

# Comparing Inhaled Ultrafine versus Fine Zinc Oxide Particles in Healthy Adults

## A Human Inhalation Study

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**Rationale:** Zinc oxide is a common, biologically active constituent of particulate air pollution as well as a workplace toxin. Ultrafine particles ( $< 0.1 \mu\text{m}$  diameter) are believed to be more potent than an equal mass of inhaled accumulation mode particles ( $0.1\text{--}1.0 \mu\text{m}$  diameter). **Objectives:** We compared exposure-response relationships for respiratory, hematologic, and cardiovascular endpoints between ultrafine and accumulation mode zinc oxide particles. **Methods:** In a human inhalation study, 12 healthy adults inhaled  $500 \mu\text{g}/\text{m}^3$  of ultrafine zinc oxide, the same mass of fine zinc oxide, and filtered air while at rest for 2 hours. **Measurements and Main Results:** Preexposure and follow-up studies of symptoms, leukocyte surface markers, hemostasis, and cardiac electrophysiology were conducted to 24 hours post-exposure. Induced sputum was sampled 24 hours after exposure. No differences were detected between any of the three exposure conditions at this level of exposure. **Conclusions:** Freshly generated zinc oxide in the fine or ultrafine fractions inhaled by healthy subjects at rest at a concentration of  $500 \mu\text{g}/\text{m}^3$  for 2 hours is below the threshold for acute systemic effects as detected by these endpoints.

**Keywords:** air pollution; metal fume fever; particulate matter, ultrafine; zinc

Ambient air pollution particles occur in three major size distributions. Common combustion processes generate some primary ultrafine particles ( $< 0.1 \mu\text{m}$  diameter), which can rapidly coalesce into larger accumulation mode particles ( $0.1\text{--}1.0 \mu\text{m}$  diameter). The third fraction is the coarse mode particles, from 1 to  $100 \mu\text{m}$  in diameter, which are often generated by mechanical breakdown of the earth's crustal minerals. A given mass of ultrafine particles has a markedly higher ratio of total surface area to weight than does the same mass of accumulation mode particles. In experimental animals, inhaled ultrafine particles exhibit greater lung inflammatory and systemic activity than an equal mass of larger particles, and these particles may also have greater effects in adult human subjects (1–3). With ambient particle air pollution exposure, certain respiratory effects have been more closely associated with the number of ultrafine particles than with the total mass of particle exposure (4).

Zinc is a common element in the earth's crust and an essential mineral in human nutrition. Zinc oxide may be found in ambient

air particles from combustion sources (5–7), and it is generated in high concentrations in industrial processes, such as brass founding and welding or cutting galvanized sheet metal. Actual exposures of welders and others depend on the conditions in the workplace, but the permissible exposure limit for occupational exposure to zinc oxide in the United States is  $5.0 \text{mg}/\text{m}^3$  respirable dust. Zinc is one of several transition metals that have been proposed to contribute to the biological activity of ambient combustion particles. Freshly generated zinc oxide causes a self-limited, febrile, and inflammatory response known as metal fume fever, subtle findings of which have been detected in one study after inhaled concentrations of  $2.5 \text{mg}/\text{m}^3$  over 2 hours (8). This finding may be because of the presence of a high proportion of ultrafine particles in the circumstances in which zinc oxide fume is created.

The degree to which ultrafine particles are more potent than an equal mass of fine particles is an area of active scientific investigation and controversy, and has not previously been tested by direct comparison in human subjects. On the basis of the known effects and the previously established safety of inhaled zinc oxide, we compared ultrafine to fine zinc oxide in a human inhalation study to test whether ultrafine zinc oxide particles are more potent than an equal mass of fine zinc oxide particles.

## METHODS

### Study Protocol

The study was a three-factor comparison of the effects of inhaling  $500 \mu\text{g}/\text{m}^3$  zinc oxide in the ultrafine versus fine modes with a clean-filtered air control session. The study was approved by the Research Subjects Review Board of the University of Rochester, and informed, written consent was obtained from subjects. Exposures were by mouthpiece at rest for 2 hours from approximately 8:00 to 10:00 A.M. on 3 exposure days. The exposure concentration was chosen with the anticipation that subtle effects would be seen with fine zinc oxide particles and significantly greater effects would be seen with ultrafine particles at the same mass concentration. This is based on findings of previous animal inhalation toxicology studies in which ultrafine particles produced significantly greater response than the same mass of fine particles, possibly because of the greater total surface area of the ultrafine particles. We thus chose a low dose of exposure for safety considerations. We used the orthogonal Latin square design for three treatments, which included all six possible sequences. The order of exposures was balanced so that equal numbers of subjects inhaled ultrafine zinc oxide, fine zinc oxide, or clean air on their first, second, and third exposure days. The order of exposures was assigned randomly to subjects, and exposures were separated by a wash-out period of 3 weeks or more. Exposures were conducted in a double-masked fashion in which neither subjects nor technicians testing the subjects were aware of the exposure conditions. On each exposure day, subjects came to the clinical research center at approximately 7:00 A.M., breathed through the mouthpiece while wearing a nose clip from approximately 8:00 to 10:00 A.M., with a 10-minute break off the mouthpiece between the first and second hour of exposure, and remained for follow-up testing until 4:00 P.M.

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They were given a symptoms questionnaire, activity log, and a digital thermometer to record symptoms and oral temperature at 9:00 p.m. (~ 11 hours after exposure). Subjects returned to the laboratory the following morning, approximately 23 hours after exposure, for final testing and sputum induction for total and differential cell count. Subjects were not studied within 6 weeks of a respiratory infection.

## Subjects

Twelve subjects (six women, six men; one woman and one man in each exposure sequence) with a mean age of 35 years (range, 23–52 years) were recruited by advertisements in local newspapers and on the university campus. Inclusion criteria were as follows: age from 18 to 55 years and good general health, with no chronic medical conditions or chronic medications. A preliminary sputum induction was performed before participation in the full study, and only subjects who were able to produce more than  $7 \times 10^5$  total cells entered the study. Exclusion criteria were any tobacco smoking in the past year or a history of occupational exposure to zinc oxide. Women of child-bearing potential were given a pregnancy test before study enrollment and before each inhalation exposure to ensure that they were not pregnant during the study.

## Zinc Oxide Particle Exposures

Subjects underwent the identical exposure protocol on three mornings from approximately 8:00 to 10:00 A.M. Exposures were conducted in the environmental exposure facility, using a one-pass dynamic flow exposure system, in the University of Rochester General Clinical Research Center, an in-hospital research facility for clinical studies. Details of particle generation and the use of this mouthpiece exposure system have been described elsewhere (9, 10). We used the same exposure system as for our ultrafine carbon particle studies, except for using a zinc rather than a carbon electrode for particle generation. Briefly, zinc oxide particles were generated by an electric arc discharge system (Palas generator; Palas, Karlsruhe, Germany) between two consumable zinc electrodes (zinc, 99.99% pure; ESPI, Inc., Ashland, OR) in an argon gas environment with added oxygen, and then mixed with air before inhalation. The distance from the particle generator to the mixing chamber was 1 m of a 0.5-in diameter pipe with a flow rate of 6 L/minute and calculated transit time of 5 seconds. The distance between the particle mixing chamber and the mouthpiece was 0.5 m through a 1.25-in pipe, at a flow rate of 80 L/minute, which required approximately 1.2 seconds.

Real-time measurements of particle number concentration were made continuously and recorded every 5 seconds during exposure on both inhaled and exhaled air using two condensation particle counters (Model 3022a; TSI, Inc., St. Paul, MN). The sampling port is 2 in from the mouth port. We have measured particle size in the mixing chamber and directly before the mouthpiece and found no change in size between these points. We have measured inspired air temperature during particle generation just before the mouthpiece at 24°C with 11% humidity.

Ten-minute size measurements were made periodically on both the inspired and expired sides with an electrostatic classifier (Model 3071a; TSI, Inc.). Inspired mass concentration measurements were taken continuously (recorded at 1-minute intervals) using a tapered elemental oscillating microbalance (Model 1400a; Rupprecht and Patashnik, Albany, NY) and verified using gravimetric measurements of filters (Nucleopore, 47-mm diameter, 0.22- $\mu$ m pore size [Nucleopore, Inc., Pleasanton, CA]) that had collected a known volume (e.g., ~ 275 L) every half hour. A scanning mobility particle sizer (TSI Inc., Shoreview, MN) was used to relate the number of particles deposited to the mass of inhaled and exhaled particles by an estimation calculation. The target exposure mass concentrations were 500  $\mu$ g/m<sup>3</sup> for both ultrafine and fine zinc oxide exposures. An oxygen sensor placed in the mixing chamber measured inspired oxygen concentrations to ensure the particle mixture was maintained at 21% inspired oxygen. Breath-by-breath analysis of V<sub>T</sub> and respiratory rate acquired using a pneumotachograph was used to calculate minute ventilation.

The fine zinc oxide particles were generated identically to the ultrafine particles using consumable pure zinc electrodes, but they were allowed to coalesce to create functionally larger particles. To achieve this, an 8-ft-long, 4-in-diameter copper tube served as an aging chamber. Particles were passed through the tube at a rate of approximately

6 L/minute, or for a residence time of 3.3 minutes, which is enough time for a significant amount of coagulation to occur. Particle number concentration dropped from  $4.8 \times 10^7$  to  $1.9 \times 10^5$  particles/cm<sup>3</sup>, for an increase in count median size from 40 to 260 nm, while keeping the mass concentration constant. Size measurements for the fine particles were partially in the aerodynamic region, making our measurement by electrostatic classification less accurate, so that calculating the deposition fraction of the fine particles by size or mass is an approximation.

## Responses to Inhaled Ultrafine and Fine Zinc Oxide Particles

Measures of effects known to be associated with metal fume fever caused by inhaled freshly generated zinc oxide, including oral temperature, blood pressure, ECG, blood cells, cytokines, clotting parameters, and induced sputum cells and cytokines, were studied for 24 hours after each of the three exposure conditions.

## Symptoms

Subjects repeatedly completed a symptom questionnaire asking them to rate degree of symptoms on a 10-cm Likert scale marked “None” at the left end and “Worst I have ever experienced” at the right end. Symptoms were scored by measuring the distance in centimeters along the Likert scale. Twelve symptoms previously associated with metal fume fever and one negative control symptom were measured.

## Physiology

Heart rate and blood pressure were measured by an automated blood pressure cuff (Dinamapp; GE Medical Systems, Milwaukee, WI). Oral temperature was measured by a digitally reading thermometer (Filac; Sherwood Medical, St. Louis, MO). Oxygen saturation at rest was measured by digital pulse oximetry (Nellcor N-395; Nellcor, Hayward, CA).

## Hematologic and Immune System

Blood cell differential counts and expression of activation markers and adhesion molecules, coagulation factors, and markers of inflammation were studied. Fresh heparinized whole blood was stained with fluorochrome-labeled monoclonal antibodies (Becton Dickinson, Mountain View, CA). Leukocytes were stained with the desired monoclonal antibody conjugated to fluorescein isothiocyanate, and simultaneously stained with both CD14-PE and CD45-PerCp. Red blood cells were lysed, and cells were analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW argon ion laser at 488 nm. Ten thousand events were collected from each sample in list mode using Cell Quest software (Becton Dickinson). Lymphocyte subsets were determined as a percentage of gated cells and were multiplied by the concentrations of leukocytes from the complete flow blood count to express subsets as concentrations of cells.

Interleukin 6 (IL-6), serum amyloid A, and other markers of inflammation were determined using commercial enzyme-linked immunosorbent assays that were validated using dilution and add-back experiments. For these assays, venous blood was collected in heparin anticoagulant, and aliquots of plasma stored at -80°C before analysis.

## ECG Parameters

ECG parameters reflecting cardiac electrophysiology were measured using a continuously recording 12-lead Holter monitoring system (Mortara Holter 12-Scribe System; Mortara Instruments, Milwaukee, WI). The 24-hour recordings started before exposure, encompassing the exposure time and the subsequent 15 to 16 hours after exposure. ECG parameters were analyzed based on 10-minute samples obtained in resting supine position to minimize the influence of activity and body position on the studied parameters. Recordings were sampled before and immediately after exposure, and at 3, 6, 11, and 23 hours after exposure. These sample recordings were analyzed for multiple characteristics of electrical activity of the heart, including heart rate, heart rate variability, ventricular repolarization, and cardiac arrhythmias.

Heart rate variability was analyzed using time and frequency domain parameters. The following time domain parameters were calculated: (1) SDNN (SD of all normal to normal beat [NN] intervals), (2) rMSSD (the square root of the mean of the sum of the squares of differences between adjacent NN intervals), and (3) pNN50 (NN50 count divided by the total number of all NN intervals). Frequency-domain heart rate

variability (HRV) parameters (computed with fast Fourier transformation) included total power, very-low-frequency power, low-frequency power, high-frequency power, and low-to-high-frequency ratio. Low- and high-frequency power were normalized to adjust for intersubject differences in total power.

Repolarization duration was analyzed using QT interval duration, which was measured manually in lead II, and corrected for heart rate using both Bazett's and Fridericia's formulae. Repolarization morphology was quantified using T-wave amplitude, measured as median, from leads I, II, V2–V5, and using T-wave complexity calculated using principal component analysis. Repolarization variability was quantified using the SD of QT duration and QT peak duration, and using the SD of T-wave complexity. In addition, levels of ST segment were measured in leads II, V2, and V5. Presence and frequency of supraventricular and ventricular arrhythmias were also analyzed.

### Sputum Induction

Respiratory tract cells from large bronchi of the central airways (11) were sampled by saline-induced sputum induction approximately 24 hours after the end of each exposure. We followed the method described by Hargreave and colleagues (12). Subjects unable to produce an adequate sample (at least  $7 \times 10^5$  cells with  $\geq 70\%$  nonepithelial cells) were excluded from the study. Sputum was processed on ice using the "plug selection" technique (13). Induced sputum samples were tested for cell count and differential by light microscopy.

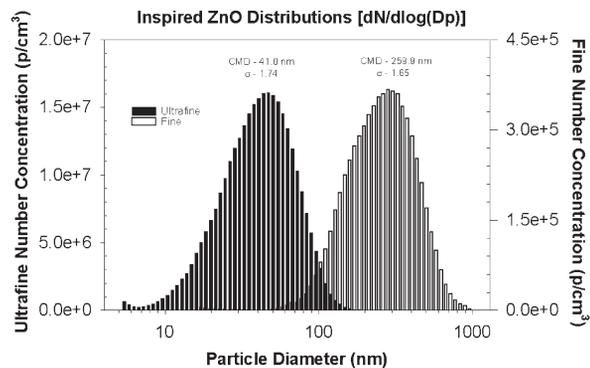
### Statistical Analyses

Data were first expressed as the mean for each exposure category and time, graphed and inspected. *t* tests were applied for differences between exposure conditions at each time point. As suggested by Jones and Kenward (14), the "mixed model" approach was used to further analyze the data. There were two types of covariance pattern among measurements from the same subject: dependencies among measurements in the same treatment period, and among measurements from different treatment periods. It was assumed that the between- and within-period covariance structures were separable. To accommodate between-period dependencies, "subject effect" was included in the mixed model. For the remaining within-period dependencies, an autoregressive moving average process with order (1,1) correlation structure was assumed. Using the mixed procedure (SAS, Carey NC), the model included terms on effects for exposure (three levels: ultrafine zinc oxide, fine zinc oxide, and control), period (three levels), carryover (three levels), sex (two levels), time, sex by exposure interactions, and baseline adjustment. A level of 5% was required for statistical significance. Because of the multifaceted nature of this study, a fairly large number of significance tests were performed. Our strategy for interpretation was to rely on the pattern of the significance tests and on concordant effects among biologically related variables, rather than on individual *p* values. Data are shown as means  $\pm$  SE unless otherwise indicated. As part of the statistical analysis, we computed confidence intervals (CIs) for the differences between ultrafine and fine zinc oxide for a series of endpoints. The CIs give a range of effect sizes that are all consistent with the data. The true effect may be anywhere in this interval. If the *p* value is not significant, then the CI will include 0, and the difference between the two exposures does not lie outside the interval with 95% certainty.

## RESULTS

### Particle Size, Surface Area, Mass, and Respiratory System Deposition

The ranges of inspired zinc oxide particle distributions for ultrafine and fine particles are shown in Figure 1. For the ultrafine particle exposures, the count median diameter was  $40.4 \pm 2.7$  nm geometric standard deviation (GSD) 1.7 or approximately 0.04  $\mu\text{m}$ , whereas for the fine particle exposures, the count median diameter was  $291.2 \pm 20.2$  nm GSD 1.7, or approximately 0.29  $\mu\text{m}$ . The inspired particle number concentrations had a median of approximately  $4.6 \times 10^7$  for ultrafine, and  $1.9 \times 10^5$  for fine particles (Table 1). This indicates that subjects breathed more than 200-fold the number of ultrafine as fine particles during an



**Figure 1.** Frequency distribution of inhaled zinc oxide particles for the two exposures conditions (left: ultrafine zinc oxide; right: fine or accumulation mode zinc oxide) as measured by number concentration (number of particles per volume of air) measured in the inspiratory circuit of the human mouthpiece exposure system during the studies. CMD = count median diameter;  $dN/d\log(D_p)$  = the normalized particle number concentration;  $P/\text{cm}^3$  = particles per cubic centimeter of inspired air. Overlapping distributions at a particle diameter of 100 nm indicate that in ultrafine exposures there was a small percentage by number above the ultrafine size.

exposure, whereas the mass was the same. We have examined particles generated for this study on a filter medium under electron microscopy, where we can see single particles (magnification,  $\times 5,000$ ) that have accumulated and aggregated on the filter medium (Figure 2). Assay of these particles was done in the laboratory of Dr. Bice Fubini (Università di Torino). Assay of zinc oxide particles collected from this generator using x-ray diffraction and Reitveld quantification showed them to contain 99.8% zinc oxide and 0.2% zinc. To verify accuracy of our real-time tapered elemental oscillating microbalance measurements of inspired (but not expired) zinc oxide concentration, we compared tapered elemental oscillating microbalance versus the weight of filtered inspired mass concentrations of fine and ultrafine zinc oxide particles. The mass concentration by tapered elemental oscillating microbalance was on average slightly lower than mass concentration measured by filter, but there was good agreement. Mean tapered elemental oscillating microbalance measurements for ultrafine particles were within a range of 11 to 18% of mean filter mass measurements at each of four time points in the 2-hour exposure, as seen in Table 1.

Table 1 also shows the inspired and expired number concentrations of the ultrafine particles. The difference between inspired and expired number concentrations represents the particles deposited in the respiratory system beyond the mouthpiece. Deposition fraction of particles varies by size, but here we have taken the total deposition fraction, which is approximately 75% for these ultrafine particles. This deposition fraction is much higher than that seen for larger particles.

### Subject Responses to Exposures

Statistically significant exposure effects were, with minor exceptions, not seen either with fine or ultrafine zinc oxide when compared with clean-filtered air. This indicates that the inspired concentration of zinc oxide,  $500 \mu\text{g}/\text{m}^3$  in either the ultrafine or fine fraction, was below the concentration at which systemic effects of the kind tested can be detected. Table 2 shows *p* values for an effect of exposure for selected variables. Over 170 variables were studied. All those variables (described in METHODS) showed no significant difference between exposures and controls at the

TABLE 1. MEAN ZINC OXIDE INHALATION EXPOSURE RESULTS (N = 12)

Time Range (min)	TEOM Mass Concentration ( $\mu\text{g}/\text{m}^3$ )	Filter Mass Concentration ( $\mu\text{g}/\text{m}^3$ )	Inspired Particle No. Concentration ( $\text{particles}/\text{cm}^3 \times 10^6$ )	Expired Particle No. Concentration ( $\text{particles}/\text{cm}^3 \times 10^6$ )	No. Deposition Fraction
Ultrafine					
0–30	483.5 $\pm$ 89.5	536.0 $\pm$ 60.0	46.1 $\pm$ 5.6	10.0 $\pm$ 2.4	0.78
30–60	480.4 $\pm$ 81.6	516.5 $\pm$ 46.5	48.8 $\pm$ 7.6	10.1 $\pm$ 2.7	0.79
60–90	465.8 $\pm$ 60.3	515.8 $\pm$ 72.5	48.9 $\pm$ 6.9	9.8 $\pm$ 2.6	0.80
90–120	470.1 $\pm$ 70.9	517.3 $\pm$ 71.1	48.3 $\pm$ 6.8	9.6 $\pm$ 2.6	0.80
Fine					
0–30	461.1 $\pm$ 39.1	473.7 $\pm$ 49.3	0.191 $\pm$ 0.015	0.12 $\pm$ 0.11	0.33
30–60	464.1 $\pm$ 47.5	466.9 $\pm$ 73.9	0.191 $\pm$ 0.013	0.12 $\pm$ 0.10	0.35
60–90	452.5 $\pm$ 35.5	468.8 $\pm$ 58.1	0.193 $\pm$ 0.013	0.12 $\pm$ 0.07	0.35
90–120	452.9 $\pm$ 40.6	513.7 $\pm$ 60.9	0.192 $\pm$ 0.014	0.12 $\pm$ 0.07	0.36

Definition of abbreviation: TEOM = tapered elemental oscillating microbalance.

$p < 0.05$  level. Because there were so many statistical tests and because the Bonferroni correction or a similar conservative approach was not used, we would anticipate some statistical tests to show a significant  $p$  value by chance alone. Although there were several positive  $p$  values and borderline  $p$ -value exposure effects, when we examined the raw data, some cases showed that these  $p$  values were caused by preexposure baseline differences on days of exposure. Additional cases were seen as inconsistent with other data measuring the same parameters. For example, a significant treatment effect was seen in the percentage of peripheral white cells identified as monocytes by flow cytometry, yet no such difference was seen in the same parameter when cells were measured by a Coulter counter (Table 2). Examination of the data in the cases with  $p < 0.05$  values did not support a true exposure effect. We believe in some cases that the  $p$  values for comparison of ultrafine to fine particles were caused by chance alone because of the large number ( $> 170$ ) of statistical comparisons made.

## DISCUSSION

Higher concentrations of freshly generated zinc oxide given in previous human inhalation exposure studies can produce symp-

tomatic, physiologic, and hematologic effects, as well as elevations in certain peripheral blood and bronchoalveolar lavage cytokines (15–17). The current U.S. Occupational Safety and Health Administration permissible exposure limit for zinc oxide is  $5.0 \text{ mg}/\text{m}^3$  inspired air, fume or respirable dust, as an 8-hour time-weighted average, over a 40-hour working week (18). In one study of previously unexposed subjects, a subtle response in symptoms, oral temperature, peripheral blood, and bronchoalveolar lavage cells and cytokines were seen at both 2.5 and  $5.0 \text{ mg}/\text{m}^3$  for 2 hours. In a study in which exposure to freshly generated zinc oxide was expressed as cumulative dose ( $\text{mg} \times \text{minutes}/\text{m}^3$ ) an effect was seen on bronchoalveolar lavage cytokines at a mean cumulative exposure of  $537 \text{ mg} \times \text{minute}/\text{m}^3$ . This exposure is equivalent to a concentration of  $4.5 \text{ mg}/\text{m}^3$  for 2 hours, and is similar to the cumulative exposure in our previous study in which effects were seen at  $5.0 \text{ mg}/\text{m}^3$  for 2 hours (equivalent to  $600 \text{ mg} \times \text{minute}/\text{m}^3$ ) (19).

However, other studies have found effects only at significantly higher exposure concentrations. In studies of subjects exposed to zinc oxide at a median concentration of  $33 \text{ mg}/\text{m}^3$  for 10 to 30 minutes, bronchoalveolar lavage tumor necrosis factor  $\alpha$  was higher at 3 hours after inhalation than at 20 hours, indicating an early role for tumor necrosis factor  $\alpha$  in the lung response to zinc oxide fume. In a human inhalation exposure to fine and ultrafine magnesium oxide at a mean of  $137 \text{ mg}/\text{m}^3$  for 15 to 45 minutes, there were no significant differences in bronchoalveolar lavage cells and cytokines, pulmonary function, or peripheral blood neutrophil counts 18 to 20 hours after exposure (22).

In the current study, a mean zinc oxide exposure concentration of one-tenth the permissible exposure limit, or  $500 \mu\text{g}/\text{m}^3$  of inspired air by mouthpiece over 2 hours at rest, produced no measured responses in symptoms, or physiologic, hematologic, or cardiac electrophysiologic parameters studied. At this exposure concentration of fine zinc oxide, we did not observe an effect, and we did not observe any increased response to ultrafine zinc oxide versus fine zinc oxide. Because there was no measured response to the larger or finer particles, we cannot determine at this concentration whether inhaled ultrafine particles are more potent than fine particles in healthy adults.

The number deposition fraction measured in this study was 0.75 for the ultrafine particles that had a count median diameter of  $0.04 \mu\text{m}$ . This also corresponded to a mass deposition fraction of 0.78 using a scanning mobility particle sizer to relate number to mass and following a method of calculation previously described. The deposition fraction values were also corrected for losses in the delivery valving. The number deposition fraction for fine particles with a count media diameter of  $0.289 \mu\text{m}$  was 0.343. This number deposition fraction for ultrafine particles of twice

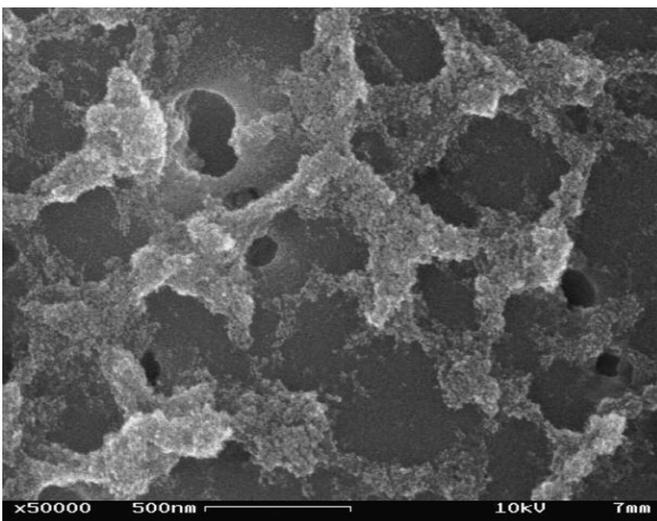


Figure 2. Scanning electron photomicrograph of ultrafine zinc oxide particles collected on filter medium from our exposure system. Particles have coalesced on the filter medium, but unit particles appear to be irregular in shape. Bar at bottom shows length of 500 nm or  $0.5 \mu\text{m}$ . Scanning electron microscopy by Brian McIntyre.

**TABLE 2. COMPARISONS OF SELECTED VARIABLES TESTED FOR DIFFERENCES BETWEEN ULTRAFINE AND FINE ZINC OXIDE EXPOSURES**

Variable	Effect Size	p Value	95% Confidence Interval
12 Symptoms	0.11	All p > 0.05	(-24.52, 24.75)
Physiologic variables			
Systolic BP, mm Hg	-2.45	0.97	(8.93, 4.01)
Diastolic BP, mm Hg	3.20	0.79	(-1.82, 8.23)
Mean BP, mm Hg	-4.49	0.36	(-10.77, 1.78)
Heart rate, beats/min	5.35	0.30	(-3.18, 3.89)
Temperature, °F	-0.07	0.48	(-0.46, 0.32)
O <sub>2</sub> saturation, %, pulse oximetry	0.47	0.11	(-0.19, 1.13)
Minute ventilation at rest, L/min	-0.32	0.16	(-1.14, 0.49)
Respiratory rate at rest, breaths/min	-0.53	0.67	(-2.37, 1.31)
V <sub>T</sub> at rest, ml	9.68	0.34	(-67.78, 87.15)
Induced sputum			
Total cells × 10 <sup>6</sup>	0.30	0.34	(-2.11, 2.73)
Viability, %	-2.91	0.38	(-9.13, 3.31)
Macrophage, %	1.82	0.71	(-14.15, 17.80)
Lymphocyte, %	0.26	0.98	(-0.50, 1.03)
Neutrophils, %	-4.93	0.13	(-23.65, 13.79)
Epithelial, %	3.24	0.26	(-12.87, 19.36)
Eosinophils, %	-0.63	0.82	(-2.06, 0.79)
Peripheral blood analyses by Coulter counter			
White blood cells, 10 <sup>3</sup> /μl	-0.14	0.97	(-0.76, 0.47)
Red blood cells, 10 <sup>6</sup> /μl	0.02	0.81	(-0.10, 0.15)
Hemoglobin, g/dl	0.15	0.64	(-0.05, 0.36)
Neutrophils, %	2.18	0.60	(-3.83, 8.19)
Lymphocytes, %	-0.40	0.74	(-7.51, 6.71)
Monocytes, %	-0.83	0.68	(-2.99, 1.31)
Eosinophils, %	0.17	0.96	(-0.90, 1.25)
Basophils, %	-0.05	0.79	(-0.43, 0.31)
Platelets, 10 <sup>3</sup> /μl	5.01	0.26	(-3.50, 13.53)
NK cells, % total lymph	-0.55	0.85	(-1.95, 0.83)
CD8+ cells, % total lymph	0.31	0.98	(-1.66, 2.30)
B lymphocytes, % total lymph	0.18	0.55	(-1.67, 2.05)
Null, % total lymph	-0.43	0.34	(-1.00, 0.12)
CD3+ CD25+, % gated	-2.32	0.29	(-9.59, 4.94)
Blood cell surface markers on neutrophils, lymphocytes, monocytes			
		All p > 0.05	
Coagulation factors			
Factor VII, ng/ml	-6.38	0.53	(-19.83, 7.07)
Fibrinogen, mg/ml	0.02	0.75	(-0.11, 0.16)
Plasminogen tissue activator, ng/ml	-0.01	0.20	(-0.06, 0.03)
Von Willebrand factor, % of normal	19.84	0.94	(-21.86, 61.55)
Soluble blood cytokines, markers			
IL-6, pg/ml	2.54	0.15	(-0.46, 1.51)
Protein C, μg/ml	-729.72	0.80	(-0.35, 0.18)
ECG analyses			
Normal to normal beat interval, NN, ms	2.5448	0.31	(-36.93, 42.02)
Total power, ms <sup>2</sup>	-729.72	0.54	(-3,748.29, 2,288)
Manual R to R wave interval, s	-0.02	0.22	(-0.10, 0.04)
Very low frequency, ms <sup>3</sup>	4.50	0.96	(-1,792.13, 1,801.13)
High frequency, ms <sup>3</sup>	-321.84	0.07	(-1,153.50, 509.82)
Low frequency, ms <sup>4</sup>	-324.76	0.60	(-1,347.37, 697.85)

Definition of abbreviations: BP = blood pressure; IL = interleukin; NK = natural killer.

Effect size represents the difference in mean effect value for ultrafine particles minus fine particles. (For the variables not shown, p values were all > 0.05.)

that for fine particles implies that the mass deposition for the ultrafine particles in this system is also twice that for the fine particles, although no mass deposition fraction was calculated for the fine particles in the studies.

A model to predict deposition fraction, the Multiple Path Prediction Model, was also used for comparison (23, 24). Using the average frequency, V<sub>T</sub>, FRC, and inspired mass concentration from our study, and a density of 5.6 g/cm<sup>3</sup> for zinc oxide, a predicted deposition fraction was calculated. The ultrafine particles had a predicted deposition fraction of 0.39 and the fine particles had a predicted deposition fraction of 0.47. Our ultrafine particle deposition fraction results were considerably

higher than the predicted values (0.75 actual vs. 0.39 predicted), as we have seen to a lesser extent in our previous studies (25). Our fine particle number deposition fraction agreed better with the model (actual 0.34 vs. predicted 0.47), but the mass deposition fraction was undetermined in the study. Because the count median diameter was around 0.3 μm, the mass of the larger particles will have a larger impact on the deposition fraction than the ultrafine particles. From the Multiple Path Prediction Model, the site of deposition is predicted to be greater in the alveolar region for ultrafine versus fine particles. Normalized for surface area, the tracheobronchial region is predicted to receive the highest deposition of fine versus ultrafine particles.

In our previous studies of inhaled ultrafine pure carbon particles at a lower concentration, 10  $\mu\text{g}/\text{m}^3$  in healthy adult subjects at rest, no convincing effects of exposure were found in a similar protocol. In a further study of breathing 25  $\mu\text{g}/\text{m}^3$  ultrafine carbon particles during exercise, exposure was associated with reduced blood monocyte expression of intercellular adhesion molecule-1 in a concentration-related manner, evidence for transient reductions in parasympathetic influence on heart rate variability, a reduction in repolarization (QT) interval, and a small reduction in blood monocytes and activation of T lymphocytes in healthy women (25–27). However, no change in induced sputum cells was seen at 25 or 50  $\mu\text{g}/\text{m}^3$  of ultrafine carbon particles (33).

These responses were not seen with zinc oxide particles in our study. The number concentration of fine particles in this exposure was approximately  $2 \times 10^5/\text{m}^3$ . If particle number were the only determinant of particle effects in the lungs, then giving equal numbers of particles of different sizes would be expected to produce equal responses. For example, to achieve exposure of fine particles with an equal number concentration as the ultrafine particle exposure ( $\sim 2 \times 10^5/\text{m}^3$ ) used here would require a mass concentration of approximately 125  $\text{mg}/\text{m}^3$  of fine particle zinc oxide. Such a mass concentration would be expected to produce significant symptoms and physiologic effects on the basis of previous dose–response studies (15, 19). However, considering previous animal studies (1), we believe that total particle surface area, while closely related to particle number, may be the more important characteristic defining toxicity.

Zinc appears to be more soluble in the acidic milieu of lysosomes of macrophages and epithelial cells than in water, with a retention half-time of approximately 6 hours in animal models, whereas carbon particles are less soluble (20–22). It thus seems possible that, whereas ultrafine carbon particles can pass intact from the lungs into the systemic circulation, deposited zinc oxide particles may be less likely to do so (28–32).

Exercise increases the deposition fraction and thus the total dose of inhaled ultrafine particles compared with resting exposure (25), and it is possible that inhaled carbon particles are more biologically active than inhaled zinc particles based on comparison of our findings of effects seen with ultrafine carbon particles at lower inhaled concentrations than the 500  $\mu\text{g}/\text{m}^3$  used here. However, the interaction of exercise with inhaled ultrafine particles may produce effects not seen without exercise during exposure, so that this may not be a valid comparison of the effects of carbon versus zinc oxide particles.

Our choice of the sample size ( $n = 12$ ) for the study design, which uses subjects as their own control subjects, accounts carefully for diurnal variations, and balances exposure sequences, was based on our previous similar studies in which we were able to detect clinically small differences between exposure to clean air and zinc oxide. For example, we previously detected a mean difference in oral temperature of 0.8°F, a difference in mean serum IL-6 of 1.6 pg/ml, and a mean difference in symptom score for cough of 1.1 on a scale of 10 with this sample size and design (8).

This previous experience demonstrated our ability to detect very small differences in responses using this exposure design. If ultrafine zinc at this concentration caused a clinical response different from fine zinc, we would thus expect to detect it using a crossover design with 12 subjects.

The CIs in Table 2 indicate that there is 95% certainty that the true value for this difference lies between two confidence limits. The interval between the confidence limits represents the magnitude of difference that may exist between the ultrafine and fine exposures. Given that the p values are not significant,

all the confidence intervals include the possibility of no effect (difference = 0).

The table shows that we are able to exclude a difference in oral temperature (CI,  $-0.469, 0.327$ ) of  $\pm 0.5$  or more degrees Fahrenheit, in oxygen saturation (CI,  $-0.192, 1.134\%$ ) of  $\pm 1.2\%$ , for fibrinogen (CI,  $-0.116, 0.169$ ) of  $\pm 0.17$  mg/ml, for serum IL-6 (CI,  $-0.469, 1.515$ ) of  $\pm 2$  pg/ml or more, and for the symptom of cough (CI,  $-0.982, 1.048$ ) of 2 on a scale of 10.

True differences of a smaller magnitude may be present, but we believe that the CIs exclude clinically important differences in effects of major interest between ultrafine and fine zinc oxide at this low mass concentration. Statistical analysis comparing fine and ultrafine zinc to control clean air exposures also showed no difference between either size of zinc particle and control, indicating that at this inspired concentration there are no effects on the parameters tested.

In summary, with inhalation of 500  $\mu\text{g}/\text{m}^3$  fine zinc oxide by mouth for 2 hours at rest, we saw none of the effects on symptoms, body temperature, or white blood cell count we previously found with exposure to 5.0 and 2.5  $\text{mg}/\text{m}^3$ . Thus, for the outcomes measured, this was too low a dose to show any effect, or a “no observable effect level” of exposure. This was true for both an ultrafine and a fine particle distribution of zinc oxide.

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