8-2019

Predictive Biomarkers Demonstrating the Effect of Levels of Copper and Zinc from Exposure to World Trade Center 9/11 Particulate Matter on Human Esophageal Cells in vitro

Rossara Nunez
Montclair State University

Abstract:

The World Trade Center Attack on September 11th, 2001 was the largest environmental attack that has ever happened in New York City. In the aftermath of the collapse of the twin towers, many first responders and rescue workers were exposed to the resulting clouds of dust. This toxic material has been shown to be responsible for membrane damage in human lung cells and can possibly become the cause of increased oxidative stress. The underlying factors that produce these findings are thought to the synergistic effects of the many components found within the market street sample. Complete analysis of the heavy metal found in this toxic material were determined by Paul Lioy of Rutgers University and his team of twenty plus scientists. Since Copper and Zinc, found in known quantity in the dust are together known to be antioxidants, studies were performed to determine changes in reactive oxygen species (ROS) identified under conditions of increases of these particular heavy metals. Most recently gastroesophageal related diseases have been seen in many first responders. Previously only diseases of the lungs were of great concern. Many first responders have developed Gastro-esophageal reflux disease (GERD) symptoms since 2005 and long-standing reflux symptoms that are not treated will lead to Barrett's esophagus and esophageal cancer. This study will compare human esophageal and lung cells and will try to determine if high concentration of zinc and copper can reduce or increase oxidative stress *in vitro* cells exposed to various concentrations of WTC dust.

MONTCLAIR STATE UNIVERSITY

Predictive biomarkers demonstrating the effect of levels of Copper and Zinc from exposure to World Trade Center 9/11 particulate matter on human esophageal cells in vitro

by

Rossara Nunez

A Master's Thesis

Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of

the Requirements for the

Degree of

Master of Science

August 2019

College/School: College of Science and Mathematics

Department: Biology

Thesis Committee:

[redacted]

Thesis Sponsor: Dr. Ann Marie DiLorenzo

[redacted]

Committee member: Dr. Lee H. Lee

[redacted]

Committee member: Dr. Kirsten Monsen

PREDICTIVE BIOMARKERS DEMONSTRATING THE EFFECT OF LEVELS OF COPPER AND ZINC FROM EXPOSURE TO WORLD TRADE CENTER 9/11 PARTICULATE MATTER ON HUMAN ESOPHAGEAL CELLS IN VITRO

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

by

ROSSARA NUNEZ

Montclair State University

Montclair, NJ

2019

Acknowledgement:

First of all, I would like to acknowledge and thank my mentor Dr. Ann Marie Dilorenzo for the never-ending support and guidance she has given me through this whole process and for the opportunity to work in her lab and the trust she has given me. I am confident that I wouldn't be be the scientist I am today without her help. I would also like to thank Dr. Lee Lee and Dr. Kirsten Monsen for their mentorship and support through this journey. I would also like to thank Adam Parker for all the technical assistance provided. I would also like to thank my undegraduate research team, Ariel Piatt, Brian Debrah-Afful, Yveeka Marcellus and Charles Ezewanne for helping me carryout the experiments. I would also like to acknowledge Promega for the support in the educational assay kits they supplied me with. Last but not least, I would like to thank and acknowledge Dr. Paul Lioy from Rutgers University for providing us with a sample of the World Trade Center Dust therefore making this thesis project possible.

Table of Contents

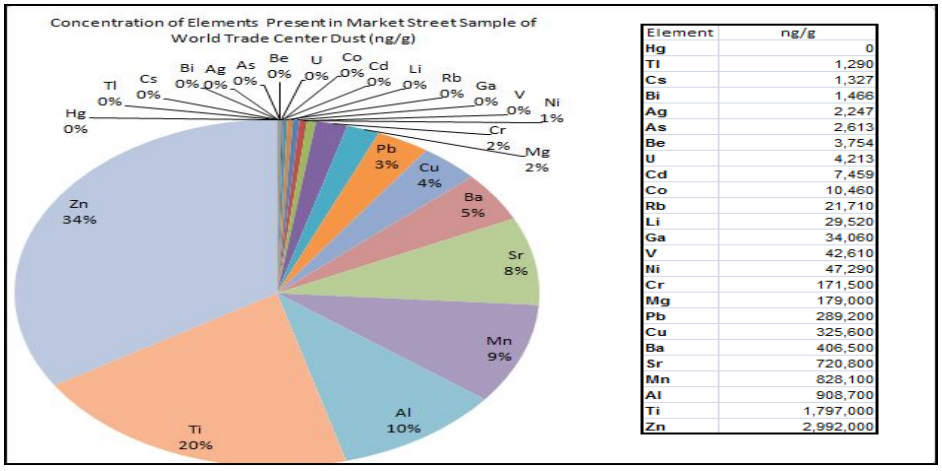
List of Figures

List of Tables

Introduction:

The WTC Environmental Attack

The World Trade Center Attack on September 11th, 2001 was the biggest terrorist attack that has ever occurred in New York City. Around 3,000 people died on 9/11 and more than 6,000 were injured including law enforcement officers, firefighters, emergency medical services and military personnel. The World Trade Center attack has induced psychological and physiological trauma to the responders and civilians present at the attack (Liu *et al* 2014). In the aftermath of the collapse twin towers, many of these first responders and rescue workers were exposed to the fall out dust. The WTC dust has been characterized as a complex mixture of many different substances including asbestos, concrete, lead, glass fibers, debris, soot, gypsum and hydrocarbons. (Lioy *et al* 2002). The WTC dust has brought a tremendous level of environment pollution/damage to the New York City area and the underlying factors that produce these findings are thought to be because of the unique chemical composition of the WTC dust (Lippman *et al* 2015). The complete analysis of the market street sample of the World Trade Center dust were determined by Paul Lioy *et al* at Rutgers University. More than 20 metals have been identified in the dust.



Element	ng/g
Hg	0
Tl	1,290
Cs	1,327
Bi	1,466
Ag	2,247
As	2,613
Be	3,754
U	4,213
Cd	7,459
Co	10,460
Rb	21,710
Li	29,520
Ga	34,060
V	42,610
Ni	47,290
Cr	171,500
Mg	179,000
Pb	289,200
Cu	325,600
Ba	406,500
Sr	720,800
Mn	828,100
Al	908,700
Ti	1,797,000
Zn	2,992,000

Figure 1. Percentage of elements found in WTC dust (Lioy et al., 2002).

Physiological Effects of WTC Dust in Lung and Esophageal Cells

More than 6,000 individuals have been affected by the toxic dust released in the 9/11 attack. 24 hours after the 9/11 attack, a persistent WTC cough was noticed in the first responders as well as shortness of breath, wheezing and asthma like symptoms (Wang *et al* 2010). After a 6-month period, many rescue workers were diagnosed with Reactive Airway Dysfunction Syndrome (RADS) including symptoms of difficulty breathing, sputum and wheezing (Lambroussis *et al* 2009). In addition, in the first year after the collapse, 45% of the first responders had been diagnosed with sudden-onset asthma (Wang *et al* 2010). This is due to WTC dust particle size in the 2.5–10 μm range, which can penetrate deeply into the lungs, causing irritation of the alveolar wall and impairing lung function (Xing *et al* 2016). Even though most research has focused on obstructive airway diseases (OAD) in correlation to the WTC dust, other organ systems have been affected including the gastrointestinal system. More than 44% of the WTC rescuers have developed gastro-esophageal reflux disease (GERD) symptoms since 2005 (Haider *et al* 2018). Long-standing reflux symptoms that are not treated will lead to Barrett's esophagus and can progress to esophageal cancer (Li *et al* 2011). It is believed that the rescue workers have developed GERD due to accidental indigestion of dust particles that damage the esophagus lining and causes irritation in the gastroesophageal tract (Lui *et al* 2017). There is an association between OAD and GERD, a hypothesis that GERD facilitates irritation of the airway and causes inflammation exacerbating OAD in first responders (Prezant *et al* 2002) yet the mechanism of it remains unclear.

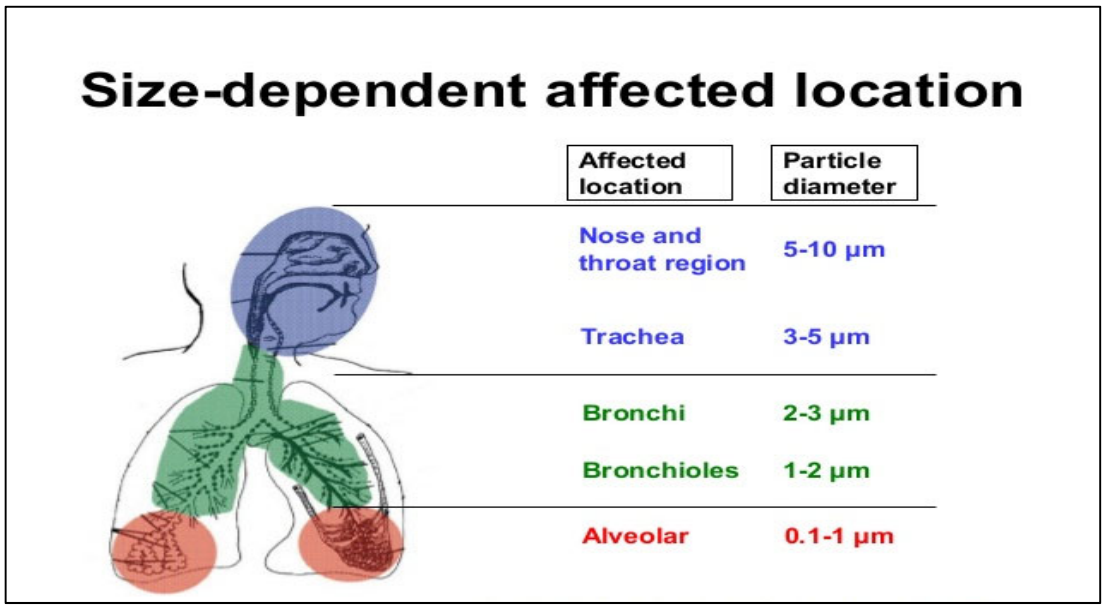


Figure 2. The Size of WTC Particulate matter that is capable of penetrating the respiratory system (Heyder, 2004; Oberdoster et al., 2005).

The Effects of the WTC Dust on a Cellular Level

Numerous studies have been conducted using WTC dust in lung fibroblast cells (MRC-5) and have shown detrimental results such as WTC dust decreases proliferation of MRC-5 *in vitro* (Hernandez *et al* 2012). It is believed that these symptoms occur due to high levels of oxidative stress. Reactive oxygen species (ROS) are the products formed when molecular oxygen is reduced. ROS are free radicals such as superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), singlet oxygen (O_2) and hydrogen peroxide(H_2O_2) are all the forms in which ROS is typically generated in the cell. H_2O_2 has the longest half-life and various ROS are converted to H_2O_2. ROS is known to be responsible of lipid peroxidation in membranes, direct oxidation of proteins, and cleavage of DNA and RNA molecules; in excess it can lead to cell damage including cancer and mutagenesis. (Nita & Grzybowski 2015).

In previous studies, it has been shown that WTC dust particles were deposited in the airways of the rescue workers causing a toxic effect on lung fibroblast cells (Cohen *et al* 2015). This increases the amount of inflammation found in the respiratory system and increases the levels of ROS. Oxidative stress is one of the central mechanisms in which particulate matter (PM) can affect the respiratory system leading to cell injury and apoptosis (Ghio *et al* 2012). Recently, oxidative stress caused by WTC dust has been associated with epigenetic changes in the lungs, as well as changes in DNA methylation, histone modifications and lung cells morphology (Sunil *et al* 2017).

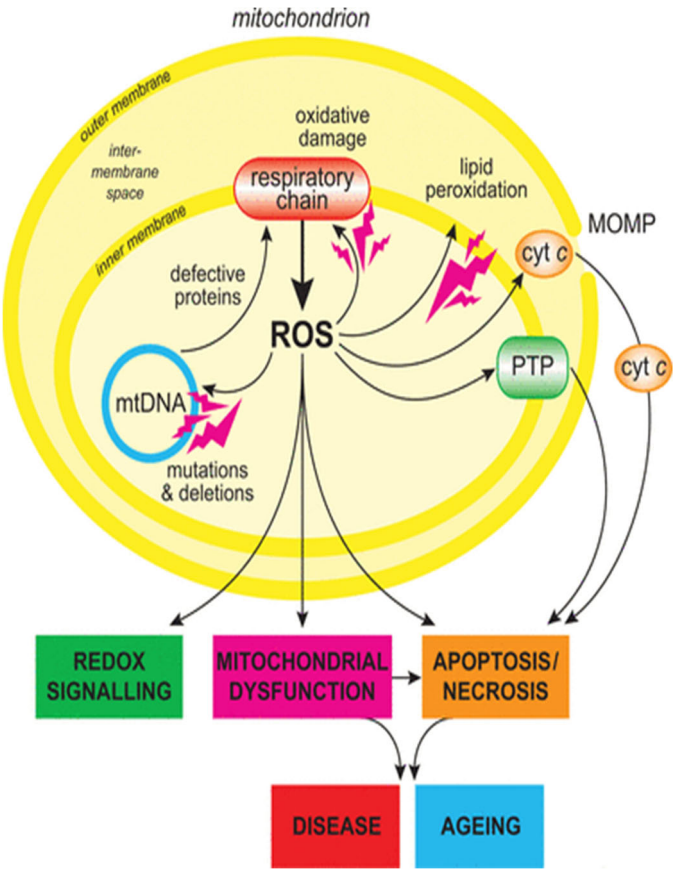


Figure 3. ROS Mechanism of action
"How Mitochondria Produce Reactive Oxygen Species."
Biochemical Journal (Murphy 2009)

Rationale for using Zinc & Copper and known effects

In Figure 1. The percentage of elements found in the WTC were analyzed by a large team of scientists lead by Paul Lioy of Rutgers University. Some of the elements analyzed were metals that generate oxidative stress through the electron transport chain to diminish levels of antioxidants (Ghio *et al* 2012). This study began with a question asking if Zinc and Copper, two metals found in the WTC dust are working synergistically to reduce or increase the oxidative stress in MRC-5 and HEF *in vitro.* Copper contributes about 4% of the WTC dust. The positive/negative role of this heavy metal will be compared to results of total WTC dust exposure. It is known that excess amounts of copper can lead to the production of ROS through the Fenton reaction mechanism which yields a hydroxyl radical and an oxidized metal ion (Dayem *et al* 2017).

Copper (Cu) is an element found in the periodic table that was discovered around 9,000 B.C. Copper is used extensively to make coins, to conduct electricity and it's also used in home heating systems. It is also found in our body as a nutrient, its main function is to be a catalytic cofactor for enzymes and its needed for many biological processes, including cellular respiration, and connective tissue formation (Zhao *et al* 2014). Copper is also useful in the production of erythrocytes, and for the regulation of neurons (Collins *et al* 2010). An adequate amount of copper is needed for iron absorption as well, due to Copper and Iron being redox-active metals. Copper is not only important for humans, but for plants, fungi and bacteria as well (Festa & Thiele 2011).

Zinc (Zn) is an element that contributes about 34% of the total WTC dust, the highest amount of any metal found in the complete analysis of the WTC dust. Zinc is also the second-most abundant mineral found in humans. Zinc is essential for the maintenance of almost 2,000

transcription factors and its needed for the proper activity of more than 300 enzymes (Zhao *et al* 2014). Zinc is needed for cellular homeostasis, and apoptosis regulation. In addition, Zinc is an important regulator of gene expression and membrane stability (Marreiro *et al* 2017).

Zinc as a possible antioxidant

Zinc, a well-studied mineral is believed to be an antioxidant instead of a toxic metal. One of its function as a possible antioxidant is the catalysis of copper/zinc superoxide dismutase (Lee 2018). In addition, Zinc can decrease ROS levels by removing Copper from its binding site where it catalyzes and forms a hydroxyl radical (Gaetke 2003). Most recently, Zinc can block and sequester copper absorption in the intestinal wall and Zinc is now being used as a treatment option for people who have Wilson's disease, a genetic disorder characterized by copper accumulation in different organs (Brewer 2014). The mechanism of action for the antioxidant Zinc is to inhibit the nicotinamide adenine dinucleotide phosphate oxidase (NADPH-Oxidase) enzyme and to activate metallothionein synthesis. Metallothioneins (MT) are highly conserved cysteine-rich binding proteins that blocks the production of hydroxyl radicals, therefore inhibiting ROS (Marreiro *et al* 2017).

Project Description

My thesis project began with a concern of the toxic metals in the World Trade Center Dust and questions whether other organs apart from the lungs were being affected by the toxic WTC dust. Previous experiments have shown that membrane damage, oxidative stress, cell death and mutagenic effects have occurred *in vitro* in human lung cells even after 18 years of the World Trade Center attack. Epidemiological studies demonstrated that more than 40% of the World Trade Center responders had GERD symptoms and some of them were diagnosed with Barrett's esophagus and esophageal cancer (Haider *et al* 2018). This sparked my interest into the correlation

of lung and esophageal cells in the presence of different toxic metals in the WTC dust. In this study, different concentrations of WTC dust were used on healthy human fibroblast lung cells (MRC-5) and healthy human esophageal fibroblast cells (HEF). In addition, concerns of the damage caused by Copper and as possible damage or benefits of Zinc were studied in both cell types. Cell proliferation and Ros-Glo H_2O_2 were the assays of choice.

Objectives of this Study

1) To determine the WTC dust effects in esophageal cells at a cellular level, not just epidemiologically.

2) To determine the effects of Copper and Zinc in a greater depth.

3) To determine if Copper or Zinc is more detrimental in MRC-5 or HEF cells.

Materials & Methods

Dust Sample Preparation:

One gram of WTC dust was placed and weighted out in a fume hood. The dust was then sterilized under a laminar flow hood with Ultraviolet (UV) light. Sufficient media was used to dissolve the dust in order to create a 100 mL solution of the stock. WTC. The stock solution was then diluted to the desired experimental concentrations (50ppm,125ppm and 250ppm). The LD 50 has been previously determined to be 250ppm and therefore that is the highest concentration used in this experiment.

Media Preparation

The stock WTC media was prepared aseptically using Eagle's Minimal Essential Media (MEM), 1% of Penicillin Streptomycin (PS), Glutamax (G), Kanamycin Sulfate (K) and 10% Fetal Bovine Serum (FBS). It was then exposed to UV radiation for sterilization purposes. The dust was weighted out in a Sartorius analytical scale to make a 500pmm stock solution. From the

stock, dilutions were calculated to make 50ppm,125ppm, and 250ppm solutions. The stock and dilutions were then stored in -18˚C freezer.

Cell Culture Maintenance

MRC-5 (ATCC #CCL-171) are male fibroblast cells found in human lung tissue that were cultured in T-25 flasks containing MEM and 10% FBS that contains 1% PSKG. The conditions of the cells were monitored daily using phase contrast/Inverted AO microscopes. Once the MRC-5 cells were attached and confluent, subculture was done using aseptic technique. Then, the cells were plated in white tissue culture treated clear bottom 96-well plates and incubated at 37 ºC for 24 hours for monolayer formation to occur within each well, which was again confirmed by Phase Contrast/Inverted microscope. MRC-5 plates had normal complete media that was replaced with media containing different concentrations of WTC dust, WTC/Cu, WTZ/Zn, Zinc and Copper.

Human Esophageal Fibroblasts (HEF) [ScienCell #2730] are fibroblast cells found in human esophageal tissue. HEF were maintained with the same normal subculture procedure as MRC-5 cells.

Preparation of Copper and Zinc

Two grams of Copper Sulfate ($CuSo_4$) were weighted out in a Sartorius analytical scale. Sufficient media was used to dissolve the copper in order to create a 100mL solution of the stock. The copper sulfate solution was then sterilized by vacuum filtration leaving the copper residue at the bottom of the flask. The stock solution was subdivided using complete media and 10% Fetal Bovine Serum (FBS) and then diluted in complete media to the desired experimental concentrations (25ppm,62.5ppm and 125ppm).

Two grams of Zinc Sulfate ($ZnSo_4$) was weighted out in a Sartorius analytical scale. The zinc sulfate was then added to complete media and sterilized by vacuum filtration leaving the

zinc residue at the bottom of the flask. Sufficient media was used to dilute the copper in order to create a 100 mL solution of the stock. The stock solution was subdivided using complete media and 10% Fetal Bovine Serum (FBS) and then diluted to the desired experimental concentrations (50ppm,125ppm and 250ppm).

Cell Proliferation Assay

MRC-5 and HEF cells were plated 24 hours before experiment. Confluency had been confirmed for all the wells. After 24 hours 100µl of the specific experimental metals and WTC dust replaced the normal complete media that had allowed normal proliferation to occur. This experimental media was in contact with healthy MRC-5 and HEF cells for 24 hours. After 24 hours of exposure time the CellTiter 96 A_{Queous} One Solution Cell Proliferation Assay (PROMEGA G3582) was performed. This assay is a colorimetric method that determines the number of viable cells. It uses a tetrazolium compound called MTS (Owen's reagent) dye that combined with an electron coupling reagent phenazine ethosulfate (PES) which produces a colored formazan product that is soluble in tissue culture media. 20µl of the defrosted reagent was added into each 96-well plate. Plates were then incubated at 37^0C for 1 to 4 hours. The absorbance was then recorded at 490nm using a 96-well plate reader.

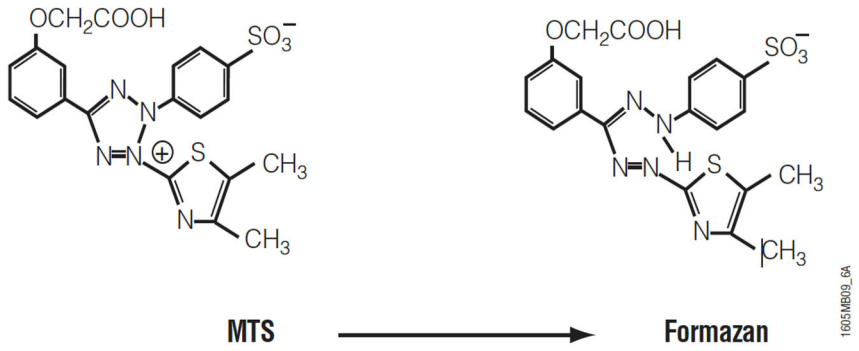


Figure 4. MTS tetrazolium conversion to formazan product

ROS-Glo™ H_2O_2 Assay

Ros-Glo H_2O_2 Assay (PROMEGA G8820) measures H_2O_2 which is the most stable and has the longest half-life reactive oxygen species (ROS). It undergoes a mechanism for H_2O_2 measurement that indicates the amount of ROS by increments of luminescence. As in the previous assay, cells were grown in 100 µl of normal complete media in white translucent 96-well plate and incubated at 37^0C for 24 hours. Confluence was determined and after 24 hours 100µl of the specific experimental metals and WTC dust replaced the normal complete media that had allowed normal proliferation to occur. This experimental media was in contact with healthy MRC-5 and HEF cells for 24 hours. After 24 hours of exposure time, the ROS-GLO H_2O_2 assay was performed. The H_2O_2 substrate was produced by diluting 10mM H_2O_2 Substrate with the 125µM H_2O_2 dilution buffer. Once the substrate solution had been made, 20µl of the H_2O_2 Substrate solution was added to each well and incubated for up to 6 hours. The Ros-Glo detection solution was prepared by adding the Reconstitution Buffer to the lyophilized Luciferin Detection Reagent to produce Reconstituted Luciferin Detection Reagent. 100µl of d-Cysteine and 100µl of Signal Enhancer Solution was also added to produce the ROS-Glo Detection Solution. 100µl of the Ros-Glo detection solution was added to each 96 well plate and incubated for 20 minutes at room temperature. After this series of steps, the luminescence was then recorded using a 96 well plate reader.

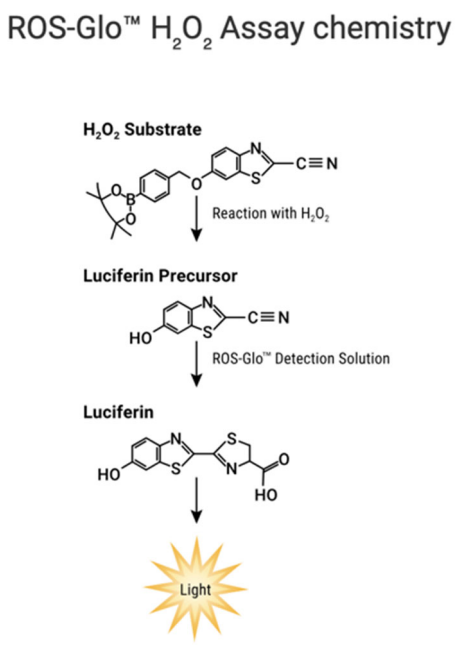


Figure 5. Ros-Glo H_2O_2 assay chemistry.

Timeline for Completion

This project was conducted at Montclair State University Biology Department. It began in Fall 2018 and finished in Summer 2019.

Results and Discussion

The collapse of the World Trade Center on 9/11 was a tragedy that continues to affect many. Previous studies have shown that WTC dust particles were inhaled and swallowed causing airway and esophageal irritation (Lippman *et al* 2015). Preliminary studies have concluded that exposure to the WTC particulate matter increases arterial blockage, inflammation and development of cardiovascular disease (Weiden *et al* 2012). The purpose of this study is to examine the effects of WTC dust, Copper and Zinc in both MRC-5 and HEF cells *in vitro*. To determine cell proliferation and oxidative stress levels in cells with various concentrations of WTC dust, Copper and Zinc and to determine if there are differences in the effects of cell physiology of human esophageal versus human lung cells.

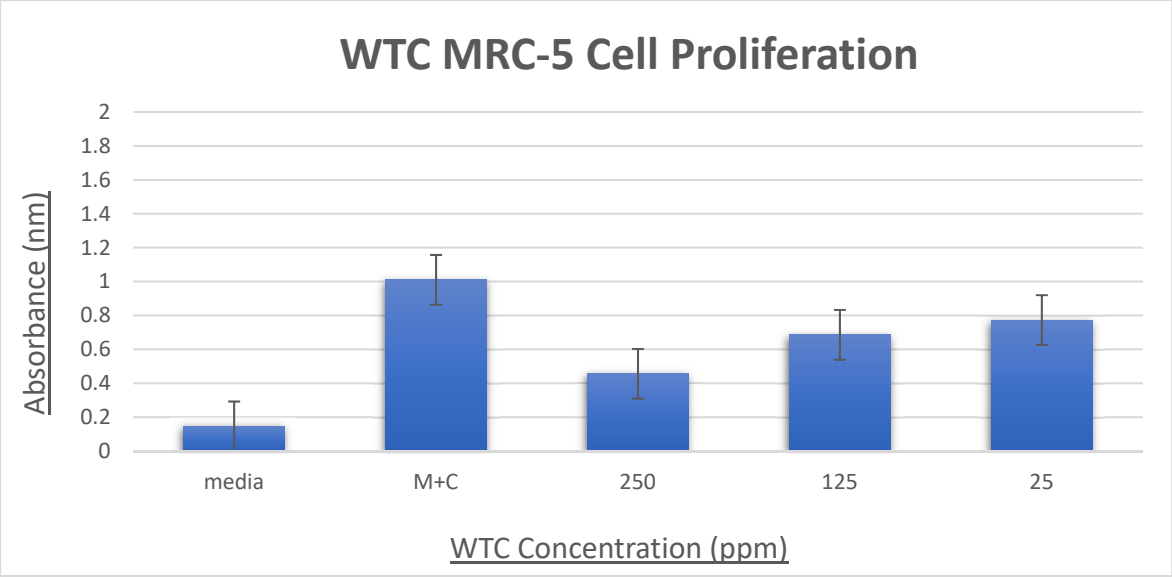


Figure 6. **Cell proliferation levels in MRC-5;** absorbance (nm) was measured using a 96-well plate reader vs. the concentration WTC Dust in various ppm (250, 125, and 50 ppm) measured by the ***Cell Proliferation One Solution Assay***. In the figure shown, the higher amount of absorbance, the higher amount of cell viability.

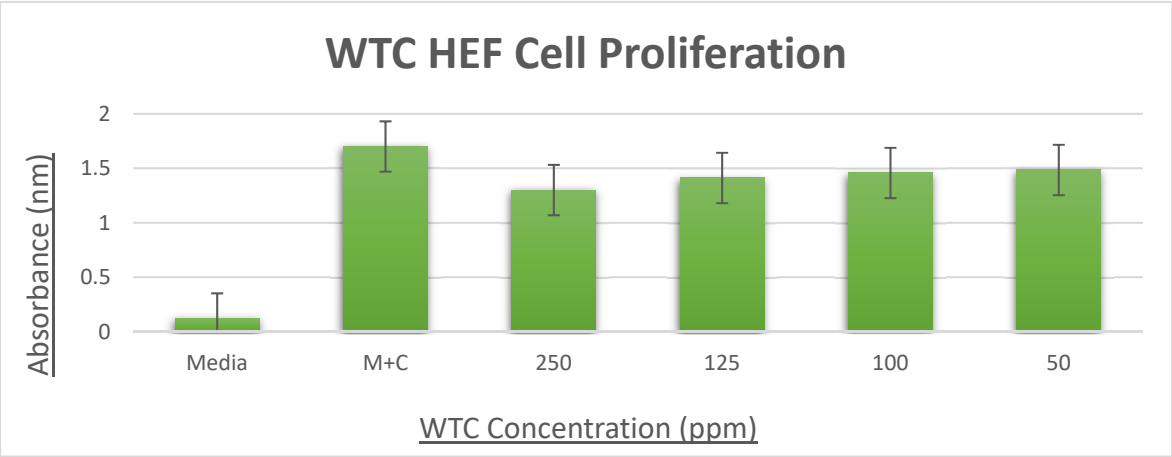


Figure 7. **Cell proliferation levels in HEF;** absorbance (nm) was measured using a 96-well plate reader vs. the concentration WTC Dust in various ppm (250, 125, 100 and 50 ppm) measured by the ***Cell Proliferation One Solution Assay***. In the figure shown, the higher amount of absorbance, the higher amount of cell viability.

Cell proliferation One Solution assay determines the level of viability this is measured as a colorimetric change as absorbance increases. Figure (6) shows that cell proliferation of MRC-5 cells with a concentration of 250ppm is highly decreased, demonstrating that the effect of WTC dust in lung cells is highly detrimental as prior studies have shown. While interestingly, in figure (7) HEF proliferation levels are also decreased but not to the extent of MRC-5, correlating to previous studies demonstrating the association between WTC dust exposure and lung disease (Wu *et al* 2010).

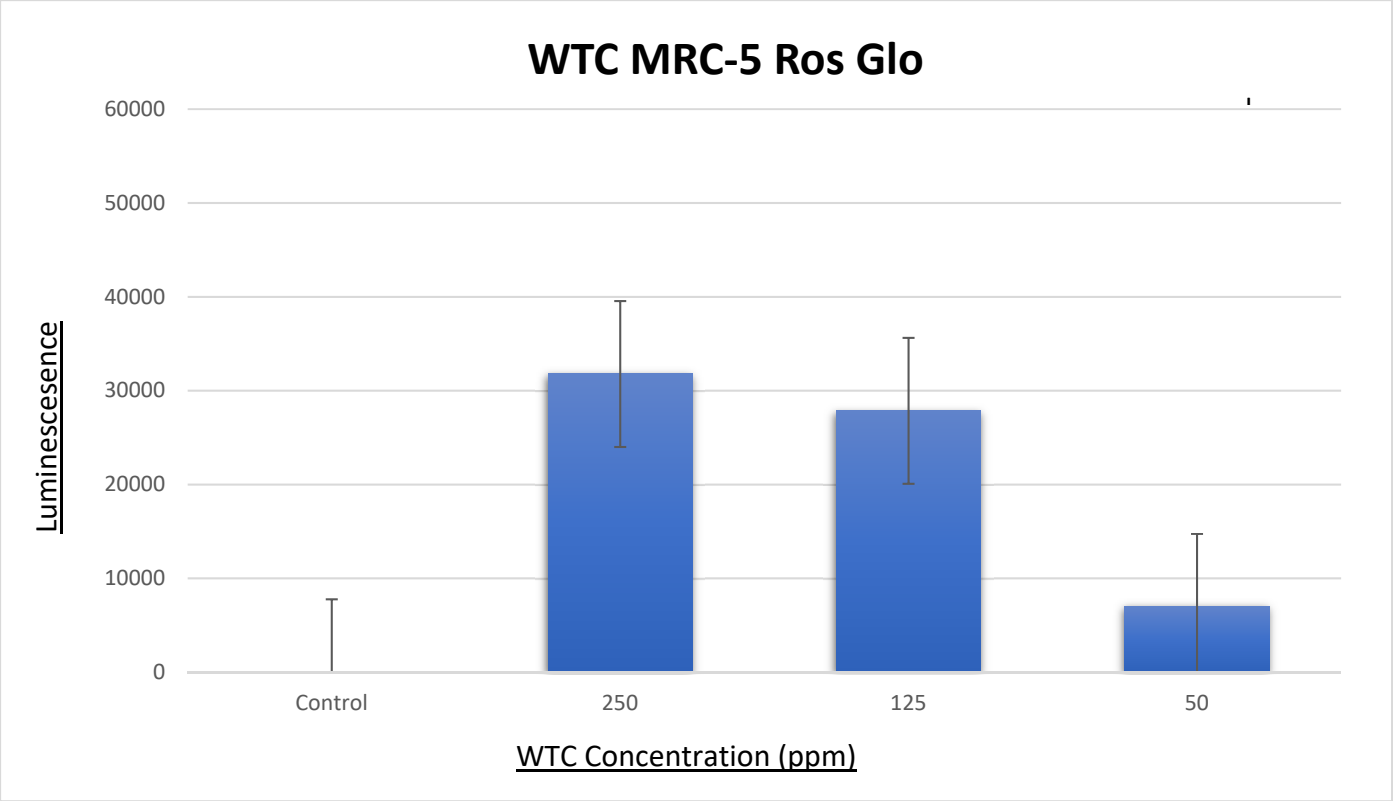


Figure 8. **Levels of H_2O_2 in MRC-5/WTC;** luminescence (a.u.) was measured using the luminometer vs. the concentration WTC Dust in various ppm (250, 125, and 50 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

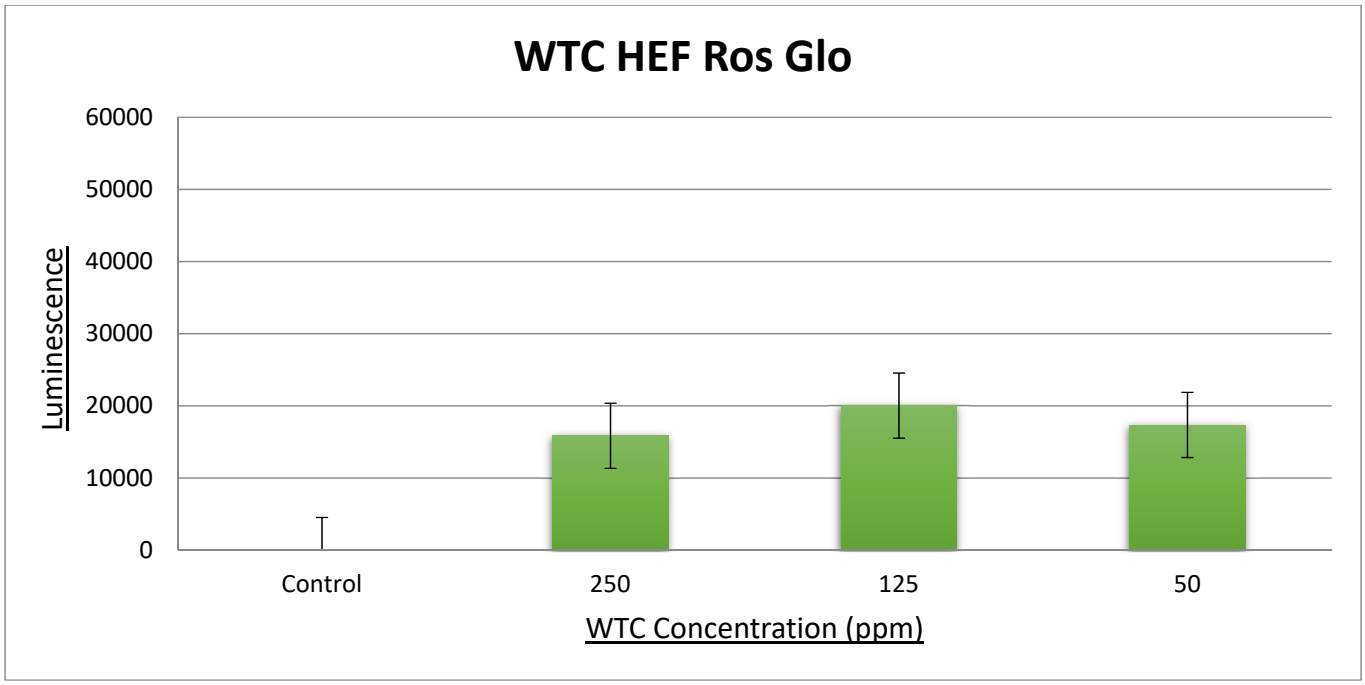


Figure 9. **Levels of H_2O_2 in HEF/WTC;** luminescence (a.u.) was measured using the luminometer vs. the concentration WTC Dust in various ppm (250, 125, and 50 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

Concentration (ppm)	WTC MRC-5	WTC HEF
250	31777	15848.5
125	27857	20033.5
50	6958.33	17352.2

Table 1. ROS Concentrations of WTC MRC-5 (blue) and WTC HEF ROS-Glo assay (green)

ROS-Glo H_2O_2 assay determines the levels of ROS produced as shown by changes in measurable luminescence. Figure (8) shows that ROS levels are extremely high at a 250ppm concentration yet as the concentration lessen, ROS levels decreases as well. Yet in figure (9) the concentration of ROS is the highest at 125ppm in HEF suggesting that smaller amounts of WTC dust appear to be

needed to damage the esophageal lining. Figure (8) correlates with the results found in figure (6) and previous studies, where WTC dust exposure exacerbates airways diseases.

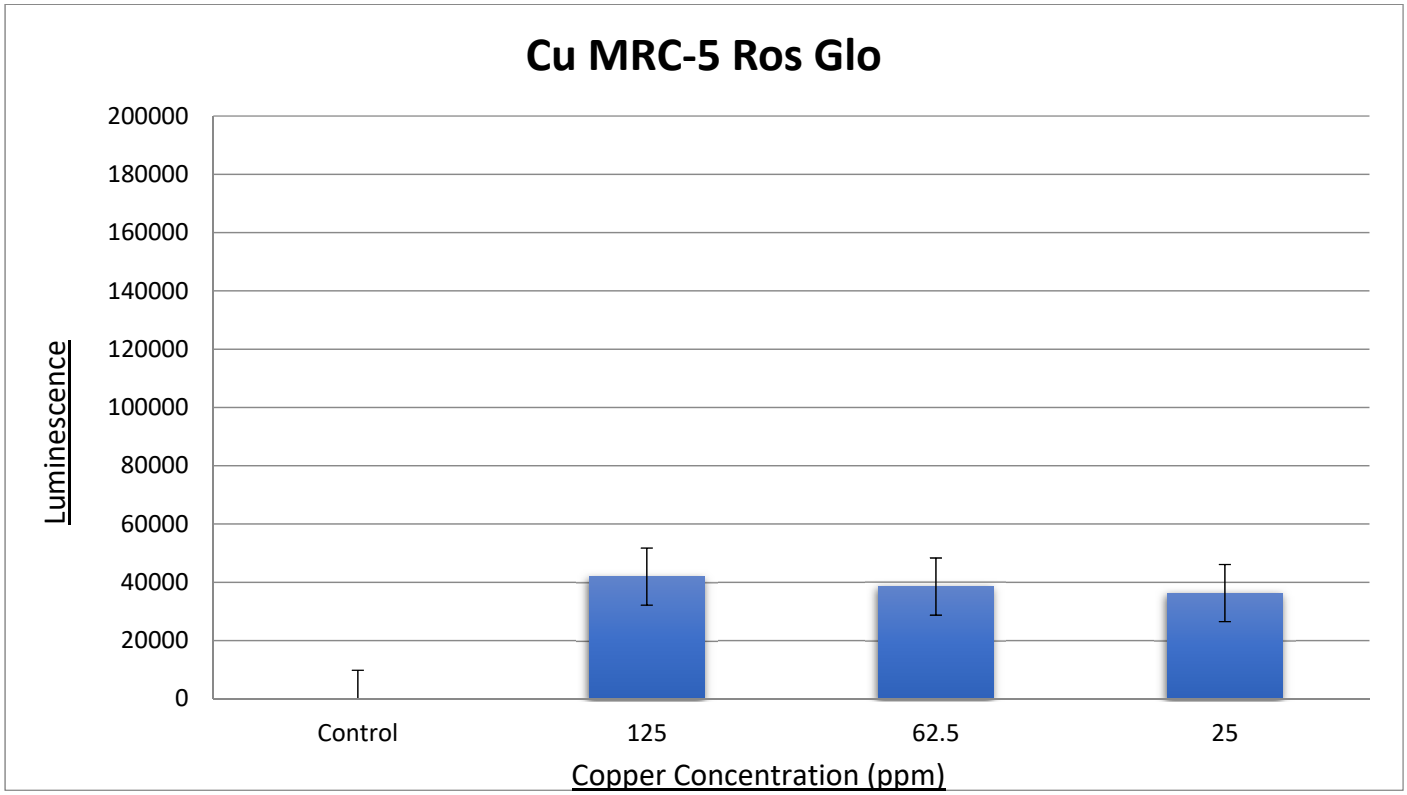


Figure 10. **Levels of H_2O_2 in MRC-5/Copper;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of Copper (125, 62.5 and 25 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

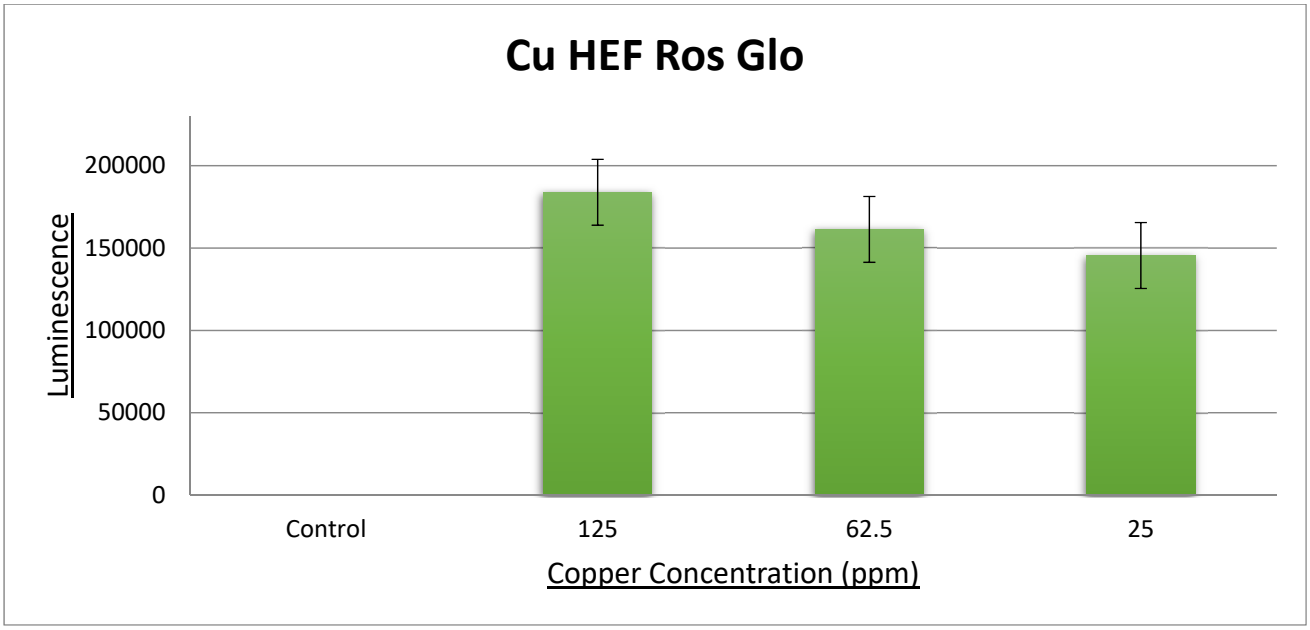


Figure 11. **Levels of H_2O_2 in HEF/Copper;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of Copper (125, 62.5 and 25 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

Concentration (ppm)	Cu MRC-5	Cu HEF
125	41968	183866
62.5	38522	161339
125	32691	145479

Table 2. ROS Concentrations of Cu MRC-5 (blue) and Cu HEF ROS-Glo assay (green)

Figure (10) shows that Copper causes a slight increase of ROS levels in MRC-5 while in fjgure (11), Copper causes ROS levels to be extremely high especially at 125ppm suggesting that high concentrations of Copper is extremely harmful in HEF cells**.** This might be due to the anatomy of the esophagus, suggesting that less quantity of Copper is needed to harm HEF rather than MRC—5 correlating to preliminary studies that indicate that many rescue workers have GERD symptoms due to particle ingestion.

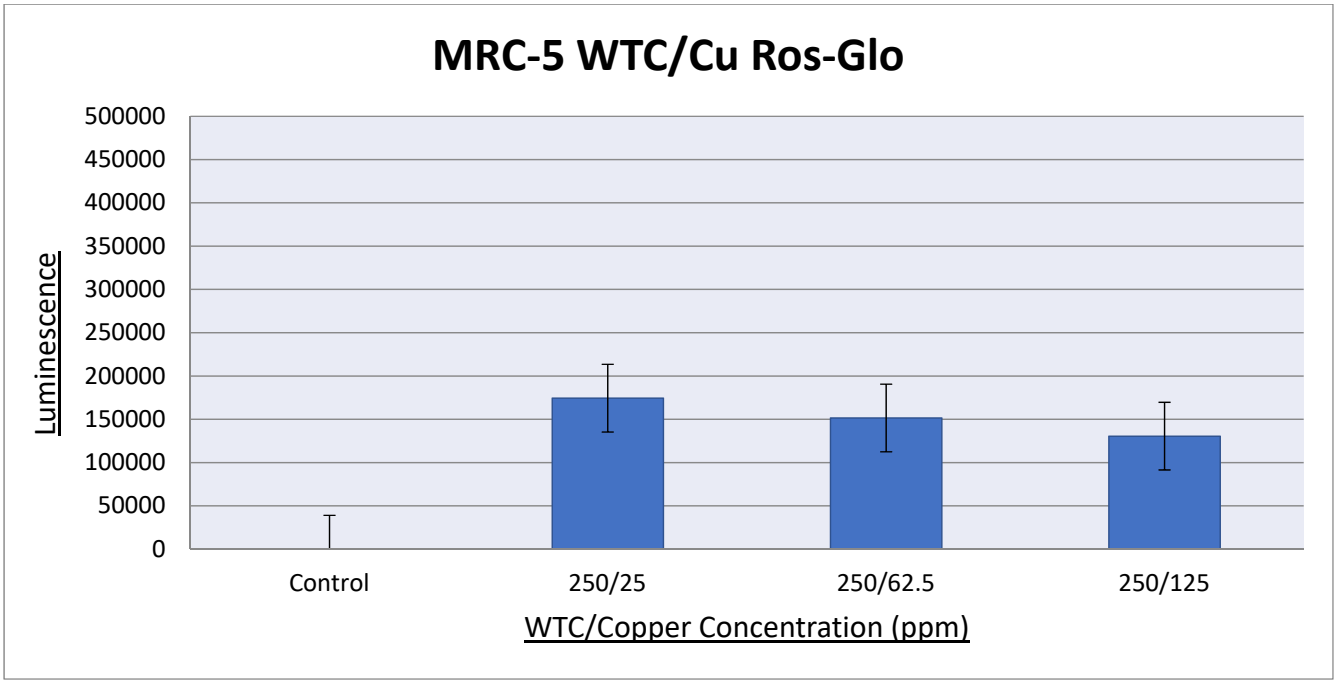


Figure 12. **Levels of H_2O_2 in MRC-5/WTC+Copper;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of WTC and Copper (250 WTC/25 Cooper, 250 WTC/62.5 Copper and 250 WTC/25 Copper ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

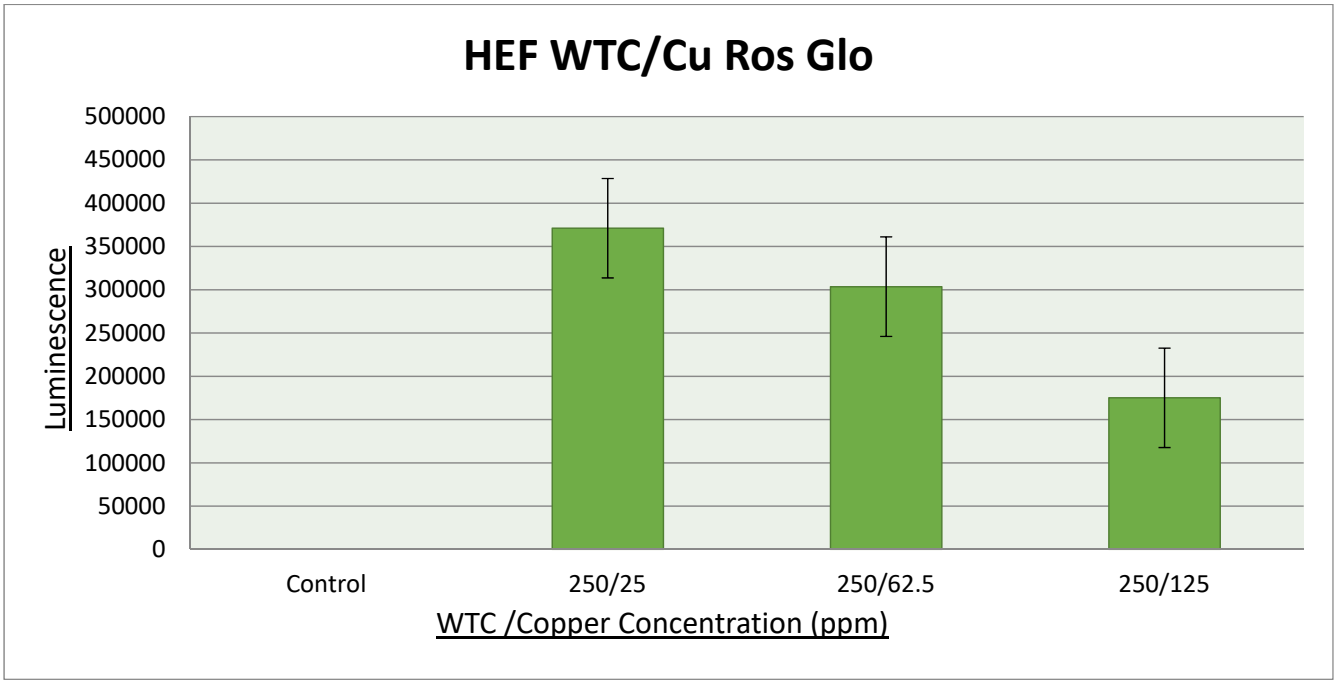


Figure 13. **Levels of H_2O_2 in HEF/WTC+Copper;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of WTC and Copper (250 WTC/25 Cooper, 250 WTC/62.5 Copper and 250 WTC/25 Copper ppm) measured by ***ROS-Glo^{TM} H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress.

Concentration (ppm)	**WTC/Cu MRC-5**	**WTC/Cu HEF**
250/25	174416.5	371059.8
250/62.5	151457	303500
250/125	130492.5	175140.2

Table 3. ROS Concentrations of WTC/Cu MRC-5 (blue) and WTC/Cu HEF ROS-Glo assay (green)

Figure (12) shows that a combination of WTC and Copper in MRC-5 cells increases the levels of ROS when compared to figure (8) and figure (10). Yet as the concentration of Copper increases, oxidative stress decreases, suggesting that Copper might be beneficial to lower the harmful effects of the WTC dust in lung cells. While in figure (13) a combination of WTC and Copper in HEF cells indicates a high amount of ROS levels especially in 250/25ppm combination giving the impression that even a small amount of Copper is detrimental in HEF cells. Copper can induce oxidative damage at a cellular level (Gaetke *et al* 2014) and may enhance apoptosis by activating caspase 3,8 and 9.

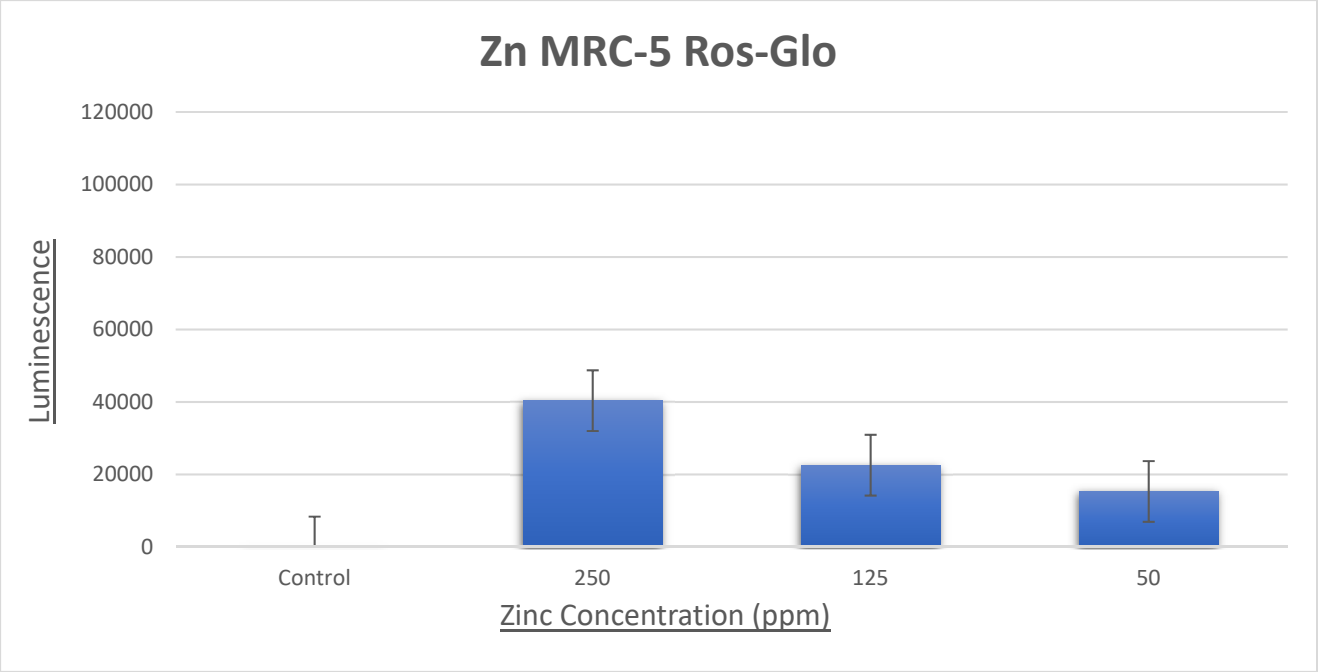


Figure 14. **Levels of H_2O_2 in MRC-5/Zinc;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of Zinc (250, 125 and 50 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

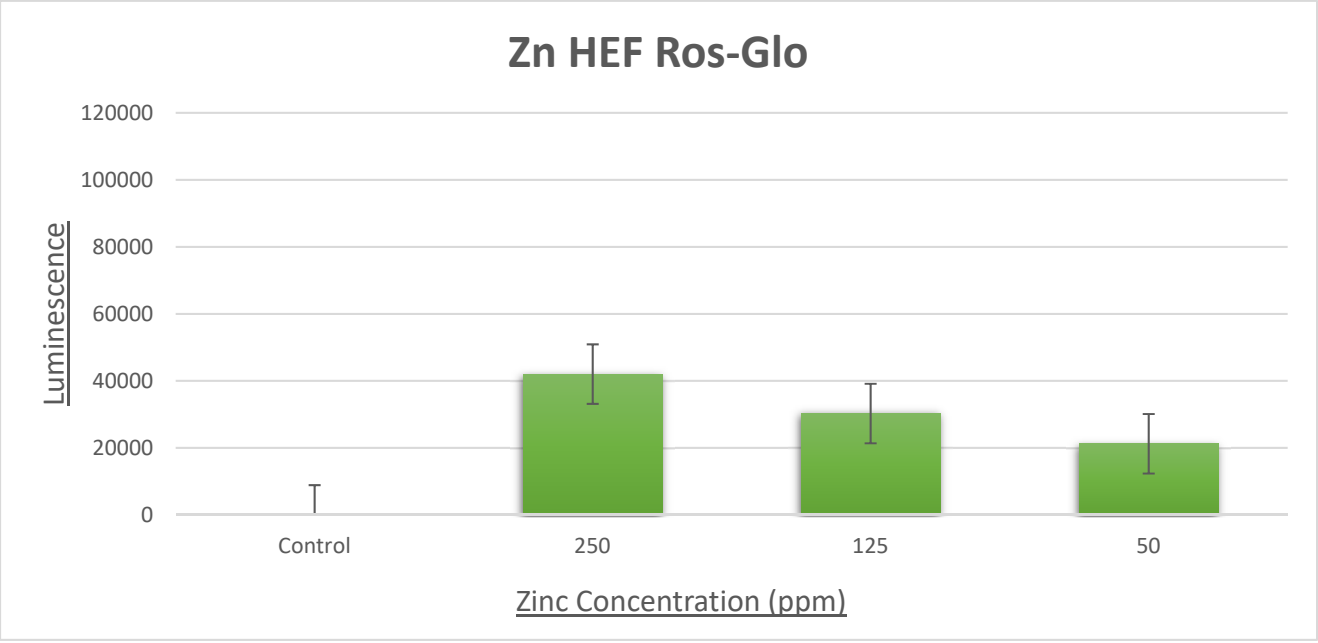


Figure 15. Figure 15. **Levels of H_2O_2 in HEF/Zinc;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of Zinc (250, 125 and 50 ppm) measured by ***ROS-Glo^{TM} H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

Concentration (ppm)	**Zn MRC-5**	**Zn HEF**
250	40363.7	42032
125	22510.8	30250.7
50	15324	21232.5

Table 4. ROS Concentrations of Zn MRC-5 (blue) and Zn HEF ROS-Glo assay (green)

Figure (14) shows that high amounts of Zinc (250ppm) increases ROS levels slightly yet figure (10) demonstrates that a smaller amount of Copper (125ppm) has the same damaging effect as 250ppm.While in figure (15) 250ppm Zinc increases ROS levels higher in HEF cells than MRC-5 and even though ROS levels are increased as Zinc concentration is higher, a lower amount of Copper concentration is still more damaging to HEF cells *in vitro* suggesting that Zinc might have antioxidant properties (Prasad 2014).

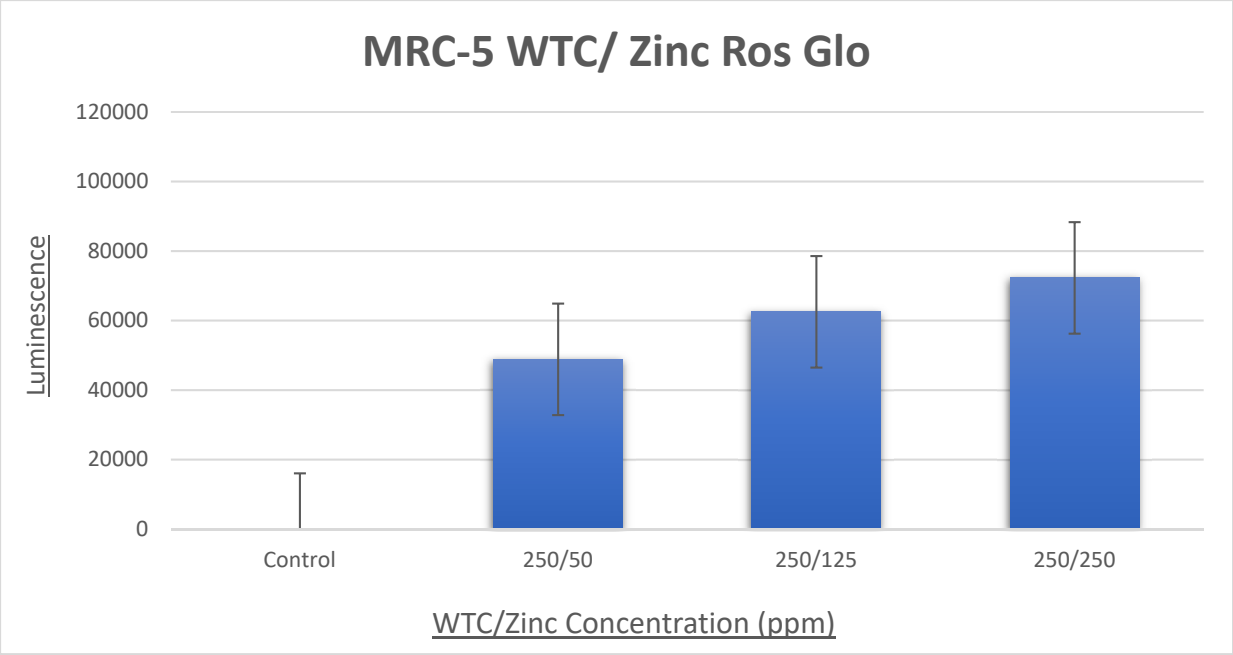


Figure 16. **Levels of H_2O_2 in MRC-5/WTC+Zinc;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of WTC and Zinc (250 WTC/50 Zinc, 250 WTC/125 Zinc and 250 WTC/250 Zinc ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

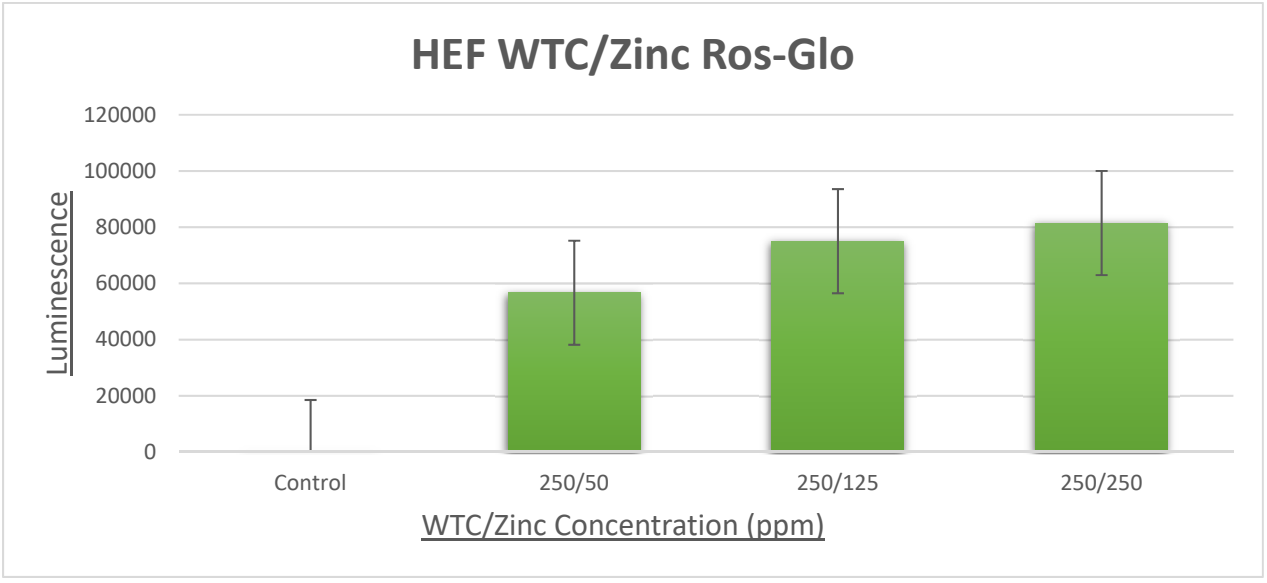


Figure 17. **Levels of H_2O_2 in HEF/WTC+Zinc;** luminescence (a.u.) was measured using the luminometer vs. the various concentrations of mixture of WTC and Zinc (250 WTC/50 Zinc, 250 WTC/125 Zinc and 250 WTC/250 Zinc ppm) measured by ***ROS-Glo^{TM} H_2O_2 Assay***. In the figure shown, the higher amount of light, the higher amount of oxidative stress. (Numbers were normalized).

Concentration (ppm)	WTC/ Zn MRC-5	WTC/ Zn HEF
250/50	48849.3	56672
250/125	62518	75011.5
250/250	72171.7	81477.3

Table 5. ROS Concentrations of WTC/Zn MRC-5 (blue) and WTC/Zn HEF ROS-Glo assay (green)

Figure (16) shows a combination of WTC and Zinc in MRC-5 cells, as the concentration of Zinc increases, ROS levels become higher suggesting that high amounts of Zinc can be harmful yet in comparison to figure (12), WTC and Zinc combinations are much lower than WTC and Copper suggesting that such high amount of Zinc in the WTC dust might actually lessen the damaging effects of the WTC dust (Pattnaik 2013). Figure (17) demonstrates that a combination of WTC and Zinc increases oxidative stress higher than MRC-5. This indicates that HEF cells are more sensitive to metals. Yet in figure (13), WTC and Zinc combination is less than WTC and Copper combination correlating to previous studies were Copper can form hydroxyl radicals that can convert to H_2O_2. leading to higher amounts of ROS levels in Copper than in Zinc.

Conclusions

The results of this study confirm that the WTC dust exposure has contributed to the progression of lung disease. Additionally, the WTC dust is affecting the esophagus. Epidemiological results have demonstrated that GERD is a disease that many first responders have, nevertheless, there has not been any molecular research investigating the mechanism behind GERD in correlation to the WTC dust. The predominant finding of this study has shown that HEF cells are showing higher levels of oxidative stress when exposed to Copper, rather than MRC-5. Furthermore, this study has showed that Zinc is acting as a possible antioxidant lowering cell damage and oxidative stress by decreasing the effects of WTC dust as a whole and its Copper components. This study suggest that the esophagus is more sensitive to Copper and Zinc due to its anatomical structure. It suggests that less amount of particulate matter (PM) is needed to damage the esophagus rather than the lungs yet the exact mechanism is still unclear. The complete WTC dust effects is still unknown, first responders are still suffering ailments and side effects of the WTC dust. A continued study on HEF cells should be done to elucidate the reasoning behind Copper and Zinc toxicity prevalence in HEF rather than MRC-5.

Future Research

Future research should focus on the role of Copper and Zinc in NADPH inhibition using the NADP/NADPH-Glo Assay Protocol because Zinc inhibits the NADPH-oxidase enzyme, reducing chronic inflammation (Marreiro *et al* 2017). Future studies should also look at the role of Zinc in the regulation of cell death because Zinc is an inhibitor of apoptosis (Ruttkay-Nedecky et al 2013). Future work should also look at Zinc and Copper together and repeating the Ros Glo H_2O_2 assay to confirm these preliminary findings. Future studies should also look at other metals

in the WTC dust that have not been analyzed in depth yet and seeing if specific combinations can reduce or increase the effects of ROS in MRC-5 and HEF cells.

Bibliography

1. Brewer, George J. "Alzheimer's Disease Causation by Copper Toxicity and Treatment with Zinc." *Frontiers in Aging Neuroscience*, vol. 6, 16 Mar. 2014, doi:10.3389/fnagi.2014.00092.
2. Cohen, Mitchell D., et al. "Acute High-Level Exposure to WTC Particles Alters Expression of Genes Associated with Oxidative Stress and Immune Function in the Lung." *Journal of Immunotoxicology*, vol. 12, no. 2, 9 June 2014, pp. 140–153., doi:10.3109/1547691x.2014.914609.
3. Collins, James F, et al. "Metabolic Crossroads of Iron and Copper." *Nutrition Reviews*, vol. 68, no. 3, Mar. 2010, pp. 133–147., doi:10.1111/j.1753-4887.2010.00271.x.
4. Dayem, Ahmed Abdal, et al. "The Role of Reactive Oxygen Species (ROS) in the Biological Activities of Metallic Nanoparticles." *International Journal of Molecular Sciences*, vol. 18, no. 1, 10 Jan. 2017, p. 120., doi:10.3390/ijms18010120.
5. Festa, Richard A., and Dennis J. Thiele. "Copper: An Essential Metal in Biology." *Current Biology*, vol. 21, no. 21, 8 Nov. 2011, doi:10.1016/j.cub.2011.09.040.
6. Gaetke, Lisa et al."Copper toxicity, oxidative stress, and antioxidant nutrients." *Toxicology*, Volume 189, Issues 1–2. July 15, 2003.
7. Gaetke, Lisa M., et al. "Copper: Toxicological Relevance and Mechanisms." *Archives of Toxicology*, vol. 88, no. 11, 9 Nov. 2014, pp. 1929–1938., doi:10.1007/s00204-014-1355.

8. Ghio, Andrew J., et al. “Composition of Air Pollution Particles and Oxidative Stress in Cells, Tissues, and Living Systems.” Journal of Toxicology and Environmental Health, Part B, vol. 15, no. 1, 2012, pp. 1–21., doi:10.1080/10937404.2012.632359.
9. Haider, S. H., Kwon, S., Lam, R., Lee, A. K., Caraher, E. J., Crowley, G., Nolan, A. (2018). Predictive Biomarkers of Gastroesophageal Reflux Disease and Barrett’s Esophagus in World Trade Center Exposed Firefighters: A 15 Year Longitudinal Study. *Scientific Reports, 8*(1). doi:10.1038/s41598-018-21334-9
10. Hernandez, M., Choi, S., & DiLorenzo., A. (2012). Study Of pH And Cellular Response in Pulmonary Fibroblasts After Exposure To Market Street World Trade Center Particulate Matter. *OnLine Journal of Biological Sciences,12*(4), 142-148.
11. Lambroussis, Constantino G., et al. “Indications of Potential Toxic/Mutagenic Effects of World Trade Center Dust on Human Lung Cell Cultures.” *OnLine Journal of Biological Sciences*, vol. 9, no. 3, 2009, pp. 81–85., doi:10.3844/ojbsci.2009.81.85.
12. Lee, Sung Ryul. “Critical Role of Zinc as Either an Antioxidant or a Prooxidant in Cellular Systems.” *Oxidative Medicine and Cellular Longevity*, vol. 2018, 20 Mar. 2018, pp. 1–11., doi:10.1155/2018/9156285.
13. Lippmann, Morton et al. “Health effects of World Trade Center (WTC) Dust: An unprecedented disaster's inadequate risk management.” *Critical reviews in toxicology* vol. 45,6 (2015): 492-530. doi:10.3109/10408444.2015.1044601
14. Li, J., Brackbill, R. M., Stellman, S. D., Farfel, M. R., Miller-Archie, S. A., Friedman, S.,Cone, J. (2011). Gastroesophageal Reflux Symptoms and Comorbid Asthma and Posttraumatic Stress Disorder Following the 9/11 Terrorist Attacks on World Trade

Center in New York City. *The American Journal of Gastroenterology, 106*(11), 1933-1941. doi:10.1038/ajg.2011.300

15. Lioy, Paul J, et al. “Characterization of the Dust/Smoke Aerosol That Settled East of the World Trade Center (WTC) in Lower Manhattan after the Collapse of the WTC 11 September 2001.” *Environmental Health Perspectives*, vol. 110, no. 7, 2002, pp. 703–714., doi:10.1289/ehp.02110703.
16. Liu, Bian, et al. “World Trade Center Disaster Exposure-Related Probable Posttraumatic Stress Disorder among Responders and Civilians: A Meta-Analysis.” *PLoS ONE*, vol. 9, no. 7, 2014, doi: 10.1371/journal.pone.0101491.
17. Liu, Xiaoxue, et al. “The Effect of World Trade Center Exposure on the Timing of Diagnoses of Obstructive Airway Disease, Chronic Rhinosinusitis, and Gastroesophageal Reflux Disease.” *Frontiers in Public Health*, vol. 5, 8 Feb. 2017,doi:10.3389/fpubh.2017.00002.
18. Marreiro, Dilina, et al. “Zinc and Oxidative Stress: Current Mechanisms.” *Antioxidants*, vol. 6, no. 2, 29 Mar. 2017, p. 24., doi:10.3390/antiox6020024.
19. Murphy, Michael P. “How Mitochondria Produce Reactive Oxygen Species.” *Biochemical Journal*, vol. 417, no. 1, 2009, pp. 1–13., doi:10.1042/bj20081386.
20. Nita, Małgorzata, and Andrzej Grzybowski. “The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults.” *Oxidative Medicine and Cellular Longevity*, vol. 2015, 17 Nov. 2015, pp. 1–23., doi:10.1155/2016/3164734.

21. Pattnaik, Niharika, and Ann Marie, DiLorenzo. “Comparison of World Trade Center Dust with Zinc Acetate and Lead Oxide Combinations to Determine Damage to Human Lung Cells.” *Journal of Toxicology and Environmental Health Sciences*, vol. 5, no. 4, 5 Mar. 2013, pp. 60–65., doi:10.5897/jtehs2012.0001.
22. Prasad, Ananda S. “Zinc Is an Antioxidant and Anti-Inflammatory Agent: Its Role in Human Health.” *Frontiers in Nutrition*, vol. 1, 1 Sept. 2014, doi:10.3389/fnut.2014.00014.
23. Prezant, David J., et al. “Cough and Bronchial Responsiveness in Firefighters at the World Trade Center Site.” *New England Journal of Medicine*, vol. 347, no. 11, 12 Sept. 2002, pp. 806–815., doi:10.1056/nejmoa021300.
24. Ruttkay-Nedecky, Branislav, et al. “The Role of Metallothionein in Oxidative Stress.” *International Journal of Molecular Sciences*, vol. 14, no. 3, 14 Mar. 2013, pp. 6044 6066., doi:10.3390/ijms14036044.
25. Sunil, Vasanthi R., et al. “World Trade Center (WTC) Dust Exposure in Mice Is Associated with Inflammation, Oxidative Stress and Epigenetic Changes in the Lung.” *Experimental and Molecular Pathology*, vol. 102, no. 1, Feb. 2017, pp. 50–58., doi: 10.1016/j.yexmp.2016.12.005.
26. Wang, Shang, et al. “Roles of MAPK Pathway Activation during Cytokine Induction in BEAS-2B Cells Exposed to Fine World Trade Center (WTC) Dust.” *Journal of Immunotoxicology*, vol. 7, no. 4, 24 Aug. 2010, pp. 298–307., doi:10.3109/1547691x.2010.509289.

27. Weiden, Michael D., et al. “Cardiovascular Biomarkers Predict Susceptibility to Lung Injury in World Trade Center Dust-Exposed Firefighters.” *European Respiratory Journal*, vol. 41, no. 5, 16 Aug. 2012, pp. 1023–1030., doi:10.1183/09031936.00077012.
28. Wu, Maoxin, et al. “Case Report: Lung Disease in World Trade Center Responders Exposed to Dust and Smoke: Carbon Nanotubes Found in the Lungs of World Trade Center Patients and Dust Samples.” *Environmental Health Perspectives*, vol. 118, no. 4, Apr. 2010, pp. 499–504., doi:10.1289/ehp.0901159.
29. Xing, Yu-Fei et al. “The impact of PM2.5 on the human respiratory system.” *Journal of thoracic disease* vol. 8,1 (2016): E69-74. doi:10.3978/j.issn.2072-1439.2016.01.19
30. Zhao, Lu, et al. “Zebrafish in the Sea of Mineral (Iron, Zinc, and Copper) Metabolism.” *Frontiers in Pharmacology*, vol. 5, 6 Apr. 2014, doi:10.3389/fphar.2014.00033.