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### A Preliminary Investigation of World Trade Center (WTC) Dust Effects on MRC-5 Lung Fibroblasts and the Cellular Inflammatory Response

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## **Abstract:**

The World Trade Center (WTC) attack on September 11th, 2001 was the biggest terrorist attack that has ever occurred in New York City. About 3,000 people died on September 11th, 2001 and more than 6,000 were injured including law enforcement officers, firefighters, emergency medical services and military personnel. The building collapse generated a huge cloud of dust and first responders have suffered from long-term lung related illnesses. The present study evaluated three questions: 1.) does WTC dust treatment of MRC-5 lung fibroblasts induce an inflammatory response as evidenced by the production of nitric oxide? 2.) Do heavy metals such as Copper or Manganese (two major components of WTC dust) affect lung fibroblast viability and induce oxidative stress? 3.) What are the possible effects of EGCG-S (green tea, GT) on these cellular inflammatory processes? The results showed that the World Trade Center Dust treatment did induce Nitric Oxide (NO) production, indicative of an inflammatory response. Manganese (Mn) or Copper (Cu) treatment decreased cell viability and increased ROS production. Treatment with EGCG-S demonstrated the generation of intracellular ROS (Reactive Oxygen Species). Manganese-induced ROS (Reactive Oxygen Species) activation was potentiated in the presence of EGCG-S.

A Preliminary Investigation of World Trade Center (WTC) Dust Effects on MRC-5 Lung Fibroblasts and the Cellular Inflammatory Response

By:

Katelyn Anna Kerod

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  
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College/School: College of Science and Mathematics

Department: Biology

Thesis Committee:



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Dr. Ann Marie DiLorenzo, Thesis Sponsor



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Dr. Lee H. Lee, Committee member



\_\_\_\_\_  
Mary Lou Gubler, Committee member

A PRELIMINARY INVESTIGATION OF WORLD TRADE CENTER (WTC) DUST EFFECTS ON MRC-5  
LUNG FIBROBLASTS AND THE CELLULAR INFLAMMATORY RESPONSE

A THESIS:

Submitted in partial fulfillment of the requirements

For the degree of Master of Science

By:

KATELYN ANNA KEROD

Montclair State University

Montclair, NJ

2020

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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 throughout this whole process and for the opportunity to work in her lab. I am confident that I wouldn't be the scientist I am today without her help. I would also like to thank Dr. Lee Lee and Dr. Sandra Adams for their mentorship and support throughout this journey. I would also like to thank Adam Parker for all the technical assistance provided. I would also like to thank Dr. Ueli and Mary Lou Gubler for helping me design experiments, and conduct all the experiments needed. I would also like to thank Promega for all the kits they supplied. 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 as well as Dr. Lee Lee for supplying the EGCG-S compound. Lastly, I would like to thank the Graduate Assistants of the Biology Department at Montclair State University for all of their help and support.

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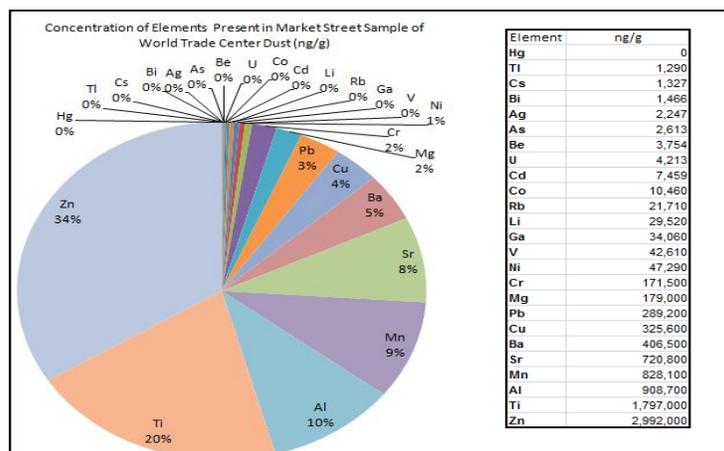
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## **Introduction:**

### The World Trade Center Attack:

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

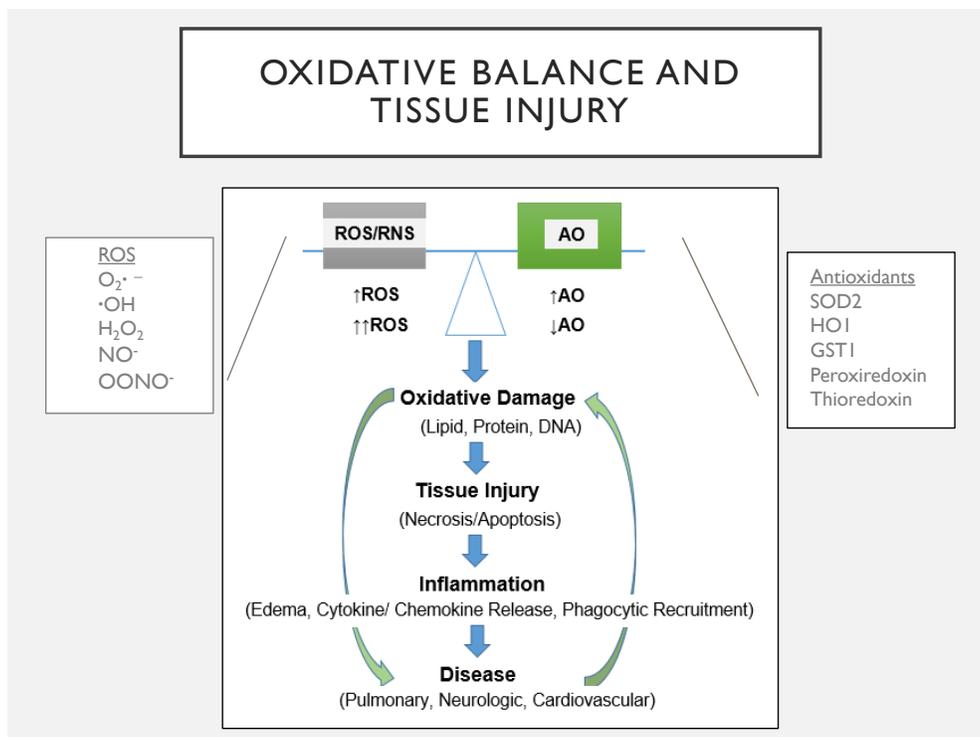


**Figure 1:** This figure shows the percentage of elements found in WTC dust as determined by Dr. Lioy's Lab (Lioy et al., 2002).

The Effects of the WTC Dust on a Cellular Level:

Numerous studies have been conducted using WTC dust in lung fibroblast cells (MRC-5). Negative effects on the cells have been demonstrated, such as a decrease in proliferation *in vitro* (Hernandez et al., 2012). It is believed that these effects are due to high levels of induced oxidative stress. Reactive oxygen species (ROS) are formed when molecular oxygen is reduced. ROS are free radicals, typically generated in cells as superoxide anions ( $O_2^-$ ), hydroxyl radicals ( $OH^\bullet$ ), singlet oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ).  $H_2O_2$  has the longest half-life and various types of ROS are converted to  $H_2O_2$ . ROS is known to be responsible for 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 toxic effects in lung fibroblast cells (Cohen et al., 2014). This deposition increases the amount of inflammation found in the respiratory system and increases the levels of ROS. Oxidative stress is one of the central mechanisms with which particulate matter (PM) can affect the respiratory system, ultimately leading to cell injury and apoptosis (Ghio et al., 2012).

Recently, oxidative stress caused by WTC dust has been associated with changes in the lungs, changes in DNA methylation, histone modifications and lung cell morphology (Sunil et al., 2016). Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation. NO is considered as a pro-inflammatory mediator that induces inflammation due to over production in abnormal situations.

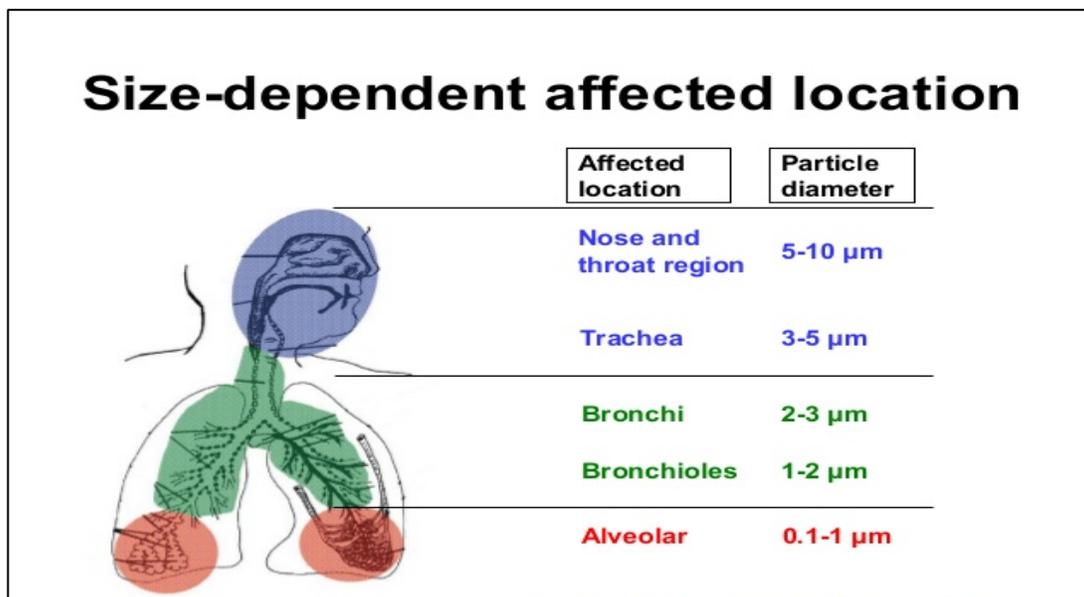


**Figure 2:** This figure shows the Oxidative Balance and Tissue injury (M. Hernandez, Personal Communication).

Physiological Effects of WTC Dust in Lung Cells:

More than 6,000 individuals were affected by the toxic dust released in the 9/11 attack. 24 hours after the attack, a persistent cough was noticed in the first responders, accompanied by 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 difficulty of breathing, and wheezing (Lambroussis et al., 2009). In addition,

during the first year after the collapse of the towers, 45% of the first responders were diagnosed with sudden-onset asthma (Wang et al., 2010). These effects are due to WTC dust particles with a size in the 2.5–10  $\mu\text{m}$  range. Such particles can penetrate deeply into the lungs, causing irritation of the alveolar wall and impairing lung function (Xing et al., 2016). Obstructive airway diseases (OAD) were found to result from dust exposure as well.

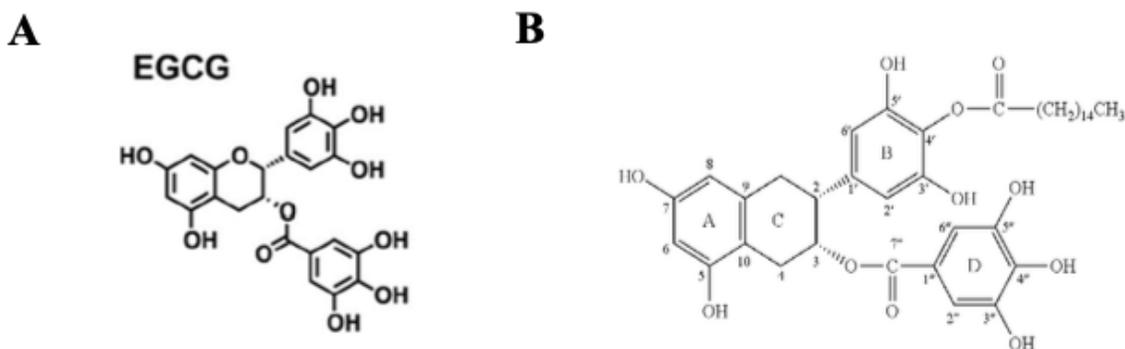


**Figure 3:** This figure shows the size of WTC particulate matter that is capable of penetrating the respiratory system (Heyder, 2004; Oberdoster et al., 2005).

EGCG-S (Green tea, GT) Effects on Lung Cells:

Tea is one of the most popular beverages in the world and its health promoting effects are well recognized and well-studied. The positive effects of tea are assigned to its polyphenolic compounds, particularly the catechins. Epigallocatechin-3-gallate (EGCG) is regarded as the best bioactive polyphenol in green tea with remarkable antioxidant and anticancer bioactivity (Nishikawa et al., 2006). EGCG is used in foods, cosmetics, and dietary supplements owing to its

health benefits for humans (Nagle et al., 2006). Decades of research have shown that EGCG possesses tremendous biological activities (Fechtner et al., 2017; Gan et al., 2018; Nikoo et al., 2018; Shin et al., 2016; Zhang et al., 2015). The compound is currently being investigated in the clinic as a potential long-term treatment of diseases caused by viruses, such as Herpes simplex virus, and as possible treatment for certain chronic diseases. Since EGCG is hydrophilic, recent pharmaceutical developments have made it more stable and bioavailable with enhanced lipophilicity (Zhong & Shahidi, 2011). The structure of EGCG was modified by esterification to produce an EGCG-acyl ester derivative containing stearic acid, also termed EGCG-Stearate (EGCG-S) (Zhong & Shahidi, 2011). Due to these modifications EGCG-S is more potent and can be used in medical formulations (Zhao et al., 2013). This form of the catechin may overcome the challenges of poor host cell absorption and susceptibility to *in situ* metabolic changes (Colpitts & Schang, 2014). Epigallocatechin-3-gallate (EGCG), the major catechin in green tea, has also shown a potential protective effect against heavy metal toxicity in humans (Ohyoshi et al., 1999; Okuda et al., 1982).



**Figure 4:** This figure shows the structure of EGCG (A) and how it was modified to make it more stable by addition of an ester bond and a stearate residue to generate EGCG-S (B). (Colpitts & Schang, 2014 & Hsu, 2014)

## **Project Description:**

My thesis project began with two questions. 1.) does WTC dust treatment of MRC-5 cells induce an inflammatory response as evidenced by the production of nitric oxide? 2.) If Nitric oxide production is detected after WTC treatment, are cytokines (IL-6, IL-8) also induced? If so, can subsequent treatment with EGCG affect this nitric oxide production or subsequent cytokine release? EGCG has many beneficial properties affecting the health of the cells and also the health of the whole person. Previous experiments have shown that membrane damage, oxidative stress, cell death and mutagenic effects have occurred *in vitro* in human lung cells even 18 years after the World Trade Center attack. This finding sparked my interest in exploring the effects of EGCG and different toxic metals present in the WTC dust on cells in culture. My goal was to try and evaluate the response of EGCG to see if there was a rescue or preventative effect on the cells against the heavy metals and the WTC Dust. In the present study, different concentrations of WTC dust were used to treat healthy human fibroblast lung cells (MRC-5). In addition, toxic effects of copper and manganese were studied. Assay readouts were cell viability, and determination of cellular nitric oxide and ROS production.

## **Timeline for Completion**

This project was conducted at the Montclair State University Biology Department. It began in the fall semester of 2018 and was completed in the summer semester of 2020.

## **Materials/Methods:**

### Media and Dust Sample Preparation:

#### Standard Media (MEM) Preparation:

The stock 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). Media with FBS was stored at +4 C.

#### Dust Sample Preparation:

50mg of World Trade Center Dust (WTC) were weighed out. The dust was then sterilized under the laminar flow hood ultraviolet light. Standard MEM media was used to suspend the dust in a final volume of 50 mL, giving a solution concentration of 1,000ppm. This stock solution was then diluted to the desired experimental concentrations.

#### EGCG-S Sample Preparation (Green Tea, GT):

The EGCG-S compound was supplied by Dr. Lee Lee. A 10mM solution was prepared in 100% dimethylsulfoxide (DMSO). Subsequent dilutions were made using standard MEM. Experimental EGCG-S concentrations were 50uM, 25uM and 12.5uM.

#### Preparation of Copper and Manganese:

A stock solution of 7.95mM manganese chloride was prepared in standard MEM and sterilized by vacuum filtration. The correspondingly prepared solution of copper sulfate had a

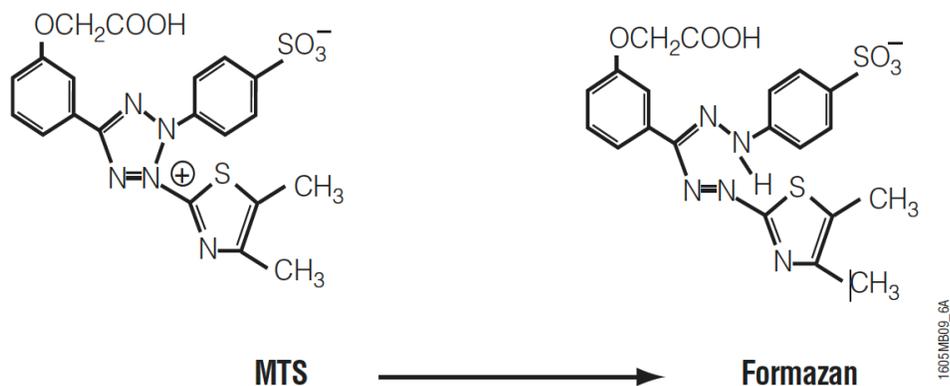
concentration of 6.26mM. Final experimental concentrations for the two metals were 100uM, 200uM, 400uM and 800uM.

#### Cell Culture Maintenance:

MRC-5 cells (ATCC #CCL-171, male lung fibroblast cells) were cultured in T-25 flasks containing standard MEM. Cells were plated in 100ul of standard MEM in white-walled tissue culture treated clear bottom 96-well plates and incubated at 37 °C for 24 hours to reach confluency. Standard medium was then replaced with experimental medium containing different concentrations of WTC dust, Cu, and Mn. All experiments were carried out 3 times and the mean and the standard deviation was calculated.

#### Cell Viability Assay:

The CellTiter 96 A<sub>Queous</sub> One Solution Cell Viability Assay (PROMEGA G3582) is a colorimetric method that determines cell viability. This assay requires incubation of a reagent with a population of viable cells to convert a substrate to a colored or fluorescent product that can be detected with a plate reader. It uses a tetrazolium compound called MTS (Owen's reagent) that, combined with an electron coupling reagent, phenazine ethosulfate (PES), produces a colored formazan product that is soluble in tissue culture media. 20µl of the reagent was added into each well of an experimental 96-well plate. Plates were then incubated at 37°C for 1 to 4 hours. The absorbance in the wells was recorded at 490nm using a 96-well plate reader.

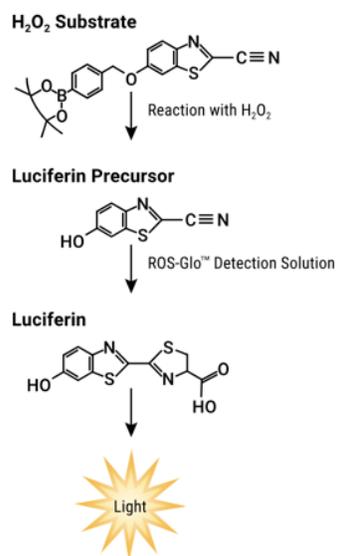


**Figure 5:** This figure shows the MTS tetrazolium conversion to the formazan product.

ROS-Glo™ H<sub>2</sub>O<sub>2</sub> Assay:

The Ros-Glo H<sub>2</sub>O<sub>2</sub> Assay (PROMEGA G8820) measures H<sub>2</sub>O<sub>2</sub>, which is the most stable of all the reactive oxygen species (ROS). Cells were plated in 80 μl of media in each well of an opaque white 96-well plate and incubated at 37°C to allow for attachment. After 24 hours test compounds were added and incubations were continued for 18 hours. Subsequently, 20 μl of the H<sub>2</sub>O<sub>2</sub> Substrate solution were added to each well and then incubated for up to 6 hours. During this incubation period the Ros-Glo detection solution was prepared. 100 μl of the Ros-Glo detection solution was then added to each 96 well plate and incubated for 20 minutes at room temperature. The luminescence was then recorded using a 96 well plate reader.

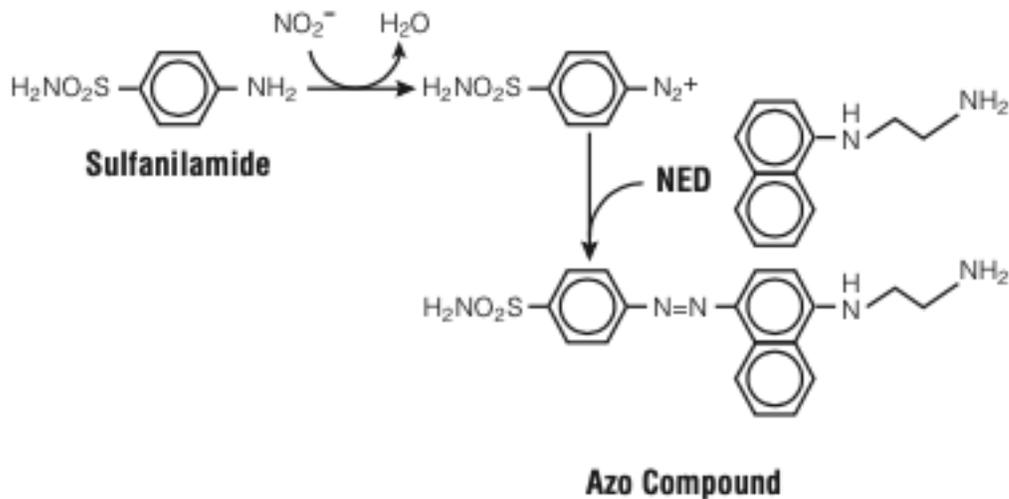
## ROS-Glo™ H<sub>2</sub>O<sub>2</sub> Assay chemistry



**Figure 6:** This figure shows the Ros-Glo H<sub>2</sub>O<sub>2</sub> assay chemistry.

Detection of Nitric Oxide (Griess assay):

This assay detects  $\text{NO}_2^-$  in a variety of biological and experimental liquids such as plasma, serum, urine and tissue culture medium. The Griess Reagent System is based on a chemical reaction, which uses Sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions (figure 7). Nitric oxide itself is unstable and spontaneously converts to nitrate and nitrite. The Griess assay detects nitrite. Media from treated cells (100ul) is removed to a new plate, 50 ul of the Sulfanilamide Solution is added and incubated for 5–10 minutes at room temperature (25 °C), protected from light. Afterwards, 50 $\mu\text{l}$  of the NED Solution is added and incubated at room temperature (25 °C) for 5 – 10 minutes, protected from light. Absorbance is read at 550nm. A standard curve with known amounts of nitrite is generated in parallel for quantification.



**Figure 7:** This figure shows the Griess Assay chemical reaction.

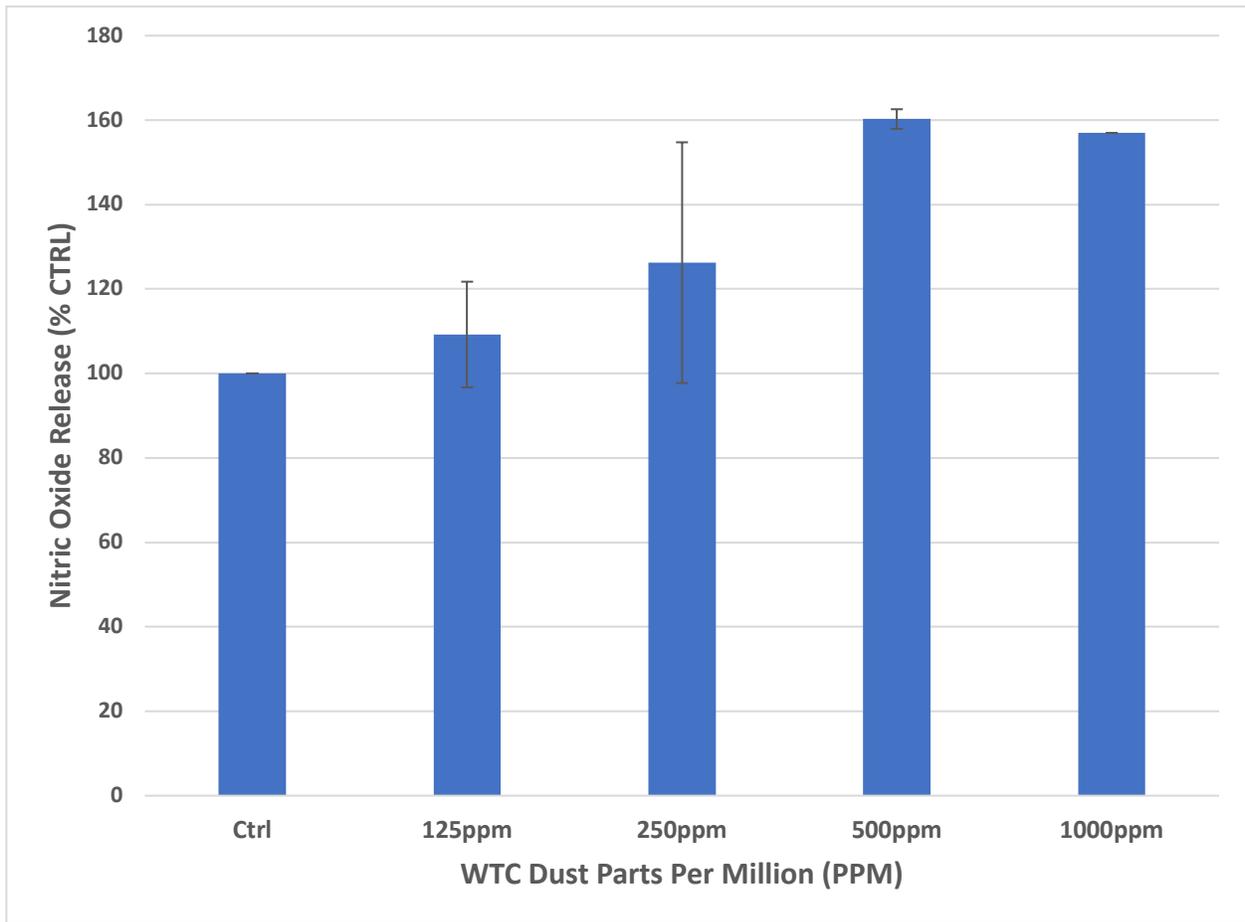
As stated above, the nitric oxide produced by the cells is unstable and converts into nitrite and nitrate. However, the Griess reaction only detects nitrite. In order to measure total nitric oxide

production, therefore in some cases an additional preincubation with nitrate reductase was performed prior to addition of Griess reagent.

**Results:**

**Nitric Oxide (NO) Release in MRC-5 Human Lung Fibroblasts**

NO production can reflect levels of inflammation. In order to evaluate the effects of WTC dust in MRC-5 lung cells, confluent cells were treated for 24hrs with increasing amounts of WTC dust and the release of NO was measured using the Griess Assay.

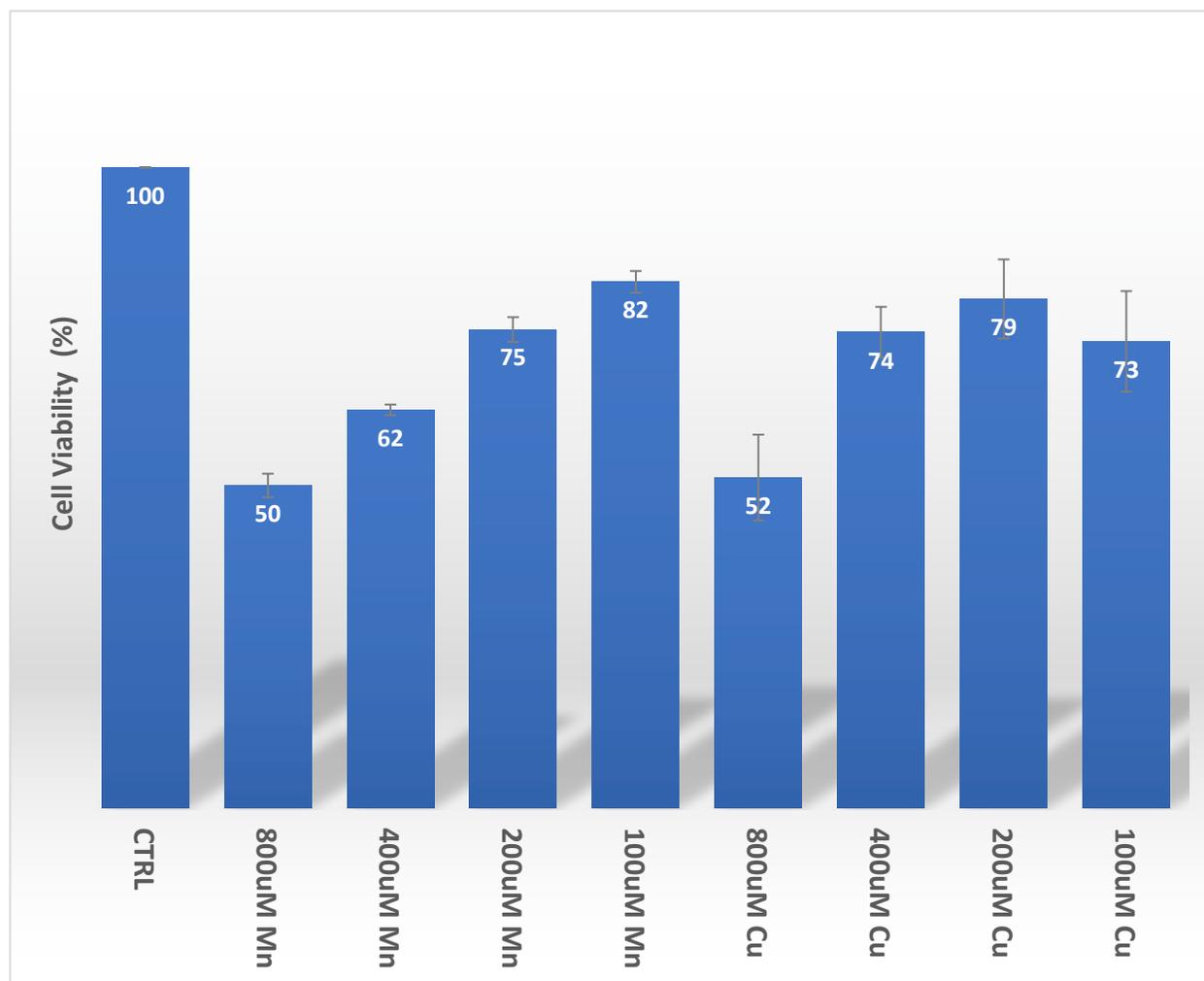


**Figure 8:** Nitric Oxide Release in MRC-5 cells after 24h exposure to WTC dust (n=3).

As it can be noted in Figure 8, NO production increased appreciably (to a maximum 60% increase) with increasing amounts of WTC dust. The cells exhibited NO release at low concentrations which increased as WTC dust concentrations increased. The increase observed at the 125ppm dose is likely not significant.

## Effect of Manganese (Mn) or Copper (Cu) on the Viability of MRC-5 Human Lung Fibroblasts

To examine the effects of Mn or Cu, two heavy metals shown to be present in high concentrations in WTC Dust, MRC-5 Human Lung Fibroblasts were treated for 24hrs with increasing concentrations of Mn or Cu ions and cell viability was determined.



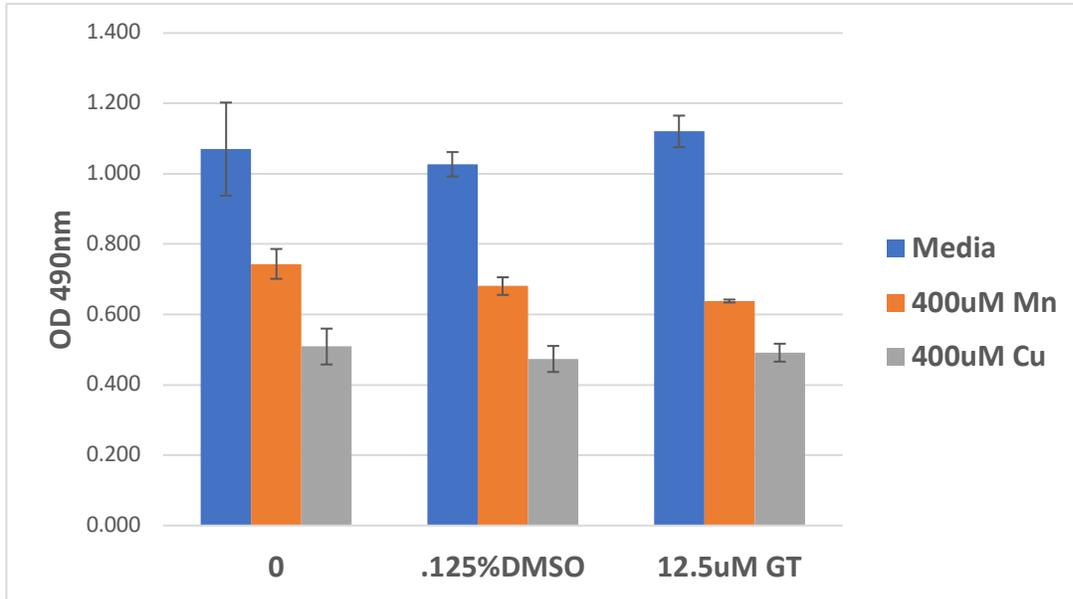
**Figure 9:** MRC-5 cells were exposed to increasing concentrations of Cu or Mn for 24 hr. Cell viability was measured by the Cell Titer 96 Aqueous One Solution Cell Viability Assay from Promega (n=3).

As shown in **Figure 9**, both Mn and Cu decreased cell viability in a dose dependent manner.

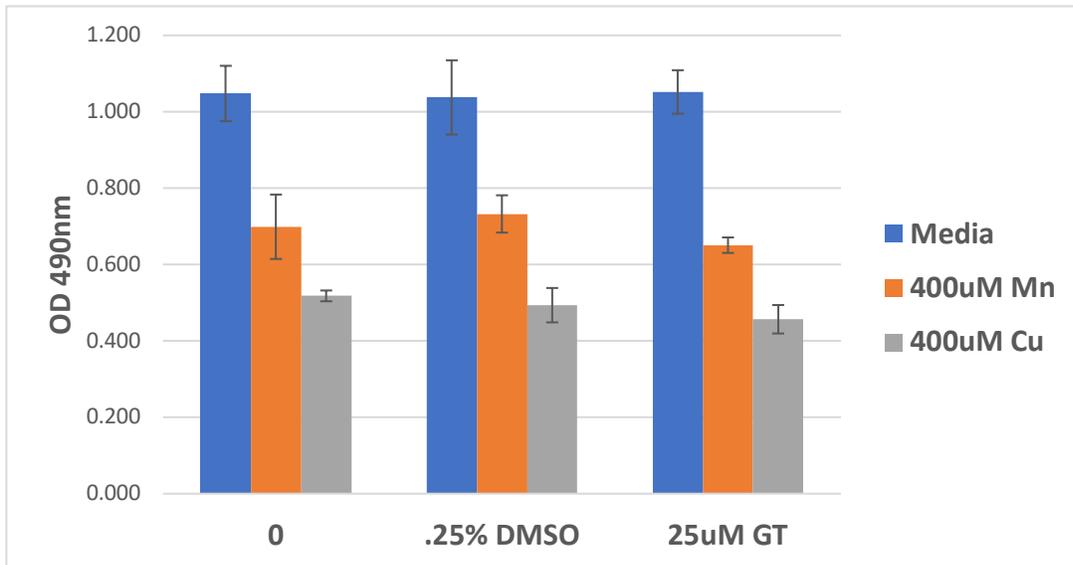
### Effects of EGCG-S (GT) on Cell Viability in Mn or Cu treated MRC-5 Human Lung Fibroblasts.

In order to determine if GT was able to reverse the negative effects of heavy metals on cell viability, MRC-5 Human Lung Fibroblasts cells were incubated with Mn or Cu +/- GT or the corresponding % DMSO vehicle control concentrations for 24hrs and cell viability was determined.

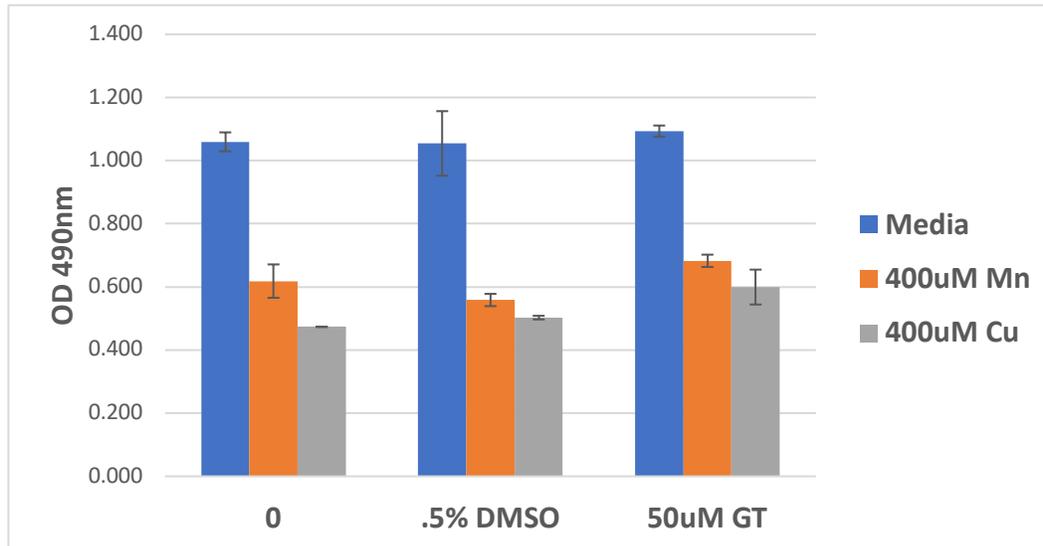
**Figure 10 A:**



**Figure 10 B:**



**Figure 10 C:**



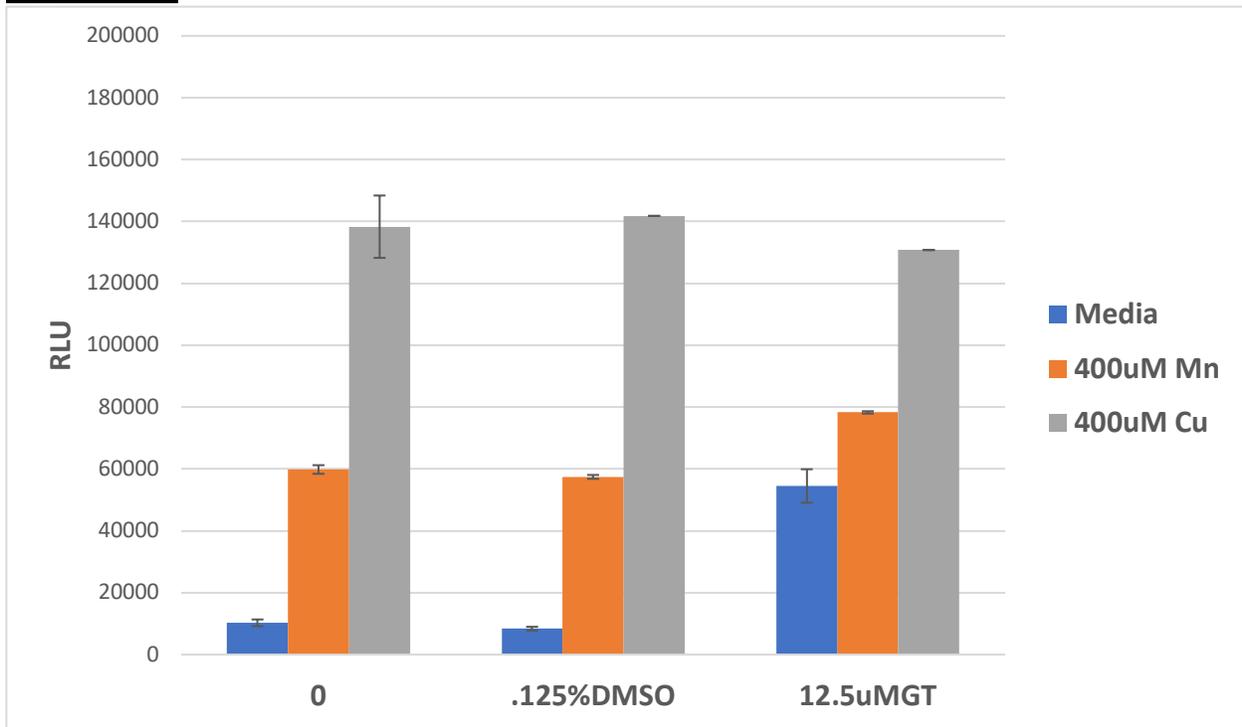
**Figure 10:** MRC-5 Human Lung Fibroblasts cells were treated for 24hrs with 400uM Mn or Cu +/- various amounts of GT. (A)12.5 uM GT, (B) 25uM GT, (C) 50 uM GT. Cell viability was measured as described previously (n=3).

Exposure to GT alone at all concentrations did not inhibit cell viability. In contrast, as previously observed in Mn or Cu treated cells, cell viability was decreased and was not changed with GT co-incubation. Mn and Cu at all concentrations show inhibition, but Cu exhibits a more pronounced inhibitory effect. Addition of GT for 24 hours has no effect.

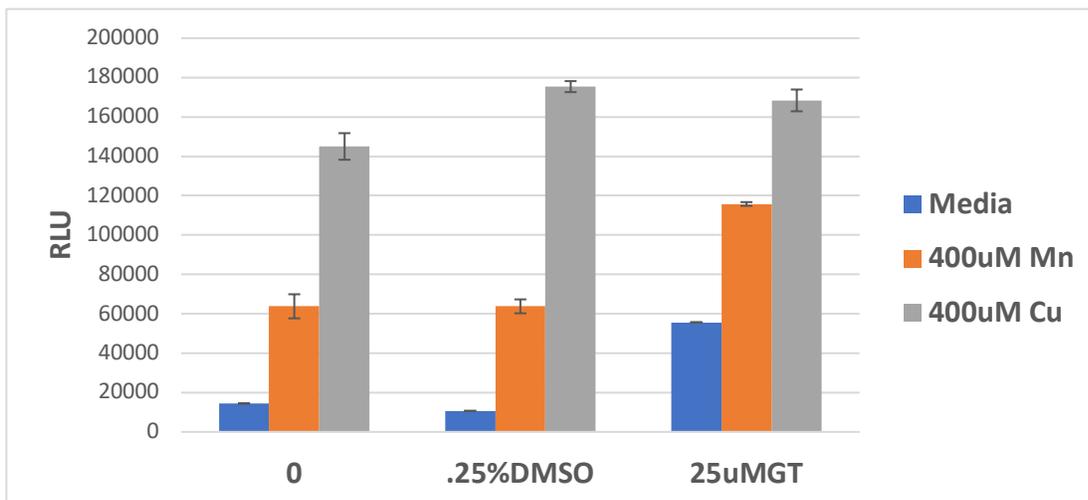
## Effect of GT on ROS production in Mn or Cu treated MRC-5 Human Lung Fibroblasts

Another indicator of oxidative stress is the production of ROS. To evaluate the effects of GT on Mn and Cu cellular damage, MRC-5 Human Lung Fibroblasts cells were incubated with Mn or Cu +/- GT for 24hrs and intracellular ROS levels were determined.

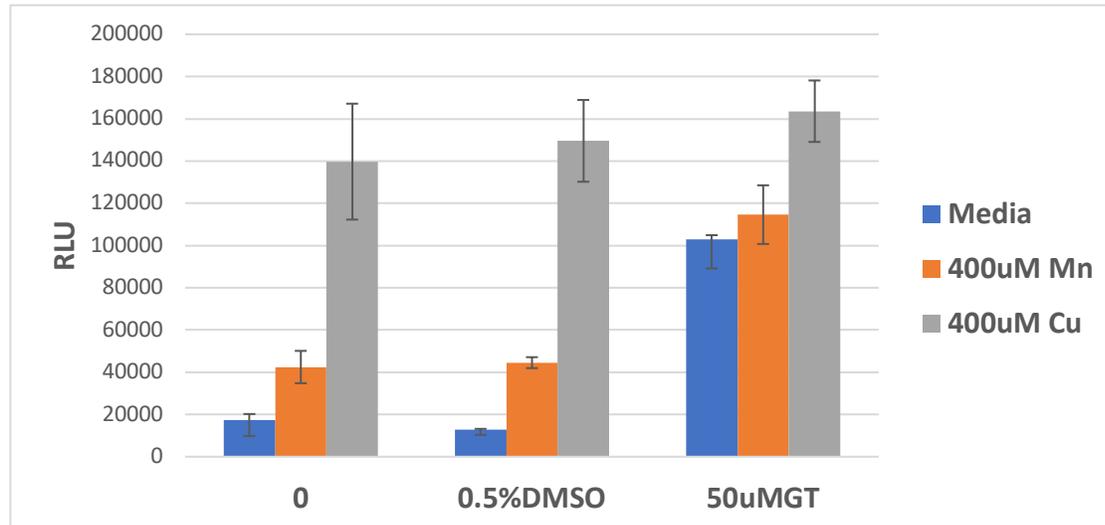
**Figure 11 A:**



**Figure 11 B:**



**Figure 11 C:**



**Figure 11:** MRC-5 Human Lung Fibroblasts cells were treated for 24hrs with 400uM of Mn or Cu +/- various concentrations of GT. (A) 12.5 uM GT, (B) 25uMGT, (C) 50uM GT. Intracellular ROS levels were measured by the ROS-Glo H<sub>2</sub>O<sub>2</sub> Assay (n=3).

As can be seen in Figure 11, exposure to GT at all concentrations increases ROS production compared to the DMSO controls. In addition, 400uM Mn or Cu also increase ROS production. This ROS production is potentiated in the presence of GT but only for the Mn treatment. Even though GT alone does not have an effect on cell viability, as previously shown in Figure 10, all concentrations of GT alone induce activation of ROS.

## **Discussion:**

With the serious environmental pollution occurring today, lung-related diseases, especially chronic airway inflammatory diseases, have become more prevalent. The World Trade Center attack on September 11th, 2001 was the biggest terrorist attack that has ever occurred in New York City. In the aftermath of the collapse of the twin towers, many of the first responders, rescue workers, and civilians were exposed to the fall out dust. The World Trade Center attack has induced physiological and psychological trauma in responders and civilians present at the location of the attack (Liu et al., 2014).

Nitric Oxide production by immune cells has been used as an indicator of the presence and extent of inflammation as well as the effectiveness of anti-inflammatory agents. Nitric Oxide is a signaling molecule involved in many physiological processes within the human body. It is regulated by Nitric Oxide synthase, an enzyme that has three different isoforms, two of which are regulated by calcium/calmodulin and phosphorylation. The third isoform is induced or expressed during inflammation, and produces higher levels of Nitric Oxide for a longer period of time. Nitric Oxide diffuses very rapidly and can easily diffuse from one cell to the next (Brown & Borutaite, 2001). Cytokines are also involved in the inflammatory response. They are part of a category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis. IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. IL-8 is produced by macrophages and other cell types such as epithelial cells, it induces chemotaxis in target cells causing them to migrate toward the site of infection. IL-8 also stimulates phagocytosis. Excessive production of Nitric Oxide in activated immune cells during inflammation can contribute to tissue

injury (Zhang et al., 2017). Various health effects such as fibrosis, asthma, cancer, etc. have been observed in first responders and the people who were exposed to WTC dust.

As shown in the first experiment (Figure 8) Nitric Oxide production increased appreciably (with a maximum of 60%) with increasing amounts of World Trade Center dust. Similarly, published studies describing silica-nanoparticle treatment of cultured cells have demonstrated an inflammatory response indicated by the production of Nitric Oxide and subsequent induction of cytokines and interleukins (Voicu et al., 2019). Taken together, these results show that Nitric Oxide production may be a strong indicator of oxidative stress that contributes to inflammation.

Metals are essential to a living cell, as they are involved in many metabolic processes and enzymatic reactions. However, in too high concentrations they can have toxic effects on the cells (Liang & Zhoue, 2007). The precise molecular mechanism of metal cytotoxicity is not known but oxidative stress has been shown to be a factor (Wu et al., 2012). An analysis of WTC dust showed that Manganese and Copper are among the many heavy metals found (Figure 1). As shown in Figure 9, both Manganese and Copper decreased cell viability in a dose dependent manner. EGCG-S has been shown to act as an antioxidant. As shown by literature data, EGCG-S inhibited viral infection, as evidenced by an increase in cell proliferation and cellular respiration, and a decrease in Reactive Oxygen Species production (Mohamed, 2017). Guided by this information, the effects of ECGC-S on Manganese and Copper treatment of cells were investigated. As shown in Figure 10, Manganese or Copper treatment of cells clearly decreased cell viability but did not change with EGCG-S co-incubation.

EGCG-S at low concentrations has been shown to inhibit DNA damage that was induced by Reactive Oxygen Species but higher concentrations of the compound may result in damage to cellular DNA and inhibit cellular functions (Wu et al., 2011). EGCG possesses potent antioxidative activity capable of protecting normal cells from various stimuli-induced oxidative stress and cell death. Co-treatment with EGCG and chromate was shown to protect human bronchial epithelial cells from chromate-induced cell death in a dose-dependent manner. (Wu et al., 2011).

In contrast, Figure 11 shows that exposure to EGCG-S alone at all concentrations tested increased Reactive Oxygen Species production compared to the DMSO vehicle controls. In addition, 400uM Manganese or Copper also increased Reactive Oxygen Species production. This Reactive Oxygen Species production was potentiated in the presence of EGCG-S only for Manganese. Even though EGCG-S by itself did not have an effect on cell viability, Reactive Oxygen Species activation was observed with EGCG-S treatment at all concentrations. With the addition of EGCG-S to both Manganese and Copper treatment, it can be seen that a 24-hour EGCG-S treatment has unexpectedly increased Reactive Oxygen Species production. This finding could be due to the length of the EGCG-S treatment; perhaps a one hour only pretreatment with EGCG-S before the addition of Manganese and Copper would prevent the Reactive Oxygen Species increase observed. In addition, a rescue effect by EGCG-S when applied **after** Manganese and Copper treatment could also be investigated. Such a rescue effect of EGCG- has been shown in the literature (Wang et al., 2019).

## **Conclusion and Future Research:**

In conclusion we have shown that the World Trade Center dust treatment did induce Nitric Oxide production, indicative of an inflammatory response. This inflammatory response could also lead to induction of inflammatory cytokines like IL-6 and IL-8, as shown in Voicu et al (2019) by the use of silica nanoparticle treatment of MRC-5 cells.

Manganese or Copper treatment decreased cell viability and increased Reactive Oxygen Species production. Treatment with EGCG-S was shown to generate intracellular Reactive Oxygen Species. Reactive Oxygen Species activation was potentiated in the presence of EGCG-S but only for Manganese. EGCG is generally considered to be an antioxidant, however, there are studies that have demonstrated EGCG to have pro-oxidative activities (Li et al., 2010), thus supporting our own findings. This pro-oxidant role may be linked to the induction of DNA damage and apoptosis.

Due to the Corona Virus (COVID-19) global pandemic, the laboratory work could not be continued. Future research might therefore involve further experiments studying the WTC-dust based induction of cytokine production and apoptosis. Such experiments might possibly shed some more light on the mechanisms underlying the pathology observed in 9/11 first responders.

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