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Short-term inhalation of cadmium oxide nanoparticles alters pulmonary dynamics associated with lung injury, inflammation, and repair in a mouse model

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Abstract

Context—Cadmium oxide nanoparticles (CdO NPs) are employed in optoelectronic devices and as a starting material for generating quantum dots as well as for medical imaging and targeting of pharmaceutical agents to disease sites. However, there are lack of data concerning short- and long-term effects of CdO NPs on the lungs.

Objective—To determine the effects of inhaled CdO NPs at an occupationally relevant concentration on pulmonary injury and repair, and on systemic immunity in adult male mice

Methods—Mice were exposed to 240 μg CdO NPs/m^3 for seven days (3 h/d) and lavage levels of pulmonary injury/inflammatory markers, bacterial uptake by circulating phagocytes, and lung histology examined either one or seven days following the final exposure.

Results—Levels of total protein, lactate dehydrogenase activity, cytokine markers of inflammation (i.e. interleukin-1β, tumor necrosis factor-α, and interferon-γ), tissue remodeling matrix metalloproteinases (MMP)-2 and -9 activity, and phagocytic activity of circulating phagocytes were significantly increased one day after the final exposure. By seven days post-exposure, MMP-2 activity decreased to control levels, while MMP-9 activity remained significantly above control values, although dropping by about half from day one.

Conclusions—This study demonstrates that short-term inhalation exposure to CdO NPs can stimulate pathways in the lungs associated with inflammation, cell injury, and tissue remodeling as well as alter immune function. Findings here demonstrate that even short-term inhalation exposure to CdO NPs in the workplace could lead to deleterious pulmonary effects in exposed workers.

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Declaration of interest
No author has any financial interest in the outcome of this study.

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Keywords

Cadmium oxide nanoparticles; inflammation; inhalation; metal oxide; phagocytosis; pulmonary injury

Introduction

Nanomaterials (NM) possess novel characteristics making them uniquely suited for a variety of applications and, as a result, the number of industries making use of nanotechnology is rapidly growing (Grassian, 2008). Nanoparticles (NPs), by definition, have at least one dimension less than 100 nm. This small size provides them a high surface area that could contribute to increased toxicity compared to their larger counterparts. Despite this potential for toxicity and lack of safety testing, NPs have been introduced into commercial goods and can be currently found in over 800 consumer products, including clothing and sunscreen (Kang, 2011; Marquis et al., 2009).

In addition to their incorporation into commercial products, NPs are released into the atmosphere as a result of road transport and engine combustion (Luparello et al., 2011). The acute effects of inhaled NPs from diesel combustion and welding fumes have been shown to cause pulmonary inflammation and fibrosis in both humans and experimental animals (Card et al., 2008). Welding fumes, in particular, are composed of a vapor of mixed metal oxide NPs which may include aluminum, chromium, copper, and cadmium (Cd) that, as a whole mixture, have been shown to increase the levels of pro-inflammatory cytokines in the lavagable extracellular spaces within the lung (Revell, 2006). Few studies have examined the persistence of such effects. However, in a study examining the effects of quantum dots (QD) on lung injury and inflammation, Roberts et al. (2013) showed that a single intratracheal instillation with either 5 or 12.5 μg QD per rat resulted in inflammation one day post-exposure that peaked after 7–14 d post-administration. The authors also reported that the levels of alveolar macrophages observed in the lungs were also elevated up to 14 d post-exposure.

Metal oxide NPs have found applications in a variety of areas including use in biosensors and medicine. In medicine, one particularly important metal oxide NP is cadmium oxide (CdO), which is used as a starting material for the generation of QD, as well as for medical imaging and targeting of pharmaceutical agents to sites of disease (Chan et al., 2005).

Acute inhalation exposure to high levels of larger-sized soluble and insoluble Cd compounds result in bronchial and pulmonary irritation (EPA, 2000). As a result, bulk Cd is considered to have acute toxicity. Inhaled soluble cadmium chloride ($CdCl_2$) causes transient bronchial inflammation, increased bronchial reactivity, and increased MMP-9 activity in lung lavage fluid in beagles (Bolognin et al., 2009). Exposure of primary cultures of rat lung alveolar epithelial cells to Cd^{+2} causes increased release of interleukin (IL)-6 and macrophage inflammatory protein (MIP)-2, while Cd-treated cultures of alveolar macrophages release increased amounts of IL-1β, tumor necrosis factor (TNF)-α and MIP-2 (Lag et al., 2010). Cassee et al. (2002) demonstrated that the toxicity of nebulized $CdCl_2$ particles increased with increasing particle size, with ultrafine-sized particles (33 nm)

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having the greatest toxicity in exposed rats. In other rodent studies, chronic exposure to inhaled soluble Cd caused emphysema-like pathology (Holden, 1980; Snider et al., 1973) and primary lung cancers after 18 months of exposure to $CdCl_2$ (12.5–50 μg Cd/m^3) (Takenaka et al., 1983). Both these conditions are associated with diminished function of the pulmonary and systemic immune systems (reviewed in Oberdorster, 1986).

Kundu et al. (2009) demonstrated that the respiratory system is especially at-risk for inhaled Cd, as up to 90% of the inhaled dose is absorbed in the lungs, compared with 5% in the gastrointestinal tract, and the remaining 5% distributed in small amounts throughout the body. In that study, examination of Swiss albino mice over a period of 15–60 d following a single inhalation exposure of 5 mg $CdCl_2$ (per kg body weight) lead to excessive lung cell proliferation and inflammation (Kundu et al., 2009). In contrast, inhalation exposure to even higher $CdCl_2$ doses (i.e. up to 80 mg/kg body weight) reduced total lung cell counts (Kundu et al., 2009). Inhalation of soluble Cd has also been reported to produce systemic immune dysfunction. In this case, exposure to aerosolized $CdCl_2$ reduced lymphoproliferation in response to lipopolysaccharide and phytohemagglutinin stimulation and inhibited primary IgM response to sheep erythrocytes five to eight days following a single exposure to Cd at 0.88 mg Cd/m^3 (Krzystyniak et al., 1987). In another study, rats were exposed to either 0.5 or 5.3 mg water-insoluble CdO/m^3 (260 and 330 nm median size, respectively) and effects were examined on lung morphology (Buckley & Bassett, 1987). At the lower concentration, exposure to CdO increased pulmonary inflammatory cell (mostly mononuclear cells and polymorphonuclear leukocytes [PMN]) numbers that returned to control levels within 7–15 days following exposure. In contrast, exposure to 5.3 mg CdO/m^3 produced areas of interstitial thickening with a large number of inflammatory cells consisting mainly of mononuclear cells, PMN, eosinophils, and basophils that decreased in number four days post-exposure.

The aim of the present study was to determine whether short-term repeated inhalation of CdO NPs (at a concentration relevant to that which may be found in an occupational setting) produces adverse pulmonary effects to an extent different than that has been reported following inhalation exposure to soluble Cd and/or to larger CdO particles. Results of the studies here will also add to the body of literature concerning the onset and persistence of such Cd NP-induced effects. Additional studies are needed to better understand the mechanisms by which inhaled CdO NPs acted to bring about the pulmonary and systemic effects, as well as the form of Cd (i.e. soluble ions versus particle) responsible for the observed outcomes.

Materials and methods

Experimental design

Mice—About 12-to-14-week-old male CD1 mice (Charles River Labs Kingston, NY) were housed individually in polypropylene cages with Cd-reduced bedding (Sani-Chips; PJ Murphy, Montville, NJ) at the New York University, Department of Environmental Medicine Animal Facility. Mice were maintained at 22°C and 55% humidity on a 12-hour light/dark cycle and provided both water and Cd-free diet (AIN-93 G; TestDiet, Richmond,

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IN) *ad libitum*. All animal studies were performed in accordance with the NYU Medical Center Institutional Animal Care and Use Committee.

CdO NP generation and exposure—Mice were acclimated to the nose-only exposure tubes for three days prior to the start of exposure. Following acclimation, animals were exposed daily for three hours/day for seven consecutive days to CdO NPs generated from a Palas spark generator as described previously in detail (Blum et al., 2012). Briefly, CdO NPs were generated in a Palas (Model GFG-1000) arc furnace (Karlsruhe, Germany) at a high voltage frequency of 80 Hz in an argon (Ar, Ultrpure, AGI, NJ) chamber with two opposing Cd metal bars (99.995% pure, ESPI, Ashland, OR) as electrodes. The generated NPs were carried by 3.5 L/min Ar flow and mixed with 23.5 L/min particle-free air. Cooled (using ice packs) carrier gas was mixed with 1 L/min oxygen (Ultrapure, AGI, NJ) resulting in a 20% oxygen level in the exposure air. Size distribution and number concentration of freshly-generated CdO NPs were determined in real-time using a nano-DMA SPMS consisting of an electrostatic classifier (model 3080), a nano-DMA (model 3085), and a condensation particle counter (model 3010) (all purchased from TSI, St. Paul, MN). Nanoparticles were collected on a pre-weighed Teflon filter (37 mm, 0.2 μm pore size) and the NP-laden filters were weighed gravimetrically on an MT5 microbalance (Mettler Toledo, Hightstown, NJ). For the studies here, experimental animals were exposed by inhalation to an average concentration of ~240±7.9 μg CdO/m^3 (mean particle size = 15.3 ± 0.1 nm) and were polydispersed with a size geometric standard deviation of 1.5. Nanoparticle size (measured at a high-voltage frequency of 40 Hz) was measured using an atomic force microscopy by Blum et al. (2012) to be 15 nm ± 2, which was ~4 nm larger than the aerodynamic diameter measured using the nano-DMA SPMS system.

The current permissible exposure limits for cadmium in the workplace is a time-weighted average (over an eight hour shift) of 5 μg/m^3 with an action level of 2.5 μg/m^3 (OSHA, 2012). Assuming an average human worker inhales 0.5 m^3 of air per hour, exposure at the regulatory limits would result in doses 20 μg or 10 μg of Cd, respectively, per day. The average mouse respiration rate is 220 breaths/min with 0.18 mL per breath (Braun et al., 2004). Under these experimental conditions, the mice received ~1.7 μg CdO NPs per day which is below the OSHA Action level.

Following the final exposure, mice were either euthanized after 24 h or allowed to recover for an additional six days prior to euthanasia. Equal numbers of mice (n = 5–7 per time point/treatment group) were exposed either to filtered particle-free carrier air (control) or to ~240 μg/m^3 CdO NPs. Both treatment groups were euthanized at the appropriate time post-exposure using SleepAway (0.3 mL diluted 1:10 in phosphate-buffered saline [PBS]) and *ex vivo* assays performed as described below.

Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was collected following euthanasia by inserting a cannula into the trachea and instilling 1 mL of Dulbecco's PBS (DPBS) without calcium or magnesium chloride (Invitrogen Corporation, Camarillo, CA). To maximize the collection of cells and lavagable proteins, lungs were lavaged three times *in situ* with a total DPBS

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volume of 1 mL. BALF samples were recovered (~1 mL), stored on ice prior to centrifugation, and were centrifuged for 10–15 min at 6400 rpm (Capsule TOMY HF-120 microcentrifuge, Peninsula Laboratories, Inc., Belmont, CA). The supernatants were aliquoted and used immediately to measure lacatate dehydrogenase (LDH) activity or stored at −20°C until used for later assays described below. The recovered cell pellets were resuspended in 0.4 mL of DPBS and the total cell number and viability were assessed using a hemacytometer and trypan blue exclusion, respectively. To determine cell profiles by differential counts, 100 μL of the cell suspension were spread on the duplicate glass slides, allowed to dry overnight, fixed with 70% methanol, and then stained with hematoxylin and eosin (H&E). Cell sub-population profiles were determined by counting 100 white blood cells per slide (two slides per mouse). Only macrophages and neutrophils were counted, as they were the only white blood cell types observed in the BALF. After lavage, lungs were fixed by instilling 1 mL of the 10% buffered formalin. Fixed lungs were then embedded in paraffin and sections cut and stained with H&E. Slides were analyzed for any major changes by a pathologist blinded to the treatment groups.

LDH activity

Lactate dehydrogenase activity was measured in BALF samples within 1 hour following collection using a colorimetric assay (Biomedical Research Service Center, University of Buffalo, Buffalo, NY) as recommended by the manufacturer. Standards were prepared from L-LDH from bovine heart (Sigma-Aldrich, Allentown, PA) in concentrations ranging from 0.125 to 1 milliunit/mL and the results were determined at 492 nm using a microtiter plate reader (HTS 7000 Bioassay Reader, Perkin Elmer, Waltham, MA). LDH activity was calculated from a standard curve and presented as LDH activity units.

Lavagable protein

Total protein concentration was measured using a Biorad DC Protein assay (Biorad Inc., St. Louis, MO) as per the manufacturer's directions using 0–1000 μg/mL bovine serum albumin as the standard.

Gelatin zymography

Matrix metalloproteinase (MMP) activity was measured using gelatin zymography as previously described (Kleiner & Stetler-Stevenson, 1994) and 5 μg of BALF proteins were used per lane. The low protein concentration of BALF measured from control mice necessitated the need for acetone precipitation prior to the addition of loading buffer. Precipitation was performed by adding five sample volumes of cold acetone to each sample, incubating them for 1 hour at −20°C, and then centrifuging for 10 min at 15 000*g* to precipitate the proteins. The supernatant was removed and pellet allowed to air dry prior to the addition of loading buffer. To normalize MMP activity between replicate gels, each gel contained a reference sample consisting of a pooled mixture of samples. After de-staining, zymograms were dried on a gel dryer and imaged using a digital imager (AlphaImager 2200; Cell Biosciences, Santa Clara, CA). Quantification was based on differences in band integrated density values (IDV) with the background subtracted.

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Quantification of cytokines in BALF

Lavage samples were thawed slowly on ice and concentrations of IL-1β IFNγ and TNFα in each recovered BALF sample were measured by ELISA using commercially available kits (eBioscience, Inc., San Diego, CA) according to the manufacturer's instructions.

Differential blood counts

Blood was collected from the abdominal aorta in a heparinized syringe following euthanasia. At the time of collection, blood was spread on duplicate glass slides and allowed to dry overnight at room temperature before fixing for one minute in methanol and staining with H&E. White blood cells were identified and counted (100 cells/slide on duplicate slides from each mouse) using a light microscopy (100×) and percentages of lymphocytes and granulocytes were determined. Plasma samples, collected by centrifugation using a protocol similar to that used for BALF samples, were aliquoted and frozen at −20°C for later use.

Phagocyte uptake assay

Blood collected at the time of euthanasia from the abdominal aorta was placed into heparinized glass collection tubes (Becton, Dickson, and Company, Franklin Lakes, NJ) and stored overnight at 4°C. The following day, blood samples were incubated with pHrodo *Escherichia coli* BioParticles (Phagocytosis Kit for Flow Cytometry, Molecular Probes, Inc., Eugene, OR), and prepared for flow cytometry according to the manufacturer's instructions. Samples were analyzed using a 633 nm argon-ion laser (MACSQuant Analyzer, Miltenyi Biotec Inc., Auburn, CA) and data were analyzed using FlowJo Flow Cytometry Analysis Software (Tree Star, Inc., Ashland, OR). Phagocytic uptake was measured as a percentage of total counted cells using a plot of forward scatter versus side scatter.

Statistical analysis

A one-way analysis of variance (ANOVA) followed by Fisher's LSD *post hoc* testing (when appropriate) was used to determine the statistical significance between treatment groups at each post-exposure time point (SPSS Inc., Chicago, IL). Treatment means were considered significantly different at $p \leq 0.05$.

Results

Effects of inhaled CdO NP on body and lung weights

Inhalation exposure of CdO NPs for seven days resulted in significantly greater weight loss (determined as percentage of initial body weight at day 1) within 48 h of the initiation of weighing (Figure 1A). Body weights of the NP-exposed mice continued to drop and remained significantly lower than control mice throughout the entire seven days exposure period. In contrast, body weights of mice in the air control group remained constant over the entire exposure period.

At 24 h post-exposure, lungs in the NP-exposed mice weighed ~40% more compared to time-matched control mice (Figure 1B). By seven days post-exposure, lung weight of the NP-exposed mice decreased by 21% compared to the earlier post-exposure time point, but

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remained significantly elevated compared to its time-matched control counterpart (Figure 1B).

Inhaled CdO NP leads to pulmonary inflammation

Short-term inhalation exposure to CdO NPs produced pulmonary inflammation associated with lymphocyte and plasma cell invasion around the small airways evident in mice 24 h following the final exposure (Figure 2); bronchus associated lymphoid tissue (BALT) was also enhanced in exposed mice at the same post-exposure time point. One week following cessation of exposure, lung inflammation decreased, but was still centered around the small airways. The amount of BALT also appeared to decrease after one week post-exposure (compared to BALT levels seen 24 h after exposure).

Effects of inhaled CdO NPs on lavagable cell number and viability

Exposure to CdO NPs increased lavagable cell numbers at both post-exposure time points compared to time-matched control mice, with the greatest increase observed seven days following the final exposure (Figure 3A). There was a small (6%) decrease (albeit, not significant) in cell viability in NP-exposed mice one day following the final exposure compared to time-matched controls. Cell viability increased significantly by seven days post-exposure compared to the treatment-paired group after one day (Figure 3B). No differences were observed between treatment groups (or between time points) in the percentages of lavagable macrophages, neutrophils, or monocytes (~99.5%, 0.36%, and 0.14%, respectively).

Effects of inhaled CdO NP on lavagable protein and LDH activity

Total protein concentrations in BALF collected from NP-exposed mice at both post-exposure time points were increased by 4.5-fold and 3.4-fold compared to time-matched control mice, respectively. At seven days, BALF protein concentration in CdO NP-exposed mice dropped to about half that measured at the earlier post-exposure time point, but remained significantly elevated above control (Figure 4A). No significant differences in control mice between time points were observed.

Inhalation of CdO NPs significantly increased LDH activity at both post-exposure time points compared to their time-matched control counterparts (Figure 4B). At seven days post-exposure, LDH activity was unchanged from that observed in mice examined 24 h after the final exposure.

Effects of exposure to CdO NPs on lavagable pro-inflammatory cytokines

Levels of lavagable IL-1β were slightly reduced (~20%) one day following the final CdO NP exposure compared to time-matched control value. On day seven, the trend reversed and levels of IL-1β were significantly higher in NP-exposed mice compared to that observed on day one, but not significantly different from time-matched control (Figure 5A). As shown in Figure 5(B), the levels of IFN-γ measured in mice exposed to CdO NPs one day post-exposure were elevated 3-fold compared to control mice. At seven days post-exposure, IFNγ values in the same exposure group decreased by ~80% reaching the time-matched control level (Figure 5B). BALF-associated TNFα levels in NP-exposed mice were 3.4-fold greater

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than that seen in control mice 24 h after the final exposure (Figure 5C). TNFα levels in BALF increased by another 160% compared to time-matched control mice over time. However, TNFα levels in control mice were also significantly elevated (~4-fold) compared to their treatment-matched control at 24 h post-exposure.

Effects of inhaled CdO NPs on BALF MMP-2 and -9 activities

Pulmonary response to inhaled CdO NPs on the activities of MMP-2 and -9 in lavage fluid are shown in Figure 6(A). Constitutive MMP-2 activity was increased (albeit, non-significantly) above control levels one day following exposure to CdO NPs. However, at seven-days post-exposure, MMP-2 activity levels in NP-exposed mice increased dramatically ($p<0.001$) compared to time-matched control mice; MMP-2 activity for the control mice remained relatively constant over time (Figure 6B). Inducible MMP-9 activity was detected only in the lavage fluid of NP-exposed mice with a dramatic increase above controls observed one day after the end of exposure (Figure 6C). Activity of this MMP remained elevated over time (compared to time-matched controls), but decreased significantly by ~70% compared to that seen after one day.

Effects of CdO NP inhalation on peripheral white blood cell profiles

Lymphocyte values were significantly decreased (compared to control) by 12% one day after the final exposure (Figure 7A). In contrast, percentages of lymphocytes increased by ~11% seven days after exposure compared to that seen for the NP-exposed mice 24 h post-exposure. There was a 4% decrease in the number of lymphocytes in control mice over this same timeframe; however, this was not a significant change ($p = 0.08$). The percentage of circulating neutrophils was increased by 43% in NP-exposed mice (compared to control mice) one-day post-exposure. By seven days, neutrophil levels had decreased significantly (8.7%) compared to time-matched control mice and by ~25% compared to NP-exposed mice at the earlier post-exposure time point (Figure 7B). Lymphocyte and neutrophil values in the control mice were not significantly different between time points.

Effects of inhaled CdO NPs on blood leukocyte populations and bacterial uptake by circulating phagocytes

Total bacterial cell uptake by activated blood phagocytes measured as change in phagocytic index (leukocytes containing phagocytized particles/total umber of cells counted) increased by six-fold in NP-exposed mice 24 h following the final exposure (compared to control mice, Figure 8). Seven days after the final exposure, phagocytic index decreased by 54% of that seen in the 24 h post-exposure group, and was no longer significantly different from either the air-exposed or the 24 h post-exposure CdO NP group. The lack of a time-dependent effect may be due to small sample size and/or large variability within each of the exposure groups.

Discussion

The effects of inhaled CdO NPs, at a time-weighted average concentration representative of the limits for bulk CdO and possibly occupationally relevant, were examined here on pulmonary injury and repair and systemic immunity in adult male mice. Findings

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demonstrate that even short-term inhalation of CdO NPs can cause persistent pulmonary cell injury and have deleterious effects on both lung histology and systemic immune function. Moreover, it appears that while induction of some pulmonary damage biomarkers were reversible, others persisted for at least seven days after cessation of exposure suggesting continued injury and/or diminished ability to repair. Persistent adverse pulmonary effects such as those observed here can represent an important occupational health threat as engineered metal oxide nanoparticles are being manufactured and used at ever-greater frequency with a large number of workers potentially exposed in the workplace.

The principle route of NP exposure in the workplace is via inhalation (Kuhlbusch et al., 2011). However, exposure limits have yet to be set for any type of NP due to limited studies on which to base such limits (van Broekhuizen et al., 2012). The permissible exposure limit for Cd as set by OSHA (2012) is a time-weighted average of 5 μg/m^3 with an action level of 2.5 μg/m^3 over the course of an eight-hour work shift, which can result in exposure levels of up to 20 μg per day. There is a second, higher level set in the case of larger (non-nanosized) CdO, the separate engineering control air limit (SECAL) (a special workplace limit where it is not possible to achieve the permissible exposure limit, such as is the case for industries involved in Cd refining and charging), which has been established at 50 μg/m^3 over an eight-hr work shift (OSHA, 2004). These limits fail to take into account greater penetration of nanosized materials into the deep lung (i.e. alveolar space) where absorption may be greater for NPs than it is for larger-sized particles.

Inhalation of CdO NPs (mean diameter ~15 nm) caused a 6–8% decrease in body weight within two days of the initiation of exposure. This finding is analogous to that observed previously by this laboratory in pregnant mice exposed by inhalation to similar sized CdO NPs and concentrations (Blum et al., 2012). Furthermore, in a study using male rats instilled intratracheally with a nanosuspension of 400 μg Cd dioxide (CdO_2) NP/kg (mean diameter 20.1 nm) daily for three and six weeks, a 5–8% decrease in body weight was demonstrated during the first week of exposure (Papp et al., 2012).

Inhalation exposure to CdO NPs for seven days resulted in a significant increase in relative lung weight 24 h after the final exposure and remained increased even after one week of recovery. A number of other rodent studies have shown similar effects with inhaled Cd NPs. Rats exposed once (via inhalation) to a two-fold higher CdO NP mass concentration (550 μg CdO NP/m^3, 51 nm modal size) than that used here also presented with increased relative lung weight one day following exposure (Takenaka et al., 2004), but persistence of change beyond the one-day exposure was not examined. Further support for CdO NP-induced increases in lung weight comes from a study by Papp et al. (2012), who demonstrated that a six week inhalation exposure to CdO_2 NP (400 μg/kg Cd) led to a nearly two-fold increase in relative lung weight. Finally, female rats exposed by inhalation to 1.08 mg CdO NP/m^3 (46–51 nm particle size) for 10 days (12 h/d) also increased relative lung weight (Alessandrini et al., 2003). However, this is the first report demonstrating the effects of inhaled Cd NPs on lung weight at an ambient concentration more relevant to that which could be found in an occupational setting. Moreover, this study is the first to reveal persistence of such effects.

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Increased lung weight can be a result of intensified protein/fluid leakage from the vasculature into the lungs mediated by inflammatory signals from pulmonary cells (Aman et al., 2011). Increases in total BALF protein observed in this study support the concept that enhanced lung weight is due to increased vascular leakage. Changes in lavageable protein content is consistent with that observed by Cassee et al. (2002) who showed that inhaled $CdCl_2$ aerosol (33 nm particle size) produced an ~4-fold increase in BALF protein concentration. Studies by Zhang et al. (2010) also showed a significant increase in BALF protein content in rats exposed by inhalation to soluble $CdCl_2$. Enhanced levels of BALF protein are also correlated with lung inflammation and increased vascular permeability (Drent et al., 1996). The reduction in BALF protein (albeit, still significantly increased above time-matched control) observed after one week of recovery, compared to that seen one day following CdO NP exposure, could indicate that vascular permeability decreased over time which could account for the concurrent drop in lung weight.

Similar to that observed for total protein, LDH activity in BALF (a measurement of lung cell injury/death) was also significantly elevated one day following short-term, repeated exposures to CdO NPs. In contrast to that seen here, a single four hour inhalation exposure to soluble $CdCl_2$ (33 nm aerosol) failed to increase LDH activity in rats; Cassee et al. (2002) attributed their observation to high LDH background levels in the controls. Unlike BALF protein levels which dropped by 50% over time in this study, LDH activity in exposed mice remained elevated one week after CdO NP exposure. This finding suggests that lung cell injury/death as a result of CdO NP exposure persisted or continued to occur over time even after injury to lung microvasculature and subsequent fluid leakage into the alveolar space may have ceased (Drent et al., 1996; Gopcevic et al., 2011).

In this study, inhalation of CdO NPs led to the development of BALT in exposed mice. Under normal circumstances, mice and humans do not produce BALT except under conditions of inflammation (Kunisawa et al., 2005) as seen here. In addition, during infection, BALT in humans and mice has been shown to have a role in the adaptive immune response (Moyron-Quiroz et al., 2004). It was further hypothesized through work with titanium dioxide NPs that particles deposited in the lung are transported to BALT after being translocated through the interstitial space to the lymph, where they are re-excreted into the alveolar lumen (Ferin & Oberdorster, 1992) as a secondary clearance mechanism. While not examined in our study, cerium dioxide NPs (Srinivas et al., 2011) and multiwalled carbon nanotubes (Aiso et al., 2010) were found in alveolar macrophages located in BALTs, suggesting a possible lymphatic mechanism for clearance of CdO NPs from the lungs.

Inflammation, a process mediated by the release of pro-inflammatory cytokines, stimulates the activation of immune cells, among other pulmonary cell types. In this study, BALF concentrations of IL-1β, IFNγ, and TNFα each showed a distinct pattern of change related to exposure. IFNγ levels, elevated 24 h after CdO NP exposure, dropped precipitously to control levels by seven days post-exposure. IFNγ is thought to be a mediator of lung injury as its release from pulmonary lymphocytes induces apoptosis in lung epithelial cells (Yamada et al., 2004). In addition, IFNγ secreted by neutrophils has been demonstrated to increase lung alveolar permeability following oxidative injury (Barazzone et al., 1998).

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Effects on pulmonary IFNγ levels could help in explaining the observed increase in lung weight and protein levels shortly following CdO NP exposure.

Release of IL-1β produced by activated macrophages in the lung airway where it functions as a pro-inflammatory cytokine (Coulter et al., 1999), is partially regulated by IFNγ (Levine et al., 1997) which could explain (at least in part) the delay in IL-1β increase release relative to IFNγ. IL-1β is partially responsible for the release of other pro-inflammatory cytokines including TNFα (Mukhopadhyay et al., 2006). Thus, it is possible that continued release of TNFα over time could be (at least in part) due to up-regulation of itself in an autocrine manner (Mukhopadhyay et al., 2006). Other cytokines not measured in this study, but which play a role in TNF regulation (e.g. IFNβ or IL-17), could also contribute to the increased release of TNF into the pulmonary extracellular milieu (Mukhopadhyay et al., 2006). Results herein suggest that short-term inhalation of CdO NPs continues to alter the release of pro-inflammatory cytokines even seven days after exposure has ceased. In contrast to what was expected based on the observed increases of pro-inflammatory cytokines and the presence of bronchus-associated lymphoid tissue patches in the lung sections of NP-exposed mice along with a strong, localized immune response, no increase in neutrophils was observed in BALF at either post-exposure time point. Decreased numbers of neutrophils could have resulted from increased adherence of the cells within the lungs, or it is possible that the level of NPs used in the study was below the threshold needed to stimulate neutrophil invasion, but was sufficient to result in a greater release of pro-inflammatory cytokines.

Inhalation of CdO NPs resulted in increased lavageable cell numbers at both post-exposure time points with the greatest effect observed after seven days. In another rodent study, Takenaka et al. (2004) reported that cell numbers recovered from lavaged rats exposed by inhalation to a twofold higher concentration of CdO NPs (than that used in the present study) were modestly increased. A similar outcome was also reported by Stosic et al. (2010), who demonstrated that the total lung leukocyte number increased in male rats injected (IP) with soluble $CdCl_2$ (i.e. 1 mg/kg). Exposure of beagle dogs, also to soluble $CdCl_2$ (0.2% solution via nebulizer for 15 min), likewise increased total cell counts in recovered BALF (Bolognin et al., 2009). Unlike the results observed here, Takenaka et al. (2004) reported that a single inhalation exposure of 550 μg CdO NP/m^3 increased the number of lavageable neutrophils in female rats examined one day following exposure. Similarly, male rats also demonstrated increased neutrophil numbers 24 h following a single IP injection of 1 mg $CdCl_2$/kg (Stosic et al., 2010). Differences between the studies could be due to discrepancies in Cd concentration/form (i.e. NP versus soluble) and/or route of metal exposure.

Changes in alveolar permeability are associated with the degradation of extracellular matrix proteins. The activities of MMPs-2 and -9 (two extracellular matrix degrading enzymes frequently found at sites of inflammation) were measured in BALF from both treatment groups to determine if the observed histopathological alterations could be, at least in part, attributable to changes in enzyme activity. Exposure to CdO NPs significantly elevated MMP-2 activity in lavage fluid (compared to control), but not until seven days after exposures had ceased which could be explained (in part) by changes observed in pro-inflammatory cytokine levels. Control mice had no detectable MMP-9 activity at either post-

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exposure time point which is contrast to those of Bolognin et al. (2009) and Zhang et al. (2010) who reported low, constitutive activity of BALF-associated MMP-9 from unexposed animals (beagle dogs and rats, respectively). The reason(s) for this discrepancy is unclear, but may relate to the amount of protein loaded for zymography in the study here.

Metalloproteinases, as well as pro-inflammatory cytokines IL-1β and TNFα are up-regulated in the presence of reactive oxygen species (ROS) (Soccal et al., 2000). In turn, IL-1β and TNFα are both able to stimulate MMP-2 activity through up-regulation of membrane-type I MMP, which functions to activate MMP-2 (Lohi et al., 1996). At seven days post-exposure, CdO NP-exposed mice had elevated levels of both TNFα and MMP-2 activity, suggesting that although exposure had ceased, the mediators for inflammation and lung remodeling remained active. In addition, TNFα and IL-1β can stimulate transcription of MMP-9, but do not cause activation of the protease (Soccal et al., 2000) which requires another stimulus such as increased levels of ROS. While Cd does not catalyze Fenton-type reactions (Waisberg et al., 2003), it can decrease the activity of several antioxidant enzyme systems including catalase and superoxide dismutase in rats (Sarkar et al., 1998) thereby indirectly increasing the levels of ROS. In addition, increased levels of ROS can also stimulate transcription of MMP-9 through activation of nuclear factor kappa B (NF-κB) (Schreck et al., 1991). While production of ROS was not analyzed in this study, such a response could underlie the observed activation of MMP-9.

The significance of elevated MMP-2 and -9 in the CdO NP exposed lung is currently unclear. However, experimental mouse models of inflammatory bowel disease, in which both MMPs are elevated, demonstrated that MMP-2 serves a protective role in maintaining the gut barrier, while MMP-9 mediated further tissue injury (Garg et al., 2009). More research in this area is clearly needed to better understand the relationship between CdO NP exposure, induction of MMPs-2 and -9, and pulmonary disease.

As previous studies in this laboratory demonstrated that Cd associated with inhaled CdO NPs is translocated from the lungs into the blood (Blum et al., 2012), effects on circulating immune cell phagocytosis were assessed. Phagocytic uptake of labeled bacteria by blood phagocytes increased in mice 24 h after exposure, a trend which was decreased seven days later. Enhanced uptake could be due to increased percentages of circulating neutrophils also seen in this study. In addition, exposure to Cd has been shown both *in vitro* and *in vivo* to alter phagocytic activity with the effect dependent on the exposure scenario and Cd dose (Gang et al., 2009; Greenspan & Morrow, 1984). In a study by Greenspan & Morrow (1984), *ex vivo* exposure of pulmonary macrophages collected from rats exposed by inhalation to 1.5 mg $CdCl_2/m^3$ transiently increased phagocytic activity, while exposure to 5 mg $CdCl_2/m^3$ decreased phagocytic index; increased phagocytic activity persisted for five days and returned to control levels by eight days later. In addition, Cd NPs have been shown to alter phagocytic uptake *in vitro* by cultured macrophage-like cells (RAW 264.7) with the effect dependent on the shape of the CdTe microcomposites (Lu et al., 2010).

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Conclusions

These studies demonstrate that even short-term inhalation of CdO NPs can produce persistent pulmonary cell injury and inflammation, as well as have deleterious effects on lung organization and systemic immune function. While some indicators of pulmonary injury were reversible over time, others persisted for up to one week after cessation of exposure, suggesting continued damage or lack of repair. Persistent adverse pulmonary effects, such as those observed here, represent an important occupational health threat as engineered metal oxide nanoparticles are being manufactured and used at ever-greater frequency, (potentially) placing a large number of workers at risk. Future studies are needed to identify mechanisms associated with CdO NP-induced pulmonary injury, as well as the onset and persistence of such effects.

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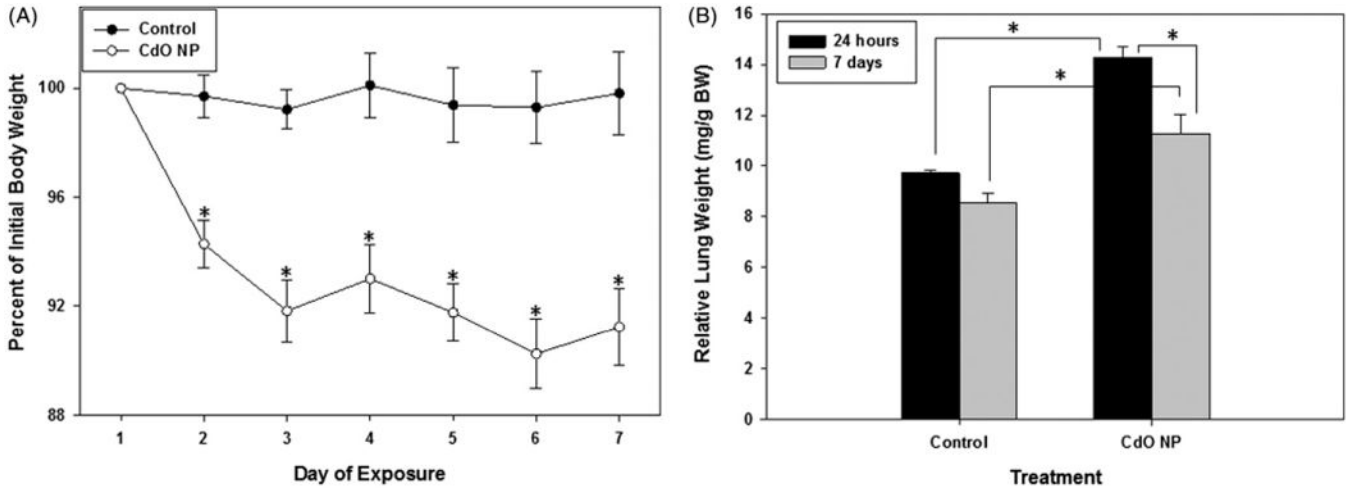

Figure 1.

Inhalation exposure to CdO NPs causes weight loss (A) and increases lung weight (B) during exposure. Male mice were weighed daily prior to exposure. Exposure day 1 was calculated as 100% for each mouse and changes in body weight were computed as percent change from day 1. Lungs were weighed following euthanasia either 24 h or seven days after the final exposure and normalized to body weight at the same time point. Data are mean ± SE from n = 5 to 7 mice for each mean. *Significant (p<0.05) loss of body weight compared to control (A) or significant (p<0.05) change in lung weight between highlighted bars (B).

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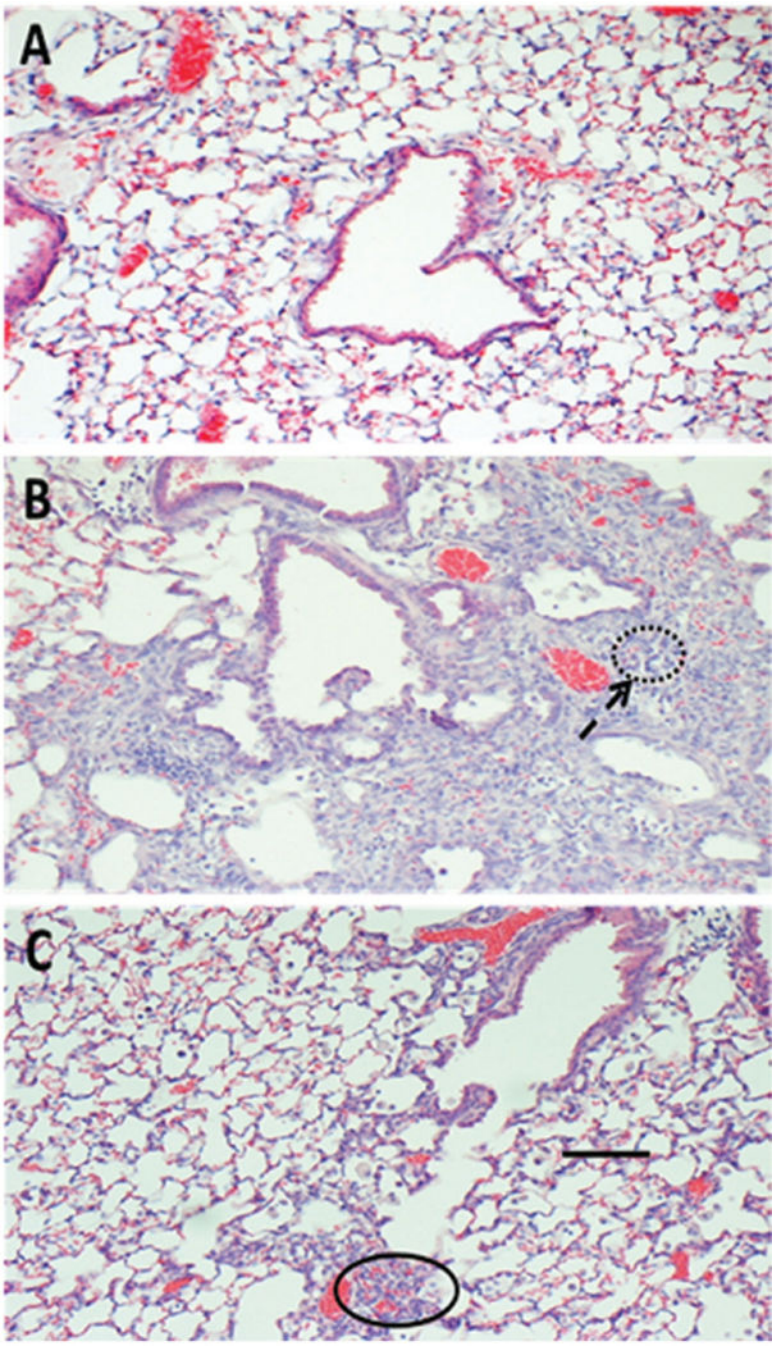

Figure 2.

Exposure to inhaled CdO NPs (240 μg CdO NP/m^3) for three hour/day for a total of seven days leads to pulmonary inflammation in male mice. Lungs were processed for histological evaluation either one or seven days after the final exposure. H&E images are representative from n = 3 mice per exposure group per time point. (A) Filtered-air exposed, (B and C) one or seven days post-cessation of CdO NP exposure, respectively. ↗, A site of fluid/inflammatory cell infiltration; —, a site of septum wall thickening; and ○, an area of increased bronchus-associated lymphoid tissue (BALT).

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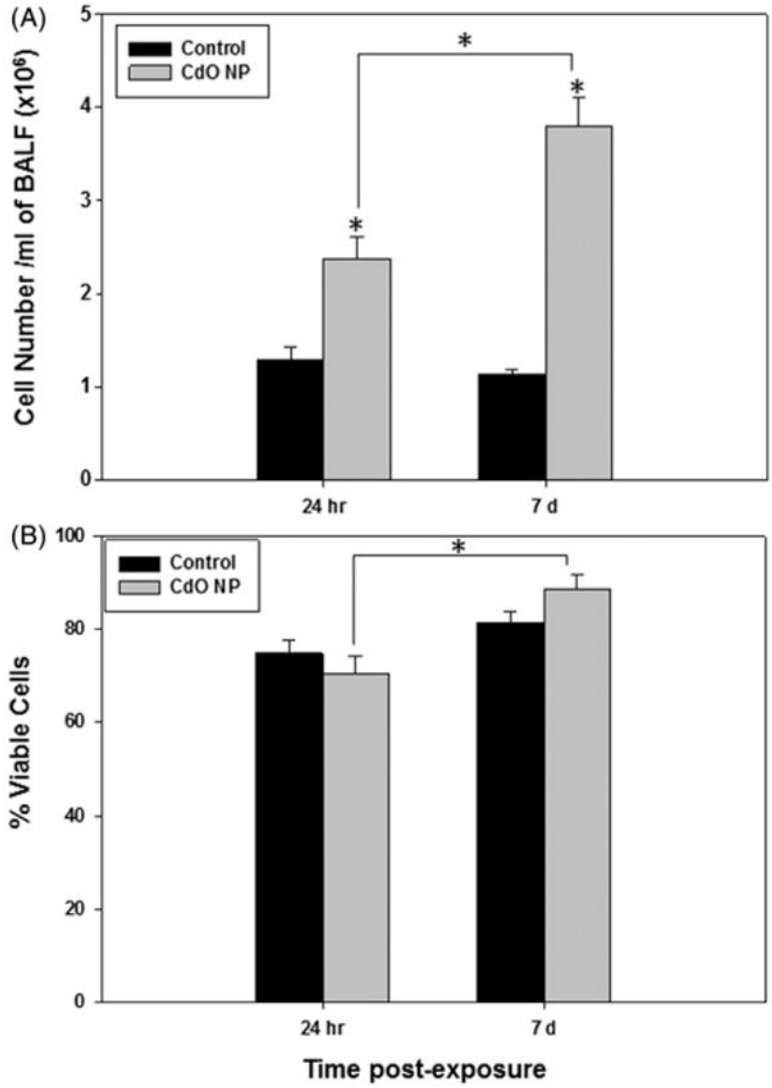

Figure 3.

Effects of inhaled CdO NPs on lavaged cell number (A) and cell viability (B) in adult male mice. At either one or seven days post-exposure, mice were euthanized and the lungs lavaged with 1 mL of PBS as described in the Methods section. Data are means ± SE from n = 5 to 7 mice/group. *p<0.05.

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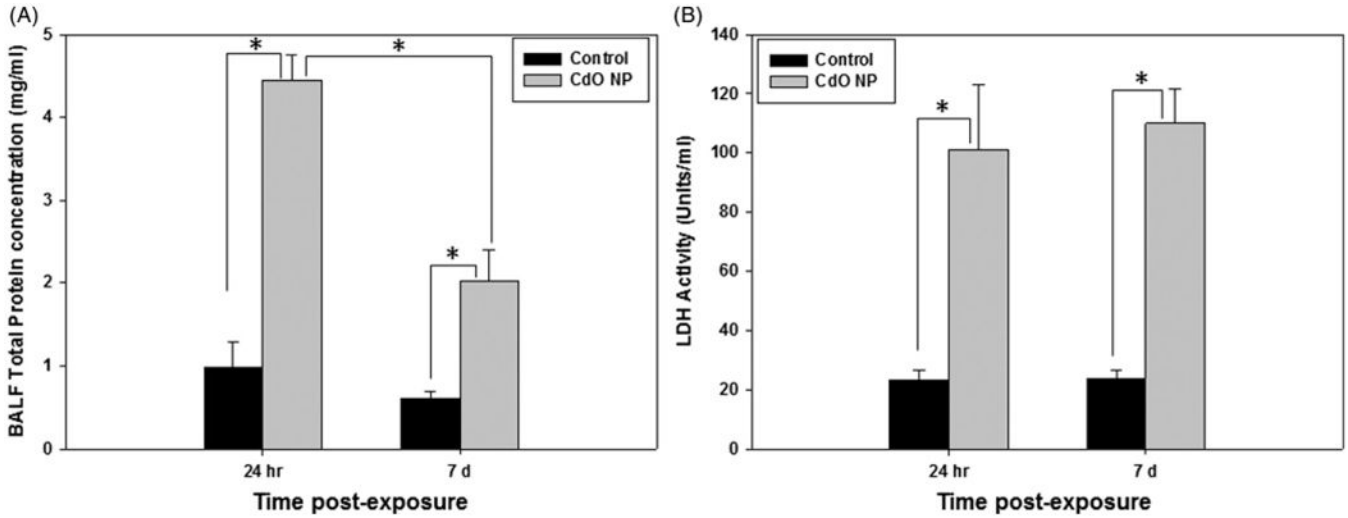

Figure 4.

Effects of inhalation exposure to CdO NPs on BALF total protein concentration (A) and LDH activity levels (B) in adult male mice. Protein concentrations were measured using the Biorad protein assay kit. LDH activity was assayed using a commercial kit against a standard curve as described in the Methods section. Data are means ± SE from $n = 5$ to 7 mice/exposure group. *$p<0.05$.

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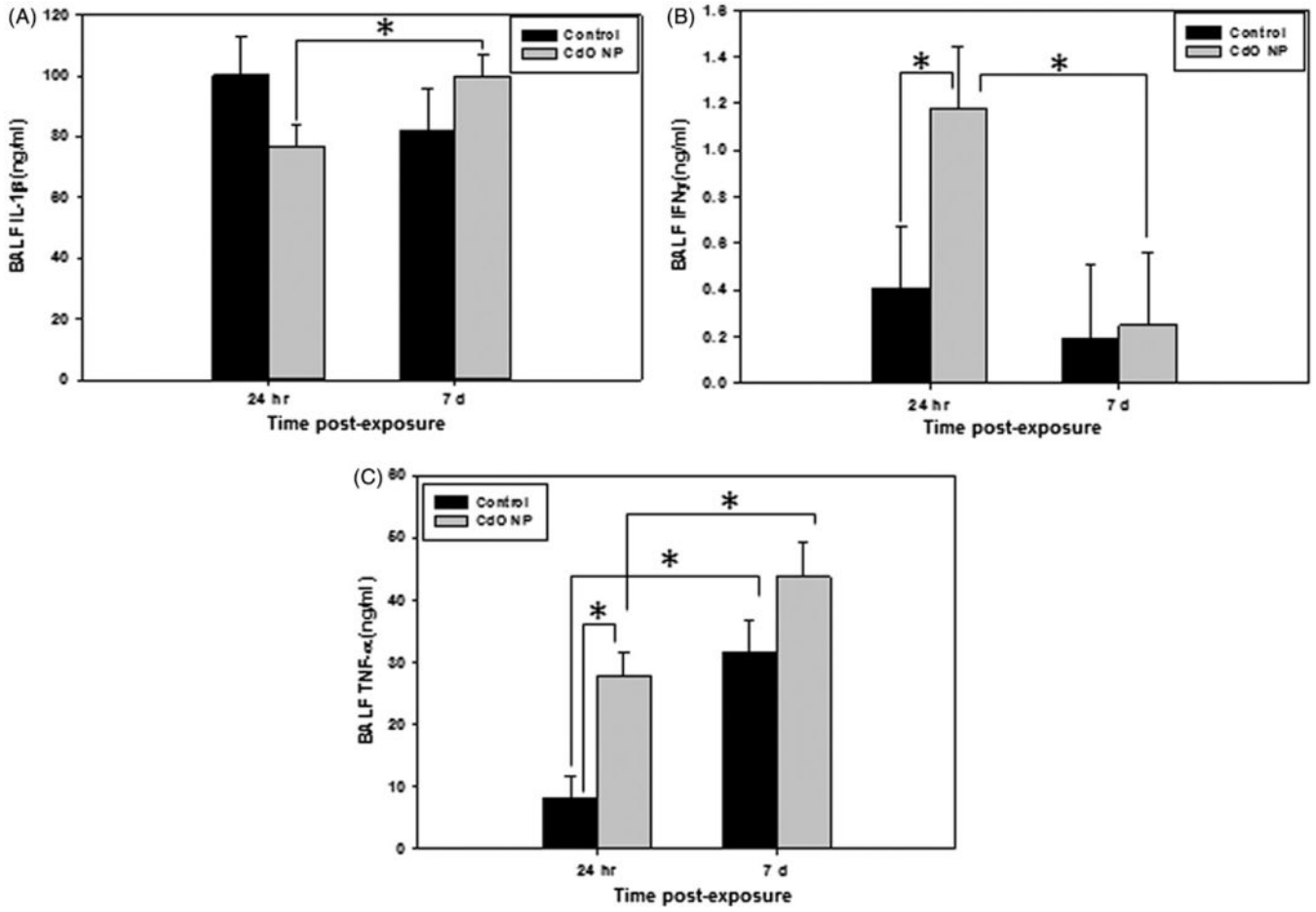

Figure 5.

Effects of inhalation exposure to CdO NPs on BALF levels of IL-1β (A), IFN-γ (B), and TNF-α (C) in adult male mice. Each analyte was quantified using a commercially available ELISA kit as described in the Methods section. Data are means ± SE from n = 5 to 7 mice/exposure group. *p<0.05.

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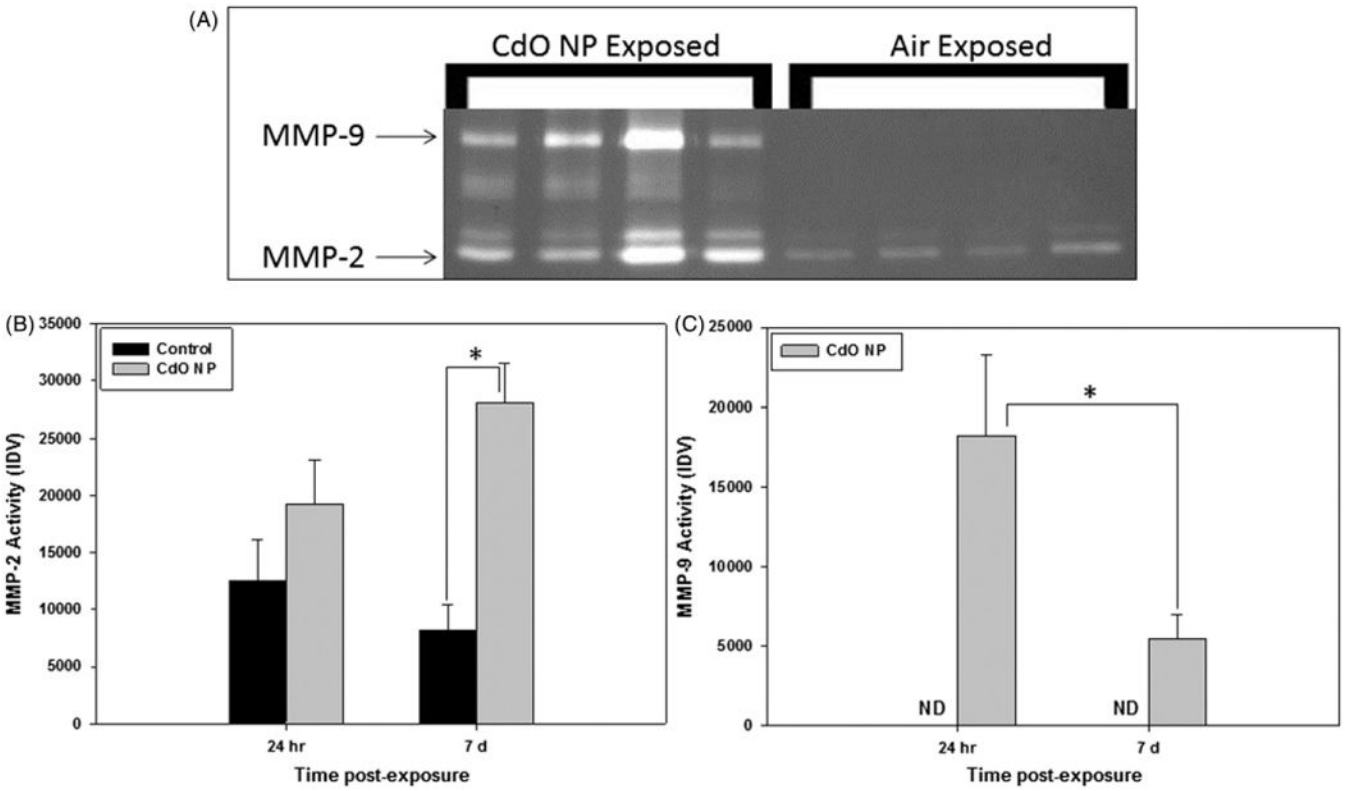

Figure 6.

Effects of inhalation exposure to CdO NPs on MMP activity one and seven days post-CdO NP exposure as determined by gelatin zymography (A). Densitometric analyses for activities of MMP-2 (B) and MMP-9 (C) were derived from integrated density values generated using the Alpha Imager software. Data are means ± SE from $n = 4$ mice/treatment/time point post-exposure. *$p<0.05$.

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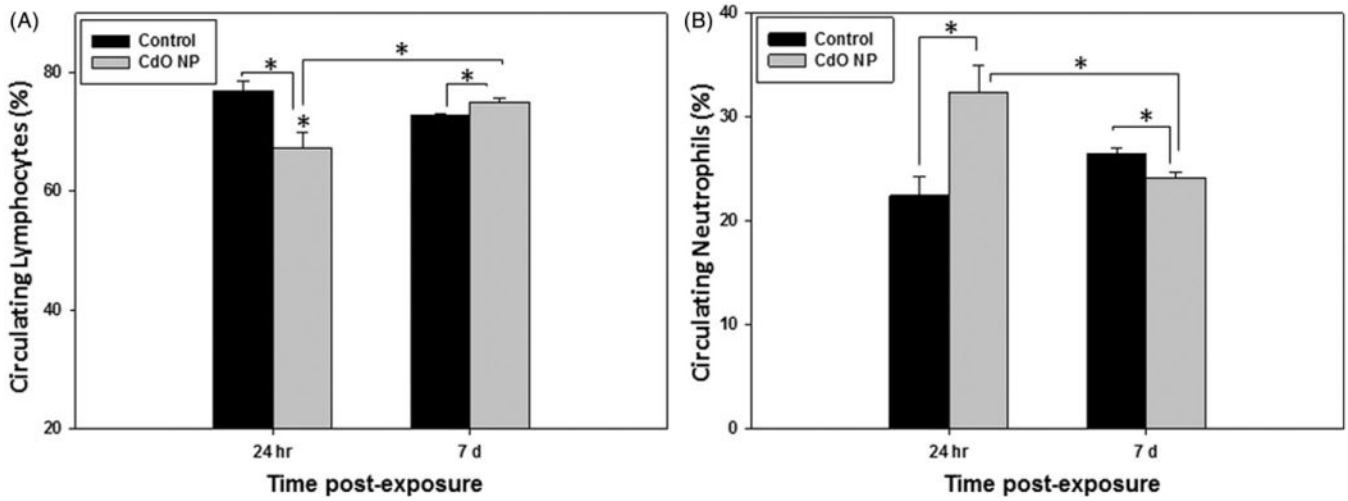

Figure 7.

Effects of CdO NPs on blood lymphocytes (A) and neutrophils (B) in adult male mice. Blood differentials were determined by counting 100 cells/slide and two slides per mouse. Data are means ± SE from $n = 5$ to 7 mice/mean. *$p<0.05$.

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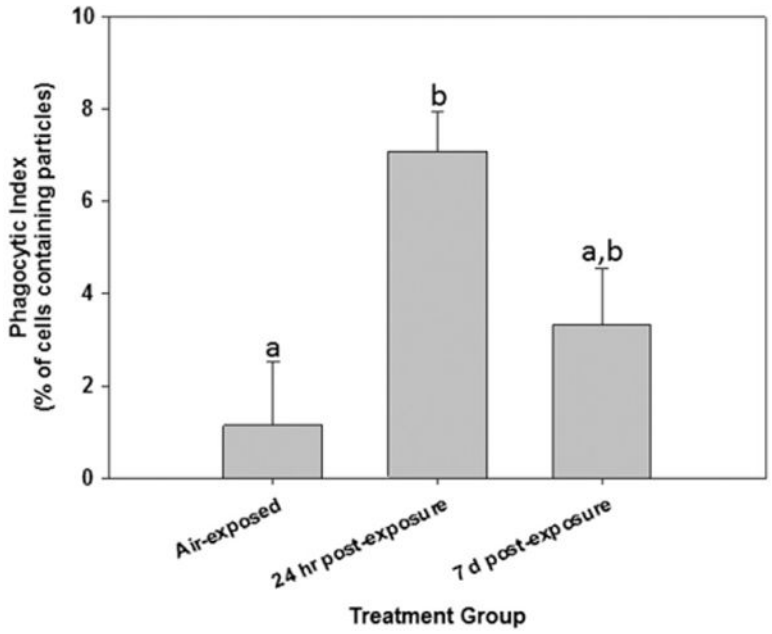

Figure 8.

Effect of inhalation of CdO NPs on the uptake of labeled bacteria by blood phagocytes. Phagocytic uptake was determined using pHrodo *Escherichia coli* labeled bacteria as described in the Methods section. Values were calculated as the difference in fluorescence between whole blood incubated with labeled particles at 37°C and whole blood incubated with unlabeled bacteria on ice. Samples were analyzed by flow cytometry and phagocytic uptake determined as described in the Methods section. Data are means ± SE from $n = 3$ to mice/determination. Means from air-exposed mice from each time point post-exposure were not significantly different and were pooled for statistical and graphical representation. Bars with different letters are significantly different ($*p<0.05$) using Fisher's LSD.