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Increased Levels of Oxidative Stress in Human Fibroblast Lung Cell Cultures and the Loss of Mitochonndrial Function Due to Exposure to Particulate Matter from September 11, 2001

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Abstract

Not only did over 2,753 individuals perish on the morning of September 11, 2001, many have suffered from mental and physical illnesses ever since. Thousands of individuals experienced breathing complications, asthma and lung cancer. Clinical trials on cancer, including a patient who was exposed to the World Trade Center (WTC) dust, have seen a decrease in the progression of tumor growth with the use of oral nicotinamide adenine dinucleotide (NADH). NADH is naturally occurring within a cell's mitochondria and aids in the production of adenosine triphosphate (ATP), the energy of the cell. NADH is an anti-aging, energy enhancing substance that also reduces fatigue and successfully treats degenerative medical conditions. NADH is becoming a possible treatment for tumor regression but it is still unknown, on a cellular level, the exact reason why NADH reacts with these cells to prevent further tumor growth. In this study, the amount of NADH produced by normal MRC-5 lung fibroblast cells will be compared to lung cells exposed to WTC dust, using a PROMEGA NAD^+/NADH-Glo assay. The same cells will be evaluated to note the decline in the levels of Glutathione to determine the amount Reactive Oxidative Species (ROS) brought onto lung cells by WTC dust. ROS is known to decrease GSH levels by causing oxidative stress that leads to apoptosis. It is important for the cells to maintain high levels of reduced glutathione (GSH) and low levels of oxidized glutathione (GSSG). PROMEGA GSH Glo Glutathione assay will be used to determine if the toxic, mutagenic and apoptotic effects of WTC dust can be shown to be the result of oxidative stress. Lung fibroblast cells will be exposed to various concentrations of WTC dust, ranging from 25-250 parts per million (ppm). It is hypothesized that the cells with the highest concentrations of this toxic particulate matter will experience the most

accumulation of oxidative stress, resulting in more NADH being oxidized to NAD+ within the mitochondria, and simultaneous decrease in protective antioxidants as shown in changes in the levels of reduced GSH. This should allow correlations to be made on a more precise cellular mechanism between exposure to this WTC dust, oxidative stress and loss of mitochondrial function seen in the diseases of the exposed first responder population.

MONTCLAIR STATE UNIVERSITY

Increased Levels of Oxidative Stress in Human Fibroblast Lung Cell Cultures and the Loss of Mitochondrial Function Due to Exposure to Particulate Matter from September 11, 2001

by

Lara Seder

A Master's Thesis

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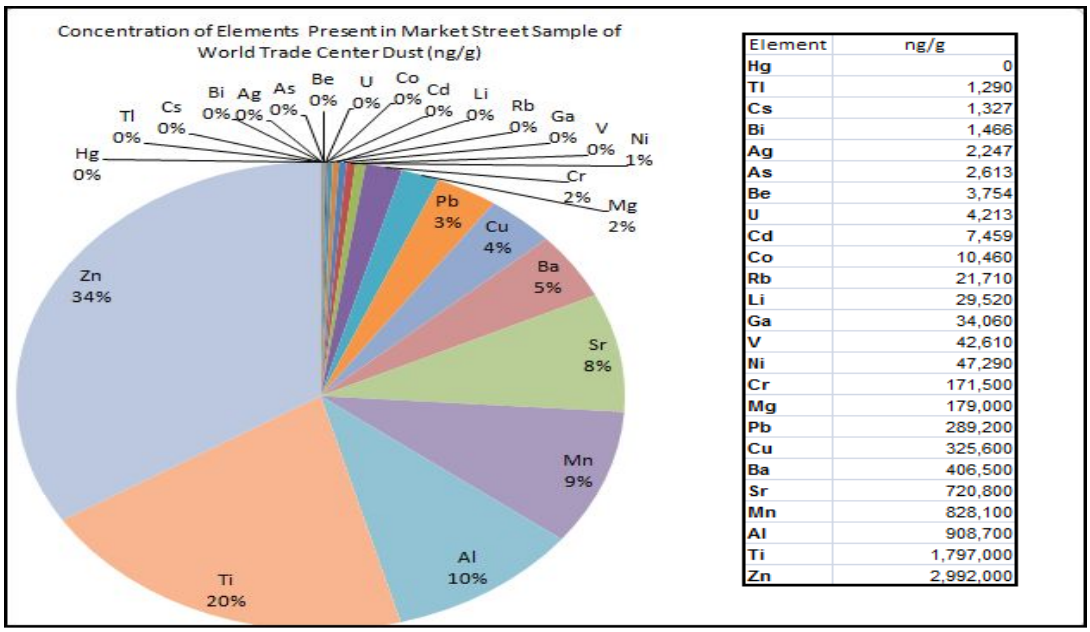
Introduction

The World Trade Center (WTC) Particulate Matter Composition

The destruction of the WTC towers that occurred on September 11th, 2001 has led to massive amounts of particulate matter and toxic dust that covered a huge portion of lower Manhattan, New York. Many residents, first responders, rescuers, passers-by and workers were exposed and affected by the exposure physically, mentally and psychologically (Welch et al., 2014). The smoke and dust eventually settled at various indoor and outdoor locations. The wind moved the plume of dust and smoke to the east then to southeast until it reached Brooklyn, New York between the first couple of hours to 18 hours after the attack. Thirteen dust samples were collected at different locations after 5 and 6 days after the collapse for determination of the physical and chemical characteristics of dust and to determine whether contaminants that are present might affect the human health by ingestion or inhalation. In the inorganic analyses of the dust samples, metals, ionic species, radionuclides, inorganic species and asbestos were identified. However, in the organic analyses of the dust samples, polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), pesticides, polychlorinated dibenzodioxins, brominated diphenyl ethers, polychlorinated dibenzofurans, concrete, gypsum and other hydrocarbons were detected. PAHs were less than 0.1% of the mass, asbestos levels ranged between 0.8%-3.0% of the mass, and lead levels were between 101-625 µg/g. The dust samples were composed mainly of soot, glass fibers, construction materials and paint. Hydrocarbons were the results of burned plastic, jet fuel, cellulose and other materials (Lioy et al., 2002). United States Geological Survey (USGS) found that the dust contained

heavy metals, glass fibers (Mg, Al, Na, Si and Ca) and slag wool (Rosati et al., 2007). WTC dust had a pH of 9.2-11.5 which means it was alkaline (Cho et al., 2014).



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. Percent composition of elements present in WTC dust (Lioy et al., 2002).

Respiratory Effects of the WTC Dust

The collapse of WTC towers has also been linked with a significant decrease in lung function of rescue workers. Many studies were done to investigate the difference between the WTC dust and the typical urban dust because the public was concerned about those workers who were exposed to the WTC dust (Rosati et al., 2008). About 90% of the particles in the dust samples were more than 10 µm in diameter, which can be filtered in the nasopharynx. Particles bigger than 2.0 µm in diameter are usually deposited in the upper respiratory airways once inhaled. Once inhaled, they cause irritation to the upper respiratory airways due to their alkaline nature. This explains the elevated incidence of upper respiratory symptoms such as throat irritation, nasal congestion, gastro-esophageal

reflux and cough which were seen highly in the exposed FDNY fire fighters (Fireman et al., 2004). Twenty percent of Manhattan population were exposed to the WTC Particulate Matter ($PM_{2.5}$) which was able to penetrate the bronchioles of many individuals. The main manifestation of upper airway was chronic rhinosinusitis (CRS). Prior to 9/11, 4.4% firefighters of FDNY reported rhinosinusitis symptoms. However, 45.1% of FDNY firefighters reported rhinosinusitis symptoms one year after 9/11 attack. This shows how rhinosinusitis symptoms increased by 10-fold. Allergic asthma, bronchitis and chronic cough were other airway immune responses to the WTC dust (Cho et al., 2014).

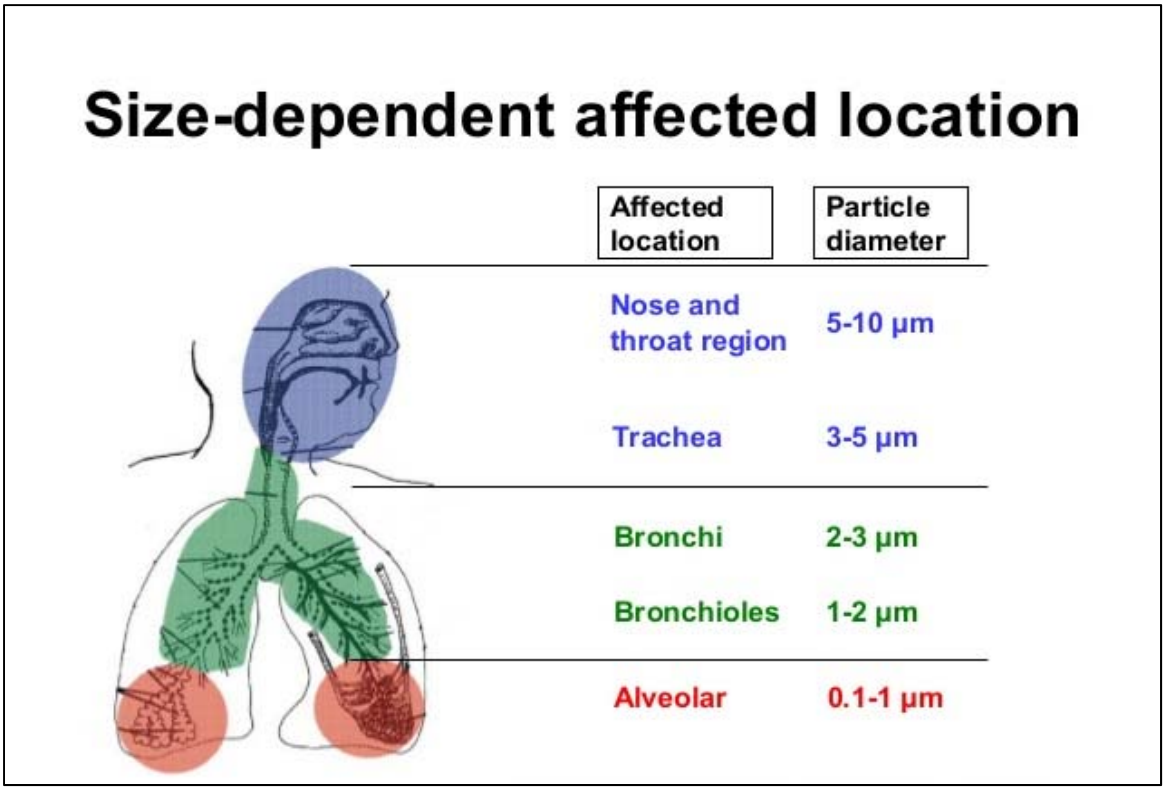


Figure 2. The size of WTC PM that is capable of penetrating the human airway. 2-3 µm PM can enter the bronchi into the lungs (Heyder, 2004; Oberdoster et al., 2005).

The WTC particulate matter was small enough to enter the lungs of recovery workers, residents and first responders. Those particles were able to reach the distal airways

to the alveoli. Many respiratory tract pathologies resulted from this dust. Sarcoid-like granulomatous inflammation was noted in recovery workers after the attack. Granulomatous inflammations are clusters of vacuolated, enlarged macrophages that are indicators of oxidative stress; which are linked to specific epigenetic modifications in the lung. This indicates some potential mechanisms that explain pulmonary toxicity (Cho et al., 2014).

Physiological and Psychological Effects of WTC Dust

Two of the toxic chemicals that were found in the WTC dust were polychlorinated dibenzo-para-dioxins as well as polychlorinated dibenzofurans (PCDD/Fs); which are byproducts of combustion reactions. Those two compounds are formed when chlorine and carbon combine at elevated temperatures. These compounds are lipophilic; hence, they accumulate in food chains. The main sources of human exposures to these compounds are breastmilk and high-fat foods. High concentrations of PCDD/Fs were discovered on the outer window surfaces and in the dust, sediment and water samples that were obtained from the WTC site. High PCDD/Fs levels were reported in plasma samples from pregnant females and in serum samples of firefighters who were first responders. According to the World Trade Center Health Registry (WTCHR), about 8,300 children who were residents of lower Manhattan or attended school there were exposed to the WTC dust. Seven years is the half-life of PCDD/Fs in adults. These compounds are associated with many health outcomes. Acute health outcomes include chloracne while longitudinal outcomes include modified immunological and productive function, cancer and diabetes in adults. However, some health outcomes in children include modified semen quality in boys and impaired behavioral and cognitive function in both genders (Kahn et al., 2017).

Prior studies have shown that the airborne particulate matter concentration in the WTC dust has been linked to higher levels of cardiovascular disease (CVD) mortality and morbidity. Acute elevations in the concentration of particulate matter of dust showed increased short-term changes in the cardiovascular outcomes (Brook et al., 2010). Another study showed higher dysfunction of left ventricle and of the isolated right ventricular diastolic among police officers 7 years after the attack of 9/11 (Croft et al., 2010). The collapse of WTC towers has caused psychological and physiological exposures that might have left 9/11 survivors with a higher risk for chronic heart disease. A study done by Jordan and his colleagues was done to investigate the relationships between psychological stress and exposure to air pollution on 9/11 survivors and new-onset heart disease. The study found that 9/11-related exposures correlates with a high heart disease risk a few years after the collapse (Jordan et al., 2011).

The collapse of WTC towers exposed thousands of people to a mixture of debris, jet fuel combustion byproducts and dust. Community members had acute exposures from the dust cloud and fires from the collapsing towers, sub-acute exposures from re-suspended dust during the first week, and/or chronic exposures from incompletely cleaned schools/homes and re-suspended dust. Pregnant women who were exposed to the WTC dust have been studied to investigate the consequences in their children and relationship to psychological stress from the attack. The WTCHR documented parental reports on the health consequences and exposure of their children. A report has showed that 45% of the survivors' children that were exposed to the WTC dust had new onsets or worsened respiratory symptoms after the exposure (Trasande et al., 2013). DNA adducts were found to be markers for oxidative stress that is due to air pollution which eventually leads to

cardiovascular disease. These DNA adducts were found to be increased in highly exposed newborns from mothers who were exposed to the WTC dust (Perera et al., 2007).

WTCHR is the registry that was made to monitor the health of the exposed survivors from the 9/11 attack (Jurek, 2016). Some studies have showed higher prevalence of PTSD and asthma among the WTC attack survivors. A study done by Shiratori and colleagues indicated that PTSD that resulted from 9/11 attack increased the probability of developing asthma by 65% after the attack. This is partially due to sympathetic adrenal medullary system and hypothalamic pituitary adrenal-axis seen in both asthma and PTSD, which leads to the disinhibition of the inflammatory responses (Shiratori et al., 2012).

Recent studies have shown that both men and women who were near the site to the WTC attack had increased risk of cognitive impairment which might be due to PTSD and WTC dust exposure and inhalation. They also had increased risk of neurodegenerative disease such as increased beta-amyloid, white matter hyper-intensities decreased brain volume. A study done by Clouston and colleagues determined that general population norms had better cognitive functioning. Also, they indicated that spending more than 5 weeks at the WTC site was linked to cognitive dysfunction that is independent of PTSD (Clouston et al., 2017). Oxidative stress that resulted from the WTC dust exposure was also a potential factor that led to Alzheimer's disease, Parkinson's disease and Huntington's disease (Dietrich et al., 2009).

The WTC dust cloud contained some persistent organic pollutants (POPs) such as perfluoroalkyl substances (PFASs) and perfluoroocatnoic acid (PFOA). PFASs were found to be elevated in first responders 5-26 months after the attack. PFASs are used as strain-resistant coatings and surfactants. They are used in construction material, buildings, carpet

and nonstick cookware. Fire-fighting materials also contain PFASs which suppress fires. Many studies have found that PFASs disrupt cardiovascular, metabolic and renal functions. PFAS exposure also elevates ROS and stimulates endothelial permeability which has an important function in ischemic renal injury. PFASs exposure has been associated with elevation in children's serum uric acid which is associated with reduced kidney function. A positive correlation has been found between concentration of PFOA and non-high-density cholesterol in NHANES, National Health and Nutrition Examination Survey (Trasande et al., 2017).

The World Trade Center (WTC) dust has been found to increase the levels of ROS in the mitochondria, causing oxidative stress on the cells and eventually leading to apoptosis (Olson et al., 2008).

The Effects of the WTC Dust on a Mitochondrial Level

The mitochondria, the power house of the cell, produce ATP from products produced from the Kreb cycle, amino acid oxidation and fatty acid oxidation. This process occurs when electron carriers such as $FADH_2$ and NADH pass their electrons through the electron transport chain and finally donating their electrons to molecular oxygen to be reduced into water. As this process happens, protons are also being pumped from the matrix of the mitochondria to the intermembrane space; which ultimately creates a proton gradient. This proton gradient allows protons from the intermembrane space to go through F_o/F_1 ATPase complex which results in ATP production. Normally, a small fraction of the electrons does not complete the entire series of the electron transport chain, but instead they leak onto molecular oxygen and lead to the production of free radicals, known as reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and

hydroxyl radical (OH^{-}) (Nelson et al., 2013). Complex I, complex III and reduced coenzyme Q (QH_2) of the electron transport chain are the main source for the production of ROS when electrons leak from these complexes and reduce molecular oxygen. Chance and colleagues provided the first evidence that mitochondria are responsible for the generation of H_2O_2 using a procedure that involves sensitive spectrophotometry (Kalyanaraman et al., 2017).

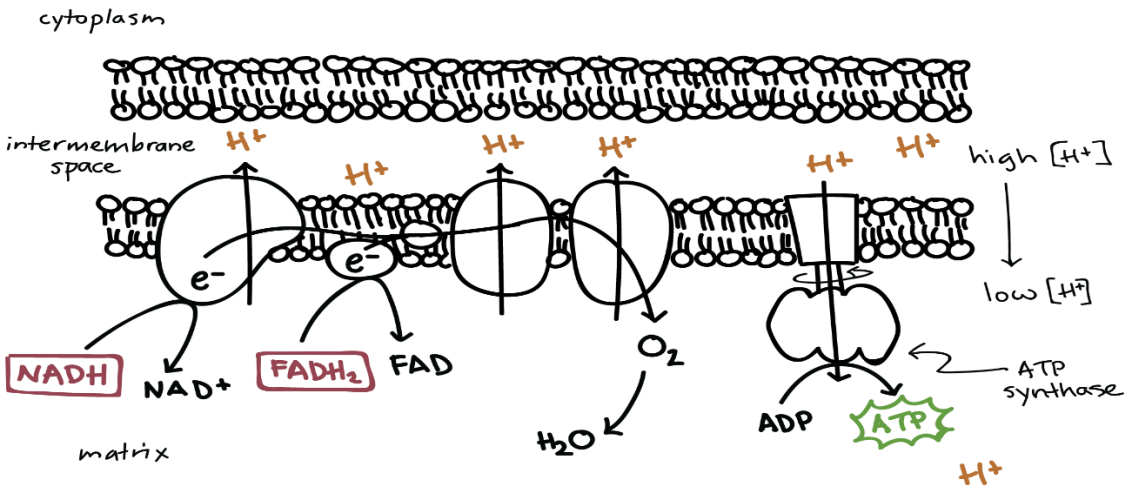


Figure 3. A diagram of the electron transport chain and ATP synthase which make up the chemiosmotic process (Khan Academy, 2018).

Extremely reactive molecules that contain unpaired outer electrons are called free radicals. ROS are in the form of free radicals. They damage cell membranes through oxidation, hence, causing oxidative stress. Oxidative stress occurs from an imbalance between antioxidants and oxidants which results in damage to the tissues and cells (Chapman et al. 2013). ROS production can be beneficial or harmful depending on quantity. ROS can be produced from many processes including the normal process of oxidative phosphorylation as explained previously. In oxidative phosphorylation, free radicals are produced in a moderate amount (Rydstrom 2006). These molecules may act as

redox messengers and play an essential role in the intracellular regulation and signaling when kept at certain cellular concentration. However, these radicals become deleterious when their concentrations are elevated above the physiological concentrations, thus, resulting in oxidative stress that might damage the cells and lead to apoptosis (Ghosh et al., 2015).

Rotenone is a molecule that inhibits the function of complex I in the electron transport chain, leading to excess leakage of electrons in the matrix of the mitochondria; which results in the formation of excess ROS such as $O_2^{\cdot-}$. Again, excess levels of ROS can cause mitochondrial damage and ultimately apoptosis. Antimycin is a molecule that inhibits the function of complex III, leading to the production of $O_2^{\cdot-}$. Inhibition of complex I and III results in lower levels of NADH, hence, lower production of ATP. The inability to produce as much ATP prevents the cell's immune system from fighting against diseases such as cancer. Antioxidants such as vitamins A, C and E can reduce free radicals by donating electrons to the free radicals. Organisms that are catalase positive reverse the production of ROS by converting them to water. However, most organisms are catalase negative; hence, they need to use different mechanisms and/or enzymes to eliminate these ROS. Some of these mechanisms include the process of superoxide dismutase (SOD), glutathione (GSH) reductase and thioredoxins (Trx2). Superoxide dismutase (SOD) is an enzyme that converts superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) which is a less reactive molecule (Chen et al., 2017).

When complex I is inhibited, nicotinamide nucleotide transhydrogenase (NNT) oxidizes NADH and at the same time transferring that proton to NADP+ to become NADPH, nicotinamide adenine dinucleotide phosphate. Usually, at normal ROS levels,

enough NADPH is produced from pentose phosphate pathway (PPP) which would be sufficient to eliminate ROS. NADPH is needed to increase the amount of reduced glutathione in the cell by using the enzyme, GSH reductase. Reduced GSH acts as an antioxidant. Glutathione peroxidase (GPx) can eliminate free radicals by reducing hydrogen peroxide into water while oxidizing the reduced glutathione (GSH) into oxidized glutathione (GSSG). The increased NADPH production by NNT leads to the reduction of thioredoxins (Trx2). Peroxiredoxin is another enzyme that oxidized thioredoxins (Trx2) to convert hydrogen peroxide into water. Also, proapoptotic proteins are found on the surface of the outer membrane of mitochondria and once ROS are produced, an increase in the permeability of the outer mitochondrial membrane is caused via lipid peroxidation; which allows cytochrome C from intermembrane space to enter cytoplasm. Cytochrome C aids in shuttling electrons between third and fourth complexes of electron transport chain and also plays an essential role in apoptosis. Cytochrome C in cytoplasm activates an enzyme called caspase which breaks down proteins, nucleases and other polymers inside the cell, hence, leading to apoptosis (Chen et al., 2017). It is hypothesized that ROS would act as inhibitors of the electron transport chain complexes. Therefore, increasing the production of ROS by increased concentrations of the WTC dust would lead to increased production of NADPH by increasing the oxidation of NADH and hence, increasing the levels of the oxidized glutathione (GSSG).

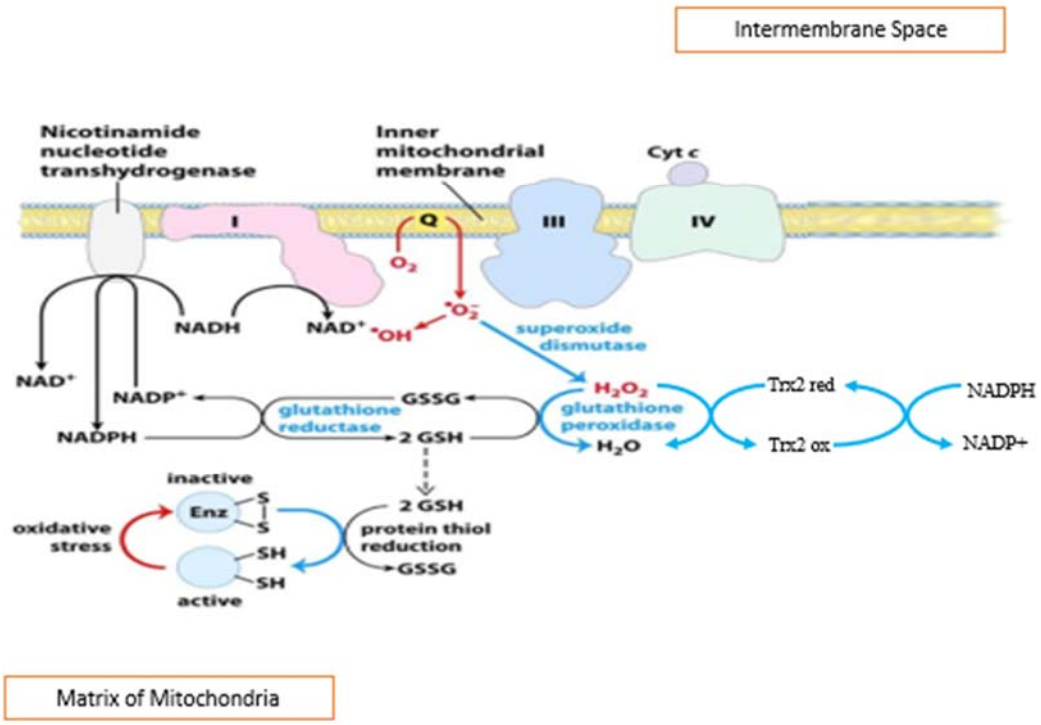


Figure 4. The formation of ROS through the electron transport chain including many redox reactions.

Carcinogenic Effects of the WTC Dust

ROS are also involved in tumorigenesis. NADPH oxidase and mitochondria oxidase are two main ROS sources found in cancer. Studies have shown that ROS, specifically H_2O_2, stimulates the proliferation of cancer cells through alterations in the signaling pathways such as PI3K/Akt. However, other studies showed that upregulation of antioxidant pathways and supplementation of antioxidants enhanced tumor survival by lowering cytotoxicity of ROS (Kalyanaraman et al., 2017). According to Dr. Birkmayer, increased levels of NADH showed increased production of ATP which allows the cell's

immune system to fight against cancer. Also, increased levels of NADH can eliminate ROS. Therefore, 40 mg to 80 mg of NADH is recommended for cancer patients to take daily to prevent the progression of cancerous cells (Birkmayer, 2017). Decreased levels of ROS activate and enhance signaling pathways for cell survival and proliferation. In breast cancer cells, inhibiting GSH pathways causes ROS to be increased to cytotoxic levels. The ROS levels and growth of cancer cells are enhanced by inhibiting glutathione peroxidase (GPx), antioxidant enzyme, via fumarate suppression in the Kreb cycle. Recent studies have found that reprogramming the metabolism of cells plays an essential role in tumorigenesis. Cancer cells need energy to proliferate through metabolic reprogramming such as lipid metabolism, glutamine addiction and altered glucose utilization. Increasing glutamine utilization is one of the major metabolic reprogramming in tumors. Modifying the pathway that produces cellular reductants (GSH and NADPH) influences the formation of ROS and tumorigenesis. It is known that ROS can cause oncogenic mutations and treatments with antioxidants inhibit the progression and initiation of certain cancer types. Antioxidants such as vitamins A, C and E or N-acetylcysteine (NAC) increase the levels of intracellular glutathione which will decrease the levels of ROS via enhanced antioxidant enzyme activity (Kalyanaraman et al., 2017).

Project Description

Even after 15 years, people continue to suffer from exposure to the WTC dust; little is known regarding the effects at the cellular level. This study presents a strategy to determine the levels of oxidative stress due to different concentrations of the WTC dust. Oxidative stress has a negative impact on the respiratory tract, cardiovascular and nervous systems because it increases the production of ROS. In this study, different concentrations of WTC dust will be used on healthy human fibroblast lung cells (MRC-5) to examine the levels of ROS, reduced GSH, NADPH and NADH in the mitochondria. These levels are crucial in determining the apoptotic effect of the WTC dust. It is hypothesized that increasing the production of ROS by increasing the concentration of the WTC dust would lead to increased production of NADPH by increasing the oxidation of NADH and hence, increased levels of the oxidized glutathione (GSSG) while reducing the levels of reduced glutathione (GSH).

Materials & Methods

Dust Sample

The World Trade Center dust sample was received from Dr. Paul Lioy at Rutgers University. The WTC $PM_{2.5}$ was chosen because the size of the dust particles that penetrated the lungs was 2.5 μm. The WTC dust contains many components including ceiling tiles, concrete, glass fibers, wallboard, cement aggregate, etc. (Lioy et al., 2002).

Figure 5. An image of the WTC dust sample obtained from Dr. Paul Lioy.

Dust Sample Preparation

One gram of WTC dust was weighed out in a fume hood. The dust was then dissolved in sufficient media to create a 100 ml solution of "stock" WTC dust media. The stock media was subdivided adjusted to the 10% Fetal Bovine Serum (FBS) level and was then diluted to each of the experimental concentrations (1.25-250 ppm).

Media Preparation

Eagle's Minimal Essential Media (MEM), 1% of Penicillin Streptomycin (PS), Glutamax (G), Kanamycin (K) and 10% (FBS) were used to prepare WTC stock dust solutions to make a 100 mg ml^{-1} stock solution. Exposure to UV radiation to the WTC dust sample was necessary for sterilization.

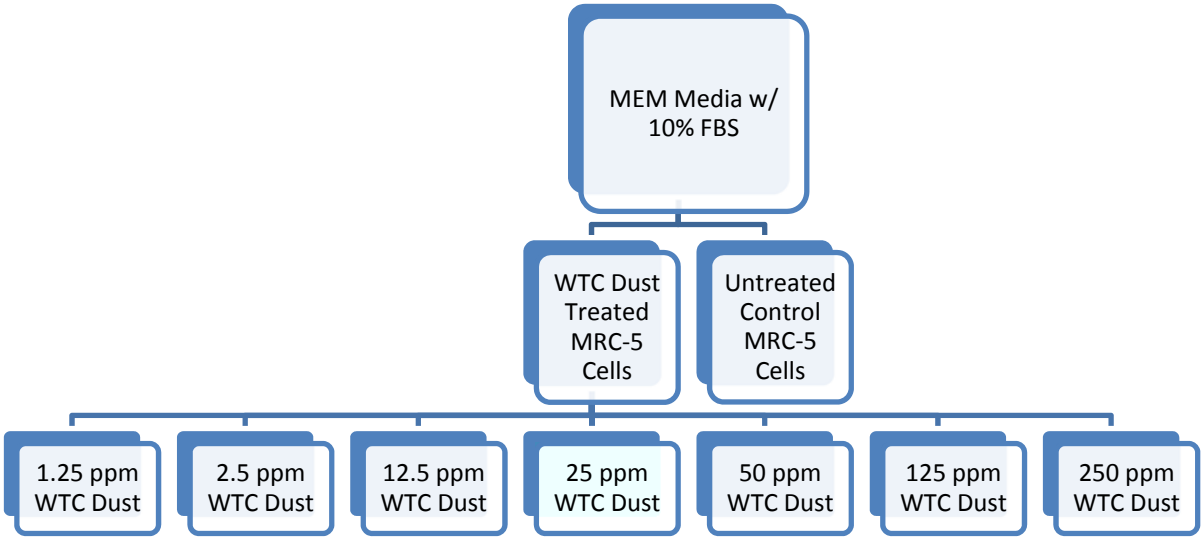


Figure 6. Various WTC dust concentrations were made using MEM media with 10% FBS.

Cell Culture Maintenance

MRC-5, fibroblast cells found in human lung tissue, were cultured in Eagle's Minimal Essential Media and 10% FBS that has 1% PSGK. Cells were then plated, sub-cultured and incubated at 37^{o}C for 24 hours. Media was then removed and replaced with media containing different concentrations of the WTC dust. Cells were then incubated for 24 hours.

ROS-Glo^{TM} H_2O_2 Assay

This assay was used to measure H_2O_2 by reacting directly with a substrate that produced a luciferin precursor. Once ROS-Glo^{TM} detection reagent was added, the luciferin precursor was converted to luciferin by the molecule, D-Cysteine. Then, luciferin reacted with the enzyme luciferase and produced a luminescent signal that was directly proportional to the concentration of H_2O_2. A 100 µl of media with cells were placed in each of the 96-wells in a white plate and incubated at 37^{0}C for 24 hours. Media was then

removed and 80 µl of the assigned "media/solution" was added to each of the 96-wells. Samples in the 96-well plate were treated with 20 µl H_2O_2 substrate solution in each well and incubated at 37^0C for 6 hours. H_2O_2 substrate solution was formed by mixing 2 µl of H_2O_2 substrate with 2.0 ml of H_2O_2 substrate solution dilution buffer. After the 6-hour incubation period, 100 µl of ROS-Glo™ detection solution was added to each well and incubated at 37^0C for 20 minutes. ROS-Glo™ was made by mixing 10ml of Luciferin detection reagent with 100 µl of D-Cysteine and 100 µl of signal enhancer solution. Luminescence was then measured using a luminometer. Some of the ROS that was produced in cell cultures include singlet oxygen, hydroxyl radical, superoxide and H_2O_2. H_2O_2 was suitable to measure due to having the longest half-life among all the other ROS. Also, most ROS in the cells were converted to H_2O_2 by SOD. If the levels of H_2O_2 change then this reflects a change in ROS levels. The amount of light was directly proportional to the amount of H_2O_2 produced by the cells (PROMEGA G8820).

GSH-Glo™ Glutathione Assay

This assay was used to measure and detect GSH levels. This assay used glutathione S-transferase to convert a luciferin derivative into luciferin if GSH was present. The luciferin produced was proportional to the amount of GSH present. A 100 ul of media with cells were placed in each of the 96-wells in a white plate and incubated at 37^0C for 24 hours. Media was then removed and 100 µl of the assigned "media/solution" was added to each of the 96-wells. Samples in the 96-well plate were treated with 50 µl of GSH-Glo™ Reagent and incubated for 30 minutes at 37^0C. GSH-Glo™ Reagent was made by mixing 100 µl of Luciferin-NT with 10 µl GSH-Glo™ Reaction buffer and 100 µl of Glutathione S-Transferase. A 100 µl of Luciferin detection reagent were added to each well of the 96-

well plate, mix briefly and incubate for 15 minutes at 37^0C. Luciferin detection reagent was made by mixing 1 bottle of luciferin detection reagent with 10 ml of reconstitution buffer with esterase. Luminometer was used to measure the luminescence. Alteration in the levels of GSH was crucial for promoting oxidative stress that might have led to cell death, apoptosis. Moreover, measuring GSH levels in tissue and cell extracts indicated oxidative stress or cell viability. The amount of luminescence produced was directly correlated to the amount of reduced glutathione (GSH) in the cells (PROMEGA V6911).

NAD^+/NADH- Glo™ Assay

This assay was used to measure the reduced and oxidized nicotinamide adenine nucleotides (NADH and NAD^+). NAD^+ cycling enzyme is an enzyme that converts NAD^+ to NADH. When NADH was present, reductase converted a pro-luciferin reductase substrate to luciferin. Luciferin was then measured using Ultra-Glo™ recombinant luciferase. The produced light was directly proportional to the amount of NADH and NAD^+ in the samples. Reductase and NAD^+ cycling enzyme cycle between NADH and NAD^+. A 100 µl of media with cells were placed in each of the 96-wells in a white plate and incubated at 37^0C for 24 hours. Media was then removed and 50 µl of the assigned "media/solution" was added to each of the 96-wells. Samples in the 96-well plate were treated with 100 µl of reconstitute luciferin detection reagent. Reconstitute luciferin detection reagent was comprised of one vial of luciferin detection reagent mixed with 10 ml of reconstitution buffer. 50 µl of NAD^+/NADH-Glo™ detection buffer was added to each well of 96-well plate, mixed briefly then incubated at room temperature for a period of 30-60 minutes. NAD^+/NADH-Glo™ detection buffer was made by mixing 55 µl of reductase, 55 µl of reductase substrate, 1.25 ml of NAD^+ cycling substrate and 275 µl of water in one vial of

NAD cycling enzyme. Luminometer was then used to measure the luminescence. NADH and NAD^+ are essential molecules for processes such as signal transduction, epigenetics and metabolism. The levels of NADH and NAD^+ are important indicators for the health of cells (PROMEGA G9071).

$NADP^+$/NADPH-Glo™ Assay

This assay was used to measure the reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH and $NADP^+$). The enzyme that converts $NADP^+$ to NADPH is $NADP^+$ cycling enzyme. When NADPH was present, reductase formed luciferin by reducing a pro-luciferin reductase substrate. Then luciferin was then measured by using Ultra-Glo™ recombinant luciferase which produced light that was proportional to the amount of NADPH and $NADP^+$ in the sample. Reductase and $NADP^+$ cycling enzyme cycle between NADPH and $NADP^+$. A 100 µl of media with cells were placed in each of the 96-wells in a white plate and incubated at 37^0C for 24 hours. Media was then removed and 50 µl of the assigned "media/solution" was added to each of the 96-wells. Samples in the 96-well plate were treated with 100 µl of reconstitute luciferin detection reagent. Reconstitute luciferin detection reagent was comprised of one vial of luciferin detection reagent mixed with 10 ml of reconstitution buffer. 50 µl of $NADP^+$/NADPH-Glo™ detection buffer was added to each well of 96-well plate, mixed briefly then incubate at room temperature for a period of 45 minutes. $NADP^+$/NADPH-Glo™ detection buffer was made by mixing 55 µl of reductase, 55 µl of reductase substrate, 1.25 ml of $NADP^+$ cycling substrate and 275 µl of water in one vial of $NADP^+$ cycling enzyme. Luminometer was used to measure the luminescence. NADPH and $NADP^+$ are essential molecules that

are necessary for cellular processes such as biosynthesis of nucleotides, lipids and amino acids and redox balance (PROMEGA G9081).

Timeline for completion

This study was conducted at Montclair State University. It began in Fall 2016 and was carried out until Spring 2018.

Results

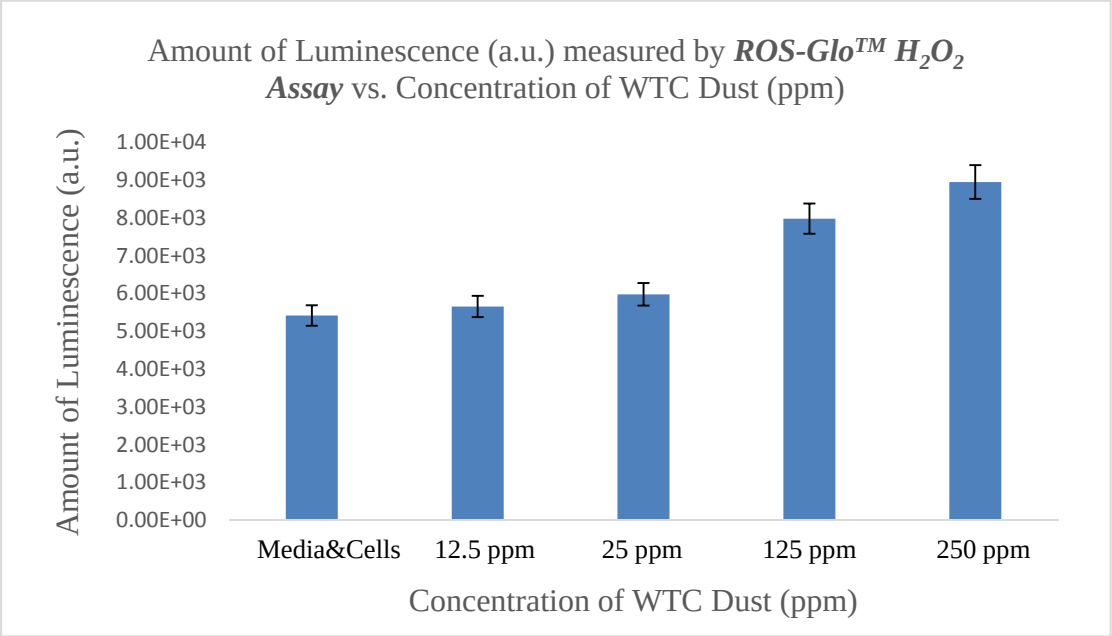


Figure 7. **Levels of H_2O_2**; luminescence (a.u.) which was measured using the Luminometer vs. the concentration WTC Dust in various ppm (12.5, 25, 125, and 250 ppm) measured by ***ROS-Glo™ H_2O_2 Assay***. As the figure shows, the amount of light increases as the concentration of WTC dust increases.

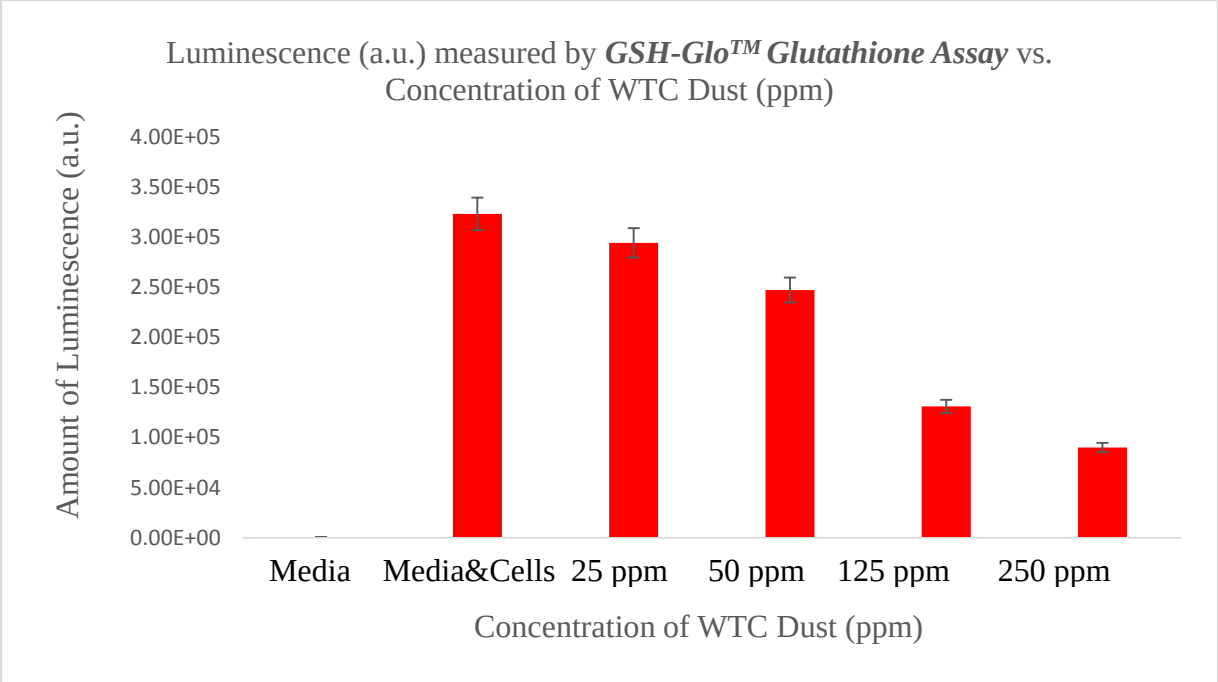


Figure 8. **Levels of reduced glutathione (GSH)**; amount of luminescence (a.u.) which was measured using the Luminometer vs. concentration of WTC Dust in several ppm (25, 50, 125, and 250 ppm) measured by ***GSH-Glo™ Glutathione Assay***. As noted, the amount of light produced decreases as the concentration of WTC dust increases. Media alone did not produce any significant amount of luminescence but once cells are added, a significant increase in luminescence is noted.

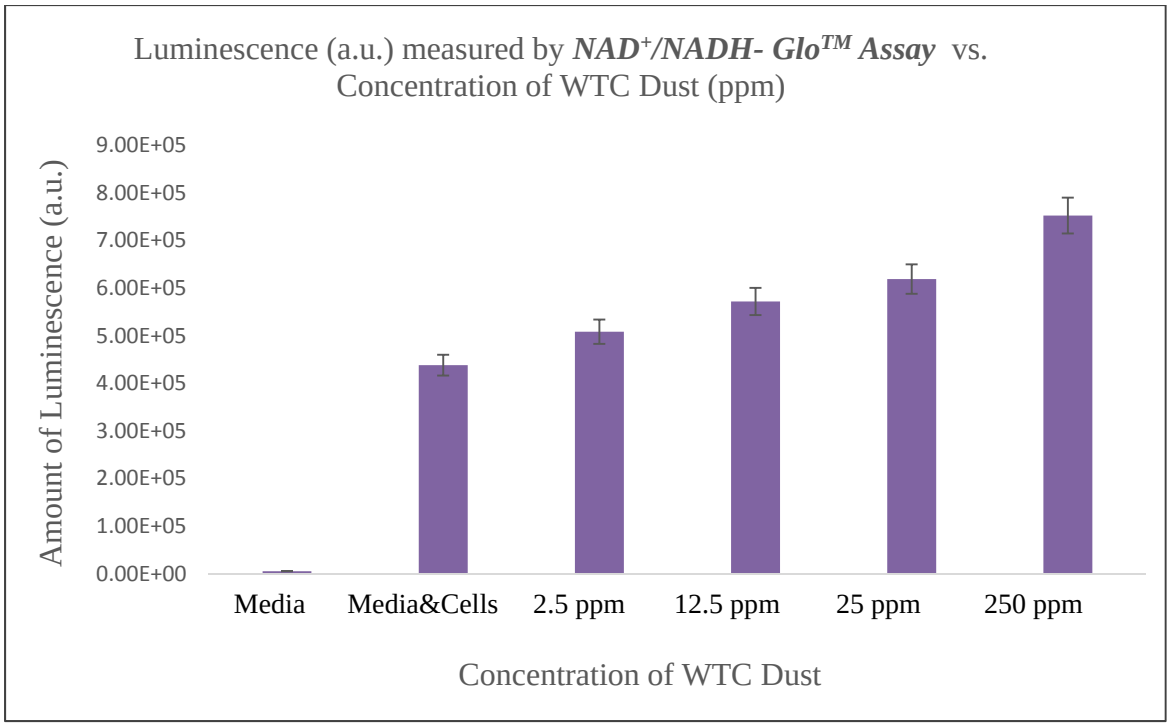


Figure 9. **Ratio of NAD$^+$/NADH**; luminescence (a.u.) measured using a luminometer vs. the concentration of the WTC dust in ppm, using ***NAD/NADH- Glo™ Assay***. As the concentration of WTC dust increases from 2.5 ppm to 250 ppm, it is noted that the amount of luminescence produced increases. When media is plated alone, barely any luminescence was produced. However, once cells are added to the media, significant amount of luminescence is produced.

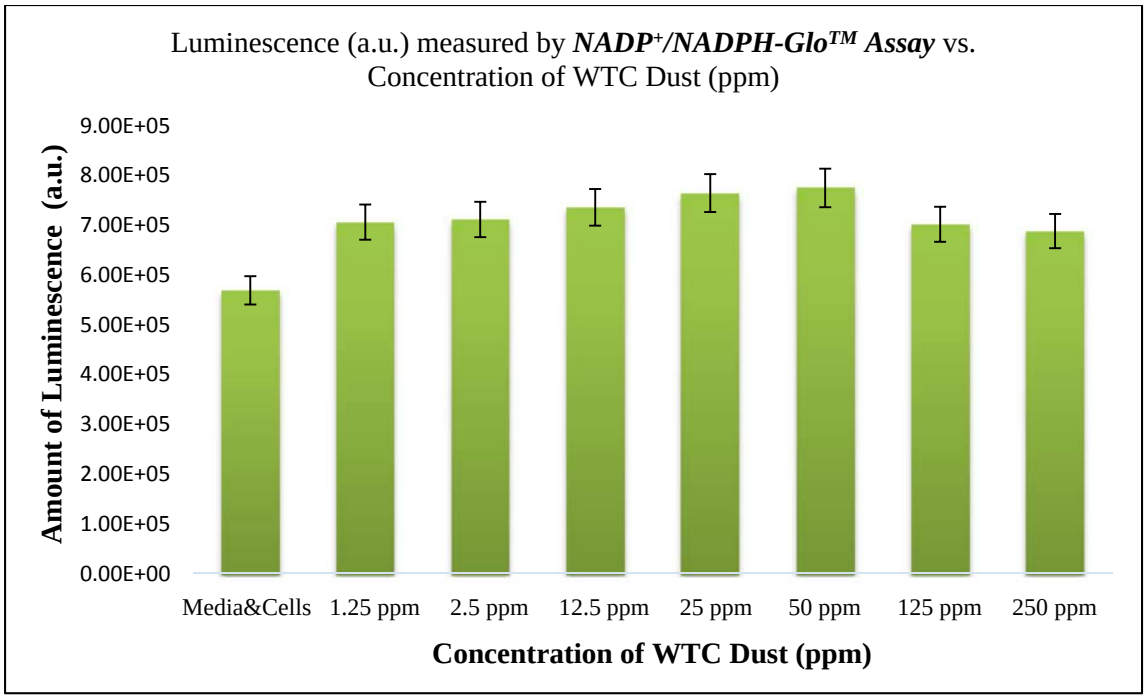


Figure 10. **Amount of NADPH**; luminescence (a.u.) was measured using a luminometer vs. the concentration of the WTC dust in ppm, using ***NADP+/NADPH-Glo™ Assay***. As noted in the figure, the amount of luminescence increases as the concentration of WTC dust increases from 1.25 ppm to 50 ppm. However, the amount of luminescence starts to leave off around 25 ppm and then decreases as the concentration of WTC dust is higher than 50 ppm.

Discussion

After the collapse of September 11th, 2001, thousands of people were exposed to the WTC particulate matter that covered a vast portion of lower Manhattan. First responders, workers, residents and stand-byers inhaled and ingested that WTC dust (Welch et al., 2014). Dr. Paul Lioy and colleagues at Rutgers University analyzed samples of the WTC dust which were found to be composed of heavy metals, gypsum, asbestos, concrete, ionic species and many organic and inorganic matter (Lioy et al., 2002). Later, it was also discovered that the size of the particulate matter that reached the lungs was about 2.5 μm which led to many respiratory illnesses (Heyder et al., 2004). Prior studies have shown that high levels of WTC dust decreased the proliferation of human lung cells *in vitro* (Hernandez et al., 2012). Thousands of people who were around that area were affected physiologically and psychologically. Many preliminary studies showed how the exposure to the WTC dust has led to increased risk of cancer, cardiovascular diseases and neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, PTSD and Parkinson's disease (Dietrich et al., 2009; Rosati et al., 2008; Brook et al., 2010; Croft et al., 2010; Shiratori et al., 2012).

The purpose of this study was to examine the effects of the WTC dust at a cellular level, focusing specifically on the mitochondria. This study used multiple cellular assays to determine the levels of oxidative stress due to various concentrations of the WTC dust and examined how the mitochondria attempts to reverse that damage.

ROS-Glo™ H_2O_2 assay determined the levels of ROS produced as the concentration of the WTC dust increases. As seen in figure (7), the amount of H_2O_2 significantly increases as the concentration of WTC dust goes from 12.5 ppm to 250 ppm. The figure shows a direct relationship between the concentration of WTC dust and the ROS

(ex. H_2O_2) produced. This indicates the WTC dust induces the production of ROS faster than being metabolized by the cells.

Next, the amount of reduced glutathione (GSH) was examined at increasing concentrations of the WTC dust. The rationale for studying the levels of GSH was to elucidate how a naturally occurring antioxidant breaks down ROS. Reduced GSH acts as an antioxidant and is found in every cell in the human body (Chen et al., 2017). As noted in figure (8), media alone did not show any GSH; however, once fibroblast lung cells were added to the media, the amount of GSH significantly increased. Also, it is noted as the concentration of the WTC dust increased, the amount of GSH progressively decreased. This was rewarding to find because as the concentration of WTC dust increases, more ROS are produced. Thus, reduced glutathione uses its antioxidant properties and begins reducing ROS into water which leads to decreased levels of reduced glutathione (GSH) and a higher level of oxidized glutathione (GSSG).

After studying the electron transport chain, it was scientific biochemical curiosity which drove this study to examine the levels of NADH. Figure (9) indicates that as the levels of WTC dust escalate, the ratio of NAD^+/NADH increases as well. This suggests that there is more NADH being oxidized to NAD^+. This may indicate that NADH is giving off its electrons to $NADP^+$ to be reduced and hence, NADPH can give off its electron to the oxidized glutathione (GSSG) to become GSH to counteract the effects of increased production of ROS.

NADPH levels were then tested to determine to further support the hypothesis of this study. Figure (10) depicts increased levels of NADPH as the WTC dust increases from 1.25 ppm until 25 ppm. It was unusual to see a plateau in the levels of NADPH around 25

ppm to 50 ppm of WTC dust, but a possible explanation might suggest that the cells can only compensate and reverse the effects within a limited threshold. This could also explain what is observed at 125 ppm and 250 ppm of WTC dust when the levels of NADPH begin to decrease. This might be happening as cells are starting to die and cannot reduce additional $NADP^+$ due to the excessively high concentrations of the toxic WTC dust.

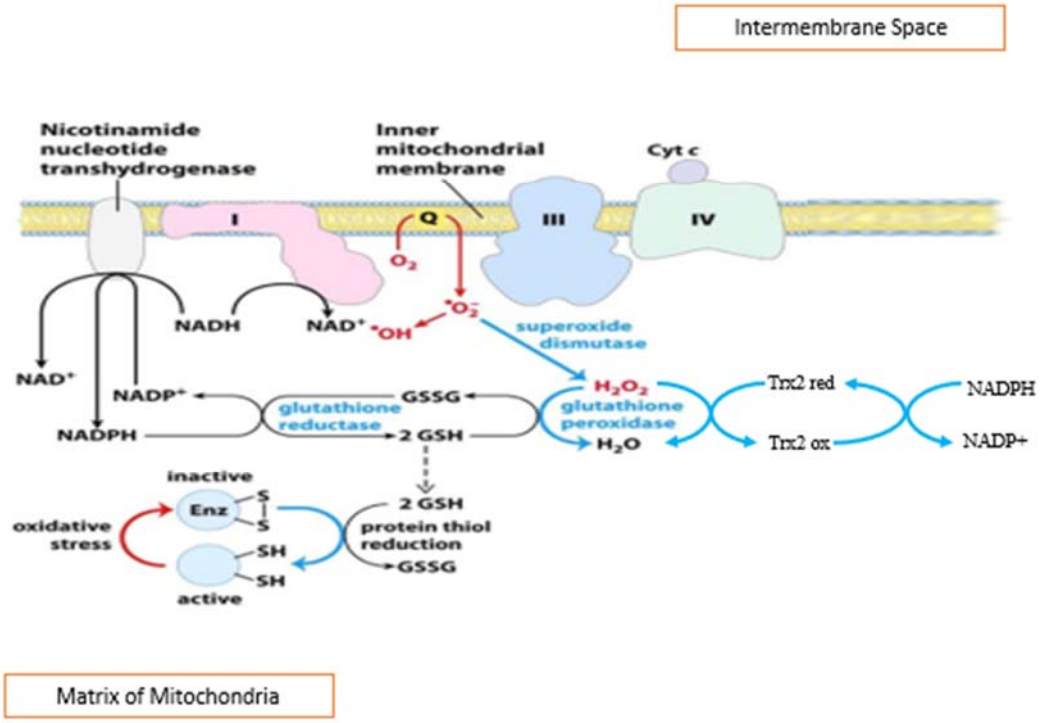


Figure 11. The electron transport chain and the mechanisms it uses to counteract the effects of ROS.

At normal cellular ROS levels, sufficient amount of NADPH is produced from the pentose phosphate pathway which is sufficient to eliminate these levels. However, when higher levels of ROS are found in the cell, NADH reduces $NADP^+$ to form NAD^+ and NADPH. NADPH is needed to increase the amount of reduced GSH in the cells by GSH

reductase. Reduced GSH acts as an antioxidant. Glutathione peroxidase eliminates free radicals by reducing hydrogen peroxide (ROS) into water while oxidizing the reduced GSH into oxidized glutathione (GSSG) as noted in figure (11). This whole process occurs in the matrix of the mitochondria during a process called oxidative phosphorylation. Thus, increased levels of ROS alter the electron transport chain and lowers the levels of ATP produced. These effects may indicate the cellular mechanisms behind the development of cancer, respiratory diseases, cardiovascular diseases and neurodegenerative diseases after being exposed to the toxic, mutagenic WTC dust.

Future Research

Future research will focus on the effects of antioxidants such as Vitamins E and C on the various concentrations of the WTC dust and hope to reduce the effects of ROS. Future experiments should study the effects of adding doses of NADH, similar to the suggestion of Dr. Birkmayer's study of administering NADH to cancer patients to inhibit the growth of cancer (Birkmayer, 2017). It would also be interesting to determine if certain proteins or tumor suppressors, such as p53, are affected by the World Trade Center particulate matter.

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