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Research paper

Immune responses during single and repeated murine endotracheal exposures of zinc oxide nanoparticles☆

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ABSTRACT

The increasing use of zinc oxide nanoparticles (ZnO-NPs) has raised concerns about their human health and environmental risks. Towards understanding their potential hazard, we investigated the *in vivo* responses of two commercially available ZnO-NPs, which have been designated as representative of manufactured materials and are used in sunscreen formulations. One such commercial sunscreen product had a zinc concentration of 10.0 ± 2.6 wt%, and the average particle dimension measured by transmission electron microscopy was 112 ± 64 nm. In comparison, ZnO-NP pristine materials appeared similar to that observed in the sunscreen, with agglomerated elongated, needle-like or prismatic structures. Healthy male BALB/c mice were exposed to either coated or uncoated pristine ZnO-NPs by endotracheal instillation with a single dose (5 μg/mouse) or with repeated doses (5 μg/mouse/week for 4 weeks). Histological examination indicated that single exposures caused some pulmonary inflammation. This was confirmed by elevated levels of pulmonary granulocytes, as well as macrophage and natural killer (NK) cells. These changes were accompanied by leukopenia and lymphopenia in the blood. After a month of weekly repeated exposures, a drop in mean body mass was observed. Pulmonary T-helper cells, NK cells, epithelial cells, and especially macrophage were elevated. Both acute and repeated exposures resulted in induction of pulmonary interleukin (IL)-6, keratinocyte chemoattractant (KC), monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α. These results demonstrate that both coated and uncoated ZnO-NPs can induce pulmonary inflammation, but that the uncoated NPs generated a stronger immune response. The acute response was macrophage and neutrophil-mediated, but repeated exposures resulted in a macrophage-dominant and possibly adaptive immunological response.

1. Introduction

Zinc oxide nanoparticles (ZnO-NPs) are utilised in many commercial products. One important property of ZnO-NPs is their translucency and effectiveness in protection against ultraviolet A and B radiation. As such, they are effective ingredients of sunscreens and moisturizers (Nohynek et al., 2007; Osmond and McCall, 2010), as well as paints, coatings and building finishing materials. ZnO-NPs have also been used as antibacterial agents in ointments, lotions, mouthwashes, food packaging and surface coatings (Jones et al., 2008; Sirelkhatim et al., 2015). More recently, ZnO-NPs have been investigated for their putative anti-cancer properties and utility for drug delivery (Zhang et al., 2011).

With the widespread use of ZnO-NPs in a variety of applications, there is increasing interest associated with their characterization and potential toxicity. The adverse effects of ZnO-NPs have been studied *in vitro* (Yang et al., 2009; Hackenberg et al., 2011; Zhang et al., 2014; Theodorou et al., 2016; Chevallet et al., 2016), *in vivo* (Sayes et al., 2007; Wang et al., 2010; Xia et al., 2011; Leite-Silva et al., 2016) and discussed in a review (Vandebriel and De Jong, 2012). Our previous *in*

☆ Relevance to Scope: this manuscript should be of significant interest to the nanotoxicology community, especially those interested in immunological effects associated with OECD-designated representative nanomaterials used in commercial sunscreen products. We present an assessment of multiple immunological indices following acute (single) and repeated (weekly for a month) exposures via the intratracheal route. The results revealed that the early response to exposure is likely targeted to sequestration and removal of the ZnO-NPs, but longer repeated exposures result in a response typical of damage repair and possibly long-term immunological adaptation. The exposure model developed will also be useful for predicting potential health hazards associated with other nanomaterials for which there is little existing information.

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vitro study, using the same ZnO-NPs, investigated the mechanism of ZnO-NP toxicity which was found to involve the generation of reactive oxygen species (ROS) and inhibition of mitochondrial function, ultimately resulting in cytotoxicity and death of both human and mouse epithelial and immune cells, even at lower concentrations tested (Zhang et al., 2014). However, current *in vitro* models cannot simulate the multitude of cellular and tissue interactions, and fail to accurately predict the toxicological behavior of the nanoparticles in living organisms (Vandebriel and De Jong, 2012).

In vivo toxicity studies of ZnO-NPs have mainly focused on dermal toxicity, since ZnO-NPs are often present within commercial products directly applied to the skin. However, other routes of exposure may be prevalent during production and processing, or through consumer use of spray products, where ZnO-NPs may be released into the ambient air resulting in inhalation exposure (Osmond and McCall, 2010). Some studies have assessed pulmonary toxicity of ZnO-NPs with instillation (Sayes et al., 2007; Xia et al., 2011) and inhalation (Warheit et al., 2009; Wang et al., 2010) exposure models. Each model has its advantages. The instillation method uses less material, and a high proportion of the desired dose can be delivered to the lung tissue in precise quantities, which may be better for investigating the mechanistic effects of NP exposure, such as that conducted here. In contrast, the inhalation model would be useful for simulating ambient air levels as would be needed for occupational exposure studies. Instillation studies in rodents have shown that ZnO-NPs are capable of inducing acute pulmonary inflammation, including neutrophil recruitment and lactate dehydrogenase (LDH) release at cumulative doses of 0.6 mg/kg to 5 mg/kg (Sayes et al., 2007; Xia et al., 2011). Another study also reported that Sprague-Dawley rats exposed to ZnO-NPs through endotracheal instillation caused neutrophilic inflammation after 24 h (Wang et al., 2008) and increased the levels of total cells, neutrophils, LDH and total protein in bronchoalveolar lavage fluid (BALF) and 8-hydroxy-2′-deoxyguanosine in blood after 72 h (Chuang et al., 2014). Inhalation exposure of 3 μm ZnO-microparticles (25 and 50 mg/m^3) and instillation of 300 nm ZnO-NPs (1 and 5 mg/kg) in rats resulted in transient inflammation as measured by increased LDH release, and elevated protein and neutrophils in BALF (Warheit et al., 2009). A repeated short-term inhalation study also demonstrated that ZnO-NPs (2.5 and 12.5 mg/m^3/day for 5 consecutive days evoked concentration-dependent transient pulmonary inflammation (Landsiedel et al., 2014). Another inhalation study showed acute lung inflammation (*i.e.*, elevated numbers of neutrophils and lymphocytes and level of total protein in BALF at 24 h) but no persistent effects (13 weeks) after a single exposure (1.31–90.5 μg/mouse) (Larsen et al., 2016). Another more recent study showed that the intratracheal instillation and the inhalation of a high dose of ZnO nanoparticles (4 mg/kg) caused a transient increase in neutrophil influx in the lung and a transient increase in concentrations of cytokine-induced neutrophil chemoattractant-1 and 2, and heme oxygenase-1 in BALF in the acute phase. These parameters returned to control level in the chronic phase, and reversible inflammation of neutrophils in the lung was observed by both approaches (Morimoto et al., 2016). Yet, details about their systemic biological effects remain insufficient and often controversial since the different materials and high doses of ZnO-NPs were used (Morimoto et al., 2016; Jacobsen et al., 2015; Saptarshi et al., 2015) and few studies have focused on standard reference ZnO nanomaterials used in the production of sunscreens. Animal studies using these NPs would reduce uncertainty on the quality of the material and ensure relevance for occupational or consumer exposure.

In the present study, we followed our *in vitro* study (Zhang et al., 2014) by using two commercially available ZnO-NPs (coated and uncoated), which have been designated as representative of manufactured materials by the Organisation for Economic Cooperation and Development and investigated the *in vivo* response of two ZnO-NPs. These ZnO-NPs have been used in various sunscreen formulations. Although the sunscreen product itself was not used in animal exposures, representative pristine ZnO-NPs were delivered to male mice by endotracheal instillation with single and repeated exposures. Given data from our previous study, which demonstrated disruptions in epithelial and immune cell function, we hypothesize that pulmonary exposure to Z-COTE will result in altered immune responses, which are dependent on the presence of coating and distinct from effects from bulk material. For this, local immune effects were assessed with respect to alterations in lung cell populations and induction of inflammation. Furthermore, potential systemic effects were monitored by measuring serum immunoglobulins, changes in haematological markers, tissue histology and other non-immunological markers.

2. Materials and methods

2.1. Characterization of ZnO-NPs and ZnO-NP-containing sunscreen product

Representative ZnO-NPs were Z-COTE (uncoated) and Z-COTE HP1 (coated with triethoxycaprylylsilane) from BASF (Mississauga, Canada). Fine ZnO (bulk control particles, micro-sized) was purchased from Sigma-Aldrich (Oakville, Canada). The coated, uncoated and micro-sized particles have been characterized extensively in our previous study (Zhang et al., 2014). The particles were ultrasonically dispersed (15 min at 30% amplitude in deionized water) as previously described (Zhang et al., 2014), and working dilutions were made freshly before use.

A sunscreen product was compared with pristine ZnO-NPs. A commercial sunscreen formulated with Z-COTE was purchased from Amazon. The morphology and chemical composition of the particles within the sunscreen were examined and measured by scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM/EDS). A portion of the sunscreen cream was smeared onto a carbon planchet and allowed to air-dry. This specimen was analyzed with both the JOEL microscope and a Hitachi S-4800 field-emission SEM operating at 2 and 15 kV accelerating voltages. EDS analysis was conducted using Thermo Noran System with six EDS detectors.

Quantitative analysis of total Zn in the sunscreen product samples was carried out by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 3000DV Emission Spectrometer with axial viewing and modified background correction. Calibration standards were prepared from a SPEX Claritas 1000 ppm zinc single element standard (SPEX Certiprep, Metuchen, NJ). Samples were transported to the ICP torch using a Ryton® spray chamber crossflow nebulizer and a 2.0 mm alumina injector. Zn concentrations were confirmed using analytical wavelengths of λ = 206.200 nm and 213.857 nm. Rhodium (1 ppb) was added to the sample dilution to serve as an internal standard.

2.2. Animal

All procedures involving animals were approved by the Health Canada Animal Care Committee. Male BALB/c mice between 18 and 22 g (8–10 weeks) were purchased from Charles River Laboratories Inc. (Saint-Constant, Canada), and acclimated for at least 1 week prior to experimentation. Animals were housed in cages under a 12-h light/dark cycle at a constant room temperature (20 ± 2 °C) and relative humidity of 50–70%. The commercial pellet diet and deionized water were available *ad libitum*.

2.3. Experiment design

Due to potential interference from the other ingredients in the sunscreen formulation, the sunscreen product itself was not used in animal exposure experiments. Instead, the pristine ZnO-NPs used were the same source as that used in sunscreen formulations. The exposure schedules are shown in Fig. 1. To investigate the status of pulmonary