1. Methods

1.1. Welding fume characterization and composition

Three welding fume samples were generously donated by Lincoln Electric (Cleveland, OH). All fume collection performed was consistent with the F1.2. methodology listed by the American Welding Society (https://pubs.aws.org/Download_PDFS/F1.2-2013PV.pdf). At least 2 grams of welding fume particulate was provided, and run with 95% argon (Ar) and 5% carbon dioxide (CO_2) gas shielding (with the exception of SMAW-SS), using the following electrodes: gas metal arc welding using a MS ER70S-3 electrode (GMAW-MS), gas metal arc welding using a ER308L Si SS electrode (GMAW-SS), and shielded metal arc welding using an Excalibur 307L-17 SS electrode (SMAW-SS).

In order to determine the amount of Cr(VI) present, Lincoln Electric performed ion chromatography, consistent with OSHA method ID-215. The detection of all other elements was also determined by Lincoln Electric using x-ray fluorescence.

1.2. Welding fume particle size

The distribution of welding fume particle sizes in suspension [50% dispersion medium (DM), 50% ultra-pure sterile water] was measured using Nanosight NS300 Nanoparticle Tracking Analysis software (Malvern, UK). The DM was prepared as described by Porter *et al*. and consisted of a final concentration of 0.6 mg/ml mouse serum albumin (Millipore Sigma, St. Louis, MO) and 0.1% v/v 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (Millipore Sigma, St. Louis, MO) in phosphate buffered saline (PBS), pH 7.4. Glucose was not added to the DM since RPMI medium already contains 2g/L glucose.

1.3. Welding fume preparation

In order to separate the solubilized fraction from the non-solubilized fraction, welding fumes were prepared at 1 mg/ml stock solutions in DM and incubated for 24 h at 37 °C in a shaking water bath.

DM was used since the high salt concentration of phosphate buffered saline has been shown to enhance welding fume particle agglomeration, as does the presence of fetal bovine serum and other proteins normally found in culture medium. The following morning, welding fume samples were centrifuged at 12,000 x g for 30 min. The supernatants (soluble fraction) were carefully recovered and filtered using a 0.22 µm polyethersulfone membrane filter (Millipore Corp., Bedford, MA) so as not to disturb the welding fume pellets at the bottom of the tube (non-soluble fraction). This method has been used by other investigators. The pellets were rinsed, weighed and re-suspended in dispersion medium to obtain 1 mg/ml solutions. In order to determine the amount of metals present in the soluble fraction, the United States Environmental Protection Agency Method 200.7, Version 4.4 was performed, using inductively coupled plasma-optical emission spectroscopy (ICP-OES) instrumentation [26].

1.4. Endotoxin analysis

In order to test for the presence of gram-negative bacterial endotoxin on all the welding fume samples, the endpoint chromogenic limulus amebocyte lysate (LAL) assay was used (Lonza; Walkersville, MD). Each welding fume was mixed with LAL and incubated for 10 min at 37 °C. At the end of the incubation, a chromogenic substrate was added, and samples were incubated for an additional 6 min. An acidic “stop” solution was added, and the absorbance was spectrophotometrically determined at 405 nm. Absorbance was directly proportional to the amount of endotoxin present. The standard curve reflected endotoxin unit/mL (EU/mL) using *E. coli* 0111:B4 endotoxin.

1.5. Cell culture

The HTR-8/SVneo cell line (ATCC; Manassas, VA) is often used to study placental function since the cell population consists of normal trophoblasts, and not placental choriocarcinoma cells. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 50 mg/ml of

penicillin/streptomycin (Invitrogen Life Sciences; Grand Island, NY). Cells were maintained at 37 °C in a 5% CO_2 in air incubator and passaged using 0.25% trypsin/0.53 mM EDTA (Sigma-Aldrich, St. Louis, MO). Four independent experiments were performed with 3 replicates of each treatment in each experiment, and assays were performed in duplicate. Values from each experiment were averaged resulting in a final sample size of n=4 for each condition.

1.6. Scanning electron microscopy

Particles were diluted 1:100 in filtered distilled water. An aliquot of 0.5 ml was vacuum filtered onto a 0.2 micron polycarbonate filter, and the filter was affixed onto a 13 mm aluminum stub mount using double stick carbon tape. The mounted filter was then sputter coated with gold-palladium for 2 min. The particles were imaged using a Hitachi S4800 field-emission scanning electron microscope (Tokyo, Japan) at 5 kV.

2.7. Transmission electron microscopy

Suspended, fixed cells were pelleted and embedded in 4% agarose. The cells were then post-fixed with osmium tetroxide followed by en-bloc staining with 1% tannic acid and 0.5% uranyl acetate. A graded series (50%, 70%, 90% and 100%) of alcohols were used for dehydration. Propylene oxide served as an infiltrating agent before embedding the cells in epoxy resin, and polymerizing in a 60 °C oven for 48 h. The resulting blocks were cut with a Leica EM UC7 Ultramicrotome at 70 nm thickness. The sections were placed on 200 mesh copper grids and stained with 4% uranyl acetate and Reynold's lead citrate. The samples were imaged using a JEOL 1400 transmission electron microscope (Tokyo, Japan).

2.8. Cellular viability

The water-soluble tetrazolium (WST-1) assay was used to determine the effect(s) of welding fume exposure on the viability of cells (Millipore Sigma, St. Louis, MO). Cells were seeded at a density of 6 x 10^4 cells in 96-well plates. After a 24 h growth period, cells were incubated with welding fume at final concentrations of 10 μg/ml and 100 μg/ml for either 4 h or 24 h.

2.9. Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) trapping with 5'5-dimethylpyrroline N-oxide (DMPO) was used to detect the presence of short-lived free radicals. The ability of MS and SS welding fumes to produce the hydroxyl radical ($^{\bullet}OH$) under Fenton-like reaction conditions in cultured cells was determined using a quartz flat cell assembly and Brüker EMX spectrometer (Billerica, MA). HTR-8/SVneo cells were used at a final concentration of 2 x 10^6 cells/ml, along with 250 μg/ml welding fume, and 100 mM DMPO, which were mixed with sterile PBS and incubated at 37 °C for 5 min before being loaded into the flat cell for analysis. Peak heights were representative of relative levels of spin-trapped $^{\bullet}OH$ radicals.

2.10. Intracellular ROS

Cells were seeded at a density of 6 x 10^4 cells/well in 96-well plates and incubated with 2',7'-dichlorohydrofluorescin diacetate (DCFH-DA), a cell permeable fluoroprobe, at a final concentration of 1 mM in serum-free medium for 45 min at 37 °C. Cells were washed twice in sterile PBS and medium was subsequently added back into the wells along with 10 μg/ml or 50 μg/ml of welding fumes, or 1 mM Cr(VI) as a positive control. Cells were then incubated for 0.5, 2, 4, and 6 h at 37 °C. Plates were read at 485 nm excitation/520 nm emission at the end of each respective time point to measure

ROS production. For negative controls, medium and welding fume were plated in wells in the absence of DCFH-DA, and readings were subtracted from those taken when exposed cells were present to account for any autofluorescence.

2.11. Cytokine production

A V-plex, pro-inflammatory MULTI-SPOT assay system (Meso Scale Deliveries, LLC; Rockville, MD) was used to measure the production of IL-1β, TNFα, IL-6 and IL-8 in culture supernatants. Samples were processed according to the manufacturer's instructions and results were quantified using electrochemiluminescence with a MESO Quickplex SQ120 instrument.

2.12. Invasion assay

A CytoSelect™ 24-well kit was used (Cell Biolabs, Inc.; San Diego, CA) for invasion assays. Cells were plated at a concentration of 7.0×10^5 cells/ml in serum free medium on inserts coated with a uniform layer of basement membrane matrix solution, and welding fume at a final concentration of 50 µg/ml was added to inserts for 24 h. Medium containing 10% fetal bovine serum was used in the lower chamber of the invasion plate to serve as the chemoattractant. After a period of 24 h, invaded cells were stained and quantified using an Olympus IX 70 inverted microscope (Olympus; Waltham, MA) and Simple PCI software was used to obtain images. Inserts were then treated with an extraction solution and allowed to incubate for 10 min at room temperature on an orbital shaker. From each sample, 100 µl was then transferred to a 96-well plate and the OD was measured at 560 nm.

2.13. Statistical Analysis

Statistical analyses of results were run using either one-way or two-way analysis of variance (ANOVA) models using Tukey's post hoc comparisons on GraphPad Prism version 8.02 (San Diego, CA). Differences were regarded as significant when $P < 0.05$.