MATERIALS AND METHODS

Thermal Spray Coating Aerosol Generation and Exposure System

A computer-controlled thermal spray coating generator and inhalation exposure system was constructed to perform animal studies to mimic workplace exposures in a laboratory as previously described (Afshari et al., 2022; Kodali et al., 2022). Rats were exposed by inhalation to aerosols (target concentration: 25 mg/m^3 x 4 hr/d x 4 d) generated from electric arc wire thermal spray coating using different consumable wires (Polymet Corporation, West Chester, OH), including a stainless-steel wire (PMET731; settings of 60 psi, 30 V, 200 A), a Ni-based wire (PMET885; settings of 60 psi, 30 V, 250 A), and a Zn-based wire (PMET540; settings of 60 psi, 23 V, 200 A). Control animals were exposed to filtered air in a separate but identical exposure chamber. The different thermal spray coating aerosols were generated in a closed spray booth and transported to an animal exposure chamber where they were collected and characterized.

The thermal spray coating exposure system was divided into two separated areas (Figure 1): (A) an enclosed room where the spray coating occurred that contained a compressed air tank, thermal spray machine with wire holder and feeder unit (AT-400 Wire Arc Spray System; Thermach, Inc., Medina, WI), the rotary and reciprocating system that holds and rotates the stainless-steel pipe to be spray coated in an up and down manner, and a spray coating booth that housed the torch gun and rotary sample holder system; (B) the animal exposure chamber with different particle characterization and chamber condition devices and air flow controllers.

Within the spray booth, different consumable thermal spray coating metal wires were fed independently into the spray gun. The wires were then charged, and an arc was generated between them. The heat from the arc melted the incoming wires, and the molten metal particles were suspended in a jet of air from the gun. The suspended molten metal particles were deposited onto the metal pipe substrate with the help of compressed air. A rotary motor rotated the feedstock pipe in circular and up-and-down directions to allow for continuous, sequential coating during the 4-hr exposure time within the spray booth. The spray gun was controlled by a computer program and was fired at selected intervals to produce a target concentration of 25 mg/m^3 in the exposure chamber.

The aerosols generated during thermal spray coating were delivered to the exposure chamber using slight negative pressure. After leaving the booth the aerosol passed through a large particle trap then mixed with an automatically adjusted amount of diluted air. After the aerosol and dilution air mixed, it passed through a home-made large particle trap and mixed with diluted air before passing through a cyclone (URG-2000-30EP, URG Corp, Chapel Hill NC) to further remove large particles. The cut size of the cyclone was 6 μm at a flow of 31 L/min. The

flow into the cyclone was maintained at 31 L/min, by using a mass flow controller connected to house vacuum on the exhaust side of the air-tight exposure chamber. Large particles were removed so that the animals would receive only the respirable portion of the particles.

The mass concentration in the chamber was monitored by a real-time aerosol monitor (DataRAM, MIE, Inc., Bedford, MA). The sensors and measurement devices were managed and controlled through a custom computer software programed written in LabVIEW (National Instruments Corporation, Austin, TX). To maintain a constant particle concentration in the exposure chamber, the software would adjust the amount of dilution air that made up the 31 L/min entering the exposure chamber. Particle mass concentrations inside the animal chamber were determined by collecting airborne particles with two, 47-mm closed-face cassettes loaded with polytetrafluoroethylene filters followed by gravimetric analyses. This filter data was used during every exposure run to calibrate the DataRAM.

Additional ports were located on the top of the chamber and used to measure chamber pressure and to collect additional particle samples for size distribution, chemical composition, and electron microscopy analyses. The air pressure and temperature and relative humidity inside the chamber were continually measured during the exposure period (Vaisala Temperature-Humidity Probe, model# HMP60; Woburn, MA). The levels of generated carbon dioxide (Vaisala CO_2 Probe, model# GMP252; Woburn, MA) were continuously monitored in the chamber during animal exposures and maintained below 5000 ppm.

Thermal Spray Coating Aerosol Characterization

Particle size and morphology. The size distribution of the different thermal spray coating aerosols inside the exposure chamber was determined using a Micro-Orifice Uniform Deposit Impactor (MOUDI; MSP Model 110, MSP Corporation, Shoreview, MN). Particles were collected between the size ranges of 0.056 – 18 μm that were separated into 11 stages where each stage was loaded with a 47-mm aluminum foil filter except for the filter stage. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the aerosols were determined from gravimetric measurements. To assess particle morphology, the aerosolized particles from thermal spray coating were collected using 47-mm cassettes loaded with polycarbonate filters (0.2 μm pore size; Whatman, Clinton, PA). The filters loaded with the collected particles were mounted onto aluminum stubs using double-stick carbon tape and viewed using a Hitachi S4800 field emission scanning electron microscope (SEM; Hitachi High-Tech America, Boston, MA).

Metal composition. Particle samples were collected inside the exposure chamber onto 5 μm pore size polyvinyl chloride membrane filters (SKC, inc., Eighty Four, PA) in 37-mm cassettes during thermal spray coating using the different consumable wires. The collected samples were analyzed

for metal components by the NIOSH contract laboratory using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) according to NIOSH Method 7303 modified for hot block/HCl/HNO_3 digestion (NIOSH, 1994). Metal content of blank filters also were analyzed for control purposes.

Electron Paramagnetic Resonance. To detect and measure short-lived free radical intermediates, electron paramagnetic resonance (EPR) spin-trapping was used. To assess whether the thermal spray coating particle suspensions were capable of producing hydroxyl radicals ($OH^{\cdot}$), they were exposed to H_2O_2 through a Fenton-like reaction. Final concentrations were 100 mM of the spin-trap DMPO (5,5'-dimethylpyrroline N-oxide, Sigma-Aldrich, St. Louis, MO), 5 mg/ml the thermal spray coating particle samples, the gas metal arc- stainless steel (GMA-SS) and manual metal arc- stainless steel (MMA-SS) welding fume samples (positive metal particle controls) or potassium dichromate [2 mM; Cr (VI) positive control], and 1 mM H_2O_2 suspended in PBS and mixed in the order listed. All reagents were mixed in test tubes for 3 min at room temperature, filtered through a Titan3 nylon 0.45 mm filter to halt the reaction and remove any metal particles. The sample was then transferred to a quartz flat cell for EPR measurement in a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA). For each sample, the instrument was set to run 3 scans with a 41 sec scan time, a receiver gain of $1.0x10^4$, a 40 msec time constant, 1.0 G modulation amplitude, 63.4 mW power, 9.751 frequency, and 3515 G magnetic field center. Samples were run in independent experiments (n = 3). Sample data were attained and processed as previously described (Stefaniak et al. 2009; Leonard et al. 2004). Briefly, signal intensity (peak height) from the 1:2:2:1 spectrum (characteristic for. OH) was used to measure the relative proportion of the short-lived radicals trapped.

Animals

Animals. Male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) were used for the project and weighed 250-300 g upon arrival. They were free of viral pathogens, parasites, mycoplasmas, *Helicobacter*, and CAR bacillus and were provided HEPA-filtered air, irradiated Teklad 2918 diet, and tap water *ad libitum*. The rats were acclimated for up to one week after arrival. All animal procedures used were reviewed and approved by the Centers for Disease Control and Prevention (CDC), Morgantown-NIOSH Animal Care and Use Committee (ACUC). The animal facilities are specific pathogen-free and environmentally controlled. The program and the facility are accredited by the AAALAC International (Frederick, MD).

Lung Toxicity Study

Inhalation Exposure. The rats were exposed to the respirable portion of aerosols (target concentration: 25 mg/m^3 x 4 hr/d x 4 d) generated from electric arc wire- thermal spray coating

using (1) a stainless-steel consumable wire (PMET731), (2) a Ni-based consumable wire (PMET885), and (3) a Zn-based consumable wire (PMET540). Control animals were exposed to filtered air. Different sets of exposed animals were harvested at 4 and 30 d after the last exposure. Animal body weights were measured during the exposure period and throughout the 30 d post-exposure period.

The aerosols were generated in a closed spray booth and transported to the exposure chamber. The justification for the target particle concentration used in the study was that it fell in the range measured in workplaces where thermal spray coating occurred (Darut et al., 2021; Huang et al. 2016; Petsas et al, 2007; Chadwick et al., 1997). Also, it is a comparable concentration used in similar animal inhalation studies exposed to other metal particles, such as welding fumes (Antonini et al., 2020; Antonini et al., 2017; Antonini et al., 2007). The actual daily mean ($\pm$ standard deviation) exposure chamber concentrations for the 4-d exposures were as follows: PMET731 (21.5 mg/m^3 $\pm$ 8.1), PMET885 (24.6 mg/m^3 $\pm$ 5.8), and PMET540 (24.9 mg/m^3 $\pm$ 3.1).

Bronchoalveolar Lavage. At 4 and 30 d after the final inhalation exposure, bronchoalveolar lavage (BAL) was performed to assess lung injury and inflammation. Animals were euthanized with an overdose of a pentobarbital-based euthanasia solution (>100 mg/kg, IP; Fatal-Plus Solution, Vortech Pharmaceutical, Inc., Dearborn, MI) and then exsanguinated by severing the abdominal aorta. The left lung was tied off, and the right lung was first lavaged with a 1 ml/100 g body weight aliquot of calcium- and magnesium-free PBS, pH 7.4. The first fraction of recovered BAL fluid (BALF) from the right lung was centrifuged at 500 x g for 10 min, and the resultant cell-free supernatant was saved for lung cell damage analysis. The right lung was further lavaged with 6-ml aliquots of PBS until 30 ml were collected. These samples also were centrifuged for 10 min at 500 x g and the cell-free BALF fraction was discarded. The cell pellets from all washes for each rat were combined, re-suspended in 1 ml of PBS buffer, counted, and differentiated.

Assessment of Lung Injury and Inflammation. Lactate dehydrogenase (LDH) was measured in the first fraction of the cell-free supernatant recovered from the BALF as a general marker for lung toxicity. LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurements were taken with a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ). For the determination of lung inflammation, total cell numbers recovered by BAL from the right lung were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cell suspensions ($5x10^4$ cells) were spun using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England) for 5 min at 800 rpm onto a slide. Cells (200/rat) were identified after labeling with

Leukostat stain (Fisher Scientific, Pittsburgh, PA) as monocytes/alveolar macrophages (AM) and polymorphonuclear leukocytes (PMN).

Lung Deposition and Clearance Study

Inhalation Exposure. The rats were exposed to the respirable portion of aerosols (target concentration: 25 mg/m^3 x 4 hr/d x 1 d) generated from electric arc wire- thermal spray coating using (1) a stainless-steel consumable wire (PMET731; settings of 60 psi, 30 V, 200 A), (2) a Ni-based consumable wire (PMET885; settings of 60 psi, 30 V, 250 A), and (3) a Zn-based consumable wire (PMET540; settings of 60 psi, 23 V, 200 A). Control animals were exposed to filtered air. Rats (n = 6/group) were harvested at days 0 (immediately following the exposure), 1, 4, and 30 d after the exposure. Due to space limitations, two exposures were performed for each of the three consumables. Exposure for the first two time points (days 0 and 1) were performed on one day, and the exposure for the second two time points (days 4 and 30) were performed on the next day. The actual daily 4-hr time-weighted average exposure chamber concentrations were as follows: PMET731 (exposure 1: 19.3 mg/m^3, exposure 2: 23.9 mg/m^3), PMET885 (exposure 1: 22.1 mg/m^3, exposure 2: 24.3 mg/m^3), and PMET540 (exposure 1: 19.7 mg/m^3, exposure 2: 23.6 mg/m^3).

Lung Deposition and Clearance of Metals. To assess the pulmonary clearance of deposited thermal spray coating particles, the metal content present in the lungs was measured on days 0, 1, 4 and 30 after the single 4-hr exposure. The concentration of specific metals deposited in the lungs at each time point was determined by ICP-AES according to NIOSH method 7300 (NIOSH, 1994). Non-lavaged whole lungs were collected from each animal, weighed, and lyophilized. The lung tissue samples were transferred to beakers for digestion. The sample containers were rinsed with concentrated nitric acid and three washings of deionized water and transferred to the respective digestion beakers. The samples were treated with 25 ml of concentrated nitric acid and 2 ml of concentrated perchloric acid, covered, and refluxed at 150^o C until complete dissolution. The sample residues were dissolved in a dilute solution of 4 % nitric acid/1 % perchloric acid and then analyzed for trace metals by ICP-AES.