Supplement Article

# Chemical Elements in Electronic Cigarette Solvents and Aerosols Inhibit Mitochondrial Reductases and Induce Oxidative Stress

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## Abstract

**Introduction:** Chemical elements and their toxicity were evaluated in electronic cigarette (EC) solvents, fluids, and aerosols.

**Aims and Methods:** Element identification and quantification in propylene glycol (PG), glycerin (G), refill fluids before and after use, and aerosols was done using inductively coupled plasma optical emission spectrometry. Cytotoxicity and oxidative stress were evaluated using in vitro assays.

**Results:** Seven elements were present in PG, G, and popular refill fluids, and they transferred to aerosols made with ECs. Selenium was in all products (0.125–0.292 mg/L), while arsenic, aluminum, and tin were frequently in solvent and refill fluid samples at lower concentrations. Iron, chromium, copper, nickel, zinc, and lead were only detected in fluid after EC use, indicating they came from heated atomizers. Elements transferred most efficiently to aerosols made with second-/third-generation ECs. Of the elements in fluid, selenium and arsenic were the most cytotoxic to human bronchial epithelial cells (BEAS-2B) and pulmonary fibroblasts in the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Selenium increased superoxide production in mitochondria and nucleoli and elevated selenoprotein H in nucleoli of BEAS-2B cells at concentrations found in EC aerosols (10 nM or 0.002 mg/L).

**Conclusions:** Elements in EC aerosols came from both e-fluids and atomizing units. Within second-/third-generation products, transfer became more efficient as power increased. In vitro responses occurred at concentrations of selenium found in some EC aerosols. Human exposure to chemical elements in ECs could be reduced by regulating (decreasing) allowable EC power and by improving the purity of PG and G.

**Implications:** PG, G, refill fluids, and e-fluids contained potentially toxic chemical elements that transferred to aerosols. Transfer was more efficient in second- and third-generation EC products and increased as power increased. Selenium and arsenic were the most cytotoxic of the elements tested in the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Selenium tetrachloride-induced oxidative stress in BEAS-2B cells, but not in human pulmonary fibroblasts. All fluids contained selenium above the concentration that induced oxidative stress in human bronchial epithelial cells. Selenium increased superoxide in mitochondria and nucleoli and increased selenoprotein H, a redox responsive DNA-binding protein that is upregulated by superoxide and an indicator of nucleolar stress. EC users are exposed to elements in aerosols, which may with chronic exposure contribute to diseases associated with oxidative stress.

## Introduction

The Food and Drug Administration and Centers for Disease Control recently expressed concern about the safety of vaping given the rapid increase in "electronic cigarette (EC) or vaping associated lung injury" (EVALI).[1,2] While the CDC found vitamin E acetate in the lungs of some EVALI patients,[3,4] the full range of possible causes of EVALI is not known. Some EVALI patients presented with burns to their lung tissue,[5] which would not be consistent with vitamin E acetate toxicity, and some EVALI patients used products that did not contain vitamin E acetate.[6] One EVALI patient was diagnosed with giant cell interstitial pneumonia secondary to cobalt exposure,[7,8] consistent with heavy metal poisoning. EC aerosols contain elements/metals,[9–13] and some, such as chromium, nickel, and lead, can cause serious health effects.[14,15]

Most metals in EC aerosols originate in the atomizers,[9–13,16] and their aerosol concentrations are often highest in second- and third-generation ECs, which operate at higher power.[11,17,18] However, some elements in EC aerosols, such as arsenic, selenium, and manganese, have not been found in atomizers.[9,12,16,19] This could be because the methodology used to characterize metals in atomizers is about 100 times less sensitive than that used to quantify metals in EC aerosols.[9,10,12,13,19,20] It is also possible that the EC solvents, which have rarely been studied,[12] are a source of some aerosol elements.

Our purpose was to identify and quantify the elements in propylene glycol (PG) and glycerol (G), commercial refill fluids, and cartomizer fluids (e-liquid) before and after use in an EC and determine how efficiently these elements transfer to aerosols. Elements identified in fluids and aerosols were tested for toxicity using bronchial epithelial cells (BEAS-2B) and human pulmonary fibroblasts (hPFs) to determine if the concentrations found in EC aerosols produce harmful effects on respiratory cells, which could with chronic use contribute to respiratory disease and may account for some cases of EVALI.

## Materials and Methods

### Solvents, ECs, Refill Fluids, Elements, and Reactive Oxygen Species Reagents

All solvents, ECs, refill fluids, elements, and reactive oxygen species reagents were inventoried and stored at room temperature and are listed in Supplementary Table 1. PG and G were purchased from chemical vendors. Thirty-three popular refill fluids were purchased from local vape shops. "Breezy Shake" was purchased on three separate occasions to compare element variations within one product. Four brands of cartomizers, five batteries, four tanks, and two replaceable dripping atomizers (RDA) were used to generate aerosols.

### Preparation of Solvents, Refill Fluids, E-liquids, and Aerosols for Elemental Analysis

The concentrations of elements in solvents, refill fluids, and cartomizer e-liquids were analyzed before and after vaping. 500 μL of e-liquid was dissolved in 9.5 mL of 98% deionized water/2% nitric acid.[16] Samples were stored in nitric acid-washed 15 mL conical vials at 4°C until analyzed. For ECs, three e-liquid samples were analyzed before and after use. Elements in 2% nitric acid served as a blank and were subtracted from test samples.

Aerosols and room air controls were generated using a smoking machine, collected using two glass impingers in tandem, and analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 7300 DV ICP-OES, Perkin-Elmer, Waltham, MA).[9–11,19] Batteries were operated at the highest voltage (2.8–5.1 V), which varied with the battery, using 4.3 second puffs. Three puffing protocols were used: continuous puffing (one puff/minute without breaks), continuous extended puffing (two consecutive continuous puffing sessions on the same device without adding e-liquid), and interval puffing (5–15-minute breaks between every 10 puffs).[11] Samples were stored in nitric acid-washed tubes. Each product was tested three times using the running conditions and standards described in Supplementary Content. Transfer efficiencies were computed to determine the percentage of an element that transferred from the fluid to the aerosol.

### Metal Reagents for Cytotoxicity Assays

The chloride salts of element stock powders were trace metal basis grade (99.99%). 1 M element ion stock solutions ($Al^{3+}$, $As^{3+}$, $Cu^{2+}$, $Fe^{2+}$, $Pb^{2+}$, $Se^{4+}$, $Sn^{2+}$, and $Zn^{2+}$) were prepared in distilled water then diluted in culture medium immediately before use for cytotoxicity assays.

### Cell Culture

Human bronchial epithelial cells (BEAS-2B) (American Type Culture Collection, Rockville, MD) were cultured in serum-free bronchial epithelial growth medium (BEGM) (Lonza Group AG, Walkersville, MD) in T-25 culture flasks (Corning Inc., Corning, NY) that were precoated with BEBM fortified with collagen (30 mg/mL), fibronectin (10 mg/mL), and bovine serum albumin (10 mg/mL).[21,22]

Human pulmonary fibroblasts (hPFs) were cultured in poly-L-lysine-coated T-25 culture flasks using complete fibroblast medium (ScienCell) containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin.[23] All cells were incubated at 37°C/5% $CO_2$ with media changes every 48 hours until reaching 80%–90% confluency. For subculturing, cells were dissociated with a 0.25% trypsin/EDTA containing 0.5% polyvinylpyrrolidone (PVP) and passaged at 70%–80% confluency to freshly coated flasks.

### Cytotoxicity

The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was used to observe mitochondrial reductase activity following treatment with elemental salts. This assay, which characterizes the cytotoxicity of chemicals, was performed as described previously.[23] BEAS-2B cells and hPFs were passaged on to 96-well plates (4000 cells/well), allowed to adhere 48 hours, then exposed to elements (concentrations ranged from 1 nM to 1 mM) for 24 hours at 37°C/5% $CO_2$ after which the MTT assay was performed, and absorbances were collected at 570 nm using a Synergy HTX microplate reader (BioTek, Winooski, VT). Each element was tested in three independent experiments. Absorbance data were normalized to the untreated control and means and standard errors of the mean (SEM) were determined using GraphPad Prism (La Jolla, CA).

### Reactive Oxygen Species

BEAS-2B cells or hPFs were attached in coated chamber slides (ibidi, Fitchburg, WI) (6000 cells/well) for 24 hours, then exposed to 1, 10, or 100 nM selenium for 1, 4, or 24 hours, after which 1 μM of MitoSOX Red (Thermo-Fisher, Waltham, MA), a selective probe for superoxide, was added for 2 minutes prior to washing and imaging at 60× with a Nikon Ellipse inverted microscope.[24] In some experiments, cells were incubated for 30

minutes with 100 µM of 2-β-mercaptoethanol (2βM), 5 mM of *N*-acetyl-ʟ-cysteine (NAC), or 100 µM of ascorbic acid prior to adding 10 nM of selenium tetrachloride. The negative control was cultured in BEGM medium. The positive control was incubated with 10 nM of selenium tetrachloride without antioxidant. Chamber slides were incubated in MitoSOX Red and imaged as described above.

CellROX Green fluoresces upon oxidation by superoxide and ·OH and subsequent binding to DNA, limiting its presence to the nucleus and mitochondria.[25] To localize and quantify CellROX Green, BEAS-2B cells were treated with 1, 10, and 100 nM selenium tetrachloride for 4 hours, followed by 5 µM CellROX Green for 30 minutes, three rinses with PBS (+), and fresh medium. Cells were imaged live using a Nikon Eclipse Ti-E microscope with a 37°C, 5% $CO_2$, and 90% relative humidity-regulated stage top LiveCell Incubation Chamber (Pathology Devices, Inc., San Diego, CA). Integrated density (sum of all the pixel intensities within a region) was determined using ImageJ ($n$ = 40–55/group). An area with no fluorescence was used to determine background, which was subtracted from the integrated density to obtain the corrected total cell fluorescence (TCF).

Selenoprotein H (SelH) was localized in fixed BEAS-2B cells and hPFs using a primary antibody followed by an Alexa Fluor 488 donkey anti-rabbit secondary antibody (Supplementary Table 1).[26]

## Results

### The Total Concentration of Elements in EC Solvents and Refill Fluids

Element concentrations were determined for six brands of PG and seven brands of G (Figure 1A and B; Supplementary Tables 3 and 4). While some products contained arsenic, tin, silicon, and sodium, all products had similar concentrations of selenium (PG = 0.125–0.201 mg/L; G = 0.129–0.292 mg/L). Tin was in all the first samples but absent or lower in concentration in the second and third samples (Figure 1A and B). Tin may have been a contaminant in the first sample or alternatively may have absorbed to the container surface between the first and second sampling. The 33 samples of refill fluids contained total element concentrations ranging from 0.102 to 1.337 mg/mL (Supplementary Table 5). The elements found included selenium, sodium, calcium, silicon, tin, aluminum, potassium, and magnesium (Figure 1C). Selenium was in all samples at concentrations (range = 0.185–0.278 mg/L) similar to those in PG and G.

Total and individual element concentrations varied between bottles of "Breezy Shake" (Black Market Manufacturing), but within a bottle, elemental signatures were similar when analyzed on different days (Figure 1D). The elements in the "Breezy Shake" samples were the same as those in PG and G (Supplementary Figure 1A). Selenium was in all samples (concentration range = 0.115–0.220 mg/L), including other "Black Market Manufacturing" refill fluids (Supplementary Figure 1B; Supplementary Table 6). The other Black-Market Manufacturing products did not contain as many other elements as "Breezy Shake" (Supplementary Figure 1B).

### Elements in EC Fluids Before and After Use and in Aerosols

To evaluate the source of elements in fluids and their transfer to aerosols, the concentrations of 16 elements were measured in fluids before and after vaping and in aerosols made with four cartomizers

(prefilled) and six clearomizers/mods filled with "Breezy Shake" (Figures 2 and 3, Supplementary Figures 2–7, and Supplementary Tables 7–14). Clearomizer/mod aerosols were made using continuous or interval puffing. Cartomizers and clearomizers are first- and second-generation ECs, respectively, while mods are third generation.[17]

Arsenic was detected in the e-fluid before and after use in similar concentrations in all brands of cartomizers (Figure 2A) and in five clearomizers/mods (Figure 2B). Arsenic transferred to the aerosol of V2 Cigs and all clearomizers/mods, except Aspire (Figure 2A and B). All clearomizers/mod aerosols with arsenic were prepared using Bottle 3, which contained arsenic (Figures 1D and 2A, B). Clone fluid was from Bottle #1, which lacked arsenic.

Before use, chromium was only in the e-fluid in V2 Cigs (Figure 2C and D). After vaping, chromium was in fluids from V2 Cigs and all clearomizers/mods (Figure 2C and D). However, chromium only transferred to the aerosols in Smok (both puffing protocols) and Tsunami (continuous) (Figure 2C and D).

Copper was detected in the fluid before and after use in BluCig Plus and V2 Cigs. In the clearomizers/mods, three products (Protank, Kanger T3S, and Smok) had "Breezy Shake" with copper in their fluid before use, while after use it was elevated in all fluids (Figure 2E and F). Copper transferred to aerosol in all clearomizers/mods, except Clone (Figure 2E and F).

Iron was in the fluid before and after use in all cartomizers, except Vuse Vibe (Figure 2G) and was elevated in two brands after use. Iron was not detected in the fluid before use in any clearomizers/mods (Figure 2H), but was in fluid after use in all brands. Iron did not transfer to any cartomizer aerosols (Figure 2G), but did transfer to aerosols made with Clone, Smok, and Tsunami (Figure 2H).

Lead was not detected in any cartomizers. In clearomizers/mods, lead was detected in the fluid before use in Protank and Smok, and in the fluid after puffing in all samples, except Tsunami (interval). Lead transferred to aerosols, often with high efficiency, in all samples of Protank, Smok, and Tsunami, and in Clone (continuous) (Figure 2I).

Nickel was in fluid before and after use and in the aerosol of only one cartomizer (V2 Cigs) (Figure 3A). Nickel was not in the fluid before use in any clearomizers/mods, but was in the fluid of all brands after use; it transferred to the aerosol of Protank (continuous, continuous extended), Kanger T3S (continuous), Smok (continuous, interval), and Tsunami (continuous, interval) (Figure 3B).

Selenium was detected in the fluid before and after vaping in all brands of cartomizers and clearomizers/mods (Figure 3C and D). Its concentration was not higher after use. In cartomizers, selenium transferred to the aerosol only in V2 Cigs (Figure 3C); however, it transferred to all clearomizers/mods aerosols, except Aspire (interval) and Clone (continuous) (Figure 3D).

Silicon was in the fluid before and after use and in the aerosols in all brands of cartomizers (Figure 3E). For clearomizer/mods, silicon was in the fluid before use in brands that were made using "Breezy Shake" Bottles 1 and 2, which had silicon (Figures 1D and 2F). It transferred to the aerosol in 9 of 13 clearomizers/mods.

Tin was in the fluid before and after use, and in the aerosol of all brands of cartomizers (Figure 3G) and clearomizers/mods, with the exception for Kanger T3S and Clone made using the continuous protocol (Figure 3H).

Zinc was in BluCig Plus fluid before and after use and in low concentrations in Mark Ten XL and V2 Cigs (Figure 3I). Zinc was not detected in the fluid before use in any clearomizer/mod except Kanger T3S, which had low levels. Zinc was in the fluid after use in
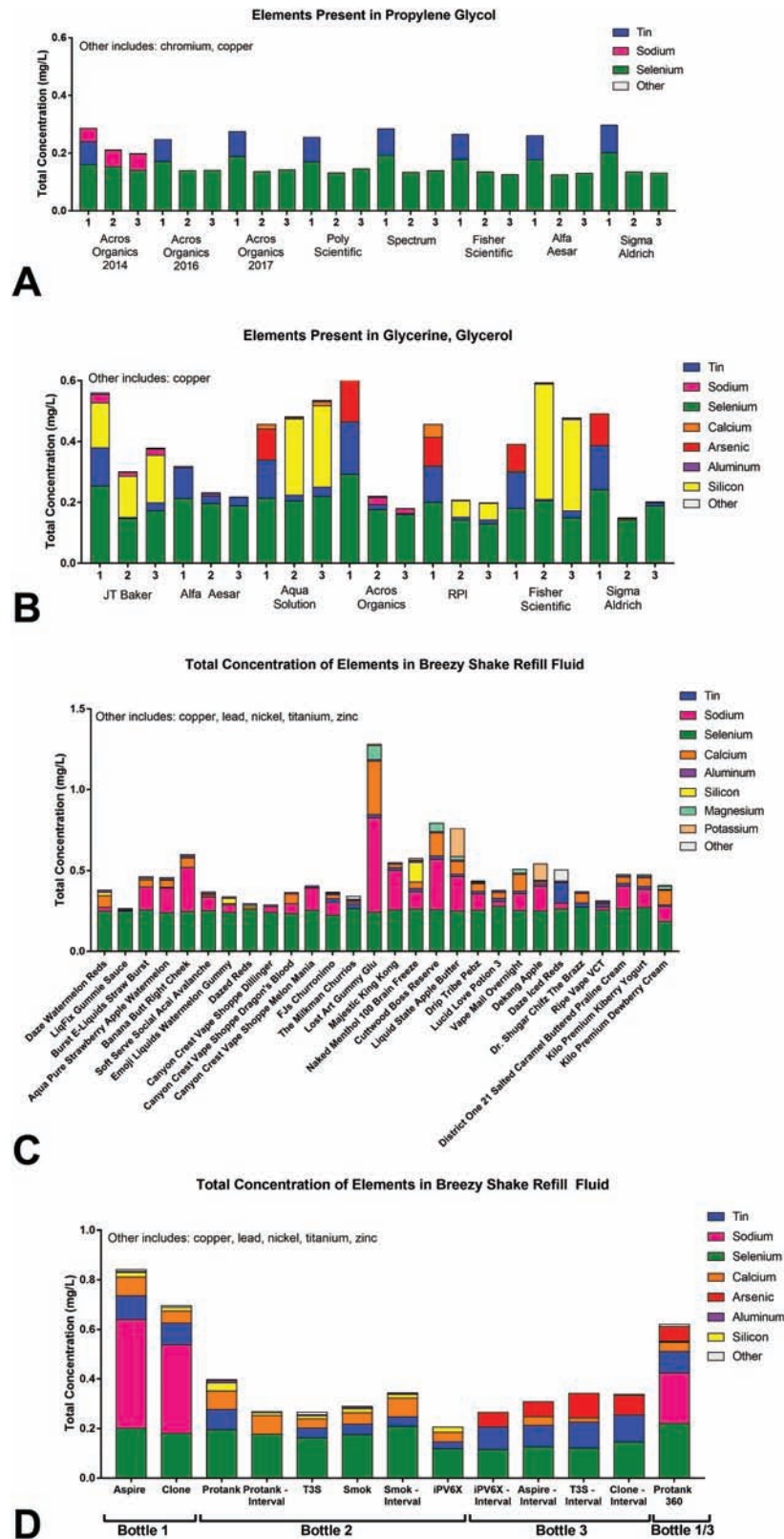
**Figure 1.** The concentrations of elements in propylene glycol, glycerin, and refill fluids. (A) The concentration of elements in six brands of PG and (B) seven brands of G. For A and B, the results of three independent trials are shown (1–3). Products were quantified on different days. Trial 1 was separated in time from trials 2 and 3, which may account for some differences. (C) The concentration of individual elements in 28 popular refill fluids. (D) The concentration of individual elements before use in three separate bottles of a popular refill fluid (Breezy Shake). G = glycerin, PG = propylene glycol.
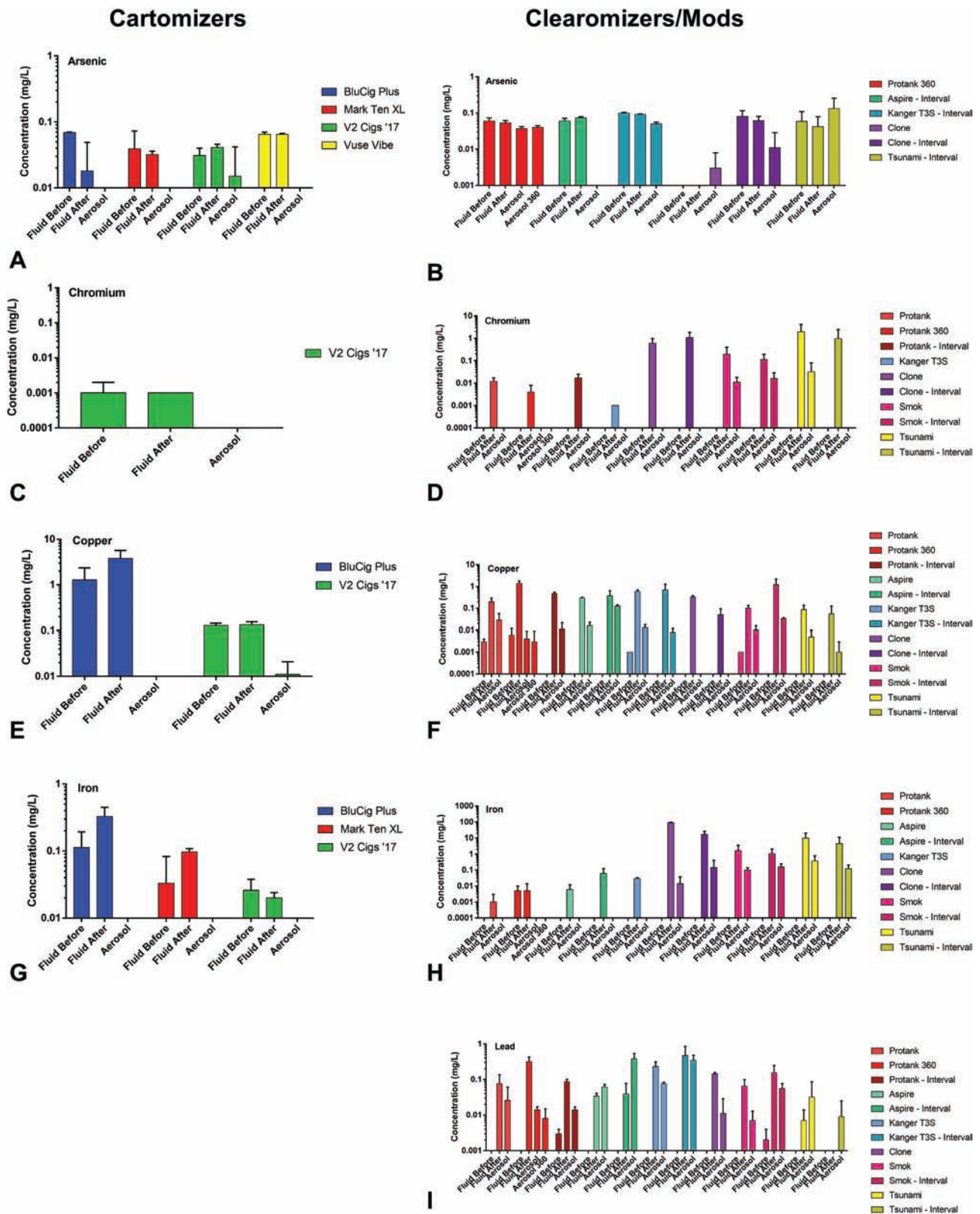
**Figure 2.** Average concentration of individual elements in EC fluids before use, after use, and in aerosols. The concentrations of arsenic (A, B), chromium (C, D), copper (E, F), iron (G, H), and lead (I) for each brand of cartomizers (A, C, E, G) and clearomizers/mods (B, D, F, H, I). Concentrations are presented in mg/L; all concentrations are the average of three independent measurements. Only brands with concentrations above the limit of detection are presented in the graphs. EC = electronic cigarette.
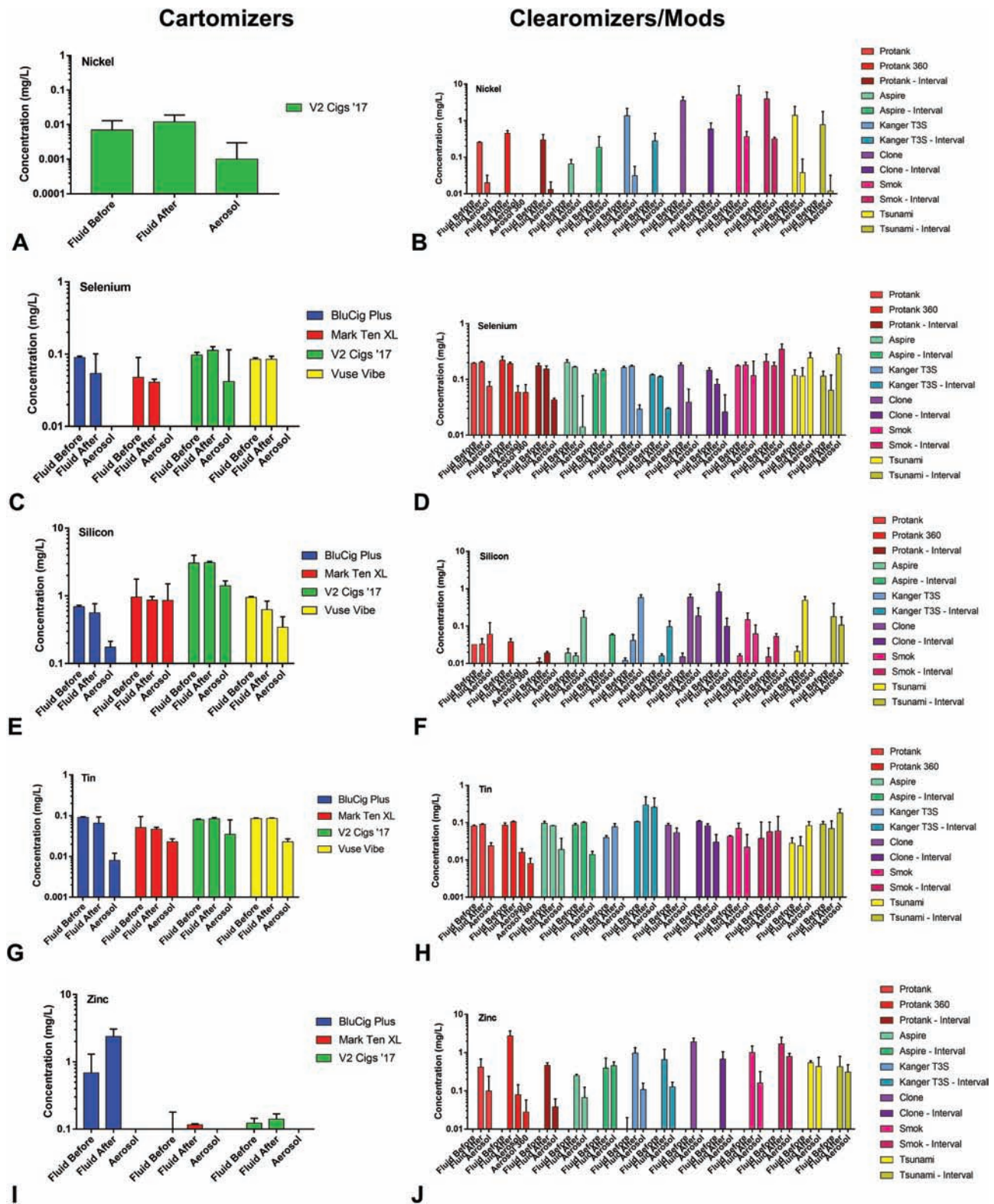
**Figure 3.** Average concentration of individual elements in EC fluids before use, after use, and in aerosols. The concentration of nickel (A, B), selenium (C, D), silicon (E, F), tin (G, H), and zinc (I, J) for each brand of cartomizers (A, C, E, G, I) and clearomizers/mods (B, D, F, H, J). Concentrations are presented in mg/L; all concentrations are the average of three independent measurements. Only brands with concentrations above the limit of detection are presented in the graphs. EC = electronic cigarette.

all clearomizers/mods (Figure 3J). Zinc did not transfer to cartomizer aerosols (Figure 3I), but did transfer to clearomizers/mods aerosols (often with ~100% efficiency), except for Clone (Figure 3J).

## Cytotoxicity of Elements in EC Aerosols

Concentration–response curves, including $IC_{50}$s and $IC_{70}$s, for each element show absorbance normalized to the percent of the control vs. the concentrations of each element (Figure 4). The $IC_{50}$ and the $IC_{70}$ values are the concentrations equal to 50% and 70% of the control values, respectively. ISO protocol 10993-5:2009(E) categorizes chemicals "cytotoxic" if they produce an absorbance in the MTT assay that is <70% of the untreated control. Both cell types were similarly affected by treatments with BEAS-2B cells being more sensitive than hPFs in some cases (eg, arsenic, selenium, and copper). The graphs in Figure 4, which are arranged from most to least potent, show that arsenic and selenium were the most cytotoxic elements for both cell types.

The effect of selenium on oxidative stress in the presence and absence of antioxidants (NAC, ascorbic acid, and 2βM) was examined in BEAS-2B cells labeled with MitoSOX Red to localize superoxide after 1 and 4 hours of treatment with 10 nM (0.002 mg/L) selenium tetrachloride (Figure 5). In treated BEAS-2B cells, but not in the controls or antioxidant treated groups, selenium induced strong fluorescence of the mitochondria, indicative of superoxide production at 1 hour. After 4 hours of exposure, fluorescence was weaker in the mitochondria, but strong in the nucleoli (Figure 5). In contrast, hPFs did not respond to selenium at concentrations of 10 nM (0.00221 mg/mL; Figure 5) or 100 nM (0.02 mg/L) (Supplementary Figure 8).

To confirm MitoSOX Red data, BEAS-2B cells were treated with CellROX Green after selenium exposure. Treatment for 4 hours with 10 or 100 nM (0.002 and 0.02 mg/L) selenium produced green fluorescence in the mitochondria and nuclei, including the nucleoli, indicative of reactive oxygen species (ROS) generation. Fluorescence was significantly elevated relative to the untreated control in both the
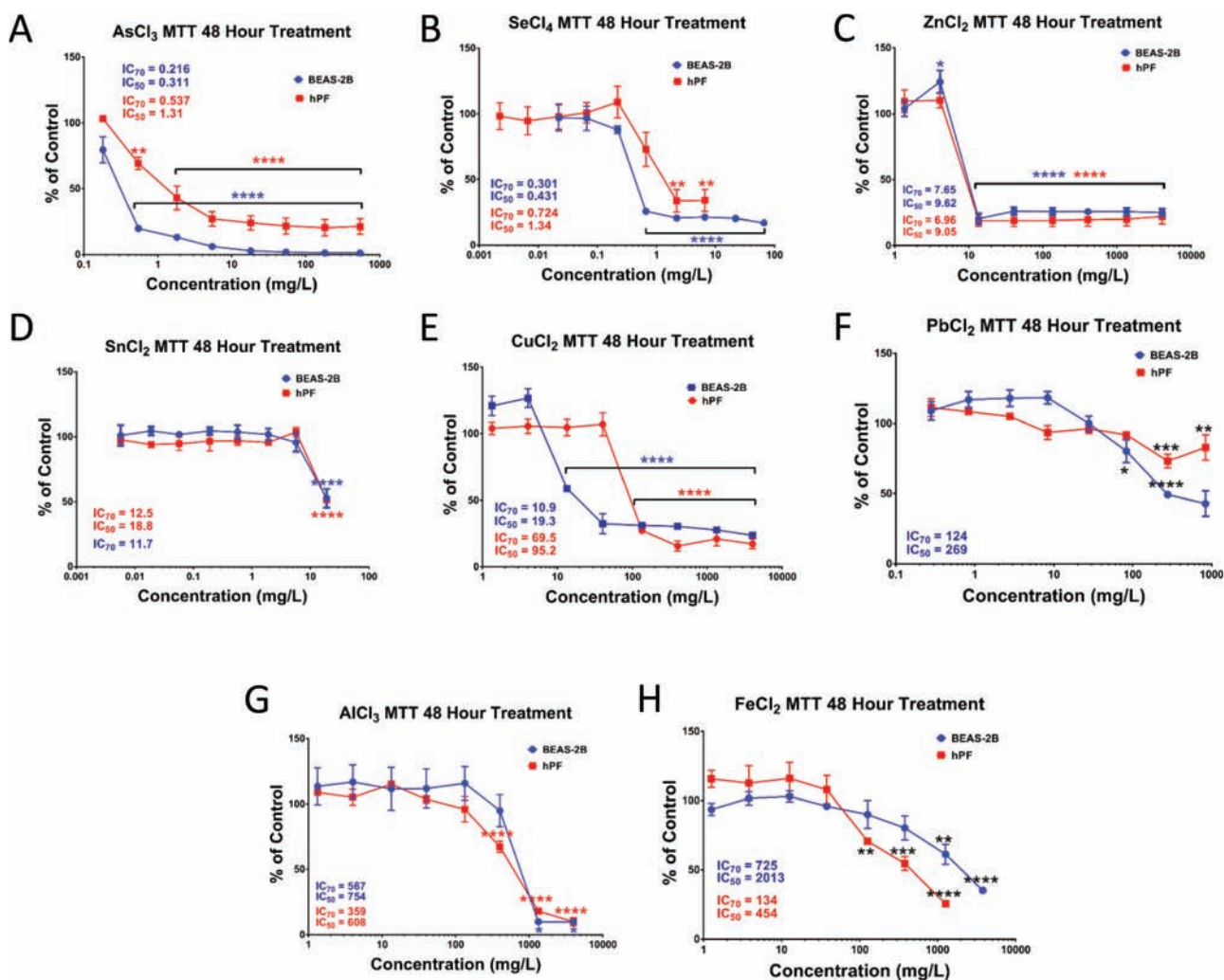


**Figure 4.** Concentration–response curves for individual elements identified in PG, G, and refill fluids. MTT assays were performed using BEAS-2B cells and hPFs treated with various concentrations of individual elemental salts: (A) arsenic, (B) selenium, (C) zinc, (D) tin, (E) copper, (F) lead, (G) aluminum, and (H) iron. Data are plotted as a percentage of the untreated controls. Each point is the mean ± SEM of three independent experiments. $IC_{50}$s were computed with GraphPad Prism software (GraphPad, San Diego, CA, USA) using the log inhibitor versus normalized response-variable slope with the top and bottom constraints set to 100% and 0%, respectively. $IC_{70}$s were read off the graphs. Means that were significantly different were determined using an analysis of variance. When significance was found, means were compared with the lowest concentration using Dunnett's post hoc test. Concentrations that were significantly different than the lowest concentration are indicated by *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. G = glycerin, hPFs = human pulmonary fibroblasts, MTT = 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, PG = propylene glycol, SEM = standard errors of the mean.
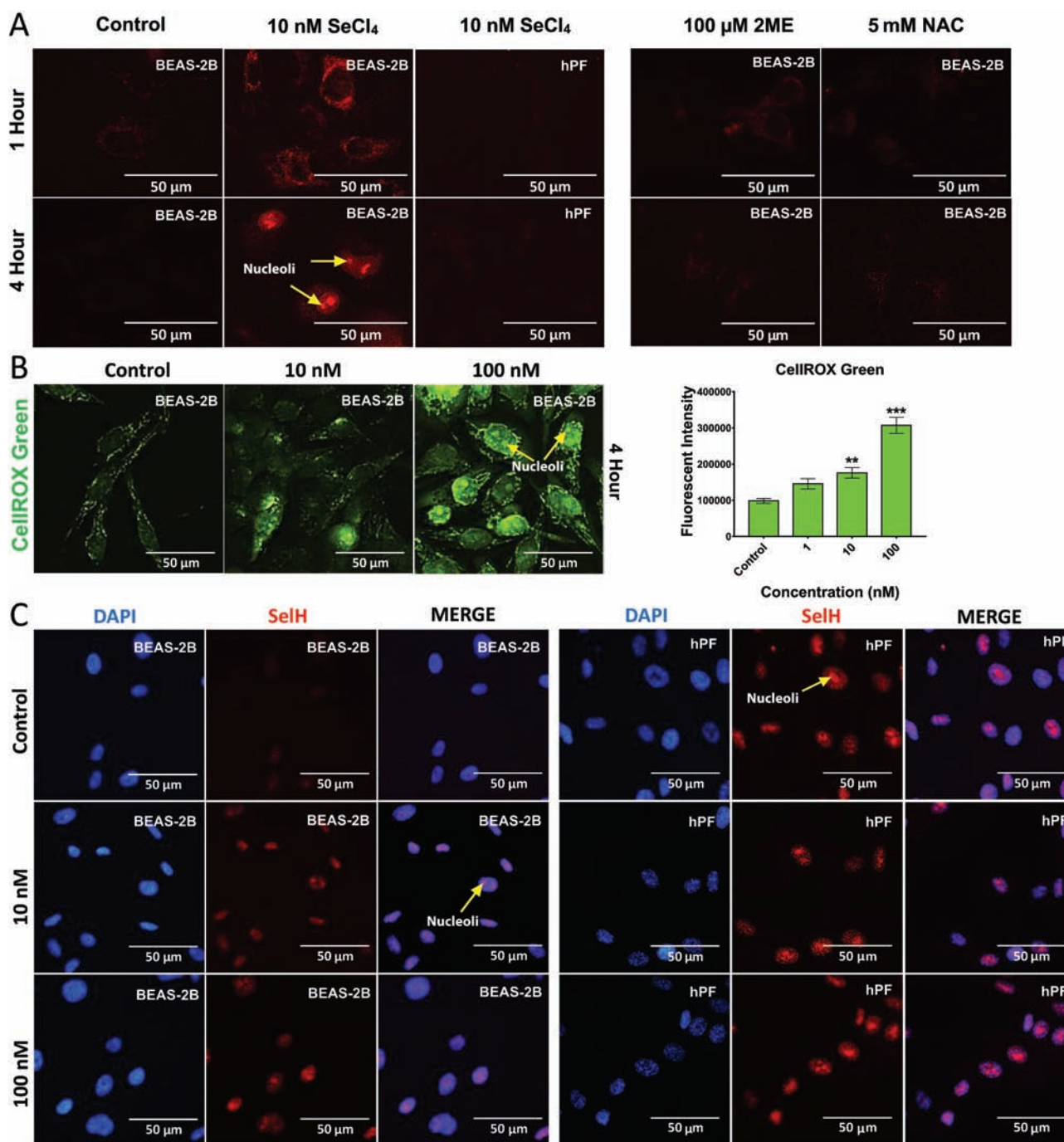
**Figure 5.** Induction of oxidative stress by selenium in BEAS-2B cells but not in hPFs. (A) Superoxide in BEAS-2B cells and hPFs treated with 10 nM (0.002 mg/L) SeCl$_4$ for 1 or 4 hours then stained with MitoSOX Red. Some BEAS-2B cells were pretreated with antioxidants (2-mercaptoethanol [2βM] or *N*-acetyl cysteine [NAC]) then treated with SeCl$_4$. (B) SeCl$_4$ treated BEAS-2B cells stained with CellROX Green (nuclear and mitochondrial ROS fluorophore) to confirm the nuclear and nucleolar fluorescent signal found with MitoSOX Red. Fluorescent intensity graphs showing means and SEM for 40–55 cells/mean. (C) Immunostaining of selenium treated BEAS-2B cells and hPFs showing SelH localization in the nucleoli of control hPFs and selenium-treated BEAS-2B cells. Arrows in A and B indicate nucleoli. hPFs = human pulmonary fibroblasts, ROS = reactive oxygen species, SelH = selenoprotein H, SEM = standard errors of the mean. Concentrations that were significantly different than the lowest concentration are indicated by **$p < .01$, ***$p < .001$.

10 and 100 nM concentrations (Figure 5B). SelH, a redox responsive DNA-binding protein which is upregulated by superoxide,[27] was detected in nucleoli of selenium treated, but not control, BEAS-2B cells (Figure 5C). In contrast, SelH was constitutively expressed in nucleoli of untreated control hPFs, and its fluorescent intensity was not increased by selenium treatment.

## Discussion

### Origin of Elements in EC Aerosols

Chemical elements, including heavy metals and carcinogens, are of interest because of their potential risk to health. The literature on this has been thoroughly reviewed recently.[28] Atomizing units in EC

have metal components that pass into aerosols upon heating.[9,11,12] However, relatively little is known about the contribution of EC fluids to the elements in aerosols.[12,29] Our data show that the solvents used in ECs (PG and G) contain chemical elements, some of which are potentially toxic (eg, As, Al, Si, Sn, and Se). All elements in solvents have also been found in aerosols, establishing PG and G as element sources. Silicon was in all G products except Acros Organics (99.5% pure) and Alfa Aeser (HPLC grade), which had the highest grade of purity (Supplementary Table 1). Selenium was in all solvent and refill fluids at similar concentrations. Selenium in refill fluids and e-liquids originated in PG and G, which are manufactured from petroleum (G is also extracted from plants) to remove elemental impurities, such as arsenic and selenium.[30] Arsenic copurifies with selenium, but was always lower in concentration than selenium and sometimes not detected. PG and G are further purified by high vacuum distillation to achieve an accepted purity standard (eg, ACS grade >95%, USP grade ≥99.5%, Analytical grade >99%). The limit on metals in USP grade PG and G is <5 ppm,[31,32] and this standard was met in our samples. Sodium and calcium in refill fluids may have been introduced with flavor chemicals or nicotine.

The three bottles of "Breezy Shake" refill fluid had similar, but not identical, elemental profiles, indicating batch variation within a product. This likely occurred because the manufacturer used different batches of solvents to compound the three bottles of Breezy Shake. Bottle #2 (Figure 1) had the lowest concentration of tin and lacked arsenic, while other products from the same manufacturer had only selenium and aluminum (Supplementary Figure 1), suggesting that better quality control during manufacture could reduce elements in refill fluids and make them safer. In addition to the elements that we report, cadmium, cobalt, mercury, manganese, nickel, lead, antimony, titanium, vanadium, and iron have been found in refill fluids.[12,29] Some refill fluids also contained trace amounts of pesticides and polycyclic hydrocarbons.[29]

## Elements in Fluid After Use and in Aerosols

In clearomizer/mod ECs, but not cartomizers, all elements, except selenium and tin, were elevated in e-fluid after use, and likely came from atomizer components.[9,20] Chromium, nickel, and iron are in filaments, while zinc, copper, and lead are in atomizer shells and airtubes.[20] After use, chromium concentrations were up to 1941× higher in second-/third-generation fluids than in cartomizer fluids. The highest element concentrations were in the two RDA products (Clone and Tsunami), which probably heat the hottest and in Smok which has a sub-ohm battery. Similar increases in element concentrations were observed in the fluid of ECs after vaping with a variety of second-/third-generation products owned by EC users.[12]

Nine elements on our target list were in EC aerosols. All of these are on the ATSDR's priority list of pollutants (Al, As, Cr, Cu, Pb, Mn, Se, Ni, and Zn)[15] and five are on the FDA's list of Harmful and Potentially Harmful Chemicals (As, Cr, Pb, Ni, and Se).[14] Element transfer efficiencies were higher in clearomizer/mod devices than in cartomizers, in agreement with other studies using second-/third-generation ECs.[28] Transfer efficiency varied among elements, with tin and selenium transferring efficiently in most products including cartomizers. Iron, zinc, copper, chromium, lead, and nickel, which accumulated in fluid during use, also transferred to the aerosols of clearomizer/mods. Chromium transfer was about 60%–75% efficient for Smok and Tsunami, which heat hotter than the Clone with 0% transfer. Selenium transferred well in clearomizer/mods and cartomizers, and in some second-/third-generation products

(eg, Smok and Tsunami interval), transfer exceeded 100% efficiency, suggesting this element was concentrated during vaping (a phenomenon also noted in some samples for arsenic, lead, and tin). Absorption of selenium and zinc from aerosols by humans is supported by the finding that both elements were significantly elevated in the urine of EC users vs. nonsmokers.[33] Selenium was also higher in EC users' serum when compared with serum from cigarette and cigar smokers[34] or when compared with serum of smokers or nonsmokers.[35]

## Cytotoxicity of Elements

Most work on EC elements has involved their identification and quantification.[28] While the toxicity of the individual elements in EC has been studied extensively in other contexts, their toxicity in EC users has not been well characterized. As a preliminary step to understanding toxicity of the major elements in EC aerosols, we conducted several experiments using mitochondrial (MTT) and oxidative stress assays. Arsenic and selenium were the most cytotoxic of the elements tested in the MTT assay, indicating inhibition of mitochondrial reductases in BEAS-2B cells at concentrations as low as 0.216 mg/L for arsenic and 0.301 mg/L for selenium. The selenium $IC_{70}/IC_{50}$s for hPFs were about two to three times higher than for BEAS-2B cells, and except for iron, this relationship held for the other elements in the MTT assay. Studies of selenium with the MTT assay have generally used cancer cells, which tolerate higher concentrations (≤1.5 to ≤12 µM)[36] than BEAS-2B cells or hPFs. A similar relationship was reported for heat-not-burn products, ie, A549 cancer-derived cells had a higher MTT $IC_{50}$ than the BEAS-2B cells.[37] Although our data cannot be extrapolated directly to EC users, the selenium $IC_{70}$ for BEAS-2B cells (0.301 mg/L) is close to the selenium concentration in Tsunami EC aerosols (0.284 ± 0.08 mg/L), suggesting it could produce an in vivo effect on mitochondria.

We focused on selenium in the oxidative stress experiments because selenium was present in all solvents, it transferred well to aerosols made with second-/third-generation ECs, and it has been associated previously with oxidative stress.[36] While selenium is required for life, cells tolerate a narrow range of selenium concentrations.[36] Since many of the antioxidant enzymes, including SelH, require selenium, too little produces oxidative stress. Paradoxically, too much selenium can generate reactive oxygen species.[38] Because the tolerated selenic range for normal cellular homeostasis is narrow, slight elevation can increase oxidative stress, which may in turn damage mitochondrial and nuclear DNA. The MitoSox and CellROX assays detected selenium-induced oxidative stress in BEAS-2B cells, elevating superoxide in both the mitochondria and the nucleoli, and further elevating SelH, an antioxidizing protein, in the nucleoli. The nucleolus is a central hub in cellular stress responses, often mediating cell cycle arrest or apoptosis.[39,40] The induction of SelH in the BEAS-2B nucleoli by selenium in conjunction with the fluorescence probe data are consistent with noncanonical "nucleolar stress." 10 nM (0.00221 mg/L) selenium, which is approximately the MitoSox $IC_{50}$, was about 195 times lower than the MTT $IC_{50}$ (0.431 mg/L) and was far lower than the concentrations found in EC aerosols made with second-/third-generation products. In contrast to BEAS-2B cells, hPFs were not responsive to selenium (even at 1 µM), probably because they have better antioxidant defenses, as indicated by the constitutively elevated SelH in their nucleoli. While we tested only selenium, other elements in EC aerosols, such as iron, chromium, and copper, can induce oxidative stress via the Fenton reaction, and some redox inactive elements (As and Pb) deplete glutathione.[41] Thus, in authentic EC aerosols, the effects of all elements

could combine to increase (or decrease) oxidative stress beyond that reported here.

### In Vivo Effects of EC Elements

While our preliminary in vitro data show evidence that chemical elements in ECs can be toxic to cells of the respiratory system and that the tested cell types differ dramatically in their ability to deal with oxidative stress, we know little about what EC elements actually do in vivo during vaping, and many factors would affect the in vivo responses. First, mixtures of elements, as found in EC aerosols, may increase or decrease toxicity above that of isolated elements, sometimes referred to as a "cocktail effect" or "mixture toxicity".[42] Moreover, elements/metals at low concentrations when tested individually, which we did, may underestimate actual toxicity of a mixture.[43] Adding to this complexity, EC elements are delivered in mixtures that also contain PG, G, nicotine, flavor chemicals, and reaction products,[44] all of which have their own toxicity profiles. For example, ethyl maltol, which is used in many e-fluids, is cytotoxic at low concentrations in the MTT assay ($IC_{50}$ ~0.1 mg/mL for BEAS-2B cells)[21] and like many flavor chemicals induces formation of free radicals.[45] Reaction products which form during aerosolization, such as acetaldehyde and formaldehyde, are often both toxic and carcinogenic.[46] Nicotine, which activates nicotinic acetylcholine receptors, can elicit opposite responses in cells depending on concentrations.[47] Further compounding this issue, some elements, such as cadmium, tend to be retained by the body, and retention can be influenced by other elements.[48] Elemental concentrations in EC aerosols are often compared with those in cigarette smoke.[9,13] While this comparison is interesting, it does not reveal information about the actual toxicity of elements in EC aerosols, since toxicity is very dependent on context and synergism/antagonism between chemicals in a mixture.

Secondly, our experiments were done in 2D cultures with continuous exposure, and single elements were tested in each assay. In contrast during vaping, elements in ECs are delivered intermittently at an air–liquid interface (ALI) as a complex mixture. Therefore, our data do not translate directly to in vivo exposures. However, controlled combinatorial testing at the ALI would be possible in future studies using information in this and other recent publications.[21,22,28,46]

Third, while there are numerous publications on elements in EC aerosol,[28] very little is known about their speciation. However, speciation is important as it affects toxicity.[49] We do not know the species of selenium in EC aerosols. It may be equivalent to what we tested, or it may be more or less toxic than reported here. It will be important in future studies to characterize the species of the chemical elements that are inhaled by EC users, determine if they build up in tissues, and evaluate the acute and chronic effects of exposure. These are not easy points to address, but should be considered in future studies on selenium and other chemical elements in ECs.

### Conclusions

Elements in EC aerosols come from both e-fluids and atomizing units. Generally, elements transferred most efficiently to aerosols made with second-/third-generation ECs, although silicon and tin also transferred well in cartomizer products. Within second-/third-generation products, transfer became more efficient as power increased. Arsenic and selenium were the most potent of eight chemicals tested in the MTT assay, and 10 nM selenium tetrachloride induced oxidative and nucleolar stress at nanomolar concentrations in BEAS-2B cells, but not in hPFs. The concentrations of selenium that induced oxidative

stress in vitro are within the range found in EC aerosols; however, in vivo toxicity will be affected by factors not evaluated in this study including: speciation, mixture toxicity, air–liquid interface exposure, and user topography. Human exposure to EC elements could be reduced by regulating (decreasing) EC power to minimize element transfer to aerosols and by improving the purity of PG and G.

### Supplementary Material

A Contributorship Form detailing each author's specific involvement with this content, as well as any supplementary data, are available online at https://academic.oup.com/ntr.

### Declaration of Interest

*The authors have no conflicts of interest. Dr. Talbot has received a grant from the National Institutes of Health and FDA for support of this work.*

### Author Contributions

Dr. Williams and Dr. Talbot had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: Williams, Loza, Ventura, and Talbot. Acquisition, analysis, or interpretation of data: All authors. Drafting of the manuscript: Williams, Ventura, and Talbot. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: Ventura. Obtained funding: Talbot. Administrative, technical, or material support: All authors. Supervision: Talbot.

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