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Selenium Supplementation Increases Antioxidant Response In Vitro

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SELENIUM SUPPLEMENTATION INCREASES ANTIOXIDANT RESPONSE IN VITRO

by

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THESIS

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of Wayne State University,

Detroit, Michigan

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for the degree of

MASTER OF SCIENCE

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MAJOR: NUTRITION AND FOOD SCIENCE

Approved by:

_________________________  ________________________
Advisor                    Date
DEDICATION

I dedicate this work to my parents. Thank you.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Diane Cabelof, advisor of my thesis research. Dr. Cabelof provided me with direction, enthusiasm and determination of my subject area and her thoughtful guidance was needed for completion of this thesis. I would also like to thank the other members of my advising committee, Dr. Zhou and Dr. Heydari. Further thanks to Hiral Patel and Kirk Simon for their exceptional lab advice and assistance during my research. Finally, I would like to convey my appreciation to Debra Zebari for helping me with the endless administrative aspects of my graduate education.
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SPECIFIC AIMS

Although the relationship of selenium and oxidative stress has been comprehensively studied for the past 25 years, there is still much to be discovered. The curiosity of selenium and its connection with aging and oxidative damage is still high. I have tested the following hypothesis: Increase selenium increases glutathione peroxidases activity, which may increase oxidative damage repair. The specific aims in my thesis research are as follows;

Specific Aim 1. To determine if selenium supplementation up-regulates GPx activity and increases oxidative stress response.

Specific Aim 2. To determine if an environment change of 3% oxygen or 20% oxygen has an impact on GPx activity.

Specific Aim 3. To determine whether GPx response is cell line specific.

Specific Aim 4. To determine if gene expression differs between MEF cells with and without selenium supplementation.
INTRODUCTION

Oxidative Stress

Selenium is a known antioxidant, which has been shown to reduce oxidative stress (89). Oxidative stress occurs when the production of oxidizing agents, free radicals and reactive oxygen species (ROS), surpasses the antioxidant capacity of cellular antioxidants in a biological system (1). Increased exposure to oxidants or decreased protection against oxidants can result in oxidative stress (4). These events can even occur simultaneously. Oxidative stress was first observed in bacteria but has now been documented in eukaryotes as well (2). Oxidation as a possible cause or marker for disease has been studied in both plant and animal kingdoms (3).

Damage to DNA due to oxidative stress has been established through many studies. The investigations have established the effect of oxidative stress to include: (i) base oxidation and fragmentation products, (ii) single-and double-strand breaks, (iii) interstrand/intrastrand cross-links, (iv) DNA-protein cross-links, and (v) sugar fragmentation products (7). Failure to repair or protect the genome from oxidative stress can lead to induction of mutations, microsatellite instability, and loss of heterozygosity, chromosomal aberrations, altered gene expression and, eventually, cytostasis, cytotoxicity, or neoplastic growth.

Cells have two options when defending themselves from oxidative stress. The first engages cellular molecules directly involved in preventing oxidative damage to the cell (2). These include antioxidant enzymes such as glutathione peroxidases,
Diagram 1. Reactive Oxygen Species Pathway.
A simplified illustration of two basic biochemical pathways that could lead to increased oxidative damage to proteins, lipids, and DNA. The diagram, taken from The Role of Oxidative Damage and Stress in Aging Review by Bokov A, Chaudhuri A, and Richardson A (105), shows the balance between the pathways- the production and the scavenging of reactive oxygen species (ROS). ROS could be superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals.
superoxide dismutases (SOD), catalase and antioxidant molecules such as glutathione and ascorbate. The second option for defense comprises of repair enzymes. These repair enzymes and systems remove and/or repair oxidatively damaged macromolecules. DNA repair usually involves DNA nucleases and glycosylases. **Diagram 1** demonstrates the duo of biochemical pathways that may lead to enhance oxidative stress.

Studies have shown repeatedly that there is an age-associated increase in free radical attacks on macromolecules such as proteins, lipids, and DNA in an assortment of tissues (8). Pioneering studies in oxidation have shown that oxidative damage increases exponentially with age (9). Brain, heart and skeletal muscle tissue tend to accumulate larger amounts of damage due to long-lived, post-mitotic cells than those composed of short-lived non-mitotic cells (8). ROS attack neurons and glial cells (post-mitotic cells) leading to neuronal damage (10). Reactive oxygen species such as H$_2$O$_2$ and O$_2^-$ generally increase with age. To end, data in countless past studies have shown that increased production of ROS is the primary factor for age-related buildup of molecular oxidative damage (8).

Oxidative stress has been associated with numerous diseases including rheumatoid arthritis, neurodegenerative diseases, and cancer (6). Cancer development is increased by oxidative stress due to increased cell proliferation, increased cellular migration and induced DNA damage that leads to genetic imperfections (1). Furthermore, oxidative damage to biomolecules (lipids, proteins, DNA) by way of excessive free radicals can lead to chronic diseases such as atherosclerosis, diabetes, post-ischemic perfusion injury, myocardial infarction,
cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans (10).

**Reactive Oxygen Species**

Reactive Oxygen Species (ROS) is a collective term given to describe an assortment of molecules and free radicals originating from molecular oxygen and that lead to oxidation of proteins, nucleic acids, and lipids (9, 12). ROS can be produced from endogenous and exogenous sources (13). Endogenous sources include mitochondria, peroxisomes, cytochrome P450 metabolism, and inflammatory cell activation. Mitochondria are the site of normal aerobic respiration where mitochondrion consume oxygen and reduce it to produce $\text{O}_2$, $\text{OH}^-$, and a significant generation of $\text{H}_2\text{O}_2$ as byproducts (10). It has been established that mitochondria is a major site of free radical production, thus, they are vastly abundant in antioxidants (14). Another endogenous source of oxidant generation occurs when cells are infected with bacteria or a virus, get destroyed by phagocytosis resulting in an oxidative burst of nitric oxide, $\text{H}_2\text{O}_2$, $\text{O}_2^-$, and $\text{OCl}$ (10).

Exogenous sources include environmental sources such as non-genotoxic carcinogens that can directly produce or indirectly induce ROS in cells (13). Another exogenous source is exposure to xenobiotics, which include chlorinated compounds, metal ions, barbiturates and radiation. Finally, specialized enzymes such as xanthine and NADPH oxidases major in the professional generation of ROS (84).
ROS are created continuously within the cell due to mitochondrial electron transfer processes or as a byproduct of actions of certain enzymes including lipoxygenase, xanthine oxidase, and cyclooxygenase (COX) (81). In addition, ROS can also be produced as an outcome of intracellular metabolism of foreign compounds by cytochrome P450 (CYP) monooxygenases.

Free radicals are a frequent result of normal aerobic cellular metabolism (10). Free radicals are molecules, atoms or ions with unpaired electrons in their outer orbit. Due to the unpaired electrons, radicals are highly chemically reactive. Free radicals play a crucial role in biological evolution while oxygen radicals are engaged in biochemical activities of cells such as gene transcription, signal transduction, and regulation of soluble guanylate cyclase activity (11). Our manmade environment has exposed humans to free radicals from areas like pollutants, cigarette smoke, and electromagnetic radiation. Free radicals are also found naturally in the environment from sources such as radon, cosmic radiation, and cellular metabolisms.

**Generation of Reactive Oxygen Species**

Molecular oxygen in its ground state contains two unpaired electrons in its outer shell (12). These two single electrons have parallel spins and oxygen can only react with one electron at a time, which makes these two electrons least reactive. However, if one of the two unpaired electrons becomes excited and transiently flips one of these spins, the molecule becomes a strong oxidant because the two electrons with opposing spins become quick to react with other pairs of electrons, especially double bonds. Due to the spin-restriction rule, oxygen reduction reactions usually
Diagram 2

Diagram 2. Univalent Pathway.
The univalent pathway diagram depicts the stages of 1-electron oxygen reduction. The diagram is adapted from The Broad Spectrum of Responses to Oxidants in Proliferating Cells by Davies (4). Ground state molecular oxygen contains 2 unpaired electrons. The 2 unpaired electrons have parallel spins and absorption of energy rapidly flips 1 of these spins, generating a singlet oxygen. In this oxygen reduction system, oxygen is first reduced to superoxide anion radical, then to hydrogen peroxide. The next reduction is the powerful hydroxyl radical, and another electron reduction gives water.
advance one electron at a time, referred to as the univalent pathway for oxygen reduction (4) (Diagram 2).

The univalent pathway shows that oxygen is first reduced to the superoxide anion radical and then further reduced to hydrogen peroxide (4). Another electron reduction produces a hydroxyl radical (-OH), an exceptionally powerful oxidant. Finally the pathway is complete with the production of water, which is the fourth and final electron reduction. Ground-state oxygen is a weak oxidizing agent while singlet oxygen, hydrogen peroxide, and the hydroxyl radical are even stronger oxidants.

**Reactive Oxygen Species & Antioxidants**

Antioxidant defense systems include structural defenses such as cell membranes, antioxidant enzymes such as superoxide dismutase and GPx, enzymes such as proteases and repair enzymes that replace damaged DNA and finally dietary antioxidants such as Vitamin E and C. Glutathione, selenium, arginine, taurine, citrulline, creatine, zinc, Vitamin E, Vitamin C, Vitamin A, and tea polyphenols are antioxidants that help regulate the ROS that are produced (10). Vitamin C, Vitamin E and Selenium are dietary antioxidants that are encouraged to reduce oxidative stress and risk of diseases such as cancer (27). Synergistic properties have been found to exist between antioxidant enzymes such as GPx, SOD, catalase, glutathione reductase, which help remove free radicals. Natural antioxidant systems are broken down into two key categories, enzymatic and non-enzymatic. Catalase, GPx, and SOD plus a few supporting enzymes encompass the enzymatic antioxidants. The non-enzymatic antioxidants, which consist of direct acting antioxidants, are
imperative to defend against oxidative stress. These come from a dietary source and include ascorbic and lipoic acid, polyphenols and carotenoids. Epidemiologic studies have shown that antioxidants have anti-inflammatory, antiatherosclerotic, antimutagenic, anticarcinogenic, antitumor, antibacterial and antiviral qualities to some extent (15-17).

**Selenium**

Selenium is a trace element that is essential to human health, required only in small amounts, and functions as a component of selenoproteins (18). Selenoproteins are selenium-dependent enzymes. 25 selenoproteins have been identified but metabolic functions have only been recognized in approximately half of those (29). A deficiency of selenium induces some pathological conditions including cancer, liver necrosis, and coronary heart disease (28). Selenium is an integral part of numerous enzymes including glutathione peroxidases (GPx), thioredoxin reductase (TR), and selenoprotein P (SeP), which encompass selenium as selenocystein. Thioredoxin reductase is involved in the regeneration of various antioxidants, possibly Vitamin C (29). Preservation of thioredoxin in a reduced structure by thioredoxin reductase is essential for regulating cell growth and viability.

Selenoprotein P is located in plasma and connected to vascular endothelial cells, which are cells that line the inner walls of blood vessels (30). A transport protein for selenium seems to be the principal function of Selenoprotein P. It also protects endothelial cells from damage brought on by reactive nitrogen species (RNS), such as peroxynitrite (31). Past studies have shown that selenite, a selenium
containing anion, in high doses can lead to cytotoxicity (19-24), cellular apoptosis (19-22), DNA fragmentation (23-24), and DNA oxidative damage in cells (22). In cell culture, it is established that without selenium in the media for cell culture, cells cannot proliferate nor survive (28). In spite of this, the mechanism for selenium and its role in cell proliferation is still unknown.

Five selenium-containing glutathione peroxidases (GPx) have been identified: cellular or classical GPx, plasma or extracellular GPx, gastrointestinal GPx, phospholipid hydroperoxide GPx, and olfactory GPx (29). Each of these are separate selenoproteins, but their functions are equivalent. They are all are antioxidant enzymes that lessen potentially damaging ROS to harmless substances like water and alcohols by coupling their reduction with the oxidation of glutathione. GPX-1 is predominantly rich in erythrocytes kidney, and liver (100).

Selenium & Disease

A clear link between selenium and disease has been demonstrated. For example, patients receiving total parenteral nutrition (TPN) without added selenium were prone to muscle weakness, muscle wasting and cardiomyopathy (32). TPN is a system of feeding nutrients through an intravenous (IV) line to patients with improperly functioning digestive systems (41, 42). Solutions used for TPN patients are now supplemented with selenium to prevent such problems. Selenium deficiency can also occur with patients with Crohn’s disease or people with gastrointestinal problems due to improper absorption.

Selenomethionine is the organic selenium analogue of the amino acid methionine and can be found in foods like corn, wheat and soybean (44, 45).
Selenomethionine operates as a transport for selenium storage in organs and tissues and can also be integrated into proteins and replace methionine. Selenium supplements may consist of sodium selenite and sodium selenate, which are inorganic forms of selenium. However, selenomethionate is regarded as the best absorbed and utilized form of selenium. Some yeasts also contain huge amounts of selenium in the form of selenomethionine, as much as 1,000 to 2,000 micrograms of selenium per gram (44). A large scale cancer prevention trial in 1983 showed that consuming daily supplements of 200 micrograms of selenium could lessen the risk of developing lung, prostate and colorectal cancer (46).

Plant foods are the primary dietary sources of selenium in most countries (34). The content of selenium in food correlates to the content of selenium found in the soil where the food or animal originated. Soils in the high plains of northern Nebraska and the Dakotas are very high in selenium content. Research has shown that people from that region have the highest selenium intake in the United States. Meanwhile, regions of China & Russia have very low levels of selenium in the soil and selenium deficiency is often reported because food in those areas are grown and eaten locally. Other sources of dietary selenium include some meats and seafood (35, 36). Animals that consume grains or plants that were grown in selenium-rich soil have increased levels of selenium in their muscle. Finally, some breads are a source of selenium in the United States, and certain nuts are a source of selenium as well.

To understand the different quantities of selenium, it is important to review
Table 1

<table>
<thead>
<tr>
<th>Food</th>
<th>Micrograms (mcg)</th>
<th>Percent DV*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil nuts, dried, unblanched, 1 ounce</td>
<td>544</td>
<td>777</td>
</tr>
<tr>
<td>Tuna, light, canned in water, drained, 3 ounces</td>
<td>68</td>
<td>97</td>
</tr>
<tr>
<td>Cod, cooked, 3 ounces</td>
<td>32</td>
<td>46</td>
</tr>
<tr>
<td>Turkey, light meat, roasted, 3 ounces</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>Bagel, egg, 4 inch</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>Chicken breast, meat only, roasted, 3 ounces</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>Beef chuck roast, lean only, roasted, 3 ounces</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Sunflower seed kernels, dry roasted, 1 ounce</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>Egg noodles, enriched, boiled, ½ cup</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>Macaroni, enriched, boiled, ½ cup</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>Ground beef, cooked, broiled, 3 ounces</td>
<td>18</td>
<td>26</td>
</tr>
<tr>
<td>Egg, whole, hard-boiled, 1 large</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Oatmeal, instant, fortified, cooked, 1 cup</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Cottage cheese, low fat 2%, ½ cup</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Bread, whole-wheat, commercially prepared, 1 slice</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Rice, brown, long-grain, cooked, ½ cup</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Rice, white, enriched, long-grain, cooked, ½ cup</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Bread, white, commercially prepared, 1 slice</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Walnuts, black, dried, 1 ounce</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Cheddar cheese, 1 ounce</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1. Selected Food Sources of Selenium.
This table, taken from the National Institute of Health’s official website, [http://ods.od.nih.gov/](http://ods.od.nih.gov/) (37), shows that selenium content varies in diverse foods. Plant foods are the main source of dietary selenium. The selenium content of the soil directly correlates with the selenium content of the food. The Daily Value for selenium is 70 micrograms (mcg).
Table 2. Recommended Dietary Allowances for Selenium.
Developed by the Institute of Medicine and taken from their website, [http://ods.od.nih.gov/](http://ods.od.nih.gov/) (37), the Dietary Reference Intakes (DRIs), is an expression used for a set of reference values. The 3 major categories of reference values contained in the DRIs are Recommended Dietary Allowances (RDA), Adequate Intakes (AI), and Tolerable Upper Intake Levels (UL). RDA is the average daily dietary intake level that is adequate to meet the nutrient obligations of healthy individuals in each age and gender set. An AI is set when there is deficient scientific data accessible to determine a RDA. The UL is the maximum daily intake that will not produce unfavorable effects. This table shows the Recommended Dietary Allowances for Selenium in Children and Adults.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Males and Females (mcg/day)</th>
<th>Pregnancy (mcg/day)</th>
<th>Lactation (mcg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3</td>
<td>20</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>4–8</td>
<td>30</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>9–13</td>
<td>40</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>14–18</td>
<td>55</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>19+</td>
<td>55</td>
<td>60</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 3

Table 4: Tolerable Upper Intake Levels for Selenium for Infants, Children, and Adults [12]

<table>
<thead>
<tr>
<th>Age</th>
<th>Males and Females (mcg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6 months</td>
<td>45</td>
</tr>
<tr>
<td>7–12 months</td>
<td>60</td>
</tr>
<tr>
<td>1–3 years</td>
<td>90</td>
</tr>
<tr>
<td>4–8 years</td>
<td>150</td>
</tr>
<tr>
<td>9–13 years</td>
<td>280</td>
</tr>
<tr>
<td>14–18 years</td>
<td>400</td>
</tr>
<tr>
<td>19+ years</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 3. Tolerable Upper Intake Levels for Selenium.
This table presents the Tolerable Upper Intake Levels (UL) of Selenium, set by the Institute of Medicine of the National Academy of Sciences and taken from their official website, http://ods.od.nih.gov/ (37). 400 micrograms per day is the set UL for adults to prevent selenosis. Selenosis is a condition caused by high levels of selenium (greater than 100 mcg/dL) that may cause white blotchy nails, hair loss, fatigue, gastrointestinal upsets, and mild nerve damage.
the following terms. The Institute of Medicine of the National Academies is responsible for setting the Dietary Reference Intakes (DRI’s), which is a set of reference values used for evaluating nutrient intake for healthy people (37). Recommended Dietary Allowances (RDA), Adequate Intakes (AI), and Tolerable Upper Intake Levels (UL) are three types of significant reference values incorporated in the DRI’s. The RDA advises the average daily dietary intake level that is satisfactory to meet 97-98% of healthy persons in each age and gender group. An AI is established when there is inadequate data presented to determine a RDA, and is set to meet or exceed the amount needed to maintain nutritional sufficiency in nearly all age and gender groups. On the other hand, the UL is the maximum daily intake allowed without causing unfavorable health effects.

Some evidence suggests that selenium deficiency may be a factor in the development of hypothyroidism, a type of heart disease, and a weakened immune system (38, 39). Other evidence shows that selenium deficiency can make the body more vulnerable to illnesses produced by other biochemical, nutritional or infectious stresses (40). Also, gastrointestinal problems may impair selenium absorption and lead to selenium depletion or selenium deficiency. Three particular diseases have been related to selenium deficiency. Keshan disease, which causes an enlarged heart and deprived heart function, Kashin-Beck disease, which results in osteoarthropathy, and Myxedematous Endemic Cretinism, which leads to mental retardation.

In a selenium-deficient region of China, young women and children were affected by Keshan disease (32, 33). It was first described in the early 1930’s in
China and is still occurring today in the countryside of China with selenium poor soil (40). The chronic version of the disease brings about a sudden onset of cardiac insufficiency, while, the acute version is characterized by moderate to severe heart enlargement, also paired with cardiac insufficiency (32, 33). Dietary intake in these selenium poor soil areas are less than 19 micrograms per day for men and less than 13 micrograms per day for women, substantially lower than RDA recommendations (37). Poor selenium nutrition status and exceedingly low dietary intake of selenium is the main association with getting Keshan disease.

**Selenium & Cancer**

Some studies show that people with higher blood levels or higher consumption of selenium have a lower chance of death from cancer, including lung, prostate and colorectal cancers (47-50). Regions in the United States with low selenium content in the soil have a significantly higher occurrence in nonmelanoma skin cancer (51).

Studies suggest that selenium influences cancer risk in two ways (52). The first is as an antioxidant where selenium helps protect the body from damaging effects of ROS. Also, selenium may prevent or slow tumor growth because particular breakdown products of selenium are considered to improve cell activity and curb development of blood vessels to the tumor. A selenium supplement of 200 microg/day is linked with substantial reductions in cancer risks in subjects with pre-treatment plasma Se concentrations below 120 ng/ml, found The Nutritional Prevention of Cancer Trial (52).
The following illustration, found in the article Environmental Health Impacts of Dispersed Mineralisation in South Africa by T.C. Davies and H.R. Mundalamo (107), presents a straightforward look on the health benefits of selenium.
The SU.VI.MAX study in France explored whether selenium united with at least one other dietary supplement could decrease the risk of prostate cancer in men (54). The participants received a supplement package with modest doses of Vitamin E, Vitamin C, beta-carotene, zinc, and selenium (100 μg/day) versus a placebo to compare the risk of cancer and cardiovascular disease. 5,141 men were enrolled with a starting PSA (prostate specific antigen) of <3 ng/ml, which is at normal level. The study found that the risk of prostate cancer in men who took the supplemental package was reduced by half.

Some data proposes that oxidative stress from free radicals may encourage heart disease (55-57). It is the oxidized form of low-density lipoproteins (LDL) that supports plaque build-up in coronary arteries (56). In turn, selenium is one of the antioxidants that may help limit the oxidation of LDL cholesterol and therefore, help prevent the incidence of coronary heart disease (55-57).

In addition, surveys have shown that persons with rheumatoid arthritis have decreased selenium levels in their blood (58, 59). Selenium may help relieve symptoms of arthritis by managing levels of free radicals (60). Diagram 3 shows an uncomplicated look at the multiple benefits of selenium.

Malabsorption in HIV/AIDS patients can lead to diminishing levels of nutrients, including selenium (61, 62). Decreased immune cell counts, amplified disease development, and great risk of death in the HIV/AIDS population are all correlated to selenium deficiency. As an antioxidant nutrient, selenium can help shield cells from oxidative stress, which may possibly slow development of the disease (63).
One study found that a selenium-based homolog of GPx is created by the HIV virus (64). In theory, the virus accelerates the reduction of selenium from HIV-infected lymphocytes. The study also showed that replication of HIV chronically infected T-lymphocytes was suppressed with selenium supplementation, *in vitro*. Trials showed that with 400 mcg daily of yeast-based selenium given to 19 patients with symptomatic HIV and AIDS, mean whole blood selenium levels increased in 70 days. 14 of those patients stated improvements of appetite, gastrointestinal function, and diminished repeated infections. Another study tested 125 HIV-positive men and women and established that selenium deficiency was linked to a higher death rate from HIV (65). In a different, smaller study, 24 children with HIV were observed for five years. In this study, they found that children with lower levels of selenium died at a younger age, which may imply faster disease progression (66).

**Selenium Toxicity & Deficiency**

Is there a health risk with too much selenium intake? Selenosis is a condition where blood levels of selenium are higher than 100 μg/dL (67). Symptoms of selenosis include gastrointestinal problems, garlic breath odor, hair loss, white nails, irritability, fatigue, and mild nerve damage (68). Selenium toxicity is uncommon in the United States. The rare selenium toxicity cases involve industrial accidents and manufacturing errors that led to exceptionally high concentrations of selenium in a supplement (69, 70). The UL for selenium set by the Institute of Medicine of the National Academy of Sciences is 400 micrograms per day for adults (37).

Another, more common issue is selenium deficiency. It has been established that selenium deficiency is correlated with weakened function of the immune
system (71). In two minor studies, healthy and immunosuppressed persons supplemented with 200 micrograms of sodium selenite a day for eight weeks showed an increased immune cell reaction to foreign antigens compared with those taking a placebo (72-74). This appears to show that immune response may be stimulated in healthy individuals taking selenium supplementation. Research indicates that selenium plays a role in regulating the expression of cell-signaling molecules called cytokines, which coordinate the immune response (75).

Selenium deficiency seems to enhance the development of some viral infections (77). The amplified oxidative stress that occurs due to selenium deficiency may encourage mutations or changes in the expression of certain viral genes. In an intriguing study completed at the University of North Carolina at Chapel Hill, selenium-deficient mice were inoculated with a strain of coxsackievirus. Results found that mutations happened in the viral genome, which resulted in a more virulent form of the virus. The mutations caused inflammation of the heart muscle identified as myocarditis. After its mutated, this virus also causes myocarditis in normal mice that are not selenium deficient, establishing that the enhanced virulence is due to a modification in the virus, not the effects of selenium deficiency on the host immune system. A study with GPx-1 knockout mice showed that cellular GPx supplies protection against myocarditis resulting from mutations in the genome of a formerly benign virus. Selenium deficiency causes decreased GPx activity, increasing oxidative damage and increasing the probability of mutations in the viral genome (71).
Cancer & Animal studies

Plenty of support shows that selenium supplementation at high levels decreases the occurrence of cancer in animals. In excess of more than two-thirds of over 100 published studies in 20 different animal models of viral, spontaneous, and chemically induced cancers found that selenium supplementation drastically lessens tumor incidence (78). The evidence indicates that selenium in its methylated form are the active species against tumors, and these methylated selenium structures are produced more with excess selenium intake (79). The relationships of selenium intake to cancer in humans and animals are summarized in the article titled Selenium and its relationship to cancer: an update, by Whanger (80).

To counteract oxidative stress, effective antioxidants are crucial and have become increasingly popular to aid in disease prevention and therapy (6). Compounds with glutathione peroxidase (GPx)-like activity are exceptionally fascinating because they catalytically remove oxidative stressors, and can consequently be applied in small quantities. GPx is the selenium-dependent enzyme discovered by Mills in 1957 (81). It is also the most considerably studied GPx. After its discovery, it was named GPx-1, or cytosolic or cellular GPx to differentiate from the further glutathione peroxidases enzymes that were discovered. Glutathione is the major intracelluar non-protein sulphhydryl, a functional group composed of a sulfur-hydrogen bond (-SH) (81). Glutathione is present in concentrations up to 10 mM in many cells and provides a crucial defense against oxidative stress by scavenging free radicals and taking part in the reduction of hydrogen peroxide.
As we already know, GPx catalyses the reduction of H$_2$O$_2$ as well as organic hydroperoxides to water and alcohols respectively, with the generation of oxidized glutathione (GSSG) (81).

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{HOH (82)}
\]

ROOH could be one of the many hydroperoxides, ROH, GSH is reduced glutathione and we already know that GSSG is oxidized glutathione (82). This reaction also stops further peroxide initiation of lipid peroxidation, therefore preserving the integrity of the cell. The reaction also regenerates GSH with the help of Glutathione Reductase (GR), by catalyzing the reduction of GSSG back to GSH as follows:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

GPx-1 in the human is ubiquitously expressed and is plentiful in erythrocytes, kidney, liver and mitochondria. In the cell cytosol, glutathione is synthesized by $\gamma$-glutamylcysteine synthetase (GCS) and glutathione synthetase (GS) (81). Transcriptional upregulation of GPx-1 as an adaptive response to oxidative stress has been shown in vitro. These additional peroxidases that were identified after GPx-1 are summarized in Table 4 and have been named GPx-2-GPx-5 (105). These peroxidases have at least 36% amino acid sequence similar to GPx-1.
Superoxide dismutase

Another essential enzyme that aids in antioxidant defense is superoxide dismutase (SOD). Superoxide dismutases are metallo-enzymes that scavenge possible damaging superoxide anions, O$_2^-$, and represent a crucial component of protection against oxidative stress (85). There are four classes of SODs that are known, distinguished by their metal groups Cu/Zn, Fe, Mn and Ni. The two most crucial SODs are Manganese superoxide dismutase, Mn-SOD or SOD2 and Copper/Zinc superoxide dismutase (SOD1). Superoxide dismutases are the first line of defense against O$_2^-$ (83). Superoxide dismutase can be generated by unique enzymes, like NADPH oxidases or xanthine, or as a byproduct of normal cellular metabolism (84). SOD then converts the superoxide to hydrogen peroxide through catalase or peroxidases. SOD1 is the most abundant and found in all eukaryotes and many prokaryotes (87). They form homodimers whose essential structural motif is a β-barrel (88). Found in cytosol and peroxisomes, SOD1 encodes a copper-zinc bound SOD (85). It catalyzes superoxide into dioxygen and hydrogen peroxide; (2O$_2^-$ + 2H$^+$ → O$_2$ + H$_2$O$_2$) (86). SOD2 is manganese bound SOD, which is prevalent in both prokaryotes and mitochondria (85). SOD2 occur as homoedimers and sporadically as homotetramers where the monomers fold into two helix-rich domains with Mn bound by two residues from each domain (88). The study of superoxide dismutase and glutathione peroxidase is essential to learning the mechanisms of how these enzymes work, which could lead to a better understanding of battling oxidative stress.
### Table 4

<table>
<thead>
<tr>
<th>Type of the enzyme</th>
<th>Glutathione peroxidase 1 or cytosolic GPx (cGPx/GPX1)</th>
<th>Glutathione peroxidase 2 or glutathione peroxidase 2-like GPx (gGPx/GPX2)</th>
<th>Glutathione peroxidase 3 or plasma/endothelial cell GPx (eGPx/GPX4, GPX5)</th>
<th>Glutathione peroxidase 4 or phospholipid hydroperoxide GPx (PHGPx, GPX6)</th>
<th>Glutathione peroxidase 5 or NADPH oxidase GPx (NOX GPx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene structure/chromosomal location</td>
<td>2 exons / 3αφL3 (Pseudogene: αφ1-Lq12)</td>
<td>2 exons / 3αφL13</td>
<td>5 exons / 5αq22.3 q33.1</td>
<td>7 exons / 1αφ1L3</td>
<td>5 exons / 4p22.1</td>
</tr>
<tr>
<td>Protein structure characteristics</td>
<td>Homotetramer, contains a single selenocysteine residue in each of four identical subunits (210 amino acids, 224 kDa)</td>
<td>Homotetramer, (190 amino acids, 215 kDa)</td>
<td>A glycosylated homotetramer of 63 kDa subunits (190 amino acids, 215 kDa)</td>
<td>Homotetramer (190 amino acids, 215 kDa)</td>
<td>324 amino acids</td>
</tr>
<tr>
<td>Regulation of protein synthesis</td>
<td>N-acetylation of the C-terminal glycine residue in mature GPx, inducing a conformational change in the active site of the enzyme</td>
<td>Upregulation in colon, lung and skin cancers; in p53-activated cell lines</td>
<td>In part by selenium levels.</td>
<td>The unique cysteine residue of GPx is essential for the maintenance of the active site</td>
<td>Upregulation in colon, lung and skin cancers; in p53-activated cell lines</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td>Abundant in tissues in humans with a high rate of peroxisome production (such as erythrocytes, kidney, liver, and lung); extremely low or absent in liver, kidney, heart, lung, brain, testes (33).</td>
<td>In both the liver and the lung, GPx is highly expressed in cancerous and normal tissues.</td>
<td>In most tissues both in cytosol and associated with membranes; it is targeted to the cytosol, mitochondria, or the nucleus, the major selenoprotein in sperm (M1).</td>
<td>In secretory and endocrine proteins. In colonic epithelium and mammary glands.</td>
<td></td>
</tr>
<tr>
<td>The role of the enzyme</td>
<td>Deterioxidation of H₂O₂ (a wide range of organic hydroperoxides), through the coupled oxidation of reduced glutathione; prevents cytosolic peroxide-induced oxidative damage; lipid peroxidation and protein denaturation; enhanced GPx activity is mediated by hydroperoxidases (23).</td>
<td>Protects GSH against toxicity of lipid peroxides; reduces the defense barriers against the accumulation of reactive oxygen species (ROS) (33).</td>
<td>As barrier for any hydroperoxide transfer; reduction in hydrogen peroxide and lipid peroxides is more effective in the biological fluid than GSH at the expense of glutathione in a major scavenger of reactive oxygen species (ROS) (33).</td>
<td>Its small size and hydrophilic surface have been implicated in the ability of this enzyme to reduce peroxidized phospholipids and cholesterol in membranes; protects against oxidative stress; in redox regulation, sexual maturation and differentiation (essential for fertilization, spermatogenesis) also reduces hydroperoxides in M1 and L2 (33).</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. GPx Characteristics.

Characteristics of different GPx varieties are organized by table (105). 7 varieties of glutathione peroxidases have been discovered and are divided by gene structure/chromosomal location, protein structure/characteristics, regulation of protein synthesis, tissue distribution, and the role of the enzyme. There is an extensive interest in studying the various types of glutathione peroxidase due to antioxidative and antiinflammatory properties.
MATERIALS & METHODS

Tissue culture

Tissue culture and supplementation studies were executed with the following cell lines: mouse embryonic fibroblasts (MEF) and mouse liver cells (BNL.C2). The MEF cells are SV-40 transformed mouse fibroblasts. The BNL.C2 cells are standard mouse liver cells. Both cell lines were derived from C57bl/6 mice, a common inbred strain of laboratory mice.

The cells were maintained at 37°C in an atmosphere of 5% CO₂ and 3% O₂ in humidified air. Mice fibroblasts and mice liver cells were both cultured in 175 cm² Corning tissue culture flasks (Sigma-Aldrich, USA) in Dulbecco’s modified minimum essential medium (DMEM). DMEM (Gibco- Invitrogen, CA, USA) was supplemented with Fetal Bovine Serum- 10% (Hyclone Laboratories Inc., UT, USA), Penicillin Streptomycin Antibiotic- 1% (Gibco- Invitrogen, CA, USA) to prevent bacterial contamination, Glutamax- 1% (Gibco- Invitrogen, CA, USA), and Glutamine- 0.5% (Gibco- Invitrogen, CA, USA). Both cell lines were also grown in complete media, supplemented with 30nM of selenium. The standard mean selenium content in Fetal Bovine Serum is 0.026 micrograms per milliliter.

The pretreatment with selenium was performed for 2-3 days by the addition of selenium to the medium in the form of 1000-fold concentrated stock solutions throughout the logarithmic growth phase of the cells. A selenium concentration of 30nM was selected for the supplementation experiments based on published data (91).
The culture media, with or without selenium supplementation, were changed every 3rd day. By the end of the incubation period, adherent cells were harvested by washing cells with 5ml 1X Phosphate-buffered saline and ethylenediaminetetraacetic acid (1X PBS-EDTA), and then trypsinized with 2ml of pre-warmed (37°C) trypsin for 3 minutes. Trypsin activity was ceased by the addition of 8 ml of complete media. Contents were mixed well by pipette and transferred to a labeled falcon tube. Counting was executed under microscope with the presence of trypan blue.

All assays were run in quadruplicate and repeated six to eight times. In order to ensure reliability of the results, 4 parallel cultures were used in each group setting (MEF- control, MEF- treated, MEF+Sel- control, MEF+Sel- treated) and the experiments were repeated 2-4 times. Oxidation was induced by exposing both cell lines to 250µM of hydrogen peroxide (H2O2) at appropriate times.

The standard components of basal media include a balanced salt solution, buffering systems, pH indicators, energy sources, amino acids, vitamins, hormones and growth factors, proteins and peptides, fatty acids and lipids, additional additives such as trace elements, and antibiotics (90). The balanced salt solution consists of inorganic salts that maintain osmotic pressure and buffer the medium at physiological pH. The most widely used inorganic ions are Na+, K+, Mg2+, Cl−, Ca2+, SO42−, PO43−, and HCO3− and are needed for physiological roles. These roles include the maintenance of membrane potential and acting as cofactors in enzyme reactions and in cell attachment. Buffering systems are needed in media to compensate for evolution of CO2 and the production of lactic acid from the metabolism of
carbohydrates. Media are commonly buffered with bicarbonate, which forms a buffering system with dissolved CO\(_2\) that is produced in the medium by developing cells. If cells are growing at a low cell density or are in a lag phase, they might generate insufficient CO\(_2\) to retain the required optimal pH. Due to this reason, these cell cultures must be grown in an atmosphere of 5-10% (90).

In the laboratory, we monitored the pH of our DMEM by observing the color of our medium. Phenol red is the pH indicator in DMEM. The medium appears cherry red if it is at optimal pH range for the growth of most cells (7.0-7.5). However, it turns yellow when the culture becomes too acidic and purplish when the pH is too high. Carbohydrates are the key energy source used by cultured cells and glucose is the most regularly used sugar in medium. Amino acids are needed in most animal cells, but glutamine is at a high demand in animal cells. Glutamine acts as both an energy source and as an amine donor in the synthesis of nucleic acids and other compounds. Accessory factors are also needed in the production of a successful medium. Accessory factors is an expression used to cover additional additives that are obligatory for effective cell growth. These comprise of trace elements, mainly iron, zinc, copper and selenium. Lastly, antibiotics are extensively used for protection in laboratory-scale tissue culture.

Medium was stored in 4°C temperature in the dark and was not stored for longer than 3 months. If glutamine is added to medium, shelf life is 2-3 weeks because glutamine is an unstable amino acid that decomposes spontaneously to produce ammonia, which is cytotoxic (90).
Patterns in Gene Expression

**RNA Isolation:** Cells were lysed directly in tissue culture flasks by adding 500 ml of Trizol reagent and pipetting up and down. Incubated at room temperature for 5 minutes, and then added 20 µl of Chloroform. Tube shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes, then centrifuged at 12,000 RCF for 15 minutes at 4°C. Aqueous phase collected in fresh 1.5 mL tube, not disrupting the interphase. 500 µL of Isopropyl Alcohol added, and then incubated at room temperature for 10 minutes. Centrifuged again at 12,000 RCF for 10 minutes at 4°C. Supernatant removed and pellet saved (RNA). RNA pellet washed in 1 mL of 75% ethanol and mixed by vortexing. Centrifuged at 7,500 RCF for 5 minutes at 4°C. Supernatant and any additional residue removed carefully with kimwipe. Pellet air-dried in hood for 5 minutes, and then dissolved in 30 µl of RNAase free water. Pipetted up and down and stored at -80°C.

**RNA Quantification:** RNA was quantified using the Nanodrop 1000 (Thermo-Scientific, USA). Samples were put on ice, vortexed, then centrifuged at 6,000 rpm for 2 seconds, to collect residue on side of tube. At the spectrophotometer, use nuclease-free water as standard for blank and cleaning. Pipette 2 µl of sample onto appropriate surface, close lid, and measure. Save and print results.

cDNA Synthesis: Combine 2 µg total RNA and molecular grade water to 7.0 µl final volume. In separate tube, mix 4 µl of dNTP and 1 µl Random Hexamer. Pipette 5 µl of dNTP/Random Hexamer mix to each RNA-containing labeled tube to make a total of 12 µl. Heat at 70°C in heating block for 5 minutes, then place on ice for 3 minutes. Use 2 µl 10X PCR buffer (Applied Biosystems), 4 µl of 25mM MgCl₂ (Applied
Biosystems), 1 µl of Reverse Transcriptase (Applied Biosystems), and 1 µl RNase inhibitor (Applied Biosystems), to create the master mix. Aliquot 8 µl of master mix to each RNA/hexamer containing tube for a total reaction volume of 20 µl. Vortex, spin down and keep in thermocycler at the following reaction conditions: 42°C for 1 hour, 95°C for 10 minutes, then hold at 4°C.

**PCR Purification:** To purify cDNA, use QIAquick PCR Purification Kit (Qiagen, USA). Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. pH of Buffer PB mixture should be yellow. Place a QIAquick spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60 seconds. Discard flow-through and place the QIAquick column back in the same tube. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30-60 seconds. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional minute.

**Real Time-PCR:** Using the purified prepared samples; RT-PCR was administered by Kirk Simon using the Roche LightCycler 480 II (Indianapolis, USA). Customary RT-PCR instructions were followed. PCR master mix was prepared, including Taq polymerase, specific primers, deoxynucleotides, and an appropriate buffer. Preincubation period is at 95°C for 5 minutes for 1 cycle. The amplification process begins when cDNA is denatured at 95°C, which separates the double strand, and then the sample is cooled to 60°C. The cooling is needed for the annealing process, where specific primers are annealed to a complementary site on each strand. The temperature is increased to 72°C where Taq polymerase extends the DNA from the primers. This amplification cycle is repeated 45 times. Finally, the
cooling period is 30 minutes long at 40°C for a single cycle.

**Glutathione Peroxidase Assay**

GPx-1 enzyme activity was indirectly calculated by measuring the rate of transformation of NADPH into NADP⁺ using the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, USA).

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Increased activity of GPx means more GSH is consumed and more GSSG is produced. As a result, increased NADPH is oxidized to NADP⁺ to regenerate GSH. Rate of transformation of NADPH to NADP⁺ is measured using absorbance levels. NADPH has a certain absorbance at 340 nm, referred to as A340. When NADPH turns into NADP⁺, the amount of absorbed light at 340 m is decreased, so A340 decreases. By measuring this decrease, we can deduce the rate of transformation of NADPH into NADP⁺ and therefore, GPx activity.

**Pre-Assay Preparation:** Dilute 1 ml of Assay Buffer concentrate with 9 ml of HPLC-grade water to form the GPx Assay Buffer (10x). Assay Buffer (50mM Tris-HCL, pH 7.6, 5 mM EDTA) is stored at 4°C. GPx Sample Buffer (10x) is composed of 1 ml of Sample Buffer with 9 ml of HPLC-grade water. Sample Buffer (50mM Tris-HCL, pH 7.6, 5 mM EDTA, 1 mg/ml BSA) is stored at 4°C. Vial of Glutathione Peroxidase (Control) contains a solution of bovine erythrocyte GPx. GPx should be aliquoted into several small vials and stored at -20°C. Prior to use, transfer 5 µl of the
supplied enzyme to another vial and dilute with 245 µl of diluted Sample Buffer and keep on ice. The diluted enzyme is stable for 4 hours on ice. GPx Co-Substrate Mixture is a vial that contains a lyophilized powder of NADPH, glutathione, and glutathione reductase. Each reconstituted vial will be enough reagent for 40 wells. Reconstitute the number of vials that you will need by adding 2 ml of HPLC-grade water to each vial and vortex well. The reconstituted reagent should be kept at 25°C while assaying and then stored at 4°C. The reagent is only stable for 2 days and should not be frozen. Finally, the vial of GPx Cumene Hydroperoxidase should be stored at -20°C when not in use. The reagent is ready to use as supplied.

**Sample Preparation (Cell Lysate):** Collect cells of desired samples by centrifugation (2,000 x g for 10 minutes at 4°C). Used a rubber policeman for adherent cells. Homogenize cell pellet in 10ml cold buffer (50 mM Tris HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT). Centrifuge at 10,000 x g for 15 minutes at 4°C and collect supernatant in pre-chilled tube. Remove any supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. Sample is stable for one month.

**Assay Protocol:** Using polystyrene well plate provided, designate two as background wells, two as positive control wells, and 2 for each sample. Background well is composed of 120 µl of Assay Buffer and 50 µl of co-substrate mixture to two wells. Positive control well (bovine erythrocyte GPx) is comprised of 100 µl of Assay Buffer, 50 µl of co-substrate mixture, and 20 µl of diluted GPx (control) to two wells. For each sample well, add 100 µl of Assay Buffer, 50 µl of co-substrate mixture, and 20 µl of sample to two wells. Set up plate reader according to program
instructions, and then initiate the reactions by adding 20 µl of cumene hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the cumene hydroperoxide as quickly as possible. Carefully shake the plate for several seconds to mix. Read the absorbance once every 30 seconds for 5 minutes at 340 nm using the plate reader.

**Hydrogen Peroxide Dose Response**

24 flasks all containing selenium supplemented MEF cells were grown for 2 weeks and passaged 6 times. Each group of 4 flasks were then treated with increasing concentrations of hydrogen peroxide (0 µM, 50 µM, 100 µM, 150 µM, 250 µM, and 500 µM). GPx assay was used to measure oxidative stress response at 3 time points (30 seconds, 1 minute, 1.5 minute).

**Statistical Analyses**

Results were analyzed via SPSS 21.0 using one way Analysis of Variance (ANOVA) and paired samples t-test. Duncan’s Post-hoc analyses were also used to compare outcomes using p<0.05 as a cutoff point for statistical significance. Mean and standard error of the mean were calculated for each dependent variable.
RESULTS

GPx-1 Enzyme Activity in MEF cells

Selenium increased GPx1 enzyme activity in cells growing in 3% O₂ growing with and without selenium. MEF transformed mice fibroblasts cells were exposed to doses of hydrogen peroxide to induce oxidative stress. We measured the rate of transformation of NADPH into NADP⁺ using the GPx assay kit as an indirect method to quantify GPx enzyme activity. The data presented in Figure 1 shows the increasing GPx-1 enzyme activity due to selenium supplementation. Results were statistically significant, p < 0.05, when compared with control cells. Due to the significant response shown in data, subsequent experiments were executed and results varied slightly, but a similar trend was observed. All experiments in this figure were performed in quadruplicate.

GPx-1 Enzyme Activity in BNL.C2 cells

BNL.C2 cells also had a considerable increase in GPx-1 enzyme activity in cells growing in 3% O₂ growing with and without selenium. To induce oxidative stress, BNL.C2 cells were exposed to doses of hydrogen peroxide and the rate of transformation of NADPH into NADP⁺ was measured with the GPx assay kit. The data shown in Figure 2 shows the increasing GPx-1 enzyme activity due to selenium supplementation. Results were statistically significant, p < 0.05, when compared with BNL control cells. Experiments in this figure were performed in quadruplicate to promote accuracy.
**GPx-1 Enzyme Activity in MEF Cells in 20% O<sub>2</sub>**

MEF cells were grown in 20% O<sub>2</sub> with selenium and compared to MEF cells grown in 3% O<sub>2</sub>. Cells were grown in 20% O<sub>2</sub> for several weeks so cells were accustomed to the oxygen level. MEF transformed mice fibroblasts cells were exposed to doses of hydrogen peroxide to induce oxidative stress. We measured the rate of transformation of NADPH into NADP<sup>+</sup> using the GPx assay kit as an indirect method to quantify GPx enzyme activity. The data presented in Figure 3 shows the increasing GPx-1 enzyme activity due to selenium supplementation on MEF cells grown in 20% and 3% O<sub>2</sub> respectively. Results were shown to be statistically significant, p < 0.05, when compared with cells grown in 3% O<sub>2</sub>. Due to the significant response shown in data, succeeding experiments were carried out and findings varied faintly, but a comparable trend was observed. All experiments in this figure were also performed in quadruplicate to ensure precise results.

**Gene Expression in MEF Cells**

Does selenium protect from DNA damage or attenuate DNA damage response? To see if selenium protects from DNA damage, we looked at gene expression in response to oxidative stress. 4 flasks of MEF cells with and without selenium were grown in 3% O<sub>2</sub>. An additional 4 flasks of MEF cells with and without selenium were grown in 3% O<sub>2</sub> then moved to 20% O<sub>2</sub> at 50% confluency. RNA was isolated from all flasks and quantified. Complimentary DNA was synthesized and then purified. Finally RT-PCR was performed but unfortunately, results were inconclusive. Genes that were observed were GapDH, RPL-4, B-pol, p21 and UNG. There were no apparent trends in gene response between samples. Figure 4
presents the results of cells grown in 3% O$_2$, while figure 5 illustrates the outcomes of cells grown in 20% O$_2$.

**Hydrogen Peroxide Dose Response**

As shown in figure 6, GPx-1 enzyme activity was higher at 50, 100, and 500 µM hydrogen peroxide, while 0, 150, and 250 µM hydrogen peroxide samples exhibited a lower GPx-1 activity. Results from the dose response trial were inconclusive.
Figure 1. Selenium Increases GPx-1 Activity in MEF Cells.
Selenium supplementation increased GPx-1 enzyme activity in MEF transformed mice fibroblasts. The media of the selenium supplemented mice fibroblasts were enhanced with sodium selenite (30nM) for several weeks, and then all cells were exposed to 250 μM of H₂O₂ to induce oxidative stress. The results represent the mean of each group of cells (MEF- Control, MEF + Treated, MEF + Selenium and MEF + Selenium + Treated) and error bars represent the standard error of the mean. There was a significant difference in GPx-1 enzyme activity observed in selenium supplemented cells compared to control cells with a p value of <0.05. Asterisks represent significant difference (p<0.05).
Figure 2. Selenium Increases GPx-1 Activity in BNL.C2 Cells.
Selenium supplementation raised GPx-1 enzyme activity in BNL.C2 mouse liver cells. The selenium-supplemented media was enhanced with 30nM of sodium selenite for numerous passages. All cells were exposed to 250 μM of H₂O₂ to stimulate oxidative stress response. The results show the mean of each group of cells (BNL, BNL + Sel, Ctrl and Treated) and error bars represent the standard error of the mean. There was a considerable difference in GPx-1 enzyme activity examined in selenium-supplemented cells compared to control cells. The p value was statistically significant, p < 0.05, when compared with BNL control cells. Asterisks represent significant difference (p<0.05).
Figure 3

**Figure 3. Increased GPx-1 Enzyme Activity in MEF Cells in 20% O₂.**
Selenium supplementation increased GPx-1 enzyme activity drastically in MEF transformed mice fibroblasts grown in 20% O₂. GPx-1 enzyme activity moderately increased in selenium supplemented MEF cells grown in 3% O₂. The media of all cells in this experiment were enhanced with sodium selenite (30nM) for several weeks, and then all cells were exposed to 250 μM of H₂O₂ to induce oxidative stress. The results represent the mean of both groups, WT+Sel 20% and WT+Sel 3%, and error bars represent the standard error of the mean. There was a significant difference in GPx-1 enzyme activity observed in cells grown in 20% compared to control cells grown in 3% with a p value of <0.05. Asterisk represents significant difference between groups (p<0.05).
Figure 4. Gene Expression Unaltered in MEF Cells in 3% O₂.
4 flasks of MEF cells with and without selenium were grown in 3% O₂. RT-PCR was administered and all experiments were executed in quadruplicates. Selenium supplementation did not alter samples and outcomes. Averages with and without selenium for each gene were similar. Common genes that were measured were Bpol, P21, and UNG. No visible trend was revealed and p value was not significant.
Figure 5. Gene Expression Unaltered in MEF Cells in 20% O₂.
4 flasks of MEF cells with and without selenium were grown in 3% O₂ and moved to 20% O₂ at 50% confluency. RT-PCR was administered and all experiments were executed in quadruplicates. Selenium supplementation did not alter samples and outcomes. Averages with and without selenium for each gene were similar. Common genes that were observed were Bpol, P21, and UNG. No visible trend was revealed and p value was not significant.
Figure 6. Hydrogen Peroxide Dose Response.
Selenium supplemented MEF cells were treated with increasing concentration of hydrogen peroxide. GPx assay kit was utilized to calculate oxidative stress response at 0.5, 1.0, and 1.5-minute intervals. Control (C) represents MEF cells with no addition of selenium or hydrogen peroxide. Apparent trend at 50 μM, 100 μM, and 500 μM showed response at its peak, while concentration of 0 μM, 150 μM, and 250 μM showed GPx-1 enzyme activity at its lowest.
DISCUSSION

Reactive oxygen species and free radicals are associated with the negative effects of aging. Enzymatic and non-enzymatic mechanisms can defend cell structures from free radical damage. Selenium is an essential trace element necessary for cellular functions in the human body. A positive correlation between low serum selenium levels with the morbidity and mortality of a range of cancers has been illustrated in numerous studies. Research also shows that selenium supplementation can have a chemopreventive role in carcinogenesis (94). GPx and SOD are two essential enzymes involved in the enzymatic defense mechanism. (92) Glutathione Peroxidase catalyzes a reaction in which H$_2$O$_2$ oxidizes the reduced form of GSH and forms H$_2$O. NADPH further reduces GSSG, the oxidized structure of glutathione, in a reaction catalyzed by glutathione reductase (93).

The activity of the glutathione peroxidase enzyme was indirectly measured by the rate of transformation of NADPH into NADP*. In this study, GPx levels were significantly increased in cells grown with 30nM of additional selenium. Notice the low doses of selenium used in this study, which are much lower than the toxic level. At this level, it does not induce apoptotic or necrotic cell death as previously perceived at higher selenium doses (94).

Incubation of cells with selenium for a minimum of 3 days is necessary for protection (93). All experiments had a minimum incubation period of 5-6 passages with the additional selenium prior to hydrogen peroxide exposure. This preincubation period may be needed to induce the translation of one or more
selenoproteins. According to several studies, 30 nM of sodium selenite has been shown to stimulate the methylation of selenocysteine tRNA and boost selenoprotein translation in numerous cell kinds (95-98). To achieve the maximum translational induction of GPx-1, from threefold to fivefold, 30 nM of selenium was added to the basic media for cell culture (91). It has been seen that additional benefits of elevated GPx-1 expression include suppressing apoptosis and debilitating the malignant phenotype of pancreatic tumor cells in both culture and xenografts (99).

Four general conclusions can be derived from the results of these experiments. (i) GPx-1 activity was significantly increased in selenium-supplemented culture media. (ii) By comparing MEF and BNL-C2 cells, we can determine that amplified GPx-1 does not differ between fibroblasts and liver cells. (iii) GPx-1 activity is drastically decreased from MEF cells grown from 20% to 3% oxygen. (iv) Lastly, there was no conclusive data on selenium and gene expression.

1) The GPx assay findings showed that the rate of transformation from NADPH to NAD+ amplified, which means glutathione peroxidase levels were heightened with the additional presence of sodium selenite in culture media. It is known that glutathione peroxidase mRNA levels and GPx activity are exclusively regulated by selenium (101-103). According to studies by Rotruck JT et al and Forstrom et al (101-102), selenium deficiency brings about decreased GPx activity. This is illustrated in the results, which showed an average of 5-fold increase from cells grown with the added selenium content. Results were statistically significant, p < 0.05, when compared with control cells.
ii) Since the bulk of our experiments were done with the MEF cells, we decided to test this theory on a different cell line. Using BNL-C2 cells, we achieved similar results, which determined that selenium supplementation in media augments GPx activity and is not cell line specific. Although both showed an indirect increase to GPx-1 activity, MEF cells had approximately a 5-fold increase. This can be due to the fact that MEF cells were grown longer with the additional selenium (8+ months), compared to approximately 2 months for the initial passaging of BNL cells. Also, due to time constraints, this experiment was not repeated as many times as the MEF cells. Results were statistically significant, \( p < 0.05 \), when compared with BNL control cells.

iii) According to a critical review on oxidative stress by Davies, cultured cells growing in 20% oxygen are in essence, preadapted to survive under oxidative stress conditions (4). Most researchers use 20% oxygen as a base for their studies, not taking into consideration that cells grown in 3% oxygen are much closer to physiological cellular levels (4). We factored this imperative information into our work and grew all cells in 3% oxygen, which meant the cells are more sensitive to an oxidative challenge and results more closely resemble the usual 2-5% oxygen concentration in the tissues. To further test this theory, a batch of MEF cells were grown in 20% oxygen for several weeks so they were accustomed to the oxygen level, then were treated with the usual 250 \( \mu \)M of hydrogen peroxide. You can see a noticeable difference in the results
of cells grown in 20% oxygen compared to the 3% oxygen. Cells grown in 20% \( O_2 \) had a 2-fold increase of glutathione peroxidase activity compared to cells grown in 3% \( O_2 \), which confirms past research. Results were statistically significant, \( p < 0.05 \), when compared with cells grown in 3% \( O_2 \). From this knowledge, we can assume that our findings are more accurate because they closely emulate human’s physiological cellular levels. According to Davies, we have kept our “cellular oxygen tension to a minimum”, which gives us further reliable data.

iv) To discover if selenium protects from DNA damage, we looked at gene expression in response to oxidative stress. Using all MEF mice fibroblasts, 8 total flasks were grown in 3% oxygen with and without the addition of selenium. Furthermore, the same amount of flasks were grown in 3% oxygen with and without selenium then moved to 20% oxygen at 50% confluency. There were no noticeable differences in any of the genes grown in 3% oxygen and cells moved to 20% oxygen, so the addition of selenium does not seem to alter gene expression. GapDH, RPL-4, B-pol, p21 and UNG were all genes selected for testing in this study.

In conclusion, reactive oxygen species, A.K.A. oxidative stress, represent a possible toxic environment for human bodies, which may lead to membrane dysfunction, DNA damage, and inactivation of proteins (100). Cancer, neurodegenerative stress, and arthritis could be the outcome from continual oxidative stress. Selenium, found at the core of the glutathione peroxidase enzyme, contributes in detoxification by reducing peroxides, scavenging free
radicals, or by conjugating with electrophilic compounds (100). This study is the start of a larger concept. Dr.Cabelof would like to develop a model system where it would be possible to investigate the role of selenium in antioxidative response and how selenium can impact DNA damage and DNA repair. The goal is to take this model we developed and expand it into genetic models where we can evaluate the role of specific genes on the impact of selenium, DNA damage, and DNA repair. The area of selenium and oxidative stress is an ever-expanding one where knowledge still has not reached its peak. Continuing research on this essential element and its role in oxidative response is imperative due to the positive correlation we have seen in this study.
REFERENCES


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ABSTRACT

SELENIUM SUPPLEMENTATION INCREASES ANTIOXIDANT RESPONSE IN VITRO

by

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Reactive oxygen species and free radicals are associated with the negative effects of aging. Enzymatic and non-enzymatic mechanisms can defend cell structures from free radical damage. Selenium is an essential trace element necessary for cellular functions in the human body. A positive correlation between low serum selenium levels with the morbidity and mortality of a range of cancers has been illustrated in numerous studies. Glutathione Peroxidase catalyzes a reaction in which H₂O₂ oxidizes the reduced form of GSH and forms H₂O. NADPH further reduces GSSG, the oxidized structure of glutathione, in a reaction catalyzed by glutathione reductase. Tissue culture was grown using mice fibroblasts in 3% oxygen and was supplemented with 30nM of sodium selenite. The activity of the glutathione peroxidase enzyme was indirectly measured by the rate of transformation of NADPH into NADP⁺ using a Glutathione Peroxidase Assay Kit. Results showed that GPx-1 activity was significantly increased (p<0.05) in selenium-supplemented culture media. By also comparing MEF and BNL-C2 cells, we can determine that amplified GPx-1 activity is not cell line specific. MEF cells had
approximately a 5-fold significant increase (p<0.05) to GPx-1 activity. Finally, all cells were grown in 3% oxygen, which closely resembles the 2-5% oxygen concentration in tissue. MEF cells were grown in 20% oxygen for several weeks, treated with 250 μM of H₂O₂, and compared to cells grown in 3% oxygen. Cells grown in 20% O₂ had a 2-fold significant increase (p<0.05) of glutathione peroxidase activity compared to cells grown in 3% O₂. In conclusion, reactive oxygen species, A.K.A. oxidative stress, represent a possible toxic environment for human bodies. The selenium element, found at the core of the glutathione peroxidase enzyme, contributes in detoxification by reducing peroxides, scavenging free radicals, or by conjugating with electrophilic compounds.
AUTOBIOGRAPHICAL STATEMENT

“Education is the power to think clearly, the power to act well in the world’s work, and the power to appreciate life.” – Brigham Young.

Education is the core of my being, just as the selenium element is the core of the GPx enzyme. The element drives the enzyme to work and succeed in its task, just as education helps me work and thrive at my career. Learning is an essential part of life. Not only have I learned throughout the years at my education at Wayne State University, but I am now continuing this passion by teaching others. I’m proud of my accomplishments at university and I also find it a joy to teach. So who am I? I am brave, bright, and boundless in my endeavors. I am cultured, capable, and confident in who I am. Finally, I am daring, diligent, and determined in everything that I do. I have acquired these traits on my journey through life and education.

My parents have always believed I would be successful in my life, and they have encouraged me to do my best. Since my parents did not go to college, they really wanted me to get a solid education, which would be a good foundation to have a flourishing career. I have received a Bachelor of Science in Science and Chemistry Education and I am on my way to completing my Master of Science in Nutrition and Food Science. My continuous learning makes me feel bright, capable, and diligent.

During my travels, I have had an insatiable thirst for knowledge. I always like to read whenever I can and surround myself with a wealth of information. This is how I learn and grow as a person. Also, living abroad these past few years has really taught me to be flexible, adapt to my environment, and appreciate others. Furthermore, teaching students where English is their second language has also taught me patience and understanding. My love of travel and teaching abroad demonstrates that I am brave, cultured, and daring.

To conclude, having knowledge is significant in my life. My goal in is to continue learning, feeding my passion of information by nurturing it and expanding it. As Benjamin Franklin once said, “An investment in knowledge pays the best interest.” Knowledge makes me feel boundless in my ambition, confident in myself, and gives me a positive and determined attitude on life.