Lung toxicity and gene expression changes in response to whole-body inhalation exposure to cellulose nanocrystal in rats

Materials and Methods

Studies for data collection were conducted using a rat whole-body inhalation exposure and lung toxicity model. Approximately 3 months old, pathogen-free, and healthy male Fischer 344 rats (CDF strain) purchased from Charles River Laboratories (Wilmington, MA) were used in this study. The entire animal study was conducted in an AAALAC International accredited animal facility (NIOSH, Morgantown, WV) following a protocol approved by the Institutional Animal Care and Use Committee. The rats, upon their arrival, were acclimated to the animal facility conditions for 12 days prior to their use in the study. Throughout the entire period of the study, the rats were housed in groups of 3 rats/cage and maintained on a 12-hour light-dark cycle in a temperature (68-72º F) and humidity (30-70%) controlled room. Food and water were provided *ad libitum* except when the rats were exposed by inhalation to the agents. Groups of rats were exposed by inhalation to crystalline nano cellulose (CNC) (US Department of Agriculture, Forest Products Laboratory, Madison, WI). The mass median aerodynamic diameter (MMAD) of the CNC particles in the aerosol generated for rat exposure was 3.17 mm with a geometric standard deviation (GSD) of 1.81. The CNC exposure was done at a dose of 20 mg/m^3, 6 hours/day, 5 days/week for 14 days. A separate group of rats exposed simultaneously to air served as the control. At 18 hours following the last exposure to air or CNC, the rats were euthanized, and lung toxicity determined as described in detail below:

Euthanasia of rats and collection of biospecimens: The rats were euthanized by an intraperitoneal injection of ≥100 mg sodium pentobarbital/kg body weight (Vortech Pharmaceuticals, Dearborn, MI). Blood collected directly from the abdominal aorta was transferred to Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) containing EDTA as an anti-coagulant and mixed well by rotating the tubes. The right lung of the rats was clamped off and bronchoalveolar lavage (BAL) was performed in the left lung using ice-cold PBS. The lavage was repeated 6 times, using 4 ml PBS/lavage, to a total volume of 24 ml/rat. The cellular and acellular fractions of the BAL were separated by centrifuging the samples (570xg, 15 minutes, 4ºC). The cell pellet obtained was resuspended in 1 ml PBS buffer and used for determination of pulmonary toxicity as described in the corresponding sections below. The diaphragmatic and cardiac lobes of the unlavaged right lung were inflated with 10% neutral buffered formaldehyde and stored in the same solution for histopathological analysis. The apical lobe of the unlavaged right lung was cut into pieces, weighing approximately 30 mg each, and stored in RNALater (Invitrogen, Carlsbad, CA) and used to determine the gene expression profile.

Lung histology: The lung lobes fixed in fomaldehyde were paraffin embedded, sectioned at a thickness of 5 ⬜m, stained with hematoxylin and eosin (H&E) and examined by a pathologist and the lung histopathological changes were scored.

Lactate dehydrogenase activity: Lactate dehydrogenase (LDH) activity in the acellular BAL fluid (BALF) was determined with a COBAS C111 analyzer (Roche Diagnostic Systems, Mount Clair, NJ).

BAL cell counts: The cellular fraction of BAL was resuspended in 1 ml of PBS buffer and the total number of BAL cells was determined using a Coulter Multisizer II and Accu Comp software (Coulter Electronics, Hialeah, FL). BAL cells ($5x10^4$) were spun onto microscope slides using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England) and stained with a Leukostat stain (Fisher Scientific, Pittsburgh, PA) to differentiate alveolar macrophages (AM) and PMN. 200 cells were counted per rat and percentages were multiplied back across the total cell count to obtain total number of AM and PMN.

BALF cytokines, chemokines, and growth factors: Concentrations of IL-1b, IL-10, IL-12, MIP-2, MCP-1, and TNFa which are involved in inflammation were determined in the acellular fraction of BALF using ELISA kits (Millipore Corporation, Billerica, MA).

Reactive oxygen species (ROS) generation by BAL cells: A previously described luminol-dependent chemiluminescence (CL) assay (Antonini *et al.,* 1994, Environmental Health Perspectives, 102, Suppl. 10: 37-42) was performed to determine the generation of ROS by the lung phagocytes (AM and PMN). Phorbol 12-myristate 13-acetate (PMA), stimulant of AM and PMN, and nonopsonized, insoluble zymosan, a stimulant of AM only, were used to determine the contribution of both AM and PMN to the overall production of ROS in the rat lungs. A BAL cell volume equivalent to $5x10^5$ total BAL cells or $5x10^5$ AM in a final volume of 350 ml HEPES buffer was incubated with luminol for 10 minutes at 37° C prior to stimulating the cells with either 10M PMA or 2 mg/ml zymosan, respectively. Baseline oxidant production by the BAL cells was measured in the absence of the stimulants. Measurement of CL was recorded using a Berthold LB 953 luminometer (Wildbad, Germany) for 15 minutes at 37° C, and the integral of counts per minute (cpm) per one million cells versus time was calculated. CL was calculated as the cpm of the stimulated cells minus the cpm of the corresponding resting cells, and the value was normalized to the total number of BAL cells for PMA-stimulated CL and total number of AM for zymosan-stimulated CL.

Lung gene expression profile: A piece of the lung tissue stored in RNALater was used to isolate total RNA free of contaminating DNA and proteins using miRNEasy Mini Kit (Qiagen, Inc. Valencia, CA) following the procedure, including the on-column DNase digestion, provided by the manufacturer. The integrity and purity of the RNA samples isolated were determined using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). Total RNA was quantified using a nano-drop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). Only RNA samples exhibiting an RNA Integrity Number (RIN) $\geq$8.0 were used in the gene expression studies.

One microgram total RNA/sample was used to create sequencing libraries using the Illumina TruSeq® Stranded Total RNA Library Prep Kit (Illumina, Inc. San Diego, CA) following

the protocol provided by the manufacturer. Stated briefly, following depletion of ribosomal RNA (rRNA), the RNA samples were purified and fragmented (68º C for 5 minutes). The RNA fragments were purified using a bead cleanup procedure and reverse transcribed into first strand of cDNA using reverse transcriptase and random primers. While synthesizing the double stranded cDNA, dUTP was incorporated in place of dTTP followed by the addition of a single 'A' nucleotide to the 3 prime ends to facilitate proper adapter ligation. Indexing adapters provided in the library preparation kit were ligated to the ends of the ds cDNA. After adapter ligation, the samples were amplified by PCR (12 cycles) to enrich the DNA fragments containing the adapter molecules and to enhance the amount of DNA in the library using a Veriti™ 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA). The PCR amplified cDNA library samples were quantified using a dsDNA HS Assay Kit (Invitrogen by ThermoFisher Scientific, Waltham, MA) and a Qubit 3.0 Fluorometer (Invitrogen by ThermoFisher Scientific, Waltham, MA). Average fragment size and fragment distribution of the cDNA library samples were then assessed using an Agilent 2100 Bioanalyzer with High Sensitivity DNA Reagents (Agilent Technologies, Santa Clara, CA).

Individual sample libraries were provided to the Centers for Disease Control and Prevention Genome Sequencing Laboratory (GSL; Atlanta, GA) for 2 x 100 base pair, paired-end sequencing using Illumina sequencer and reagents. Significantly differentially expressed genes (SDEGs) were identified by analyzing the gene expression data. Significantly differentially expressed genes (SDEGs) were those genes with an absolute fold change greater than 1.5-fold and an adjusted p-value less than 0.05.

Bioinformatic analysis of gene expression data: Bioinformatic analysis of the SDEGs was conducted using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Valencia, CA). The IPA program is designed to map the biological relationship of the input genes and classify them into categories of biological functions, canonical pathways, diseases, and networks.

Hematology: Various hematological parameters were determined in the unclotted blood samples using an IDEXX Procyte instrument (IDEXX Corporation, Westbrook, ME) following the procedures described in the user guide.

Statistical analysis of data: All data, with the exception of gene expression data, between the exposed and control groups were compared using a one-way analyses of variance. Post hoc comparisons were made using the Fisher's least significant difference (LSD) test. Analyses were performed using JMP version 13.2 for Windows. The level of statistical significance was set at $p<0.05$.