

2. Materials and Methods

Cell Culture

BEAS-2B cells were grown and maintained at 37°C with 5% CO₂. Culture medium (BEGM, Lonza) was changed three times per week throughout the culturing procedure. The evening prior to exposure, cells were seeded at 0.55×10^5 cells/mL (Passage 6) into 96- or 6-well plates.

Thermal Spray Exposures

Thermal spray fumes were generated by an automated electric arc wire thermal spray generator as previously described for the characterized PMET540, PMET731, and PMET885 wires. (Afshari et al., 2022; Kodali et al., 2022) Thermal spray particles for each rod type were weighed into aliquots for short-term storage and consequent preparation of stock solutions. Particles were then resuspended in 0.6 mg/mL bovine serum albumin in PBS to a stock concentration of 5 mg/mL. To prevent aggregation, stock solutions were prepared from dry aliquots, sonicated (Branson Sonifier 450 with Cup Horn in Sound Enclosure) at 70% amplitude for 30 seconds, and immediately serially diluted in BEGM cell culture medium to test conditions. For survival and LDH curves, doses were prepared at 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0 µg/mL to complement macrophage toxicity data described by Kodali et al (2022). For the subsequent exposures, three doses were selected from the toxicity curves to represent a low, medium, and high dose based on LDH response and survival data. Concentrations of 100, 25, 3.125, and 0 µg/mL were chosen due to the propensity to capture a range of cellular response. At 100 µg/mL, statistical analysis showed significant differences in toxicity; while there was no significant difference from controls at 25 and 3.125 µg/mL, lower doses were selected to illustrate potential acute changes in protein secretion prior to significant cell death.

Immediately before exposure, cell culture medium was aspirated from BEAS-2B culture plate wells, cells rinsed with 1x PBS (Ca and Mg free), and the relevant dose administered to individually cultured biological replicates. Cells were then exposed under incubation conditions for 24-hours before endpoint analysis. Post-exposure, culture medium was removed for ET-1, IL-6, and IL-8 ELISA assays. Cultured cells were used for LDH, cell counts, and COMET analyses.

Viability

Cell counts were conducted using the PromoCell Detach Kit (PromoCell, Cat. # C-41210), trypan blue, and a Countess II automated cell counter (Invitrogen) to assess, across four observations per condition, total estimated cells/mL and total live cells.

Cytotoxicity

Lactate dehydrogenase (LDH) release into culture medium was used as a proxy for cell membrane damage (Sigma Aldrich, Cat. # 11 644 793 001). LDH-dependent reduction of NAD⁺ transferred H⁺ to tetrazolium salts, producing formazan salts in solution and causing a colorimetric shift in response. Percent cytotoxicity values were calculated from absorbance readings of experimental samples and controls (Cytotoxicity Detection Kit, Roche). Colorimetric LDH tests were conducted across four technical replicates per condition and were complemented with four technical replicates for high controls by dose (Triton X-100 administered post-exposure).

Total Antioxidant Capacity

Antioxidant concentration in culture medium was assessed using the Total Antioxidant Capacity Assay Kit (Sigma Aldrich, Cat. # MAK187) according to manufacturer's protocols across three biological and three technical replicates per condition. Cellular production of non-enzymatic antioxidants was measured by protein and small molecule antioxidant reduction of Cu^{2+} by Cu^+ chelation of colorimetric probes. Absorbance values were read at 570 nm and antioxidant concentration calculated versus known values generated from the Trolox standard curve.

Endothelin-1

Endothelin-1 ELISA (Abcam, Cat. # ab133030) was conducted according to the manufacturer's protocol. Culture fluid was assessed for secreted ET-1 concentration after 4- and 24-hours post-exposure recovery periods across three biological and three technical replicates per condition. Absorbance was read at 450 nm (BioTek Synergy H1 Plate Reader, Agilent Technologies) immediately after the addition of stop solution. Absorbance values were blank corrected before calculating protein concentration (pg/mL) values from the standard curve.

Interleukin-6 and Interleukin-8

Modulation of Interleukin-6 (IL-6) and Interleukin-8 (IL-8) expression was assessed through ELISA quantification of protein concentration to examine acute immune and inflammatory response in BEAS-2B culture. IL-6 and IL-8 ELISA assays were conducted using Invitrogen Human IL-6 and IL-8 ELISA kits according to the manufacturer's protocol (ThermoFisher Scientific, Cat. # KHC0061 & KHC0081). All ELISA tests were conducted in three biological replicates with three technical replicates conducted per sample per assay. Incubation steps were carried out in the dark at room temperature. Chromogenic stop solution was added immediately before the plate absorbance values were read at 450 nm. All samples were blank corrected for background signal prior to concentration calculations from the standard curve.

COMET

COMET electrophoresis was used to draw charged DNA fragments through porous agarose medium to create an observable "tail" of DNA when imaged under fluorescent filters. After the 24-hour exposure, cells in independent treatment wells were rinsed with PBS and detached according to the PromoCell Detach Kit protocol with volumes adjusted for surface area of 6-well plate wells (Corning). Detached cells were diluted and added to agarose on glass slides as described by the manufacturer's protocol (Trevigen). Double-stranded DNA was stained with SYBR green (Invitrogen, Cat. #S-7585).

Images were taken at random locations across each slide using an Olympus BX63 Fluorescent Microscope and cellSens imaging software (Olympus). All individual cell COMETs were measured within each image using Comet Assay IV software (Perceptive Instruments). Data was not taken if COMET overlap precluded tail measurement or if a cell was truncated by the image bounds. Tail moment, calculated by taking the COMET tail length times the percent DNA within the tail, was used a metric for the average extent of DNA damage caused by each treatment condition and was automatically scored by the Comet Assay IV suite.