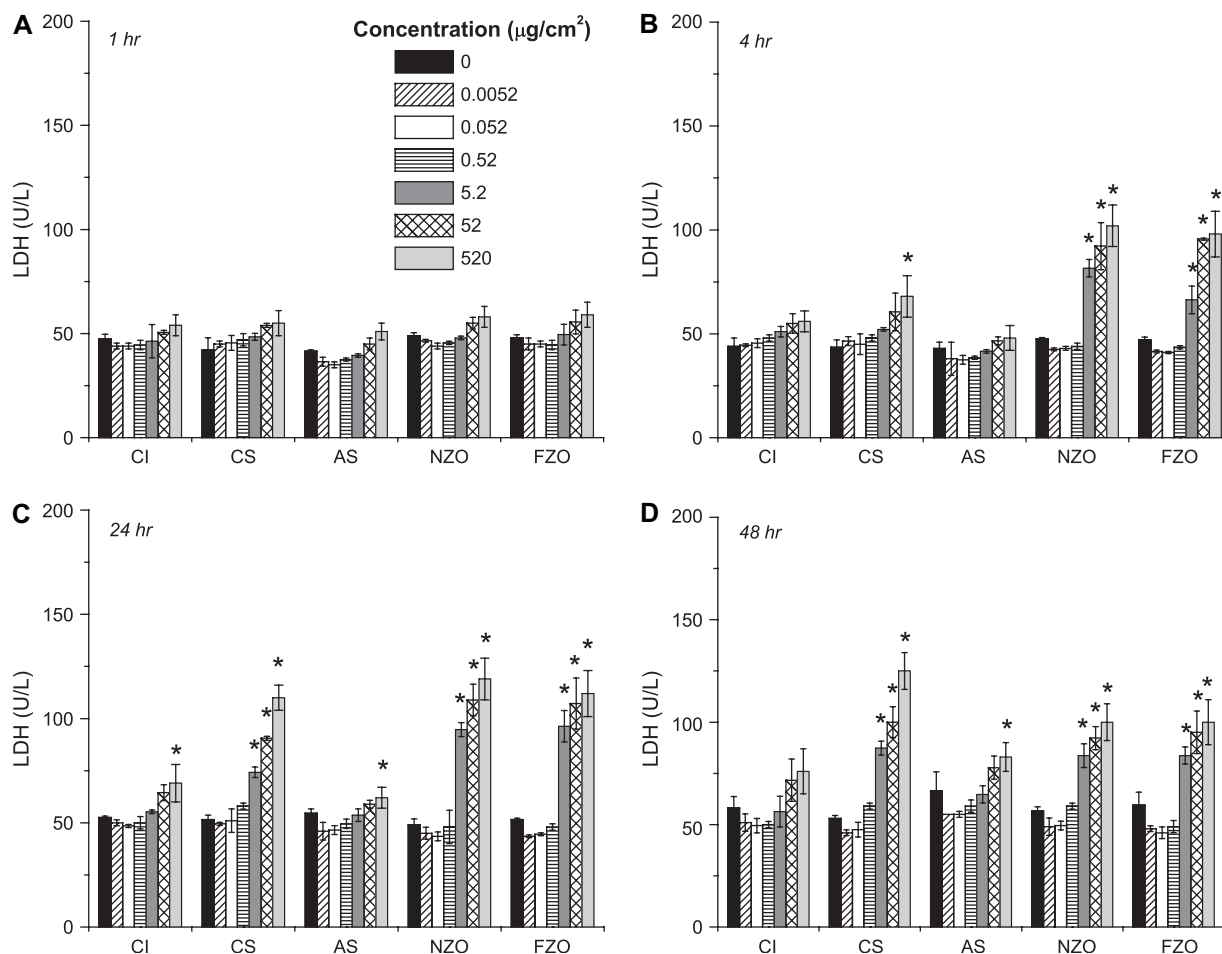


FIG. 10. Dose and time-course relationships of the cytotoxic effects in the *in vitro* coculture test system. The dose and time-response relationships of the *in vitro* coculture exposed for 1, 4, 24, or 48 h to CI, CS, AS, NZO, and FZO. Cellular response is measured in LDH release (U/L) over increasing concentrations ranging from 0.0052 to 520 µg/cm² (**p* < 0.05).

However, the TNF- α assay was not useful in discriminating differences between the three test systems studied. In addition, the IL-6 assay showed that the L2 lung epithelial cell line does not produce IL-6 when exposed to these particles.

Hemolysis Assay Results

Results from the hemolytic potential assay demonstrated the differential surface activity of each particle sample when incubated with RBCs (Fig. 15). Incubation of amorphous or CS particle types produced hemolysis of erythrocytes. However, treatment of NZO or FZO or CI particles had little or no effect on red cell hemolysis.

DISCUSSION

This study was designed to assess the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of several fine or nanoscale particles in rats. Previous studies have

reported little correlation between the relative toxicity of particles when comparing lung toxicity rankings following *in vivo* instillation versus *in vitro* cell culture exposures. In the *in vivo* component of the study, rats were exposed by intratracheal instillation to 1 or 5 mg/kg of the following particles: (1) CI, (2) CS (Min-U-Sil 5, α -quartz), (3) precipitated AS, (4) NZO, or (5) FZO. Following exposures, the lungs of exposed rats were lavaged and inflammation and cytotoxicity end points were measured at 24 h, 1 week, 1 month, and 3 months postexposure.

For the *in vitro* component of the study, three different cell culture conditions were utilized. Cultures of (1) rat L2 lung epithelial cells, (2) primary AMs (collected via BAL from unexposed rats), as well as (3) AM-L2 lung epithelial cell cocultures were incubated with the particle types listed above. The culture fluids were evaluated for cytotoxicity end points (LDH, MTT) as well as inflammatory cytokines (MIP-2, TNF- α , and IL-6). Initially, all three particle-exposed culture types were assessed for cytotoxicity biomarkers at four different time periods—1, 4, 24, or 48 h. Subsequently, MTT end points were

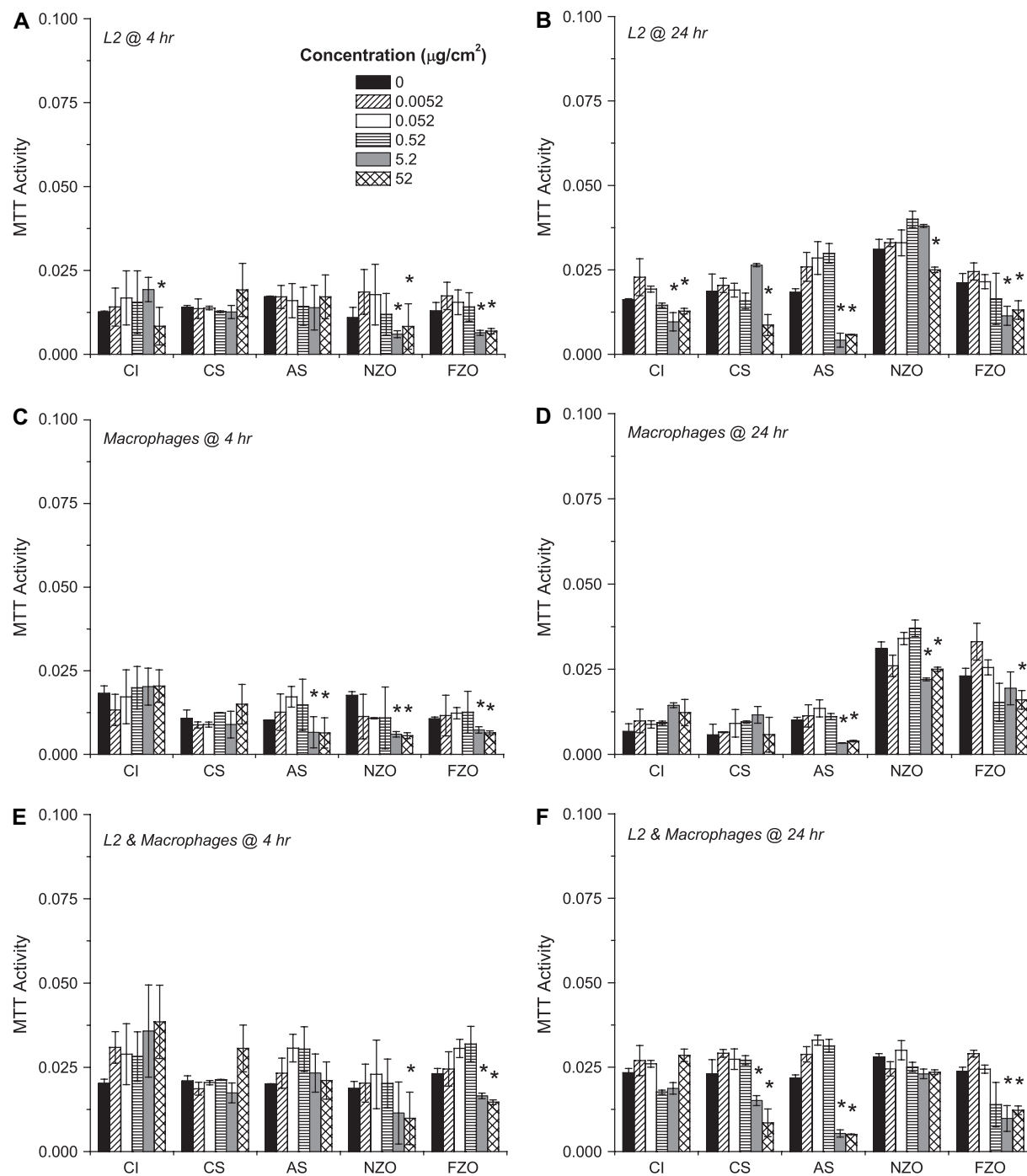


FIG. 11. Dose and time-course relationships of the MTT activity in the *in vitro* L2, macrophage, and coculture test systems. The dose and time-response relationships of the *in vitro* systems exposed for 4 and 24 h to CI, CS, AS, NZO, and FZO. Cellular viability is measured in MTT activity (absorbance at 570 nm) over an increasing concentration range from 0.0052 to 520 $\mu\text{g}/\text{cm}^2$ ($*p < 0.05$).

measured following incubation for 4 or 24 h, and the cytokine secretion into the culture fluids were measured after treatment for 24 h.

Results of *in vivo* pulmonary toxicity studies demonstrated that instilled CI particles produced little toxicity. CS exposures

produced sustained inflammation and cytotoxicity. Exposures to AS particles produced reversible and transient inflammatory responses. NZO and FZO particle instillations produced potent but reversible inflammation which was resolved at 1 month pe.

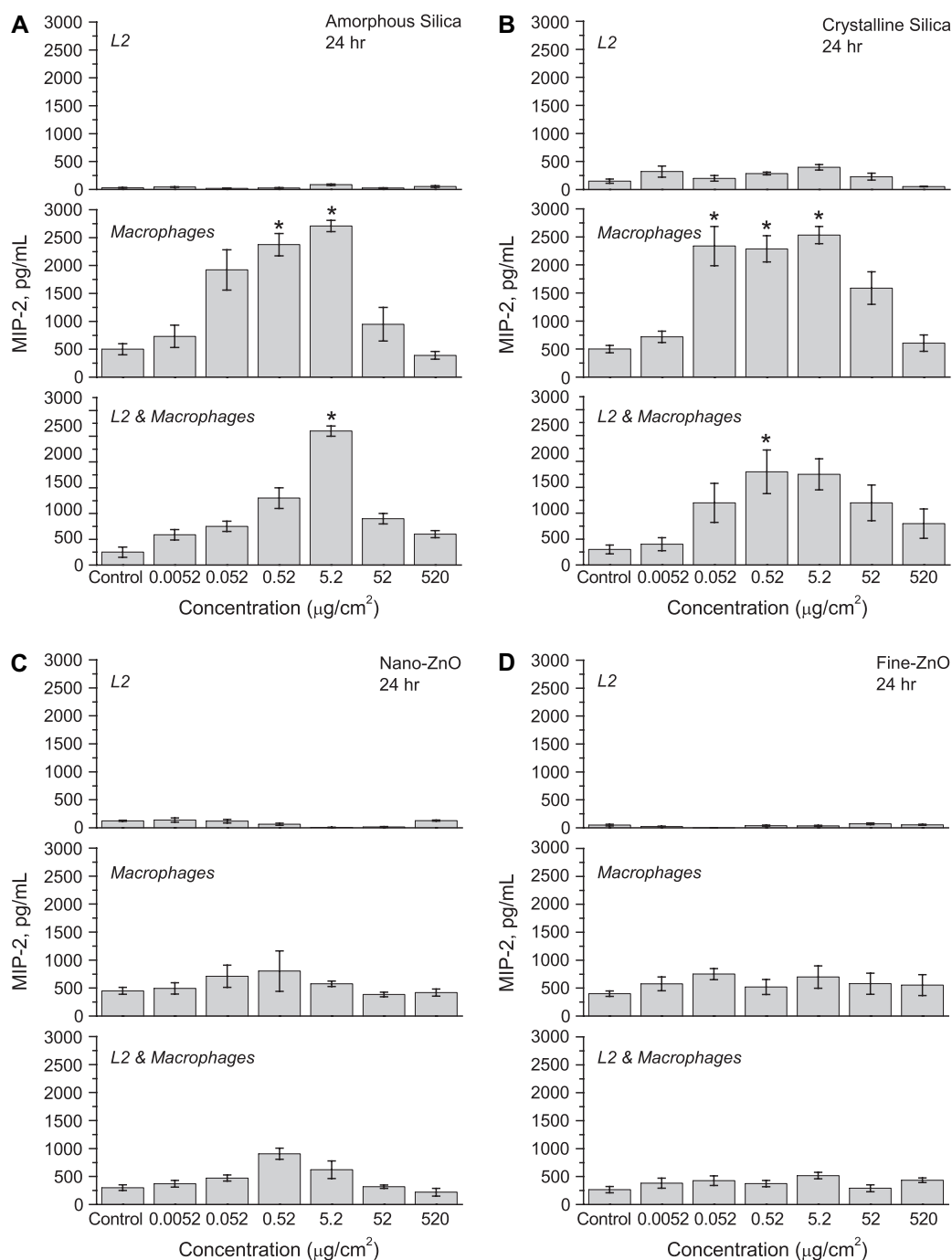


FIG. 12. MIP-2 analysis in the L2, macrophage, and coculture test systems exposed to particle types. The *in vitro* MIP-2 analyses. The production of MIP-2 in cells exposed to increasing concentrations (0.0052–520 $\mu\text{g}/\text{cm}^2$) of the different particle suspensions was measured (* $p < 0.05$).

Results of *in vitro* pulmonary cytotoxicity studies demonstrated a variety of responses to the different particle types. With respect to the LDH results, L2 cells were the most sensitive, and exposure to NZO or FZO for 4 or 24 h was more potent than exposures to crystalline or AS particles. Macrophages were essentially resistant and epithelial/macrophage cocultures generally reflected the epithelial results at 4 and 24 h

incubation, but not at 48 h incubation. MTT results were also interesting except for NZO and FZO which did not correlate well with LDH results. Results of *in vitro* pulmonary inflammation studies demonstrated that L2 lung epithelial cells did not produce MIP-2 cytokines. Crystalline- or AS-exposed AMs and cocultures, to a lesser degree, secreted these chemotactic factors into the culture media. Measurements of

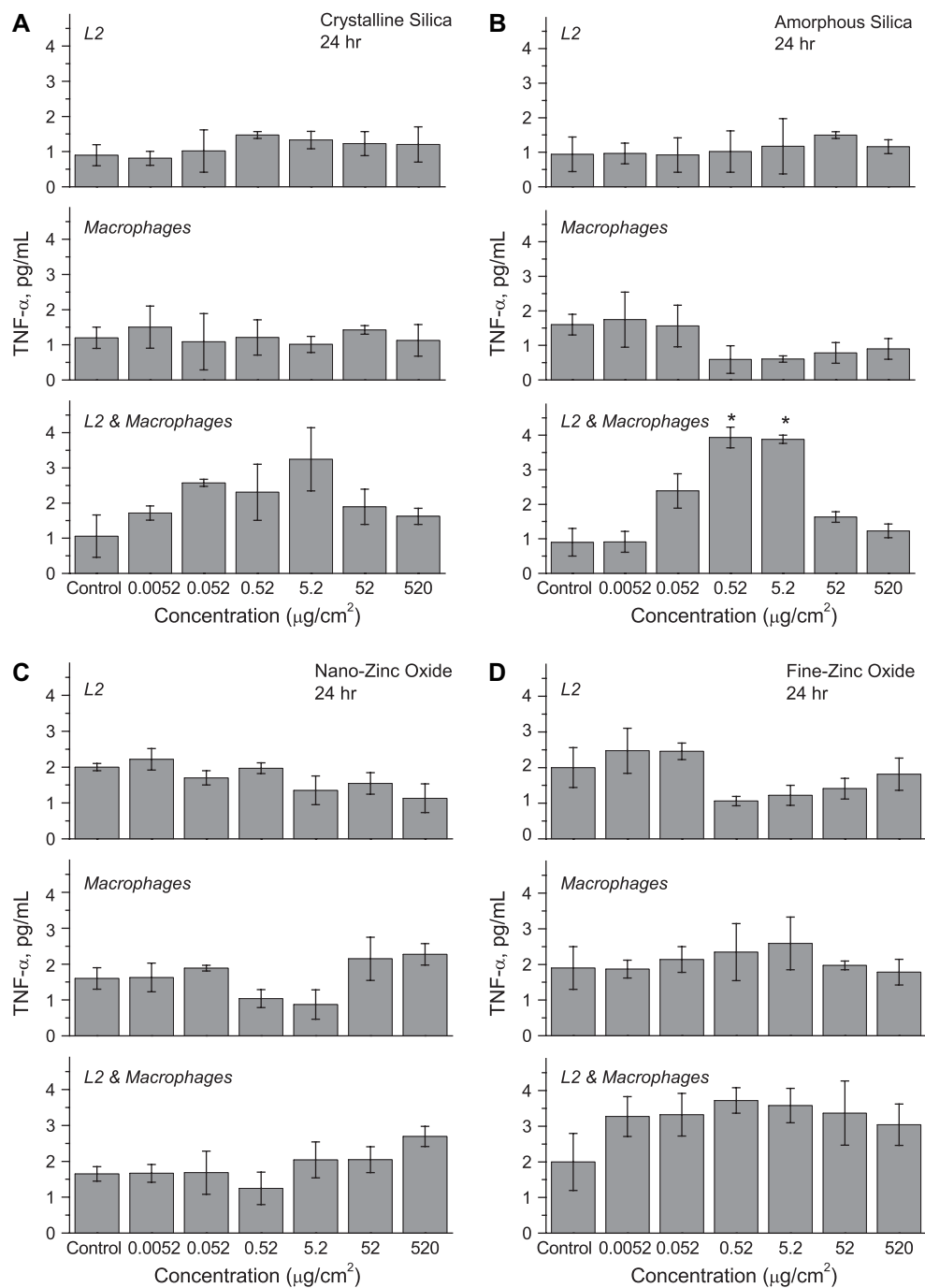


FIG. 13. TNF- α analysis in the L2, macrophage, and coculture test systems exposed to particle types. The *in vitro* TNF- α cytokine analyses. The production of TNF- α in cells exposed to increasing concentrations (0.0052–520 $\mu\text{g}/\text{cm}^2$) of the different particle suspensions was measured (* $p < 0.05$).

TNF- α in the culture media by particle-exposed cells demonstrated little activity. In addition, IL-6 secretion was measured in CS, AS, and NZO-exposed cocultures.

When considering the range of toxicity to five different particles, the comparisons of *in vivo* and *in vitro* measurements demonstrated little correlation, particularly when considering many of the variables assessed in this study, such as cell types,

culture conditions, time course of exposure, and measured end points. It seems clear that *in vitro* cellular systems will need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particles.

Seagrave and coworkers have attempted to systematically compare the results of *in vitro* and *in vivo* pulmonary toxicity

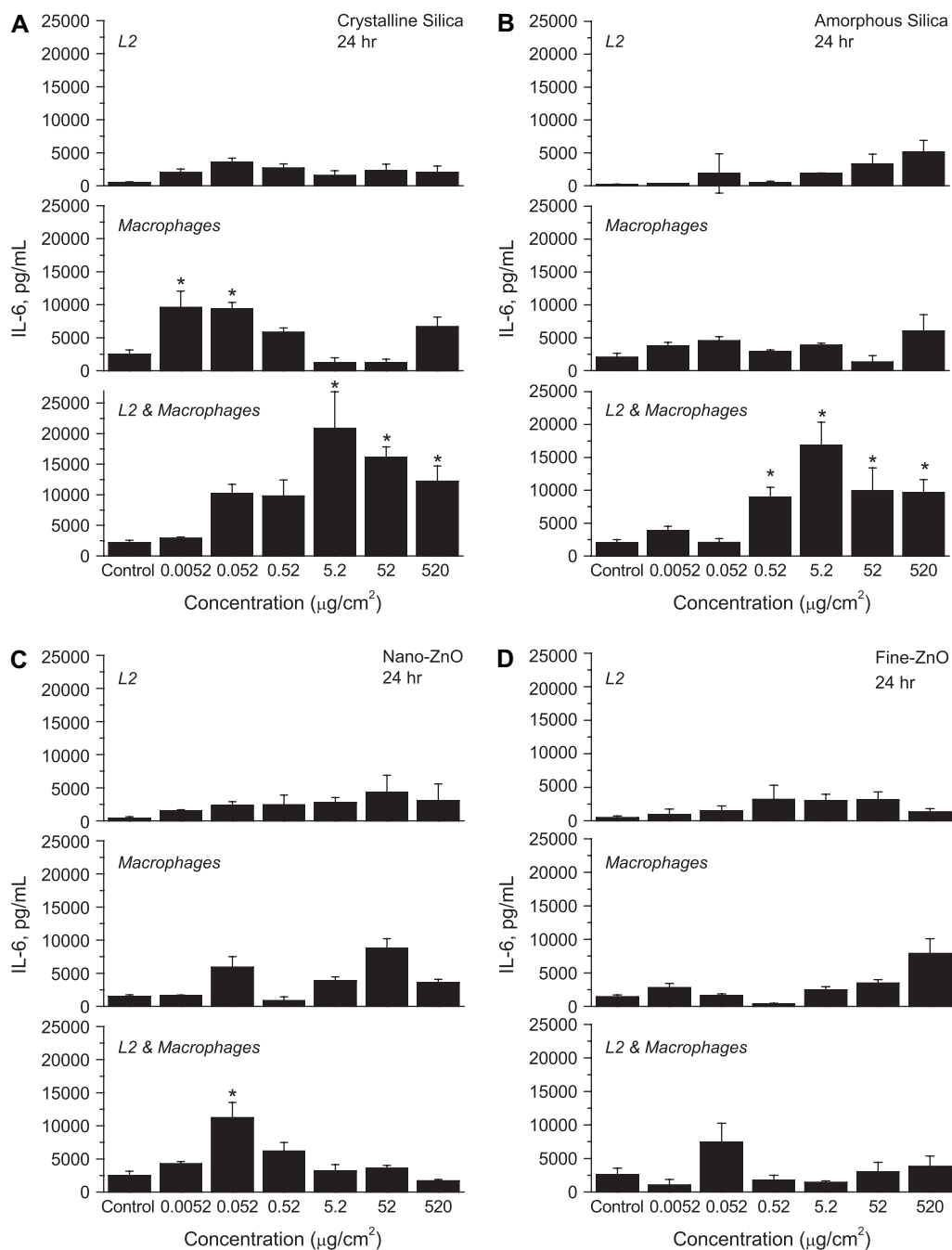


FIG. 14. IL-6 analysis in the L2, macrophage, and coculture test systems exposed to particle types. The *in vitro* IL-6 cytokine analyses. The production of IL-6 in cells exposed to increasing concentrations (0.0052–520 $\mu\text{g}/\text{cm}^2$) of the different particle suspensions was measured ($*p < 0.05$).

studies in ranking the toxicity of gasoline and diesel extract particulate samples. Initially they assessed the mutagenicity and *in vivo* toxicity of combined particulate and semivolatile organic compound (SVOC) fractions of gasoline and diesel engine emissions. In general, responses to intratracheal instillation in rats demonstrated parallel rankings of the samples by multiple end points reflecting cytotoxic, inflammatory, and

lung parenchymal changes. According to the authors, the end points selected to assess oxidative stress and macrophage function yielded little useful information. It was concluded that a subset of this panel of assays was useful in providing rapid, cost-effective feedback on the biological impact of modified technology, in the form of engine emissions. In a subsequent, companion *in vitro* study, these investigators evaluated the

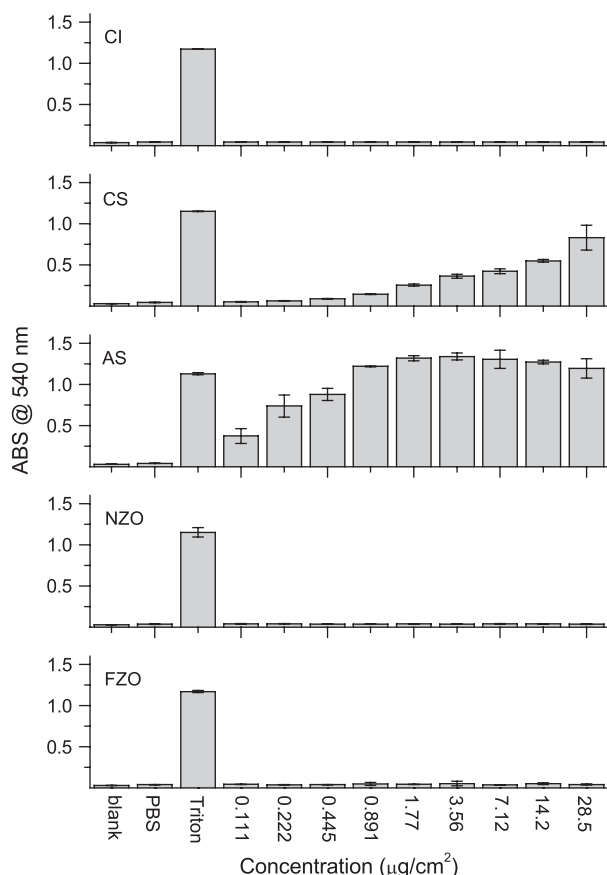


FIG. 15. The hemolytic potential of each particle suspension used in the study. Data are plotted as absorbance intensity at 540 nm (the intensity of hemoglobin released into solution) versus concentration of the particle tested. Each particle suspension was measured against a blank, PBS negative control, and 1% Triton X-100 positive control. The samples include CI, CS, AS, NZO, and FZO particles.

same samples to determine whether the *in vitro* screening studies could serve as an accurate preliminary screen to rank the toxicities of particulate samples. To facilitate this comparison, Seagrave and coworkers analyzed selected responses of cultured human lung epithelial cells and rat AMs to determine

whether the tests could discriminate among combined particulate matter and SVOC fractions of emissions collected from normal- and high-emitter, in-use gasoline and diesel vehicles. The investigators reported that macrophages were more susceptible to cytotoxicity than to epithelial cells. Samples from gasoline vehicles generally caused enhanced toxicological effects compared to the diesel engine samples. However, it is important to note that the rank order of potency from the *in vitro* assays in general did not correspond with the earlier rankings from *in vivo* comparisons of the same samples. Accordingly, the authors concluded that, while the *in vitro* assays may provide useful mechanistic information, revealing cell type-specific responses; however, in their current form, they did not accurately reflect *in vivo* comparative toxicity end points (Seagrave *et al.*, 2002, 2003, 2005).

In another comparative study, Becher *et al.* (2001) exposed *in vitro* rat lung AMs and alveolar epithelial type II cells for 20 h to various stone particles with differing contents of metals and minerals (a type of mylonite, gabbro, feldspar, and quartz). As evaluative criteria, the capacity to induce the release of the inflammatory cytokines IL-6, TNF- α , and MIP-2 was investigated. The investigators reported marked differences in potency between the various particles, with mylonite being most potent overall, followed by gabbro, and with feldspar and quartz having an approximately similar order of lower potency. The results also demonstrated differences in cytokine release patterns between the macrophages and alveolar epithelial cells. For all particle types including quartz, alveolar type II epithelial cells, demonstrated the most marked increase in MIP-2 and IL-6 secretion, whereas the largest increase in TNF- α release was observed in macrophages. To investigate possible correlations between *in vitro* and *in vivo* inflammatory responses, rats were instilled with the same types of particles, and BAL fluid was collected after 20 h. The results demonstrated a correlation between the *in vitro* cytokine responses and the number of neutrophilic cells in the BAL fluid. The investigators further assessed whether a similar graded inflammatory response would be continued in alveolar epithelial type II cells and AMs isolated from the exposed animals. Again a differential cytokine release pattern was observed between epithelial

TABLE 1
Summary of Results for the *In Vivo* Versus *In Vitro* Test Systems

Test system	LDH release	MTT activity	% PMNs	MIP-2	TNF- α	IL-6	Hemolytic potential
Animal	CS, NZO, FZO	—	CS, NZO, FZO	—	—	—	
L2 cell line	CI, CS, AS, NZO, FZO	AS, NZO, FZO		None	None	None	
Macrophages	CI, CS	AS, NZO, FZO		AS, CS	None	None	
Coculture	CS, AS, NZO, FZO	CS, AS, NZO, FZO		AS, CS	AS	CS, AS, NZO	
Human blood cells	—	—	—	—	—	—	CS, AS

Note. Abbreviation represents significant effects found when exposed to particle at any concentration. CI, carbonyl iron; CS, crystalline silica; AS, amorphous silica; NZO, nano-zinc oxide; and FZO, fine-zinc oxide. Dash indicates data not determined. "None" refers to no response/production observed by any particle tested.

type II cells and macrophages, although the order of potency between particle types was altered. It was concluded that various stone particles caused differential inflammatory responses after both *in vitro* and *in vivo* exposure, with mylonite being the most potent stone particle. The authors claimed that the alveolar type II epithelial cells were important participants in the inflammatory response following exposure to particles. The concordance between *in vitro* and *in vivo* effects was measured at only a single time point—20 h. It is noteworthy that in our studies presented herein, the major sources of MIP-2 and IL-6 secretions were the primary AM lavaged from the lungs of rats or the epithelial/macrophage coculture systems, but not the L2 (lung epithelial) cells, which are derived from alveolar epithelial type I cells. In our studies, TNF- α was rarely expressed in lung epithelial cells, macrophages, or epithelial/macrophage cocultures.

In this study, we have attempted to address several of the fundamental issues relevant to the development of a validated *in vitro* assay/cell culture system in order to best simulate the anatomical lung microenvironment concomitant with the complex biological effects (physiology) in the lungs of experimental animals exposed to inhaled particulates. These include, but are not limited to the following parameters: (1) particle dose, (2) selection of cell types for simulating the lung microenvironment (concomitant with the issue of single-cell culture systems vs. coculture systems), (3) characterization and exposure interactions of lung cells with particle types in culture versus lung fluids; (4) time course of effects (acute vs. chronic) *in vivo* versus 1, 4, 24, or 48 h incubation *in vitro*, and (5) appropriate end points for hazard evaluations (Table 1).

Particle dose is a critical issue because most *in vitro* as well as *in vivo* studies employ high exposure doses of putative toxicants in order to determine the potential (worst case scenario) hazard effect related to that particle type. Unfortunately, overload doses of particulates can stimulate an effect that may have little or no physiological relevance. In the *in vitro* component of our study, we utilized a wide range of doses to assess the dose-response relationship. In many cases, the effects were measured only at doses of 52 or 520 $\mu\text{g}/\text{cm}^2$, which should be viewed as a very high, particle-overload dose. The relative resistance of the CS-exposed L2 cells, macrophages, and cocultures to secreting LDH in the culture fluid is a significant cause for concern. Furthermore, when these cell types responded to the silica exposure, the greatest effect was usually measured only at 24 or 48 h with L2 cells and epithelial/macrophage cocultures. It seems likely that particle clearance or translocation mechanisms in the dynamic physiological state of the lung would be operative prior to 24 or 48 h. This suggests that potential hot spots of particle deposition would yield fluid fluxes or particle clearance via macrophages.

Inhaled particles are known to deposit on alveolar epithelial cells at alveolar duct bifurcations (Brody and Roe, 1983; Warheit and Hartsky, 1990). AMs are recruited to sites of particle deposition wherein they phagocytize particles (Warheit

et al., 1988). Alternatively, particles may translocate through alveolar epithelial cells from alveolar regions to interstitial sites. Therefore, the selection of epithelial/macrophage cocultures represents a more relevant simulation of the *in vivo* lung microenvironment. The macrophage component can phagocytize particles in culture but cannot simulate particle clearance from the lung. Perhaps more problematic, the *in vitro* culture system cannot simulate recruitment of inflammatory cells into the lung, which is an important component of the lung pathogenic process and has been utilized as an important toxicity biomarker for *in vivo* studies. Cytokine generation by cultured lung cells has been utilized as an indirect biomarker of inflammation. The efficacy of this approach has not been tested or validated.

To summarize our findings, we systematically evaluated a number of variables which strongly impact the ability of *in vitro* screening studies to accurately reflect *in vivo* pulmonary toxicity of several particle types in rats. The variables tested included particle dose, time course, duration of treatment exposure, and pulmonary cell types. *In vivo* effects in the lungs of rats were utilized as the barometer for comparison and validation. Under the conditions of this study, the results of *in vivo* and *in vitro* cytotoxicity and inflammatory cell measurements demonstrated little correlation. Our conclusion is that there is a need for better culture systems. In addition, more research is needed to designate the most relevant (chemically, biologically, and physiologically best) end points to test for *in vitro* toxicity. Accordingly, more sophisticated *in vitro* cell culture systems need further development and validation (relative to *in vivo* effects) to facilitate cost-effective screening systems to gauge the relative toxicity of inhaled particles.

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