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Heavy Metals in Elecronic Cigarette Aersol Can Accumulate in Lung Epithelial Cells

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HEAVY METALS IN ELECRONIC CIGARETTE AERSOL CAN ACCUMULATE

IN LUNG EPITHELIAL CELLS

By

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A capstone project submitted for
Graduation with University Honors

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University Honors
University of California, Riverside

APPROVED

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Abstract

We used human bronchial epithelial cells (BEAS-2B) to study the effect of metals in electronic cigarette (EC) aerosols on human health. The cells were exposed to metals (zinc, nickel, copper, lead) and mixtures of metals at concentrations found in EC aerosols. Cell culture media were collected before and after treatment and after recovery. High concentrations of zinc and nickel treatments decreased mitochondrial reductase activity in the MTT assay, while lead and copper had little effect. The MTT results from the metal mixture showed the greatest decrease in enzymatic activity. A Bio-Station was used to capture time-lapse images over a 24-hour period, and CL-Quant software was used to analyze cell proliferation. Lead and copper treatments did not have much effect on cell proliferation but showed evidence of cellular stress in the cell's morphology. The zinc treatments lowered the cells proliferation rate in a treatment dependent pattern. Cells treated with nickel had lower proliferation rates and underwent lamellipodia retraction. When cells were exposed with metal mixture, the effect of metal on cells proliferation decrease because metals were notifying the effect of each other. The intensity of a fluorescent zinc probe was used to quantify Zn 2+ concentrations in media and cells. There was a 30% loss of fluorescence intensity in the high treatment medium, moreover the cell lysate of the same treated showed a 29% increase in fluorescence intensity. This study shows that treatment of bronchiolar epithelial cells with metal concentrations found in EC aerosols can cause an influx of heavy metals, a decrease in mitochondrial reductase activity, and lower cell proliferation rate.

Acknowledgements

I want to thank the Talbot lad for allowing me to explore the very serious problem of electronic cigarettes in a lab setting. I want to thank Dr. Talbot for her help along the way, and I want to thank George for showing me the ropes for basic research and analytical skills. I am confident that these skills will serve me well moving forward from UCR.

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Table of Contents

Abstract………2

Acknowledgments………1

Introduction………3

Background………4

Materials and Methods………5

Results……….11

Concluding Remarks…….27

Supplementary Figures……….38

Results………. 30

Introduction

Electronic cigarette (EC) usage has become more prevalent in recent years, and they are marketed as a safe alternative to cigarettes. Unlike traditional cigarettes which combust tobacco, ECs deliver nicotine by heating internal components to aerosolize electronic cigarette fluid which is comprised of nicotine, flavor chemicals, propylene glycol and vegetable glycerin (Williams et al., 2013). The aerosols produced from ECs, often falsely labeled as only water vapor, have led to the alternative label of "vaping". With this misconception, tobacco companies cleverly advertised ECs as harm-reduction products. In some case, tobacco companies referred to their EC products as a nicotine delivery system rather than labeling it an EC.

ECs have been in the news because of their high nicotine levels, but new research has reported that metals were also leaching into the EC aerosols (Williams et al., 2013). It is suspected that these metals originate from internal metal components. When these metallic components are heated, the metals leach into the aerosol (Williams et al., 2017). With metals present in the aerosol, each puff increases both the metal concentrations delivered to the user and the potential risk associated with EC use. Metals in such high concentrations have not been seen traditional combustible tobacco products such as cigars, cigarettes, and hookahs. The long-term health effects of such high metal concentrations and ECs have not yet been determined. Nevertheless, various reports of short-term effects and in-vitro studies provide evidence of cytotoxicity associated with EC aerosols.

Most reports on EC cytotoxicity focus on EC fluids and nicotine concentrations, with very little literature regarding metal toxicity. Therefore, the purpose of this study is to determine the effect of metals found in ECs aerosols on human bronchial epithelial cells. The metal concentration used

for this study were divided into low, medium, and high concentrations which were determined by prior studies that quantify the metal concentration in ECs aerosols produced from various brands of ECs. The objectives of this study are to find (1) the effect of metals on cell proliferation, (2) the exchange of metals between the extracellular space and the cells, (3) cellular pathways that were affected by metal.

Background

In our experiment, we test the biochemical effect of metals on lung epithelial cells. The metal concentrations used in the study were determine by graduate students in the Talbot lab (Williams et al., 2013, Williams et al., 2015; Williams et al., 2017) The procedure used to determine the metal concentration is as follows:

In order to determine the average concentration of metals, four groups of electronic cigarettes were used, and these groups were chosen based off of popularity at the time. This study was interested in quantifying the abundance of tin, copper, zinc, silver, nickel and chromium. Once the groups of electronic cigarettes were chosen, the aerosolized vapor from each of the groups was collected in a 500 mL round bottom flask, which was submerged in an ice bath and covered with parafilm. Each 4.5 second puff of vapor was allowed to fully dissolve in a solution containing 10% nitric acid, 3% HCl, and 87% deionized water before the next puff was applied. (4.5 seconds was chosen because this was found to be the average time that an electronic cigarette user inhaled using the device). A total of 60 samples were taken from each of the electronic cigarettes and analyzed using inductively coupled plasma optical emission spectrometer (ICP-OES). This technique allowed us to quantify the relative abundance of the metals in each of the aerosols (Williams et al., 2013, Williams et al., 2017)

Metal Concentrations	High	Medium	Low
$ZnCl_2$ range	0.69uM	8.1uM	15.67uM
$NiCl_2$ range	0.72uM	12.4uM	17.3uM
$CuCl_2$ range	0.083uM	0.101uM	0.195uM
$PbCl_2$ range	0.0054uM	0.0316uM	0.0627uM

Table 1. this table represents the metal concentrations used during the course of the study to treat our cells (BEAS-2B). These concentrations were based off of previous studies looking at a wide variety of electronic cigarettes.

Materials and Methods:

Cell Culture

Human bronchial epithelial cells (BEAS-2B) purchased from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in bronchial epithelial cell basal medium (BEBM) (Lonza, Walkersville, MD) supplemented with 2.0 mL of bovine pituitary extract, 0.5 mL of insulin, 0.5 mL of hydrocortisone, 0.5 of mL retinoic acid, 0.5 mL of transferrin, 0.5 mL of triiodothyronine, 0.5 mL of epinephrine, and 0.5 mL of rhEGF (Lonza). Nunc T-25 tissue culture flasks were coated overnight with coating media, which comprise of BEBM, collagen, bovine serum albumin and fibronectin prior to culturing and passaging cells. At confluent, cells were washed with DPBS and detached using 0.225% trypsin with ETDA and poly-vinyl-pyrrolidone. For passaging and experiments, BEAS-2B were dispersed into single cells and seeded at density of 4000 cells/0.32 cm^2 using hemocytometers to count cells.

Cytotoxicity in the MTT Assay

Metals found in the EC aerosol were tested for cytotoxicity using MTT assay. BEAS-2B cells were plated in 96-well Falcon plates (#353072, Corning, Corning, NY, USA). The lowest and highest concentration of metal found in EC aerosol was used to determine the 9 different concentrations for individual metals. The 96-well plates layout contained control wells, vapor effect control wells (adjacent to the highest concentration), and treatment wells. MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, St. Louis, MO) dissolved in DBPS was added to control and treated cells after 24 hrs. treatment. After 3 hrs. of incubation with MTT solution, formazan crystals were solubilized in dimethyl sulfoxide, and absorbance was read at 570 nm with an Epoch plate reader spectrophotometer (Biotek, Winooski, VT). The absorbance reading was normalized back to the control group. Chemicals were considered cytotoxic when the highest concentration gave a response that was <70% of the control. Four independent experiments were done using cells from different passage numbers. Data are represented as mean ± SEM. Statistical analysis was done with two-way ANOVA with post-hoc Dunnett's test at every time points.

Proliferation Assay

BEAS-2B were cultured in 6 well plates and treated with $ZnCl_2$, $NiCl_2$ and metal mixture at 3 different concentrations (low, median, high). The plates were incubated and imaged in Nikon Biostation CT (Nikon, Melville, New York). Phase-contrast time-lapse videos were captured at 10x magnification. Cl-Quant (DR Vision, Seattle, Washington) software was used to segment the cells from the background. Segmented cells debris were excluded by size exclusion function. Area morphological feature were extracted from segmented region for each frame. Proliferation rate

was calculated by dividing area data from each time point to the first frame area data. Three independent experiments were done using cells from different passage numbers. Data are represented as mean ± SEM. Statistical analysis was done with two-way ANOVA with post-hoc Dunnett's test at every time points.

Fluo-Zin3 (Cell Permeant) Specificity Test

BEA-2Bs were grown in Ibidi 8-well chambers (Ibidi, Munich, Germany) and allow to attach overnight. Cells were incubated for 24 hrs. in individual metal (ZnCl2, NiCl2, CuCl2, PbCl2) and metal mixture solution (low, medium, high). After 24 hrs. incubation, treatment solution was aspirated from the well and DPBS was used to the wash. Fresh BEBM medium was added to the well along with FluoZin-3 acetoxymethyl ester (Invitrogen) with final concentration of 1 µM. Fluorescent images were collected using an inverted Eclipse Ti microscope (Nikon) using a 10x and 60x objective equipped with numerical apertures of 0.75 and 0.85, on high-resolution Andor Zyla VSC-04941 camera (Andor, Belfast, UK). The live cells were kept in a 37C temperature, 5% CO_2, and 90% relative humidity-regulated incubation chamber (Pathology Device Inc., San Diego, USA). Images were collected at millisecond exposures and de-convoluted using the Live De-blur feature in the NIS Element software (Nikon).

Quantifying Free Zn^{2+} with FluoZin-3 Cell Impermeant in Medium and Cell Lysate

BEAS2-Bs were grown in 6-well Falcon plates (Corning) and allow to attach overnight. Cells were incubated for 24hr in metal mixture solution (low, medium, and high). Prior to the treatment, small volume of the treatment media was collected. The sample treatment media were added into a 96-clear flat bottom black plate (Corning) and FluoZin-3 Cell Impermeant (Invitrogen) stock solution

was dilute to a final concentration of 1 μM in the treatment media to measure Zn^{2+} concentration. 485/20nm filtered light was used to excite the Zn^{2+} probe and emission fluorescence values were obtained at 528/20nm by an Epoch plate reader spectrophotometer (Biotek). After 24 hrs. of exposure, the treatment media were collected to measure Zn^{2+} concentrations. Standard curve of Zn^{2+} was made by diluting dissolved $ZnCl_2$ into media. (~0 μM - 100 μM). Prism software (GraphPad, San Diego, CA, USA) was used to interpolate fluorescence values collected from the media to uM. Data are represented as mean ± SEM. Statistical analysis was done with using two-tailed T-test comparing treatment media before and after exposure.

Quantifying Free Zn^{2+} with FluoZin-3 Cell Impermeant Cell Lysate

BEAS2-Bs were grown in 6-well Falcon plates (Corning) and allowed to attach overnight. Cells were incubated for 24hr in metal mixture solution (low, medium, and high). The treatment media were collected to be analyze (previous section). Control and treated cells were washed several times with DPBS to remove any residual treatment media and lysed using RIPA buffer containing proteasome inhibitor. Cell lysates were collected and centrifuged at 10,000 x g for 1 min at 4C and quantified using (Pierce BCA assay kit, (ThermoFisher Scientific, Waltham, MA). Lysates contained 20ug of protein was added into a 96-clear flat bottom black plate (Corning) and additional RIPA buffer was added to each well to reach volume of 100uL. FluoZin-3 Cell Impermeant (Invitrogen) stock solution was dilute to a final concentration of 1 μM in the RIPA lysate solution. 485/20nm filtered light was used to excite the Zn^{2+} probe and emission fluorescence values were obtained at 528/20nm by an Epoch plate reader spectrophotometer (Biotek). The fluorescence value was normalized by comparing back to the control cell lysate. Data are

represented as mean ± SEM. Statistical analysis was done with using two-tailed T-test comparing to a hypothetical value of 100.

Plasmids and Transient Transfection

The following plasmid was purchased from Addgene (Cambridge, MA USA) and used for transient transfections: (1) GFP-EEA1 wt (#42307). Transfections were carried out using DNA-In/CRISPER reagents (MTI-GlobalStem, Gaitherburg, MD USA). In brief, cells were allowed to reach 70% confluent prior to transfection. The plasmid and the reagent complexes were prepared according to the manufacturer recommendation.

Rab-7 Late Endosome Baculovirus Transduction

Prior to transduction cells were allowed to reach 70% confluent. Culture medium were aspirated and fresh medium containing CellLight™ Late Endosomes-RFP, BacMam 2.0 (ThermoFisher) reagent was added to reach 30 particles per cells, as suggested by the manufacturer's protocol. Cell were incubated in the medium for 18 hrs. before imaging with Nikon Eclipse.

Glutathione Experiment

GSH+GSSG/GSH Assay Kit (ab239709) was purchased from abcam (Abcam, Cambridge, MA, USA). The reagents were prepared according to the manufacturer's protocol. Cells were plated in a 6 wells plate at a density of 4000 cells/0.32 cm^2 using hemocytometers to count cells. Cells were allowed to reach 70% confluent before 24hrs $ZnCl_2$ treatment. After the treatments, 0.25% trypsin were added to each well to detached cells and $0.5x10^6$ cells were collected from each experimental group. Cells were centrifuge at 700xg for 5min at 4°C. The supernatant was discarded, and cells

pellets were resuspended and lyse over ice with the buffer reagent provided in the kit. 5% sulfosalicylic acid was added into each lysate samples and centrifuge at 8000 x g for 10mins. The supernatant was transfer into 96 wells. The reaction mixtures were prepared for two different types of results. One reaction mixture was prepared to determine the total glutathione, and another was to determine only the reduce form of glutathione. The reaction mixtures were added to the supernatant. Plate reader was using to read the absorbance from the sample at 405nm. The standard curve prepared according to the manufacturer's protocol was used to convert O.D. to determine glutathione concentration value in μg. Oxidize glutathione concentration was determine by subtracting the concentration of total glutathione by the reduced glutathione.

Nanoparticles from cell culture medium

BEA-2BS cells were grown in 6 wells plated. Cells were allowing to reach 70% confluent before treatment. Large particles depleted medium was prepared by filtering cell culture medium (BEGM) with Acrodisk 0.25 filter. Three different concentrations of $ZnCl_2$ were added into the large particles depleted medium to make the treatment solution. Cells were treated with large particles depleted treatments for 24hrs. After 24hrs, the media were collected and analysis with NS300 NanoSight instrument (Malvern Instruments, Amesbury, UK).

NanoSight measurement

Nanoparticles were analyses using NanoSight NS300 instrument (Malvern). The instruments were equipped with a 405nm laser, a high sensitivity sCMOs camera and a syringe pump. The media collected from cells culture were diluted to obtain a concentration within the recommended measurement range. Syringe pump infusion rate was set at a rate in which the nanoparticles can be captured in the field of

view for 7-9 second. All quantification of videos data was analyzed using NTA 3.1 software (Malvern). The size and intensity result of the nanoparticles were automatically determined by the software.

Results

An MTT assay was used to determine whether the metal concentrations found in ECs aerosol could be used to treat monolayer culture BEAS-2B cells. A 24-hour treatment of single metals (zinc, nickel, copper, lead) and a metal mixture treatment showed decreased mitochondrial reductase activity. The data suggest that despite the cytotoxicity, any of that these concentrations of metals can be used to treat cells without killing them (Figure 1).

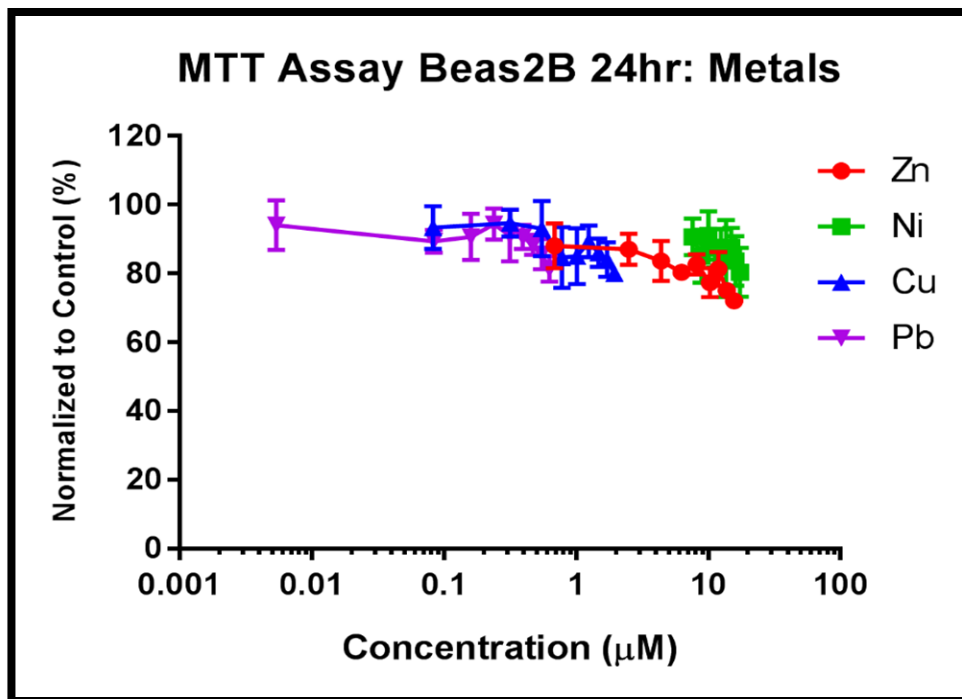
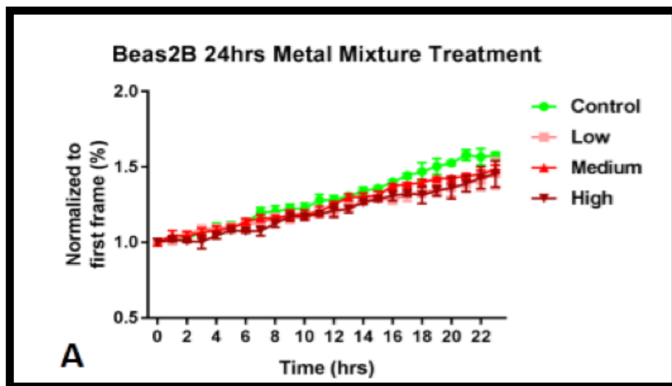


Figure 1. *MTT analysis was used to quantitatively analyze cytotoxicity of metals towards lung epithelial cells (Beas-2B). Results showed that mitochondrial reductase activity was reduced, but the lost in enzymatic activity did not reach IC50 or IC70 . Indirectly, the MTT results showed cells proliferation. Data showed that the results obtained from these predetermined electronic cigarette concentrations were not fatal to cells. MTT screening results suggest that*

the cells can be treated with any of these metal concentrations. Triplicate experiments were conducted to verify data. Statistical analysis was done with t-test compared back to hypothetical value (100%)

Biostation

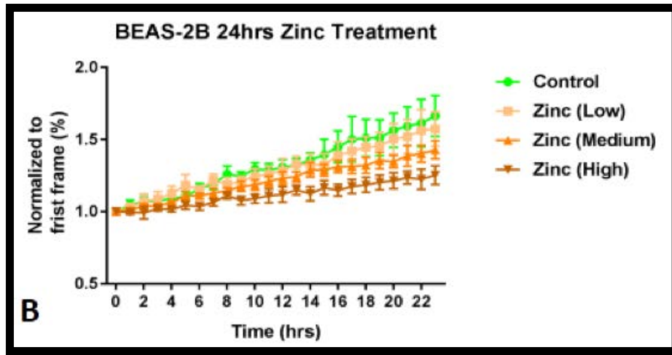
The Nikon Biostation CT helped capture time lapse images of cells as they were treated with our metal treatments. The metal treatments that we exposed our cells (BEAS-2B) to were $NiCl_2$, $ZnCl_2$, and a metal mixture. The result showed that there was a significant decrease in cell proliferation at low, medium and high concentrations at the latter part of the treatment. The data showed that cells treated with different concentration of zinc or nickel showed a stronger response pattern than cells treated with metal mixture (Figure 2). The finding indicates that metals in the mixture may interact with each other, competing for binding sites thus nullifying the effect of each metal has.

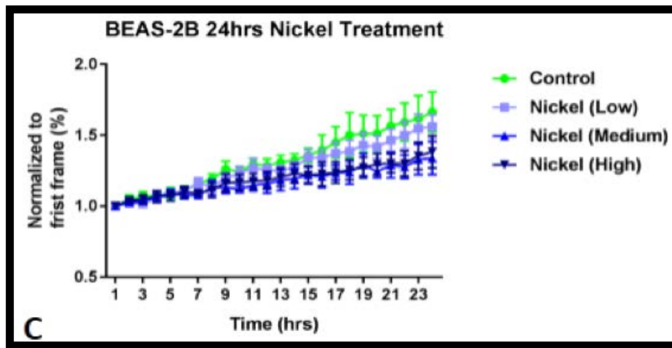


Time (Hour)	*Low*	*Medium*	*High*
17	*	-	
18	*	-	*
19	*	-	**
20	**	-	**
21	**	-	**
22	***	**	***
23	**	*	**
24	*	-	*

Time (Hour)	*Low*	*Medium*	*High*
17	-	-	**
18	-	-	**
19	-	-	**
20	-	-	**
21	-	*	***
22	-	*	***
23	-	*	****

24	-	*	*****





Time (Hour)	*Low*	*Medium*	*High*
17	-	-	-
18	-	*	*
19	-	-	*
20	-	*	-
21	-	*	*
22	-	**	**
23	-	*	*
24	-	**	-

*Figure 2 The BioStation was used to capture time-lapse images of cells treated with ZnCl2, NiCl2, and metal mixture. The purpose of the experiment was to directly analyze cell proliferation and monitor morphological changes. CL-Quant was used to segment time-lapse image and to extract useful morphological features. Cell proliferation was measured by using total cell area in the field of view. Proliferation rate dropped in highest concentrations of ZnCl2, NiCl2, and the metal concoction treatments. Raw area data subsets were normalized to their first time point. Statistical analysis was conducted with two-ways ANOVA at every time point and treatment groups were compared to the control group. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001*

Fluozin3

The cell permeant indicator Fluo-Zin3 was used in order to label the areas of the cell in which zinc entered. Fluo-Zin3 looks specifically at the presence of zinc cations within cells and in solutions. We used two different objective lenses for the experiment: 10X and 60X. The 10x objective lens provided relative localization of the probe while the 60x allowed us to narrow down the specific areas of the cell where the fluorescent marker was localized. The view from the 10X objective lens showed high probe activity in the metal mixture and $ZnCl_2$ treatments (Figure 3). In the control

cells, Zn^{2+} probe signals were mostly diffused and localized in vacuoles-like organelles. The low zinc treatment exhibited more fluorescence and more sequestering signal patterns of zinc. Cells treated with high zinc concentrations showed bright signals from vacuole-like structures (Figure 3).

In addition to the activity seen in the metal mixture and zinc treatments, there was significant activity in the $NiCl_2$ (and minimal activity seen in $CuCl_2$). This cross activity was an unexpected result. The probe was advertised specifically for zinc, but it became clear that it also worked for other metals. We observed fluorescent activity In the treatments for $NiCl_2$ and $CuCl_2$. The activity was more pronounced in the nickel treatment than it was for copper (Figure 3). Although the probe signal intensity was dimmer, the results from the $NiCl_2$ treatment were similar to the results obtained from the $ZnCl_2$ treatment. The probe signals were localized to certain vacuole regions within the cell. Although it is not exactly clear what caused the cross activity of the probe with nickel and copper, it is possible that their shared divalent character with zinc allowed the probe to pick up the activity of these metals within the cell (Ollig et al., 2016). Additionally, the cross activity of nickel may assist in a pathway that causes an influx of zinc cations in the media to enter the into the cells.

By using the zinc probe in the treatment of BEAS-2B cells we have established that there is a presence of zinc cations within the cell. The next part of the experiment focuses on whether these metals are moving into the cell as a result of our higher concentration treatments.

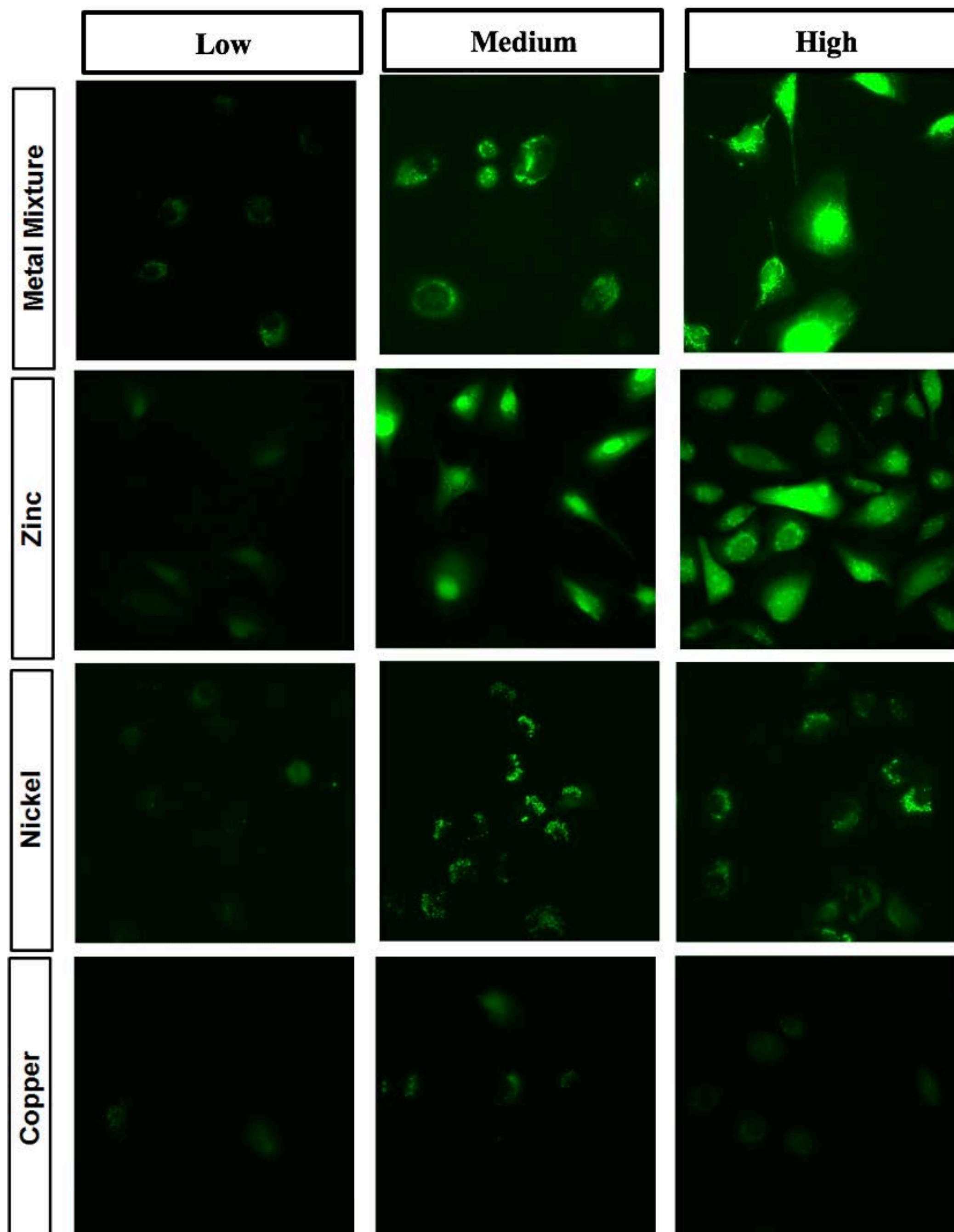


Figure 3. *The FluoZin-3 zinc probe was used as a visualization tool to mark zinc cations presence in cells and within the solution. Although the metal probe was designed specifically for zinc, our experimental data showed cross activity with other heavy metal divalent cations. NiCl2 treated cells had the strongest cross activity, while CuCl2 exhibited slightly lower cross activity. PbCl2 did not have any cross activity (Data not shown) This was purely a supplementary finding that does not impact our data quantification. Images were collected with a Nikon eclipse at 10x.*

Metal movement

In the first part of this experiment, cells were left to grow for a period of 24 hours and their media was collected. This media was labelled "before treatment" because they were collected from BEAS-2B cells cultures before they were exposed to any metal treatment. Cells were then treated for 24 hours with low, medium, and high concentrations of metal mixture media. After 24 hours, the media was collected and labeled "after treatment" because the cell cultures had been exposed to the metal mixture treatment. The "after treatment" media was treated with FluoZin-3 to monitor the concentration of the metals in the media. Our data show that there was a 30% decrease in the "after treatment" media which suggests that the metals in the treatment were taken up by the cell during the 24-hour treatment period (Figure 4). The treated cells were allowed to grow in fresh media for another 24-hour period. After the 24 hours had elapsed, the "recovery" media was collected and treated with the FluoZin-3 probe. The results showed that there was zinc present in the media. This indicates that the metals were being released from the cell back into the media.

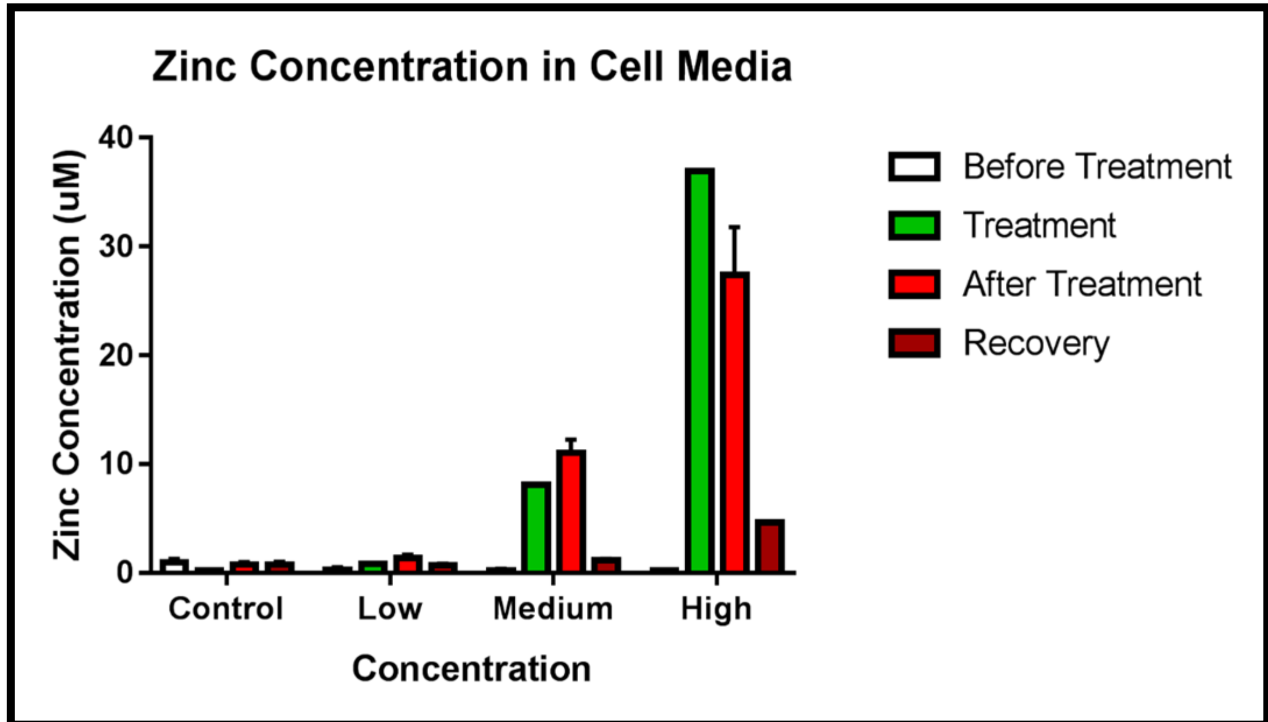


Figure 4 *The Zinc probe (FluoZin-3) was used to quantify how much zinc entered the cell. Cells were left to grow for 24 hours before the media was collected (before treatment). Cells were then immersed in a metal mixture treatment solution (low, medium, and high) for 24 hours. After this 24-hour exposure, the media was collected and FluoZin-3 was used to quantify Zn 2+ the media. It was noted that a significant amount of the Zn 2+ had left the media. Fresh media was then added after the treatment to the cell culture allowing them to recover. Zinc presence in the recovery media showed Zn 2+ was being released back into the media.*

In order to determine whether there was any zinc in the cells, we used RIPA lysis buffer to lyse the cells and quantify the amount of zinc in the lysate. Analysis of the cell lysate revealed that there was a 30 percent increase in zinc concentrations inside of the cell treated with a high concentration of metal mixture (Figure 5). The 30 percent increase in zinc concentration in the cell validates our finding from the media section which showed that 30 percent of the zinc was missing from the treatment media.

In our zinc movement experiment, the results show that zinc were released into the recovery media which had no previous metal concentration. The process by which the cell maintains homeostatic zinc concentrations is reminiscent of osmosis: If the zinc concentration in the surrounding media

is higher, then the zinc found inside the cell will also be higher. If the cell is placed into a zinc-free solution, however, the zinc then rushes out of the cell into the area of low zinc concentration. Measuring the metal concentration in the media over three different time intervals demonstrates that there was an exchange of cations between the inside and outside of the cell (Supplementary Figure 1). Furthermore, lysing the cells proved that the metal concentration lost from the high metal treatment media was present inside the cell. This finding establishes that there is a net movement of metal cations between the external environment and the inside of the cell (Figure 4,5).

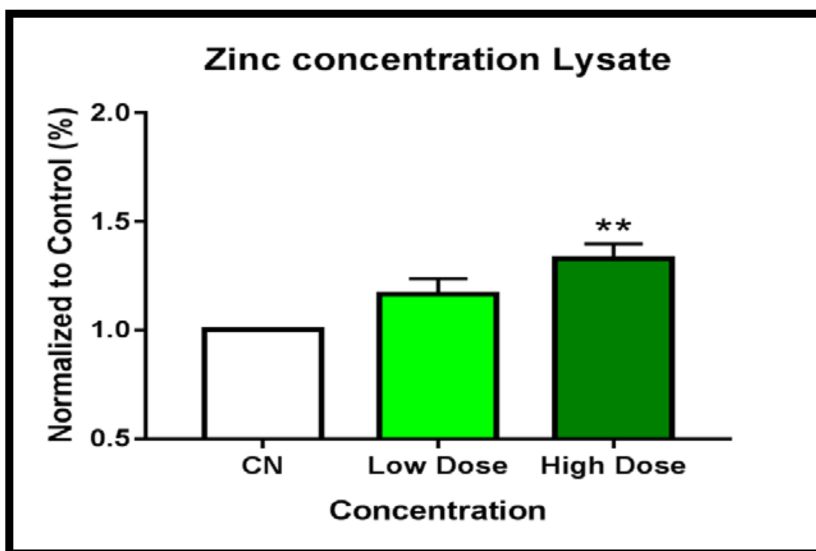


Figure 5 *RIPA lysis buffer was used to lyse cells. Lysates from different treatment conditions were subjected to Zn 2+ concentration quantification. The quantification results showed that zinc ions had entered the cells. In high metal treatment, the zinc concentration had increase by ~30% which was similar to the amount found missing in the after-treatment media. Statistical analysis was done with t-test compared back to hypothetical value (100%).* ** = $p < 0.01$

The endosome experiment:

Our screening experiments showed that zinc entered the cell in a concentration dependent pattern. The FluoZin-3 imaging data showed that zinc was localized in vacuole shaped organelle. The next

part of the paper focuses on better understanding the specific pathways zinc uses in the cell. First, we focus on the movement of zinc through the cell and then on possible interactions zinc has with proteins.

We hypothesize that zinc may entering into cells either through the endocytosis, ion channels, or both. To address this hypothesis, we performed immunocytochemistry on BEAS-2B cells treated with $ZnCl_2$. There are multiple stages in the endosomal pathway that zinc can enter into and each stage has a specific protein marker associated with it. In measuring the expression of these protein markers, we can determine whether zinc is entering the cell via endosomes and if that is the case then we can extrapolate that the zinc is entering via the endosomal process. The EEA1 protein marker was used first to visualize the effect of $ZnCl_2$ on the early endosomes, and RAB7 to visualize the effect of $ZnCl_2$ on the late endosome.

Our first endosome experiment looked at the EEA1 pattern. The cells were transfected with EEA1 and fused with GFP using DNAin before they were treated with $ZnCl_2$. Imaging done at 60x indicated that $ZnCl_2$ treatments lead to an influx of endocytosis (Figure 5). It is possible that Zn^{2+} acts as a ligand for membrane proteins that trigger receptor mediated endocytosis.

Our experiment using the EEA1 marker has established that zinc is taken up by early endosomes, so we moved forward to test whether zinc also interacts with late endosomes. RAB7 is the marker for late endosomes. The experimental layout for detecting RAB7 was similar to the protocol used to detect EEA1. Cells were transduced with Baculovirus in the CellLight reagent. The sequence inserted into cells was RAB7 fused with RFP. In addition, FluoZin-3 was used to detect zinc. Our data showed that both the Rab7 signal and FlouZin-3 were colocalized in the treated groups (Figure

6). The results from both endosomal trafficking experiments suggest that that zinc entered the cell using early endosomes and was trafficked through the cell via late endosomes. These results show one possible pathway zinc can use to travel through the cell.

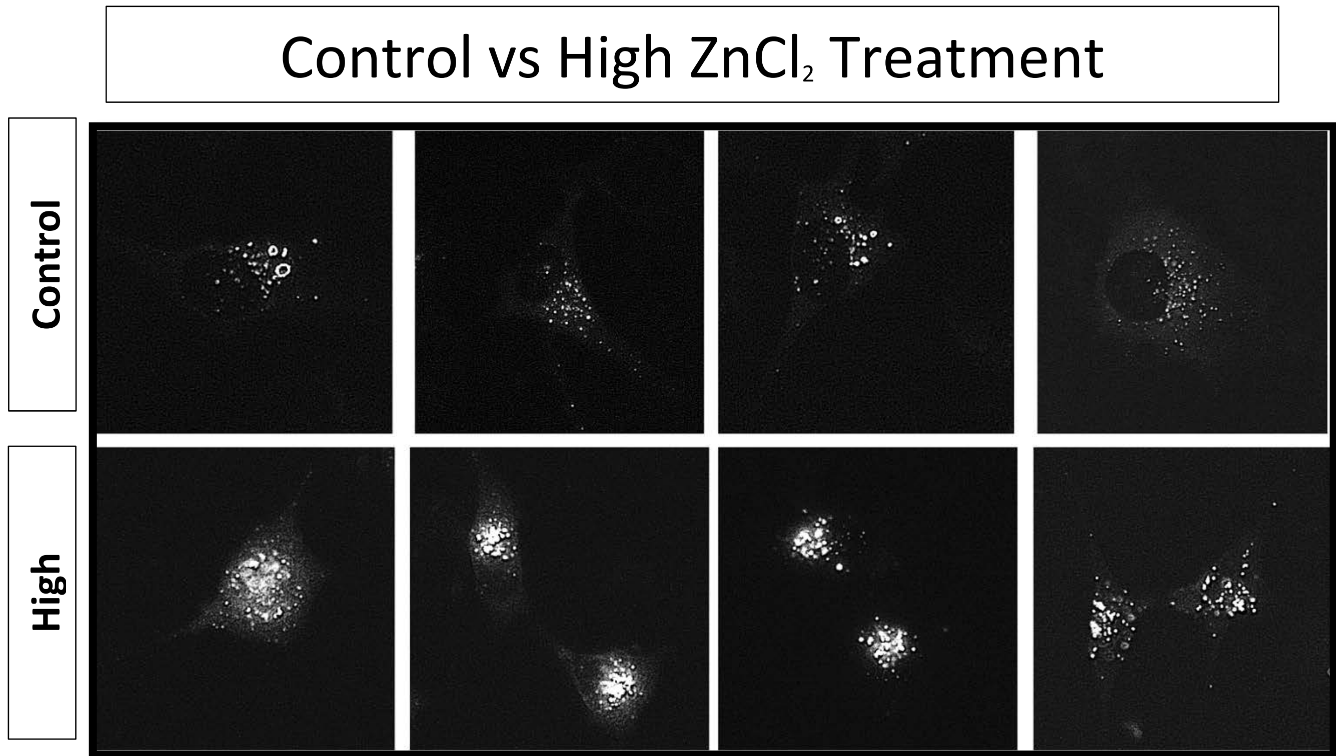


Figure 6 *The purpose of our EEA1 cell experiment was to observe EEA1 expression between $ZnCl_2$ treated cells and control cells. GFP was used to visualize the presence of EEA1 in transfected cells. The image results show a higher density concentration of EEA1 endosomes in the High treatment*

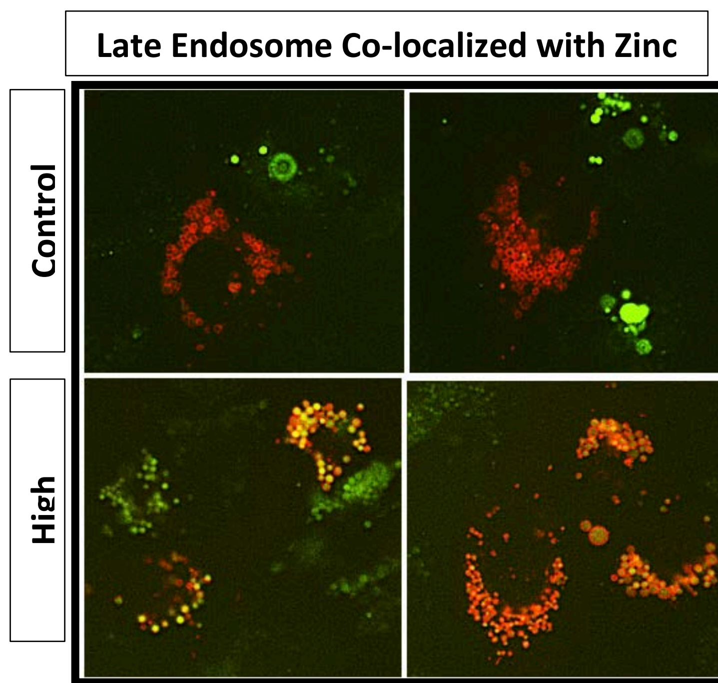


Figure 7 *The purpose of his experiment was to observe late endosome expression between $ZnCl_2$ treated cells and control cells as well as observe colocalization of zinc vacuoles with late endosomes. Zinc cations are labelled in green using FluoZin-3 and RAB7 (late endosome maker) are labelled with red using RFP from CellLight by Thermo Fisher. The high treatment of zinc appears yellow confirming that there is co-localization of zinc with late endosomes.*

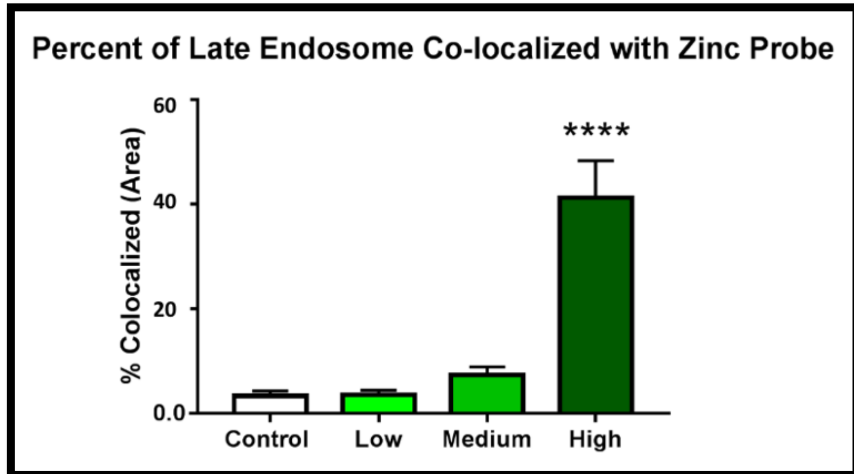


Figure 8 *The graph depicts colocalization of zinc and RAB7 using area. The high treatment has the most significant area of colocalization area at approximately 40%. Compared to the control and other treatments. Statistical analysis was one way ANOVA with Dennett's posthoc. The treated groups were compared to the control group. **** = p<0.0001*

The NanoSight experiment:

After looking at markers such as Rab7, we hypothesized the $ZnCl_2$ treatment may cause cells to form exosome in multivesticular bodies, which could package Zn^{2+} cations that can be release into the extracellular space (Gould et al., 2009). By packaging Zn^{2+} in an exosome, Zn^{2+} concentration will not increase in the medium, therefore, the exosome prevents Zn^{2+} to reenter the cells.

To determine if exosomes were released into the medium, Nano sight was used to image and quantify the number of small particles (smaller than 1mm). Prior to Nano particle imaging, the growth media of our cells was filtered with acrodisk 0.25µm to create a large particle depleted medium (Gould et al., 2009). This pre-treatment media was measured with Nano sight to determine the basal concentration of small particles. $ZnCl_2$ was added to the medium in low and high concentrations. Cells were treated with large particle depleted treatments for 24 hours after which the treatment media was collected from cell cultures and imaged using Nano sight (Figure 9a). The release of additional particles from the control

group in the after treatment was seen which indicate that cells release exosomes under normal conditions (Figure 9b). The results showed that there were more particles in the after-treatment medium (Figure 9b, 9c), and there was a greater quantity of the particulate matter found in the treatment media than was found in the control group (Figure 9c)

These results indicate that $ZnCl_2$ treatment causes exosomes to be release into the medium. The result is most notable in the high treatment condition. It is possible that in addition to packing Zn^{2+} into exosomes, these exosomes could contain signaling molecules, which can send out to other cells as a survival response to inflammation and ROS (Mathivanam et al., 2010).

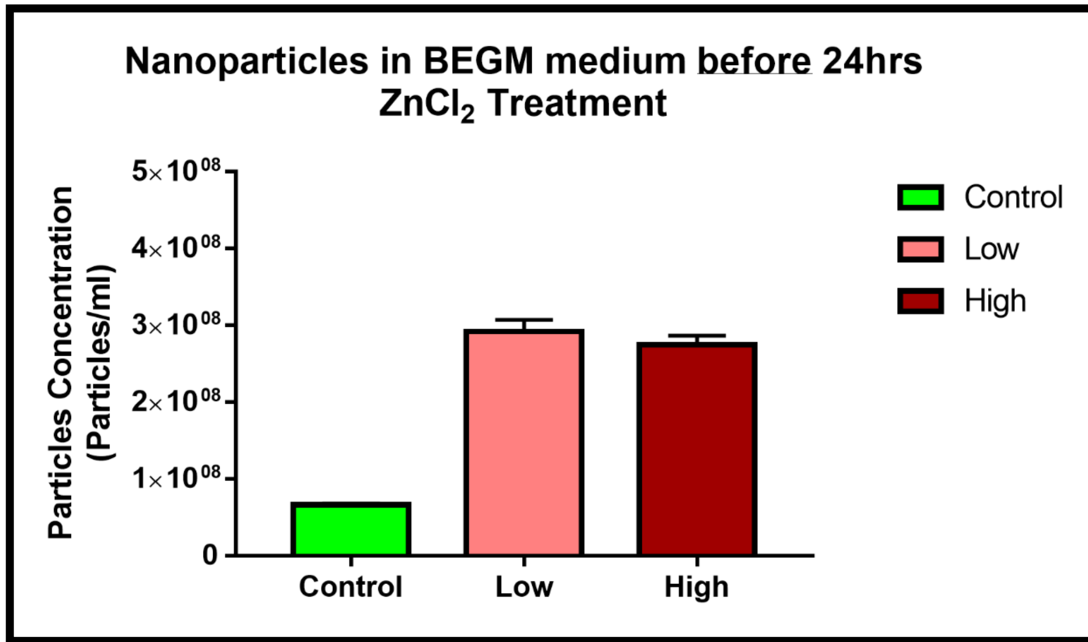


Figure 9a *determine small particles in basal*

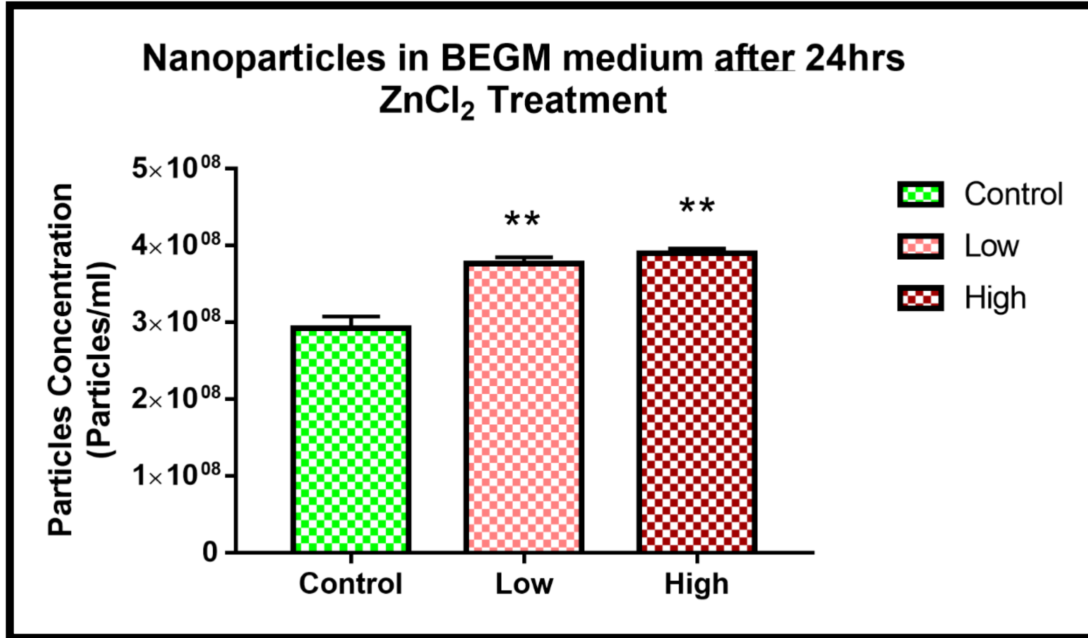


Figure 9b *Statistical analysis was one way ANOVA with Dennett's posthoc. The treated groups were compared to the control group. ** = p<0.01*

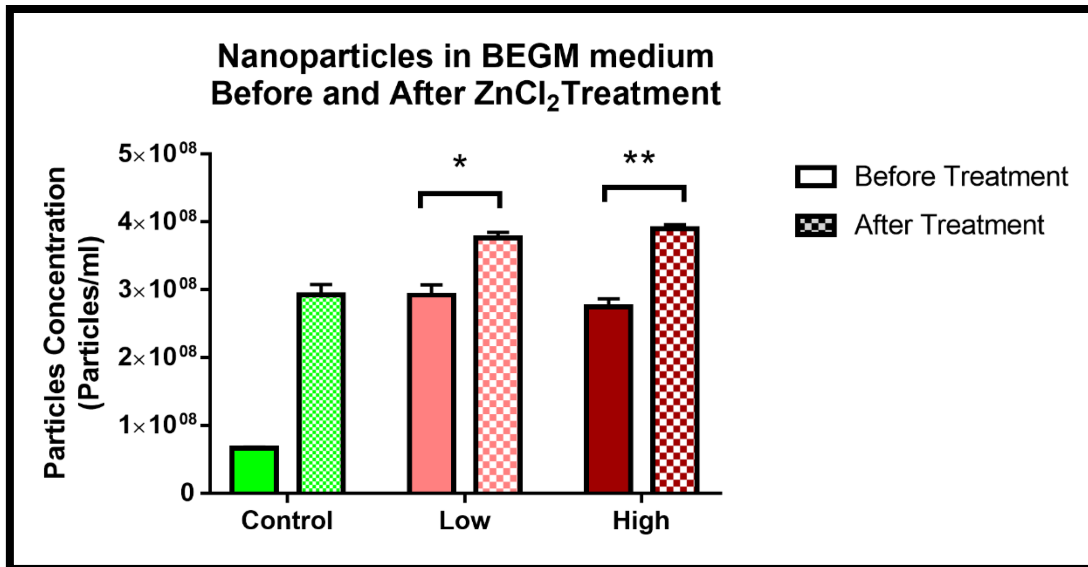
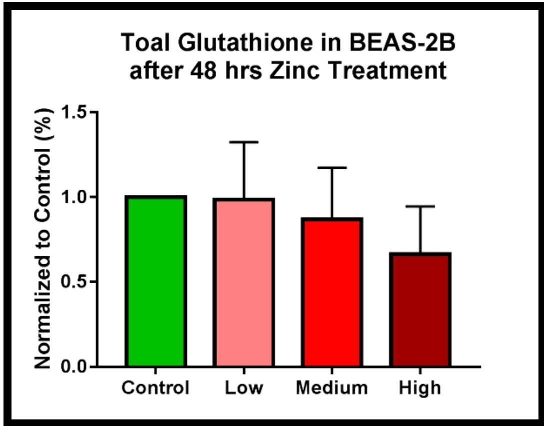


Figure 9c *Statistical analysis was two way ANOVA with sidak multiple comparison * p<0.05 **, p<0.01comparing before and after in the treated groups.*

Glutathione Experiment

We devised an experiment in which we used high zinc treatments to test the effect they had on glutathione. The cells (BEAS-2B) were treated, lysed, and then put through a GSSH/GSH assay. The results of the experiment showed that concentrations of reduced glutathione (GSH) are highest following low treatment after which they gradually recede (Figure 10). A possible explanation for this is that our low treatment is actually a naturally high threshold and sensitivity only decreases after that point. The other observation was that oxidized glutathione (GSSG) concentration increased proportionally to treatment concentrations following a 24-hour exposure period (Figure 10). This accumulating [GSSG] is interesting because it suggests that zinc's ability to affect the glutathione pathway is somehow related to this specific step (Forman et al., 2009). Further experiments must be conducted in order to make any definitive statements, but below a few schematics theorize as to what the possible effect zinc is having at this particular step in the pathway (Supplementary Figure 2)



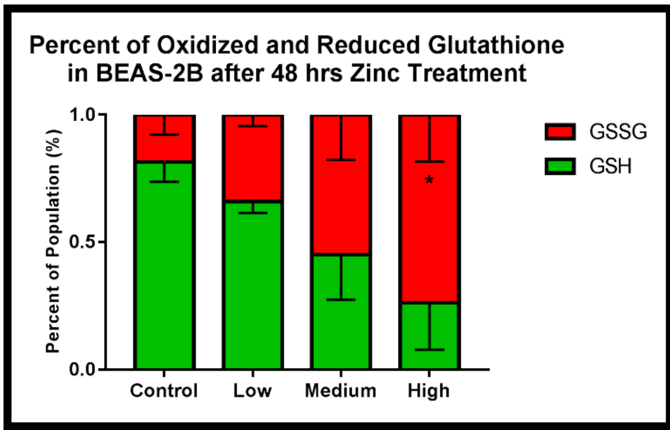
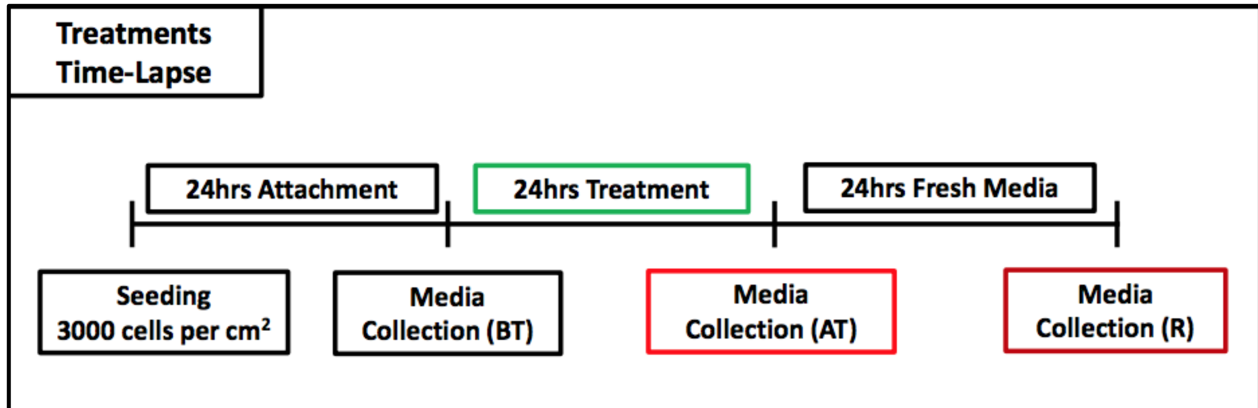


Figure 10 *The results for the GSSH/GSH assay from abcam show that the presence of oxidized glutathione increases in a concentration dependent manner. As more zinc is introduced, less of the oxidized glutathione is reduced back to GSH. The data suggest that zinc interacts with the pathway to hinder the cycle. Statistical analysis was two ways ANOVA with Dennett's posthoc. The treated groups were compared to the control group. * = p<0.05.*

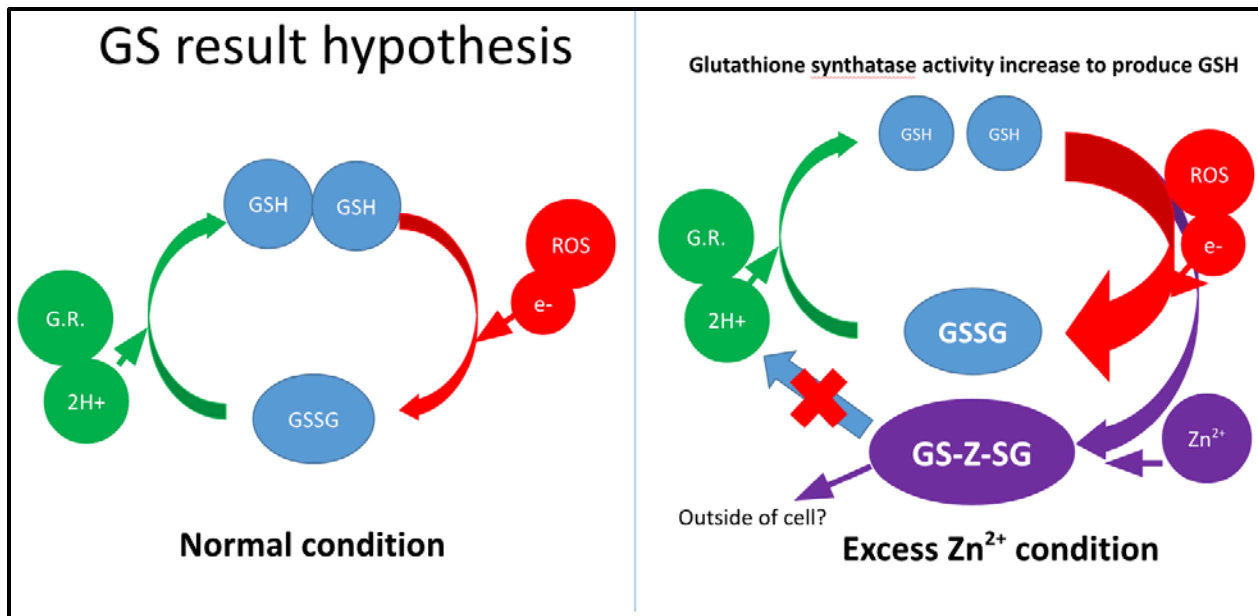
Concluding remarks

This paper builds on previous findings which reflect that there were a variety of metal concentrations present in electronic cigarettes (Olmedo et al., 2018; Williams et al. 2013; Williams et al., 2017). We applied these metal concentrations to human cells (BEAS-2Bs) in order to determine what acute effects such metals had. Our findings confirm that metals such as zinc move from the extracellular environment to the intracellular environment in a concentration dependent manner. Beyond entering the cell, we were able to identify that zinc can travel within the cell using endosomes. Our study also found that zinc has the potential to interfere with certain pathways in the cell such as Glutathione. Interference with the glutathione pathway by zinc is has potentially detrimental because interference in this pathway can allow for the buildup of ROS and other reactive species (Forman et al., 2009). Our paper is relevant because it helps fill a gap in terms of what is known about metal concentrations in ECs. The scientific community is just beginning to scratch the surface in this field and more research will be needed to fully understand what such metal concentration's effect is on human health. We focus next on identifying potential pathways that metal concentrations found in ECs can interfere with. Understanding these pathways and the effect metals have on them wil allow us to better answer the question, “Are electronic cigarettes safe?”

Supplementary Figures



Supplementary Figure 1 *The timeline above details the metal movement experiment. There were three media collections during the experiment and they were all taken after a 24 hour period. The media was the primary focus for testing whether metal was moving into the cell. The "after treatment" media collection showed that zinc was missing from the media suggesting it had been taken up by the cell. The "recovery" media showed that the presence of zinc allowing us to conclude that zinc was being let out of the cell into the media.*



Supplementary Figure 2 *The following diagram shows the hypothetical interaction taking place between zinc and glutathione in the glutathione oxidation/reduction pathway. Our hypothesis is that zinc wedges itself between glutathione's disulfide bridge which prevents it from being recycled. Since the GS-Z-SG is no longer able to be used*

for the cell to help rid its self of ROS, we hypothesize that the cell may take it out which decreases the overall concentration glutathione inside the cell which may leave the cell more susceptible to oxidative stress.

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