2008-08-13

The Antioxidant Defense Network: Synergistic Combinations to Prevent Oxidative Damage

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THE SEARCH FOR A SUPERIOR ANTIOXIDANT DEFENSE NETWORK ANALYSIS OF POSSIBLE SYNERGISTIC COMBINATIONS TO PREVENT OXIDATIVE DAMAGE

by

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A thesis submitted to the faculty of

Brigham Young University

In partial fulfillment of the requirements for the degree of

Master of Science

Department of Microbiology and Molecular

Brigham Young University

August 2008

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Amy Marie Clement

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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Date	Dr. Eric Wilson

BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

THE SEARCH FOR A SUPERIOR ANTIOXIDANT DEFENSE NETWORK ANALYSIS OF POSSIBLE SYNERGISTIC COMBINATIONS TO PREVENT OXIDATIVE DAMAGE

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One of the matchless ironies of the human body is its requirement for the highly reactive oxygen molecule, which has been clearly implicated in many diseases and the aging processes. Oxidants produced by metabolic processes damage cells by starting chemical chain reactions including oxidation of DNA and proteins as well as lipid peroxidation. Damage to DNA can cause mutations and lead to cancer if not reversed by DNA repair mechanisms. Damage to proteins causes enzyme inhibition, denaturation and protein degradation. Lipid peroxidation can cause cell lysis as well as creating mutagenic and carcinogenic by-products.

The human body contains antioxidants and enzymes that together work to prevent oxidative damage to cellular components. By and large antioxidants either prevent these reactive oxygen species from being formed or remove them before they cause damage.

There are many theories currently that tout the superior nature of diverse antioxidant combinations. One such theory is by Dr. Lester Packer of The University of California at Berkley. Dr. Packer puts forth the hypothesis that there is a superlative combination of five antioxidants that have the ability to "recharge" one another both in the blood plasma and intracellularly. This would result in a greater quality of antioxidant protection for an extended time.

The current study evaluates Dr. Packer's theory of antioxidant combination from his book *The Antioxidant Miracle*. The decay rate of the antioxidants vitamin E, vitamin C, lipoic acid, glutathione, and coenzyme Q10 alone and in combination were determined using the ORAC (Oxygen Radical Absorbance Capacity) assay. The majority of the antioxidants retained activity for longer periods of time when tested alone, rather than in combination as Dr. Packer's theory would suggest.

The assay was also preformed (using the same antioxidants and combinations) on oxidatively damaged Raji cancer cells. Cell viability and uptake of antioxidants into the cytoplasm were monitored.

Finally, a variety of multivitamins were subjected to the ORAC assay and their antioxidant capacity compared to that of the "Packer Combination". The results suggest that multivitamins are superior antioxidants than the Packer ratio listed in *The Antioxidant Miracle*.

ACKNOWLEDGMENTS

To begin with I would like to thank Dr. Kim L. O'Neill ("Coach") for his unwavering support and faith in my abilities. He always knew better than I what I was capable of, and it is a lesson well learned that I will strive to carry with me the rest of my life. Your faith in me as an eighteen year old who knew nothing about science is awe inspiring. I know that we will always be good friends.

I would also like to thank my committee for their honest appraisals and interest in my changing research endeavors. Thanks for your many suggestions as well.

I also want to thank Daniel Fuja and Amanda Hamblin, my constant cheerleaders and friends for life. I can honestly say I would not have been strong enough to stick with it unless you both were there suffering with me.

To my loving parents who saw the potential in a little girl who struggled to read and for pushing me to be what I dream of. Thank you for our forever family, financial help when we've needed it, and for always being willing to talk.

Thank you to my siblings Adam, Laurel, Aaron, Sam, and Katie (Boo) for always telling me how proud you were and at least pretending to be interested in my research. Most especially, thanks to Boo for being my best friend. The last two years have been worth the effort just so I could be here with you. We'll always be best friends, and no one will ever understand me like you do.

Thank you to the love of my life Nelson, who is so patient and loving towards me. I know I am a difficult person to live with (especially when I'm writing a thesis), but you're stuck with me for eternity, and I know you love it.

Most importantly, I want to acknowledge my Heavenly Father. He truly does make up for all that I am lacking. I know that this project succeeded only because of His guidance and blessings.

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INTRODUCTION

FREE RADICALS

The production of free radicals begins with a chemical reaction called oxidation, which is a process that transfers electrons from an atom or molecule to an oxidizing agent. Oxidation does not necessarily require oxygen, from which it is named, but is more easily illustrated as the loss of electrons from the atoms and molecules forming biological structures. The inverse reaction, reduction, occurs when a molecule gains electrons.

Free radicals (radicals) are atoms or groups of atoms with an unpaired electron(s). Normally, bonds do not split in a way that leaves a molecule with an odd, unpaired electron. However, when weak bonds split, free radicals are formed. These unpaired electrons are usually highly reactive, so radicals are likely to take part in chemical reactions trying to capture the needed electron(s) to gain stability. Commonly, free radicals “steal” an electron from the nearest stable molecule. When the electron is lost from the "attacked" molecule, it becomes a free radical itself, thus propagating the chain reaction that can result in disruption of a living cell.

When literature refers to oxidative stress, it is commonly referring to two subsets of molecules: Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). The free radicals of most interest are usually ROS, because the most biologically significant free radicals are oxygen-centered. However, not all free radicals are ROS, and not all ROS are free radicals. For instance, the ROS hydrogen peroxide (H_2O_2) is not a free radical species, but the free radicals superoxide (O_2^-) and hydroxyl radical (OH^-) are. The oxygen-centered free radicals (ROS) of greatest consequence are the hydroxyl

radical and superoxide (1). The hydroxyl radical is particularly unstable and will react quickly and non-specifically with most biological molecules.

Once formed these highly reactive radicals can initiate damaging side chain reactions such as lipid peroxidation, or they may oxidize DNA or proteins (2). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms (3)(4), while damage to proteins causes enzyme inhibition, denaturation and protein degradation (5).

There are many sources of free radicals. Radicals play an important role in combustion, atmospheric chemistry, polymerization, and many other chemical processes. Contrary to popular belief, free radicals also play an integral role in a number of biological processes, some of which are necessary for life.

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals such as the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($^{\cdot}OH$) (6). Many free radicals arise naturally during metabolism. The use of oxygen as part of the process for generating metabolic energy produces reactive oxygen species (7).

Cells can also produce free radicals as a defense mechanism. One example is the intracellular killing of bacteria by neutrophil granulocytes which produce reactive oxygen species. Further, free radicals have been implicated in certain cell signaling processes. Additionally, environmental factors such as radiation, pollution, sun exposure, herbicides and cigarette smoke can also produce free radicals.

The biological threat from free radicals comes from the damage caused when they react with important cellular components. Cells may function poorly or die if this occurs.

As mentioned previously, damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms. In fact, numerous forms of cancer are believed to be the consequence of reactions between free radicals and DNA, thus producing mutations that can unfavorably affect components such as the cell cycle and potentially lead to malignancy (1). Damage to proteins causes enzyme inhibition, denaturation and protein degradation. Lipid peroxidation can cause cell lysis as well as creating mutagenic and carcinogenic by products (1).

Damage by free radicals has been implicated as a cause of many diseases. Atherosclerosis is thought to be caused by oxidation of lipoproteins. Free radicals contribute to alcohol-induced liver damage. In fact, it is hypothesized that free radical damage could possibly cause more damage than the alcohol itself. Cigarette smoke also contains free radicals, which have been implicated in inactivation of a protein called alpha 1-antitrypsin in the lungs, promoting the development of emphysema (1). Free radicals may also be involved in schizophrenia, Alzheimer's, Parkinson's disease, and deafness. Free-radical-related symptoms include arthritis, movement disorders, psychosis, diabetes mellitus, and melanin abnormalities (8).

This involvement is not surprising as free radical chemistry is an important aspect of apoptosis, inflammation, and phagocytosis. Redox factors also serve an enormous role in other forms of cell death such as necrosis or autoschizis (9).

There have even been questions about the safety of infrequent exercise in terms of free radical damage. Endurance exercise can increase oxygen utilization from 10 to 20 times over the resting state (10). This greatly increases the generation of free radicals, prompting concern about enhanced damage to muscles and other tissues. However, the

problem only arises when individuals have not built up the body's defense network against free radicals through frequent exercise. Conversely, intense exercise in untrained individuals may overwhelm the body's defenses resulting in amplified free radical damage. Thus, individuals who are predominantly sedentary but engage in vigorous bouts of exercise occasionally may be doing more harm than good (11). There are many factors which may determine whether exercise induced free radical damage occurs, including degree of conditioning of the athlete, intensity of exercise, and diet. All of these factors would lead to building up the body's defense networks, including that of antioxidants (12).

ANTIOXIDANTS

Given that free radicals are indispensable for human life, our bodies have a number of methods to minimize free radical damage as well as repair damage which has already occurred. These defense mechanisms often come in the form of antioxidants.

Antioxidants are substances capable of slowing or preventing the oxidation of other molecules (2)(13). Antioxidants terminate free radical reactions by removing radical intermediates and inhibit other oxidation reactions by being oxidized themselves and thus becoming free radicals (2). The newly created free radicals are relatively weak and therefore are not likely to do further harm. After oxidation, an antioxidant might be regenerated by reduction, or it might be broken down and therefore need to be replaced. As would be expected, antioxidants are often reducing agents.

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (2).

Antioxidants may be synthesized in the body or obtained from the diet (13). The many diverse varieties have a broad range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone (CoQ_{10}) mostly present within cells. Other antioxidants such as lipoic acid are more evenly distributed.

The relative importance of, and interactions between, different antioxidants is a complex question, the result of the numerous enzymatic and metabolites systems having synergistic and interdependent effects (14)(15). The action of one antioxidant may therefore depend on the proper function and concentration of other members of the

antioxidant network. The protection capabilities provided by any one antioxidant will also depend on its concentration and reactivity towards the particular reactive species being considered, as well as the oxidation status of the antioxidants with which it interacts (13).

There are many different categories into which antioxidants fall. These categories help to explain the origin, structure and molecular function of the different antioxidants. Most biologically relevant antioxidants fall in one of the following three categories: vitamins, minerals, and enzymes.

VITAMINS

Vitamins are molecular, organic compounds required as a nutrient by an organism. Vitamins usually cannot be synthesized in significant quantities by the human body, and must be obtained from the diet.

The classification of vitamins does not depend on their structure but instead on their biological and chemical activity. Related chemical substances that fulfill the same specific vitamin function fall under the same category of vitamer. Vitamer categories are grouped under an alphabetized vitamin title, such as "vitamin A".

Vitamins have many biochemical functions, including: mediators of cell signaling, functioning as antioxidants, acting as hormones (e.g. vitamin D), and regulators of cell and tissue growth and differentiation (e.g. vitamin A).

The body is known to require at least 13 different vitamins (16)(17):

- **Vitamin A** - Retinol
- **Vitamin B** :
    - **B1** - Thiamine
    - **B2** - Riboflavin
    - **B3** - Niacin
    - **B5**- Pantothenic Acid
    - **B6** - Pyridoxine
    - **B9**- Folic Acid
    - **B12** - Cyanocobalamin
- **Vitamin C** - Ascorbic acid
- **Vitamin D** - Calciferol
- **Vitamin E** - Tocopherol
- **Vitamin H**- Biotin
- **Vitamin K** -Menaquinone

Basic commercial multivitamin supplement products often contain most, if not all, of the 13 vitamins required by the body in the amounts advised as the recommended daily allowance. Conversely, some components are typically much lower than RDA amounts,

often for cost reasons. For example, biotin is typically added in at only 5%-30% of the RDA in many one per day formulations because of its high cost.

Individuals taking multivitamins may risk acute over dosage if taken in amounts larger than the recommended daily allowance. Water soluble vitamins are excreted through the urine, and thus the risk of overdose is relatively low. However, fat soluble vitamins are deposited in the fatty tissue of the body before being excreted in the feces. Because of this, fat soluble vitamins can be dangerous if consumed in large amounts because of the possibility of toxicity (18).

Regardless, when taken with care, supplementing the diet with additional vitamins and minerals, multivitamins can be a valuable tool for those with dietary imbalances or different nutritional needs (19).

MINERALS

A mineral is a naturally occurring substance with a range in composition from pure elements and simple salts to very complex silicates. The body uses minerals for many different jobs, including: making hormones, building bones, regulating heartbeats, and antioxidant protection.

Essential minerals required by the body are:

- **Calcium**
- **Magnesium**
- **Iron**
- **Phosphorus**
- **Zinc**
- **Chromium**
- **Copper**
- **Iodine**
- **Manganese**
- **Molybdenum**
- **Selenium**

Basic commercial multivitamin supplement products also contain the following minerals additional to those listed above: potassium iodide, borax, and cupric.

ENZYMES

An enzyme is a biological molecule that catalyzes chemical reactions. The human body contains many enzymes, including some used as antioxidants such as: catalase, glutathione peroxidase, superoxide dismutase (SOD), and glutathione reductase (GSR) (1).

Catalase has one of the fastest turnover rates of all enzymes in the body. It functions as an antioxidant by catalyzing the decomposition of hydrogen peroxide to water and oxygen. In fact, each molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen every second (20).

Glutathione peroxidase is also used by the body to consume free peroxide in the cells. Glutathione is capable of scavenging free radicals either directly or enzymatically via glutathione peroxidase.

SOD catalyzes the breaking down of the free radical superoxide, which is a major contributor to lipid peroxidation. The end result of this reaction is oxygen and hydrogen peroxide, which can then be broken down by catalase into water and oxygen.

Glutathione Reductase catalyses the reduction of glutathione disulphide (GSSG) to glutathione (GSH), an essential antioxidant which will be discussed further later in the paper. This reaction maintains the GSH:GSSG ratio in the cytoplasm.

It is interesting that these antioxidant enzymes require metal cofactors. Iron is required as a co-factor for catalase. SOD consists of proteins co-factored with iron, zinc, manganese, or copper. It is obvious why such minerals are important in our diets and included in multi-vitamin supplements.

MEASURING ANTIOXIDANT CAPACITY

There are many ways in which antioxidant capacity is measured. Each has different benefits and drawbacks; however each assay is fundamentally similar in what it is able to measure.

TEAC:

The Trolox-Equivalent Antioxidant Capacity (TEAC) Assay is based on the suppression of radicals from 2,2'-azinobis(3-ethylbenzothiazoline6-sulfonate)(ABTS) by antioxidants in the test sample when ABTS incubates with a peroxidase (metmyoglobin) and H_2O_2 (21).

FRAP:

The Ferric Reducing Ability of Plasma (FRAP) assay measures the ferric reducing ability of plasma. Ferric to ferrous ion reduction causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (22).

TOSC:

The Total Oxyradical Scavenging Capacity (TOSC) assay measures the decrease in ethylene production caused by antioxidants (23). This assay offers the possibility to discriminate different oxyradicals, indicating the roles of these molecules or their metabolic pathways of formation, in the onset of oxidative disease (24).

ORAC:

Oxygen Radical Absorbance Capacity (ORAC) assay is a standard method of measuring the oxidative degradation of the fluorescent molecule fluorescein after being mixed with a free radical generators such as azo-initiator compounds. Azo-initiators damage the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are able to protect the fluorescent molecule from the oxidative degeneration. The degree of protection is quantified using a fluorometer. The fluorescent intensity decreases as the oxidative degeneration proceeds, and this intensity is recorded for typically 35 minutes after the addition of the azo-initiator (free radical generator). The degeneration (or decomposition) of fluorescence becomes less prominent by the presence of antioxidants. Decay curves (fluorescence intensity vs. time) are recorded, and the area under the curve is calculated.

There are benefits as well as drawbacks to using the ORAC assay to measure antioxidant capacity. One benefit of using the ORAC versus other assays is that the ORAC is able to measure antioxidant capacities for extended periods of time which takes into account antioxidants with lag phases in their protection. This is particularly important when measuring substances that are mixtures of different elements or compounds because of the possibility of slow and fast acting antioxidants. The ORAC method is the only method that takes free radical action to completion and uses the area under the curve for quantification, thus combining both the percentage of inhibition and the length of inhibition of free radical formation by antioxidants into a single quantity (25).

The drawback to the ORAC is that the nature of and substances involved in the likely free radical damaging reaction are not known (26)(27).

THE NETWORK ANTIOXIDANTS

There are many theories currently that tout the superior nature of diverse antioxidant combinations. One such theory is by Dr. Lester Packer of The University of California at Berkley. Dr. Packer puts forth the hypothesis that there is a superlative combination of 5 antioxidants that have the ability to "recharge" each other both in the blood plasma and intracellularly. This would result in a greater quality of antioxidant protection and for a much enhanced amount of time.

The current study evaluates Dr. Packer's theory of enzyme combination from his book *The Antioxidant Miracle*. The decay rate of the antioxidants vitamin E, vitamin C, lipoic acid, glutathione, and coenzyme Q_{10} alone and in combination were determined using the ORAC (Oxygen Radical Absorbance Capacity) assay.

Dr. Packer's theory can best be explained by a statement made in *The Antioxidant Miracle* text.

> Here's an example of how network antioxidants work together. When vitamin E disarms a free radical, it becomes a weak free radical itself. But unlike bad free radicals, the vitamin E radical can be recycled, or turned back into an antioxidant, by vitamin C or coenzyme Q_{10}. These network antioxidants will donate electrons to vitamin E, bringing it back to its antioxidant state. The same scenario occurs when vitamin C or glutathione defuses a free radical and becomes a weak free radical in the process. These antioxidants can be recycled back to their antioxidant form by lipoic acid or vitamin C (28).

In this study the individual antioxidants and their combinations were compared to one of the best known antioxidants resveratrol. Further, a comparison was also done to multivitamins to find if their combinations provided superior oxidative protection, because few studies have evaluated the effects of multivitamins per se rather than specific components of them (17).

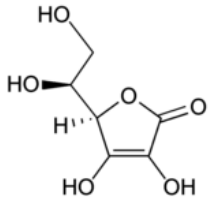
ANTIOXIDANT DETAILS AND STRUCTURES

WATER SOLUBLE ANTIOXIDANTS:

VITAMIN C

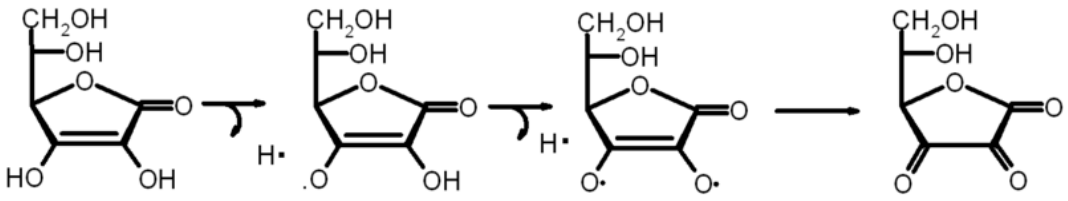
Humans have no enzymatic ability to manufacture vitamin C. However, the majority of plants and animals are able to synthesize it. While the richest natural sources are fruits and vegetables, it is also present in some cuts of meat, especially liver (29).

The North American Dietary Reference Intake recommends 90 milligrams per day and no more than 2 grams per day (30). Vitamin C is solely the L-enantiomer of ascorbate; the opposite D-enantiomer has no physiological significance. L-ascorbate, is a very strong reducing agent, which explains why it has such a short half life as an antioxidant in the body.



L-Enantiomer Vitamin C

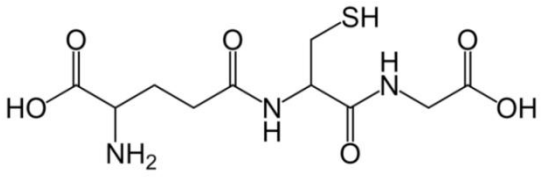
Reactive oxygen species oxidize ascorbate first to monodehydroascorbate and then dehydroascorbate. The reactive oxygen species are reduced to water while the oxidized forms of ascorbate are relatively stable and un-reactive, and do not cause cellular damage (see image below).



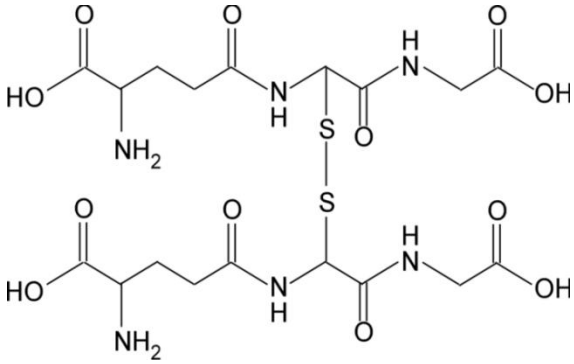
GLUTATHIONE

Glutathione is synthesized in virtually all animal cells by the two enzymes y-glutamylcysteine synthetase and GSH synthetase. Glutathione is a tripeptide, made from the amino acids cysteine, glycine, and glutamate, and is therefore not itself required in the diet. In fact, serum levels of glutathione are affected little by oral administration of glutathione (31). The oral administration of N-acetyl cysteine, on the other hand, has been shown to significantly increase levels of glutathione in serum, intracellularly (32)(33).

Glutathione can be found in a reduced (GSH) and oxidized (GSSG) states, although it is found mostly in its reduced form, since the enzyme glutathione reductase that converts it from its oxidized to its reduced form is constitutively active and inducible upon oxidative stress.



Reduced Glutathione



Oxidized Glutathione

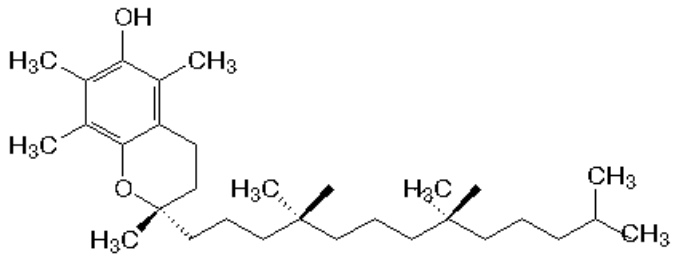
FAT SOLUBLE

VITAMIN E

Vitamin E is a fat-soluble vitamin which exists in eight different forms. Each form has its own biological activity, and α-tocopherol is the name of the most active form of vitamin E in humans (34).

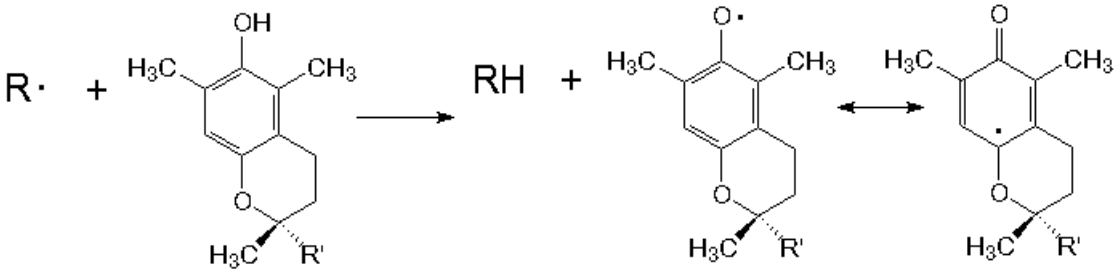
Vitamin E is made by plants and is commonly found in plant oils. Vegetable oils, nuts, green leafy vegetables, and fortified cereals are common food sources of vitamin E.

As a result of vitamin E's fat soluble property, absorption is highly dependent upon micelle and chylomicron formation, as well as bile acids. A lack of any component of these transporters will inhibit carrier formation and in turn vitamin E absorption. The fat-soluble property of vitamin E allows it to be stored within fatty tissues. In fact, over 90% of total body vitamin E is found in the adipose tissue (35)(36). The Recommended Daily Allowance is currently 15 mg a day (37).



Vitamin E (α-tocopherol)

The free hydroxyl group on the aromatic ring of vitamin E is responsible for the antioxidant properties. The hydrogen from this group is donated to the free radical, resulting in a relatively stable free radical form of the vitamin. This following figure shows tocopherol being oxidized.



Resonance stabilized radical

The antioxidant role of vitamin E is straightforward and important. As one of the body's major lipid-soluble membrane-bound free radical quenching molecules, vitamin E is vital in preventing lipid peroxidation.

COENZYME Q_{10}

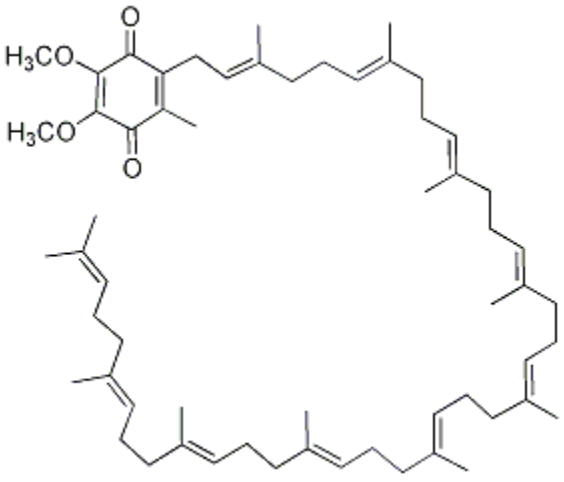
Coenzyme Q_{10} is known by many other names, such as: ubiquinone, ubidecarenone, coenzyme Q, and abbreviated at times to CoQ_{10}, CoQ, Q_{10}, or Q. The name ubiquinone is used to signify its ubiquitous distribution in the body.

As a coenzyme, this nutrient aids mitochondria, in the complex process of transforming food into ATP making it responsible for the production of the body's own energy. Ninety-five percent of all the human body's energy requirement (ATP) is converted with the aid of CoQ_{10} (38).

Virtually every cell in the human body contains CoQ_{10}, except red blood cells and eye lens cells, with cells rich in mitochondria, such as the heart and the liver, containing the most (39)(40)(41)(42).

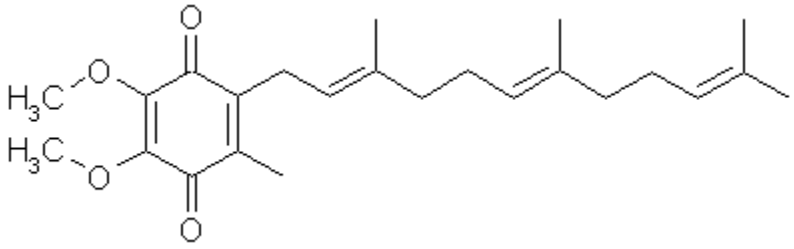
CoQ_{10} is primarily found in fish and meat; however, the human body is able to produce some CoQ_{10}. There are plenty of vegetable sources of CoQ_{10}, although the amount is significantly smaller than that found in meats.

A dose of 30 mg of CoQ_{10} is the maximum daily dose recommended by CoQ_{10} producers (43). There is no daily recommended allowance of CoQ_{10}.

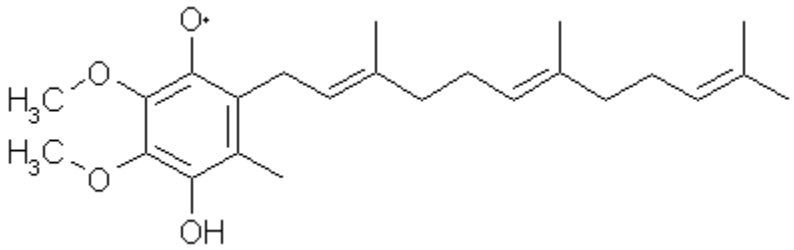


CoQ_{10} (see above structure) is a benzoquinone, where Q refers to the quinone chemical group, and 10 refers to the isoprenyl chemical subunits. The various kinds of Coenzyme Q can be distinguished by the number of isoprenoid side-chains they have.

Below is shown the reduced and oxidized quinone chemical group of the Q_3 molecule.

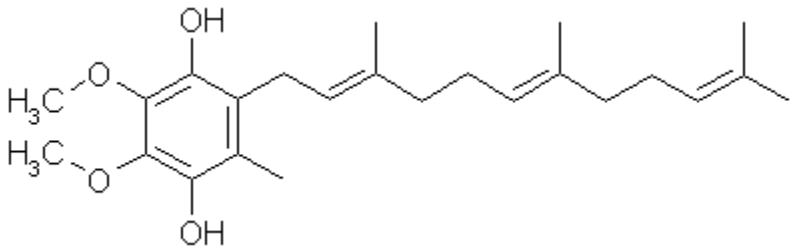


Coenzyme Q_3- oxidized



Ubisemiquinone (QH): Coenzyme Q_3- reduced

If CoQ is reduced by one equivalent, the above structure results, an ubisemiquinone, which is denoted QH. Note the free-radical on one of the ring's oxygen. Either oxygen may become a free-radical; in this case the top oxygen is shown as such. If Coenzyme Q is reduced by two equivalents, the compound becomes an ubiquinol, denoted QH_2 (see below).



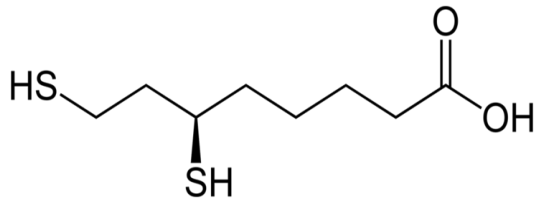
Ubiquinol (QH_2): reduced by two equivalents

LIPOIC ACID

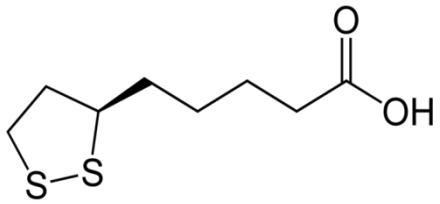
Formerly known as thioctic acid, and also referred to as simply lipoic acid, α-lipoic acid is a sulfur-containing fatty acid. Found within virtually every cell of the body, it helps generate the energy by acting alongside the key parts of the metabolic machinery.

Lipoic acid is also considered one of the most versatile antioxidants because its molecular structure is amphipathic. Unlike other antioxidants, which are hydrophobic or hydrophilic, lipoic acid functions in both aqueous body and cellular environments as well as cell membranes. By comparison, vitamin E works only in hydrophobic environments such as the cell membrane, and vitamin C works only in aqueous environments such as the blood or cytoplasm. This gives lipoic acid an unusually broad spectrum of antioxidant action.

Sources of α-lipoic acid include vegetables, meat, yeast, and supplements. Currently there are no established recommended doses for supplementation. Manufacturers of lipoic acid supplements suggest up to two 50-mg capsules daily.



α-lipoic acid: Reduced



α-lipoic Acid: Oxidized

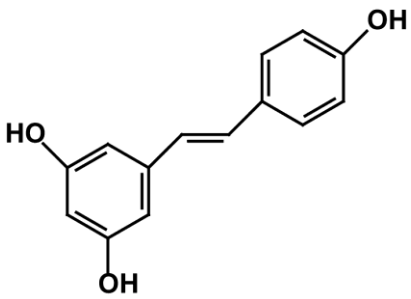
RESVERATROL

Resveratrol is a stilbenoid, a derivate of stilbene, and is produced in plants with the help of the enzyme stilbene synthase. Resveratrol is also a phytoalexin, a class of antibiotic compounds produced as a part of a plant's defense system against disease. While present in many plants, resveratrol's most abundant natural sources are the skins of red grapes and Japanese Knotweed.

Resveratrol has a high rate of oral absorption, at least 70%, but rapid and almost complete metabolism, resulting in merely trace amounts of unchanged resveratrol in the circulation. Localized accumulation of resveratrol in epithelial cells along the digestive tract and potentially active resveratrol metabolites may still produce cardiovascular and anti-cancer effects, but this is currently a matter of debate (44).

Regardless of questions pertaining to resveratrol's absorption or anti-cancer benefits, it has been found to be a far superior antioxidant in its un-metabolized form to almost all substances. Because of the supposed superior nature of the Packer ratio, it was decided that the network antioxidant combination should be compared to one of the best antioxidants known. For this reason resveratrol was used in this study.

There is currently no daily recommended allowance of resveratrol.



Resveratrol

NETWORK INTERACTIONS

According to Dr. Packer there is a dynamic interplay among these network antioxidants. In *The Antioxidant Miracle* the interplay is referred to at the antioxidant network. Packer goes on to say that the network antioxidants have special "powers" in that they can greatly enhance the antioxidant capacities of one another.

According to Dr. Packer, lipoic acid is the central antioxidant, in that it recharges all other network antioxidants. Vitamin E is the end antioxidant, in that it does not recharge any other antioxidants. Glutathione recharges vitamin C which then recharges vitamin E. CoQ_{10} also recharges vitamin E.

Dr. Packer's Network Antioxidant Interaction Theories	
Antioxidant	Recharges
Lipoic Acid	Glutathione, Vitamin C, CoQ_{10}, Vitamin E
Glutathione	Vitamin C
Vitamin C	Vitamin E
CoQ_{10}	Vitamin E
Vitamin E	Nothing

Recent research performed by laboratories other than that of Dr. Packer have produced results confirming parts of Dr. Packer's network theory. It has been found that lipoic acid, vitamin E, and vitamin C all have positive effects on the antioxidant capacity of glutathione (45)(46)(47). Another study suggests *in vivo* regeneration of vitamin E by CoQ_{10} (43). Being amphipathic (having a lipid-soluble portion and a water-soluble portion) and having two thiol groups allows lipoic acid to recharge vitamin E, vitamin C (48) and glutathione (45) according to two other studies. It has also been found that glutathione recharges vitamin C (49).

Results of Independent Laboratories	
Antioxidant	Recharges
Lipoic Acid	Glutathione, Vitamin C, Vitamin E
Glutathione	Vitamin C
Vitamin C	Glutathione
CoQ_{10}	Vitamin E
Vitamin E	Glutathione

As has been shown the results produced by independent laboratories are very similar to those of Dr. Packer's lab. No results were found demonstrating vitamin C recharging vitamin E or lipoic acid recharging CoQ_{10}.

HYPOTHESIS

For this study, we wanted to examine Dr. Packer's theory of antioxidant networking to learn if the antioxidant combination given in *The Antioxidant Miracle* has a superior antioxidant capacity compared to other single and combination antioxidants. In order to examine this, we used other sample groups for comparison to the ratio of the network antioxidants given by Dr. Packer, which were: network antioxidant components alone, network antioxidants combined in a different ratio from that given by Dr. Packer, and one of the best antioxidants on the market (resveratrol) for a comparison. Further, Dr. Packer's antioxidant network was compared to multivitamins. The purpose of this comparison was to assess if the Packer supplementation to the diet would be more beneficial in terms of antioxidant protection than multivitamin supplements.

Based on the research of other laboratories, such as Dr. Packer's, we hypothesized that combination treatments would prove to be superior antioxidants because of the "recharging" abilities that they would have on one another. We also theorized that multivitamins would prove to be superior antioxidants to the Packer combination as they contain a greater variety of vitamins and minerals.

In vivo and *in vitro* experimentation was done to assess antioxidant capacity. *In vitro* examination was done by way of the ORAC assay. *In vivo* experimentation was done using cell culture viability analysis and cell lysate analysis using the ORAC assay.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Fluorescein sodium salt was purchased from Matheson Coleman & Bell Manufacturing Chemists, Norwood, Ohio. 2,2'-azobis(2-amidinoprpane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA. Inc., Richmond, VA. L-Ascorbic Acid (99+% A.C.S reagent), CoenzymeQ_{10} (minimum 98% HPLC), Glutathione (Reduced form Minimum 98%), (+)-alpha-Tocopherol succinate, semisynthetic, (+)-alpha-Lipoic Acid, and Resveratrol (approx. 99% GC) were purchased from Sigma-Aldrich Chemie Gmbh, Steinhein, Germany.

REAGENT PREPARATION

Water soluble vitamins (VC, GSH) were dissolved in water to a concentration of 100 µM. Fat soluble vitamins (VE, LA, Q_{10} and RE) were dissolved in DMSO to a concentration of 100 µM. Multivitamins were dissolved in a solution of 50% water and 50% DMSO. Solution was mixed for one half hour.

The ratio of vitamins listed in *The Antioxidant Miracle* as being the superior combination was: 41 µl of a 100 µM vitamin C solution, 34 µl of a 100 µM vitamin E solution, 7 µl of 100 µM a lipoic acid solution, 1.4 µl of 100 µM a glutathione solution, and 1 µl of a 100 µM CoQ_{10} solution. The amount of glutathione added was determined based on physiological concentration. The average physiological concentration was used because it best represented what would be found in the body. This solution is called Packer 1 in this paper.

The test even ratio where all of the antioxidants were combined in even amounts was called Packer 2 in this paper. 50 µl of each antioxidant was combined to create the solution.

Multivitamins were diluted in the same amount of solution (50% water and 50% DMSO) as the Packer 1 combinations. This means, for instance, that if the daily recommended amounts of the Packer 1 antioxidants were diluted in 30 liters of the vehicle to create a 100 µM concentration, then the multivitamins were also diluted in 30 liters of the vehicle. This method of dilution was performed so that direct comparison of which form of daily supplementation provided more antioxidant protection was possible.

Fluorescein was made by preparing a stock solution in which 22.5 mg of fluorescein was dissolved into 50 ml of PBS. A second stock solution was made by adding 50 µl of the original stock solution to 10 ml of PBS. The final solution was made by taking 320 µl of the second stock solution and adding it to 20 ml of PBS. The concentration was then adjusted with 10 µl additions of the second stock solution until the solution produced fluorescent counts between 10,000-15,000 on the Fusion α-HT plate reader.

AAPH solution was prepared by mixing 216 mg of 2,2'-azobis(2-amidinoprpane) dihydrochloride in 10 ml of distilled water.

TISSUE CULTURE

Human Burkitt's Lymphoma (RAJI) cells were purchased from ATCC (American Type Culture Collection, Bethesda, MD). Cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Hyclone Inc., Logan, UT), 0.075%

NaHCO3, and 2mM L-Glutamine. The cells were incubated at 37˚C in a 5% CO2 atmosphere (50).

ORAC ASSAY

The ORAC assay analysis was performed on the Fusion α-HT plate reader (Alliance Analytical Inc. Menlo Park, California) with black 96 well plates with clear bottom wells. Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used.

The ORAC assay is based on the very simple idea that antioxidants protect a fluorescent molecule (fluorescein) from oxidative damage by a molecule called AAPH. The oxidization of fluorescein is supposed to represent the process of oxidization in the body, in that as the fluorescein becomes more damaged and loses its fluorescent emission, it is representative of the body being damaged by free radicals. The addition of antioxidants prevents the damage of fluorescein, and thus loss of fluorescence. The degree to which the antioxidant prevents oxidation to fluorescein is theoretically the same as the body.

EXPOSURE PROTOCOL

Cells were removed from old media by centrifugation and re-suspended in one milliliter of Hanks Balanced Salt Solution at the appropriate concentrations to create equal cells per milliliter for all samples. The cells were then placed into new flasks and exposed to a 1:10,000 concentration of hydrogen peroxide (30% aqueous solution EMD Chemical Inc., Darmstadt, Germany) for one hour at 37˚C in 5% CO_2. Cells were then re-suspended in media and antioxidant solutions (10 mM) added. Cells were then incubated

for 24 hours in the solutions. Although physiological concentrations of the Packer antioxidants ranges from 8 µM to 100 µM (51)(52)(53)(54)(55), antioxidant solutions of 10 mM were used for our *in vivo* analysis in the hopes that sufficient quantities of the antioxidants would penetrate into the cell to produce a noticeable effect on antioxidant capacities of the cell lysates. However, it is very possible that such a high concentration could actually have cause oxidation in the cells. Further experiments should be performed to assess if this concentration was too high.

Cell viabilities were obtained using trypan blue and a hemocytometer. Cells were then centrifuged and rinsed 4 times in either Hanks Balanced Salt Solution, DMSO, or both depending on if the cells were incubated in water or fat soluble vitamins. Cells were then suspended in phosphate buffer solution at 60 million cells per milliliter per sample. Samples were then lysed by freeze fracture with liquid nitrogen. ORAC analysis was then performed on the lysate.

DATA ANALYSIS

Of importance to the analysis of the results is that statistically un-significant differences between decay curves do not mean that the curves are not statistically different. Area under the curve was the analysis chosen for our results, and this leaves the possibility of vastly different decay curves having the same area under their curves. However, the vast quantity of results prevented the statistical comparison between curves at every time point. To aid in the reader's ability to analyze the area under the curve results, bar graphs were produced to demonstrate the area and their standard deviations for the antioxidants.

RESULTS

In our pursuit to examine the Packer network antioxidant ratio, we begun by performing *in vitro* ORAC examinations as described in Materials and Methods. For *in vitro* examination, all antioxidants were suspended in water, DMSO, or half water and half DMSO at a concentration of 100 uM, depending on the solubility of the antioxidants. Our first analysis was examining single antioxidants to ascertain individual antioxidant capacities. Figures 1A and 2A demonstrate the results obtained from the single antioxidant treatments. Figure 1A shows the antioxidant capacities of the fat soluble network antioxidants and compares them with that of resveratrol. Figure 2A shows the antioxidant capacities of the water soluble network antioxidants and also compares them with that of resveratrol. The results of the single antioxidant *in vitro* study found that lipoic acid and glutathione were the best fat and water soluble single antioxidants, respectively, of the Packer combination. However, neither was as good of an antioxidant as resveratrol. Vitamin E, CoQ_{10}, and vitamin C were all relatively poor antioxidants. For ease of reference, Figure B graphs were provided with all decay curves to supply a clear, visual representation of overall differences in area under the curve for each of the Figure A graphs. Figure 1 B demonstrates that resveratrol and lipoic acid had markedly higher antioxidant capacities in their samples compared to other treatments. Figure 2 B also demonstrates this finding for glutathione.

After the examination of the individual network antioxidants, we examined the antioxidant combinations to ascertain their antioxidant capacities. Figures 3A and 4A demonstrate these capacities and compare them to the superior single network antioxidants (figure 3A) and inferior single network antioxidants (figure 4A). Figure 3A

demonstrated that the network antioxidant combination in the ratio suggested by Dr. Packer (Packer 1) was inferior in terms of antioxidant capacity to not only the network combination in an even ratio (Packer 2) , but also to the best single network antioxidants. Figure 4A demonstrates that the Packer 1 combination was only found to have superior antioxidant capacity compared to vitamin C, and not to vitamin E or CoQ_{10}. The Packer 2 combination was superior to the inferior single network antioxidants. Figures 3B and 4B support our analysis of figures 3A and 4A.

Our results from the comparison of the combination treatments to the single antioxidants left us questioning which antioxidants were responsible for enhancing or diminishing the antioxidant capacity of the Packer combination. In order to ascertain which antioxidants had the greatest effect in the combination, the Packer 2 ratio was used. The design of the resulting study was to remove one of the antioxidants from the Packer 2 combination for each of the test groups. For example, the four part combination of vitamin E, CoQ_{10}, vitamin C, and glutathione in even amounts was missing the antioxidant lipoic acid. This group was termed “all but lipoic acid”. Figures 5A and 6A demonstrate the results of the four part combinational study. The results of figure 5A were expected and demonstrated that removal of antioxidants that were inferior single antioxidants caused in increase in antioxidant capacity of the sample compared to the Packer 2 control. Conversely, the removal of lipoic acid, a superior single antioxidant, caused a decrease in antioxidant capacity compared to the Packer 2 control. The results in figure 6A were somewhat unexpected in that the removal of glutathione, a superior single antioxidant, caused an increase in antioxidant capacity of the sample in comparison to the Packer 2 combination. Removal of vitamin C had the expected effect of improving

antioxidant capacity in comparison to the control. Figures 5B and 6B support our analysis of figures 5A and 6A.

The nature of our investigation led us to question the antioxidant capacities of multivitamins to use them as a comparison for the Packer combination. Seeing as the Packer combination is a recommendation for daily supplements of antioxidants for the diet, we felt that comparison to the current daily supplement recommended for the general public would be fitting. However, before our investigation could compare the Packer combination to an antioxidant, multivitamins had to be tested against one another in order to ascertain which multivitamin provided the most protection in terms of antioxidant capacity.

Our initial investigation into multivitamins began with a comparison of multivitamins of the same brand. Figure 7A demonstrates the results of this aspect of the study and shows that there is a statistically significant difference between different types of antioxidants of the same brand. The results demonstrated that Centrum Silver was the best antioxidant supplement produced by Centrum. Figure 7B supports this analysis.

Our analysis then turned to comparisons between multivitamins of separate brands to assess which was a superior antioxidant. Figure 8A demonstrates the results of this aspect of the study, which show us that multivitamins geared towards the elderly provide superior antioxidant protection compared to multivitamins geared towards the general public or children. Out of the multivitamins for the elderly, Centrum Silver was again found to be a superior antioxidant combination. Table 8 supports this conclusion.

Since there were statistically significant differences between multivitamins for the elderly and those for the general public, we became interested in comparing prenatal

vitamins to our results. Figure 9A demonstrates the results of a few of the prenatal vitamins tested and shows that there are large differences in antioxidant protection provided depending on the prenatal multivitamin selected. The Equaline Prenatal was found to be the best antioxidant combination in terms of prenatal multivitamins. Table 9 supports this conclusion.

As our analysis progressed we became curious as to how much variation we would see in different batches of the same types of multivitamins. Figure 10A demonstrates the results of this aspect of our study and shows that there is variation between different multivitamin batches, and the degree of variation depends on the brand. Figure 10 B supports our analysis.

Overall, being that the purpose of our analysis of multivitamins was to establish which multivitamin supplement was the most superior antioxidant and how it compares to the Packer combination, we decided to compare Centrum Silver to the Equaline Prenatal. Figure 11A shows the results of this comparison as well as the comparison to the Packer 1 and 2 combinations. The graph demonstrates that the Equaline Prenatal is a superior antioxidant to Centrum Silver, but that both antioxidants were far superior to either of the Packer combinations. Figure 11 B and Table 11 support this conclusion.

After having performed all of the *in vitro* experiments possible to assess antioxidant capacity, we then began using our *in vivo* model to assess the antioxidants. Our *in vivo* model used tissue culture samples for *in vivo* experimental analysis. In an effort to assess the antioxidant effects of different antioxidants and their combinations, the ORAC assay was performed on cell lysates as described in Materials and Methods. All mixtures of antioxidants were suspended in water, DMSO, or a combination at

concentrations of 10 mM depending on their solubility. The reasoning behind the use of a 10 mM solution was also explained in the Materials and Methods. Student t tests were performed using area under the curve because it best demonstrates the overall antioxidant capacity of the samples. Further, bar graphs were created of the area under the curve to demonstrate meaningful differences to the reader.

For the *in vivo* experiments, there were two types of each treatment category: damaged and undamaged. This meant that cells were either not exposed or exposed to oxidative damage with hydrogen peroxide. The different categories of treatment were based on their exposure to antioxidants or their vehicles such as water or DMSO. The control group, for instance, is the treatment group that we did not expose to any antioxidants or solution vehicles. Figure 12A demonstrates the control cells that were not damaged by hydrogen peroxide and then left in media for 24 hours (control-undamaged) had the highest viability. Cells that were damaged and then put in media for 24 hours (control-damage) had a much decreased viability, as was expected. We observed that treatment of non-damaged cells with the water vehicle had no affect on cell viability. However, damaged cells treated with water did have a decrease in their cell viability. Both damaged and non-damaged cells treated with DMSO had a large decrease in viability. Of important note in figures 12A and 13A is the general pattern of high cell viability for cells treated with water soluble antioxidants versus cells treated with fat soluble or combination of fat and water soluble antioxidants. There is one exception however to this pattern found in figures 12A and 13A. The cells that were undamaged and treated with the Packer 1 combination had a much higher viability than any other samples containing either DMSO or DMSO with water. In fact, this treatment group had

a higher viability than that of undamaged cells treated with only DMSO. This result demonstrated that there is something about the Packer 1 combination that was significantly different from any of the other treatment groups.

Our *in vivo* analysis of antioxidants then turned to the cell lysates of the samples that were read for their viability. Figures 14A and 15A show the antioxidant capacities of the lysates of undamaged and damaged cells respectively treated with the water soluble antioxidants. We observed that initially the lysates of the antioxidant treatment groups were better antioxidants compared to the control cells and water treatment groups. However, over time the lysates of the water treatment groups became better antioxidants than any of the antioxidant treatment groups or the controls. Of further importance is that the lysates of antioxidant treated damaged cells were much better antioxidants than the control lysates from the thirty minute reading on. However, examination of Figure 14 and 15 B suggests that although differences in the sample groups may be statistically significant, they might not being meaningfully different.

Figures 16A and 17A demonstrate the results of lysates of cells treated with fat soluble antioxidants. The results in figure 16A show that vitamin E and CoQ_{10} treatment on non-damaged cells resulted in a diminished antioxidant capacity of the cell lysates compared to the control cells and DMSO treatment. Lipoic acid treatment of non-damaged cells appears to have had no affect on lysate antioxidant capacity. DMSO and resveratrol treatment increased the antioxidant capacity compared to the control cells. The statistical analysis of these observation supported the findings. Figure 17A shows a dramatic initial increase on the antioxidant capacity of the cell lysate of cells treated with lipoic acid. The lysate of cells treated with CoQ_{10} improved compared to non-damaged

cells treated with CoQ_{10}. However, vitamin E treatment of damaged cells still diminished the antioxidant capacity of the lysate compared to the control cells. Resveratrol and DMSO continued to have lysates with a higher antioxidant capacity compared to the control. Figure 17 B and Table 17 support these observations.

Lastly, we examined the affects of the Packer combination and multivitamin treatment on cell lysates. Figures 18A and B demonstrates that combinational treatments had either no effect on lysate antioxidant capacity or actually affected it negatively. The Packer 1 and 2 combinations had no effect, whereas the multivitamin decreased the antioxidant capacity compared to the controls. DMSO and water treatments increased the antioxidant capacities of the lysates. Table 18 supports these findings. Figure 19A demonstrated that the Packer 1 and 2 combinations had no initial effect on the lysate of damaged cells, but eventually improved the lysate antioxidant quality compared to the control. Water was shown to increase the antioxidant capacity the most of the lysate from damaged cells in this graph. Multivitamins still decreased the antioxidant capacity of the lysates compared to the control. Although these observations were supported by the statistical analysis (Table 19), the small differences in area under the curve shown in Figure 19 B suggest that the results are not meaningfully different.

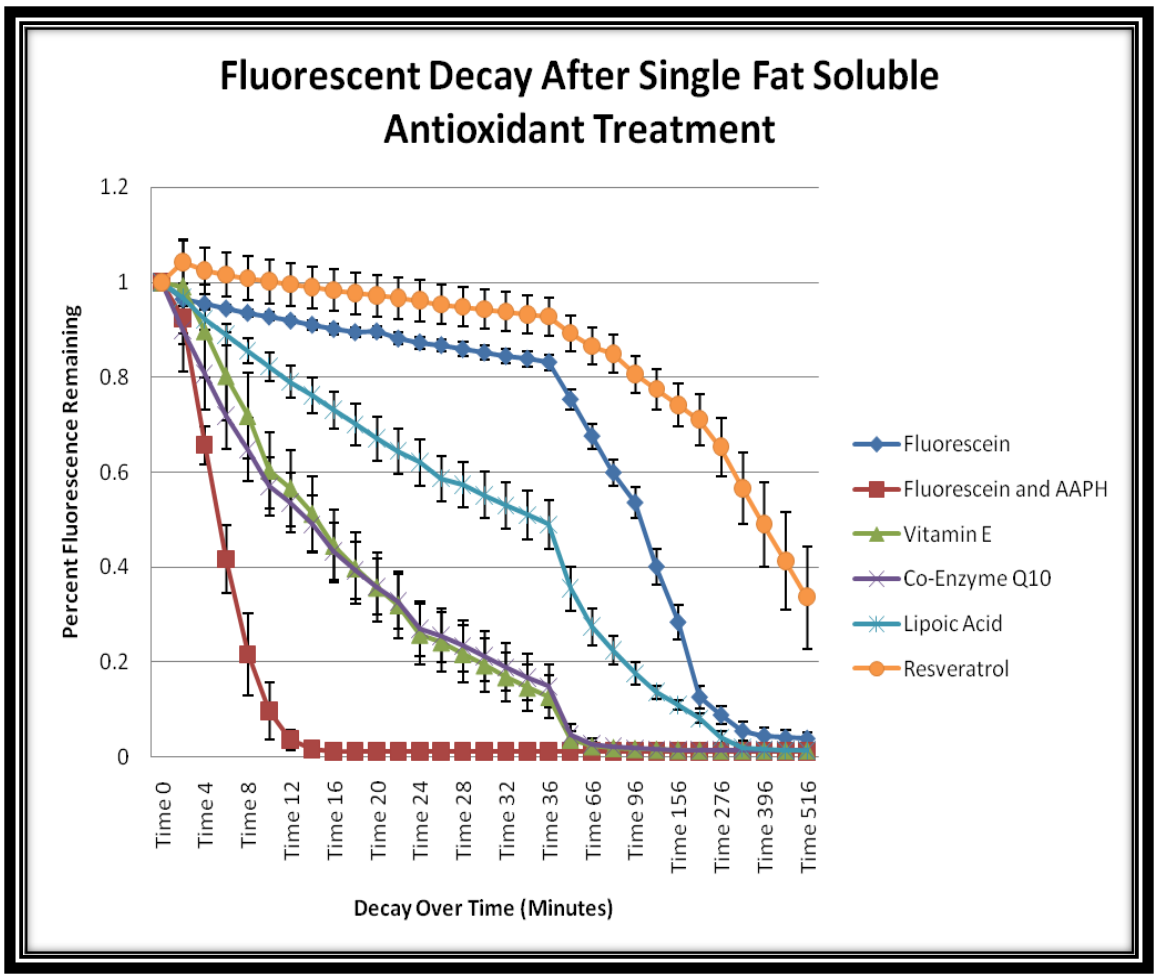


Figure 1A

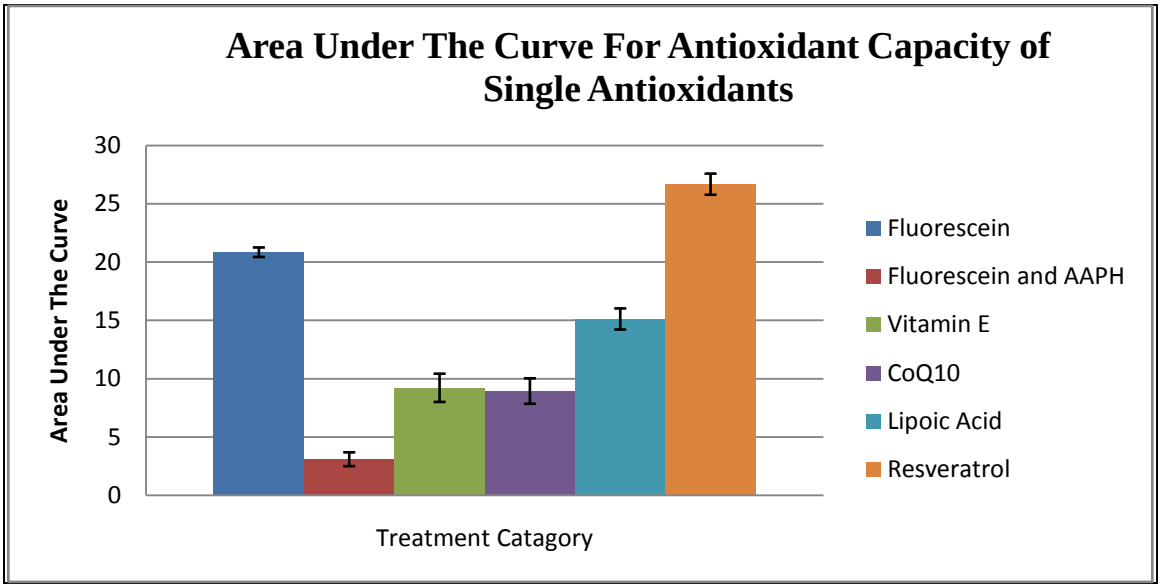


Figure 1B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
No Difference Between CoQ_{10} and Vitamin E Antioxidant Protection	p<.5
Lipoic Acid Provides More Antioxidant Protection Than Vitamin E	p<.0001
Resveratrol Provides More Antioxidant Protection Than Lipoic Acid	p<.0001

Table 1

Figures 1A & B and Table 1. Graph showing fat soluble antioxidant protection provided by individual antioxidants to fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 μM concentration. All samples but Fluorescein contained AAPH. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Results demonstrate that lipoic acid is the best fat soluble Packer antioxidant in terms of single antioxidant protection. However, resveratrol is a much superior antioxidant to any of the fat soluble Packer antioxidants. There is no statistically significant difference between the protection provided by vitamin E and coenzyme Q_{10}, which were the worst single fat soluble antioxidants.

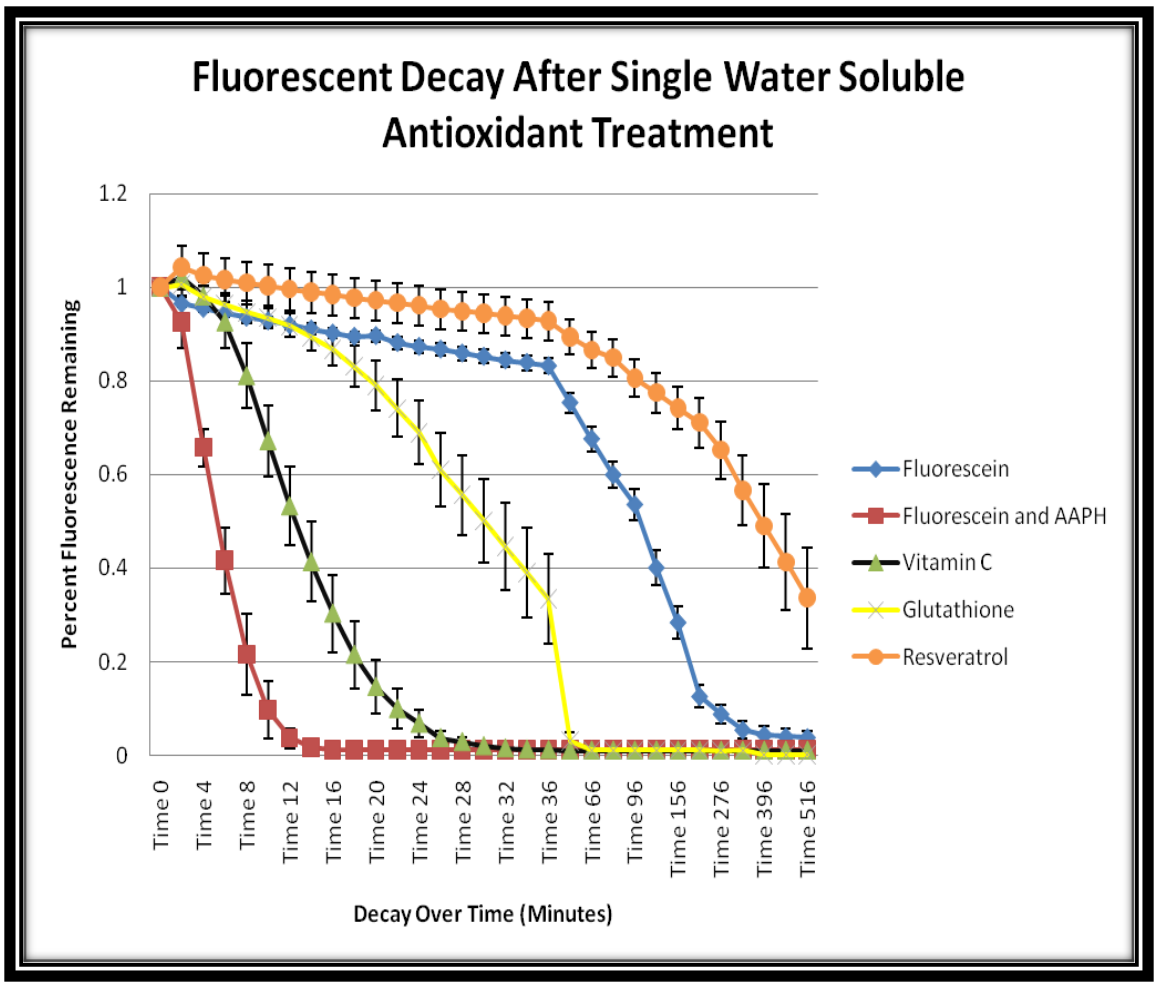


Figure 2A

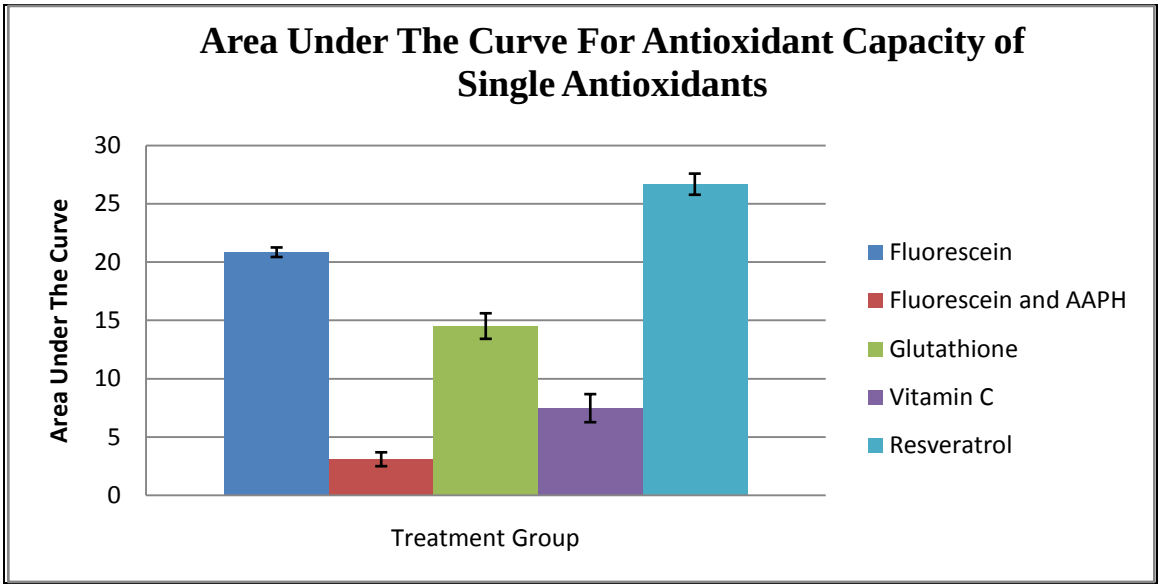


Figure 2B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
Glutathione Provides More Antioxidant Protection Than Vitamin C	p<.0001
Resveratrol Provides More Antioxidant Protection Than Glutathione	p<.0001
Resveratrol Provides More Antioxidant Protection than Vitamin C	p<.0001

Table 2

Figures 2A & B and Table 2. Results showing water soluble antioxidant protection provided by individual antioxidants to fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples but Fluorescein contained AAPH. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. The results demonstrate that glutathione is the best water soluble Packer antioxidant in terms of single antioxidant protection. However, resveratrol is a much superior antioxidant to any of the water soluble Packer antioxidants.

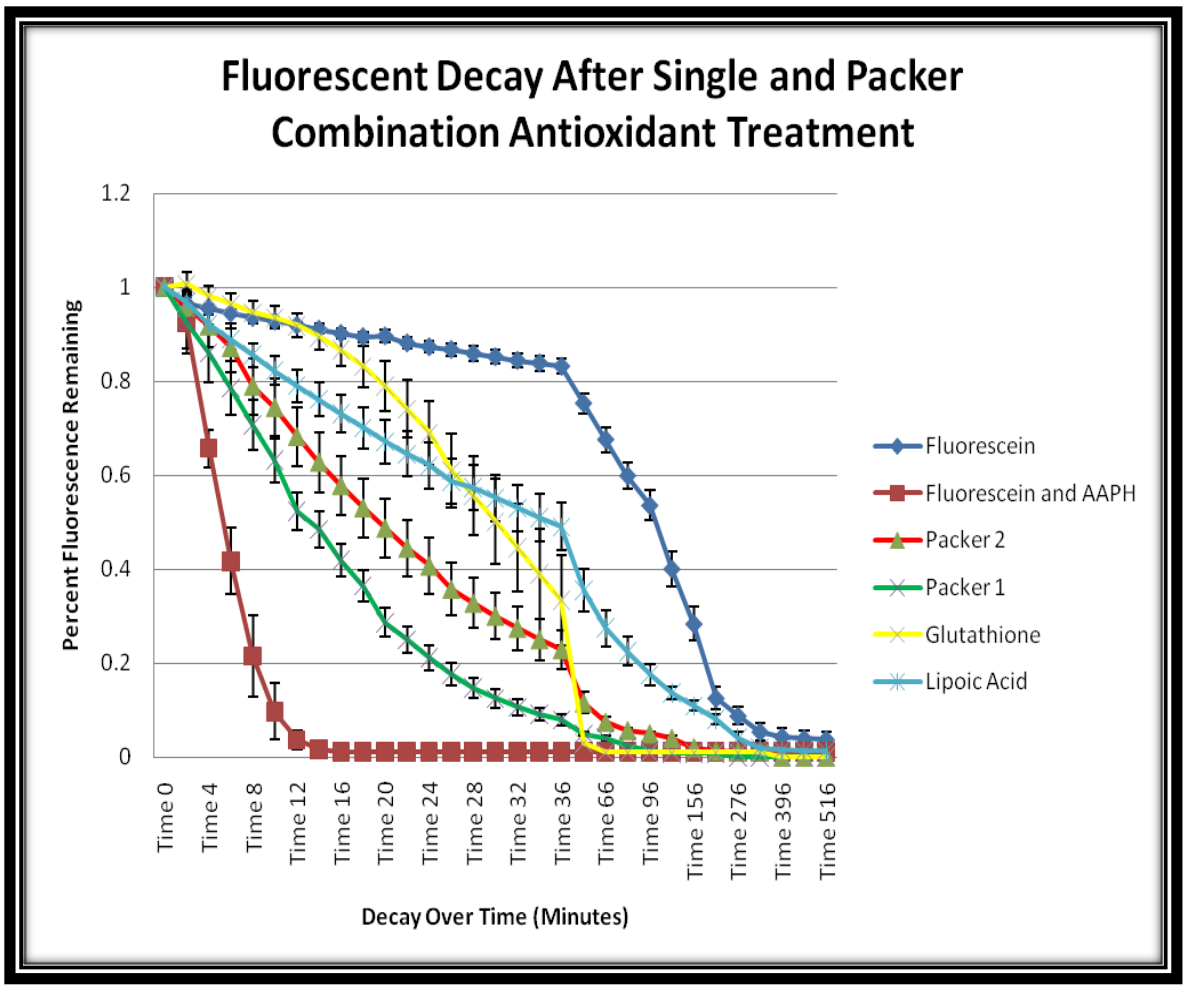


Figure 3A

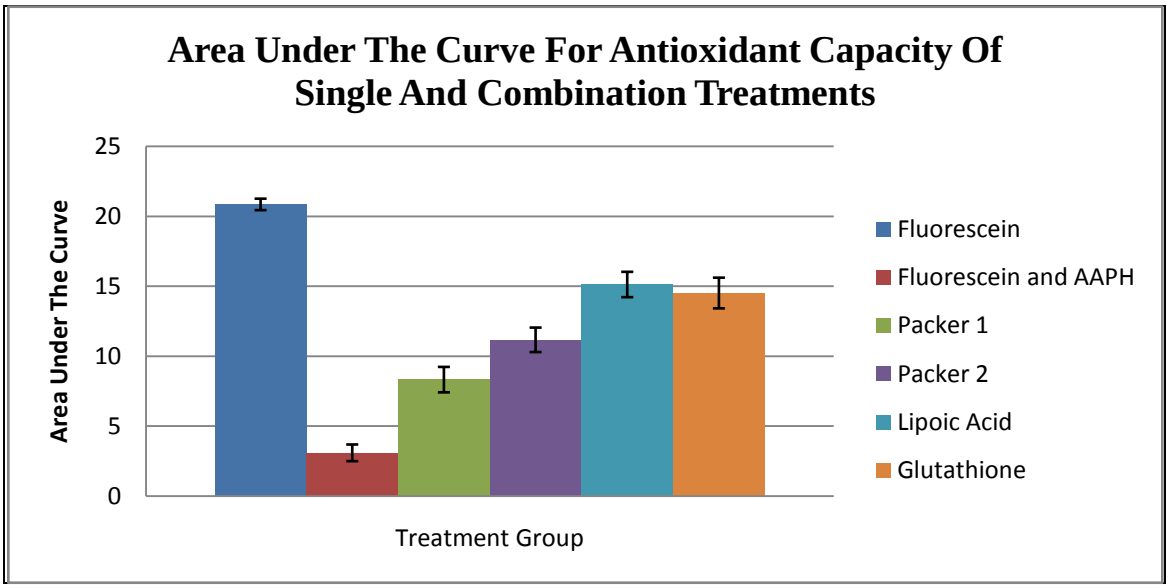


Figure 3B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
Packer 2 Provided More Antioxidant Protection Than Packer 1	p<.0001
Glutathione Provided More Antioxidant Protection Than Packer 2	p<.0001
Lipoic Acid Provided More Antioxidant Protection Than Packer 2	p<.0001

Table 3

Figures 3A & B and Table 3. Graph comparing Packer combination to the best single antioxidant protection of fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples but Fluorescein contained AAPH. Fluorescein is the positive control. Fluorescein and AAPH are the negative control. Packer 1 is a combination of the 5 Packer antioxidants in the ratio that is listed in the Materials and Methods. Packer 2 is a combination of the 5 Packer antioxidants in even amounts in the solution. The results demonstrate that the Packer 1 combination provides the least amount of protection in the ORAC assay. The results also demonstrate that the Packer 2 combination provides better protection, but is still inferior to the protection provided by the best water and fat soluble antioxidants.

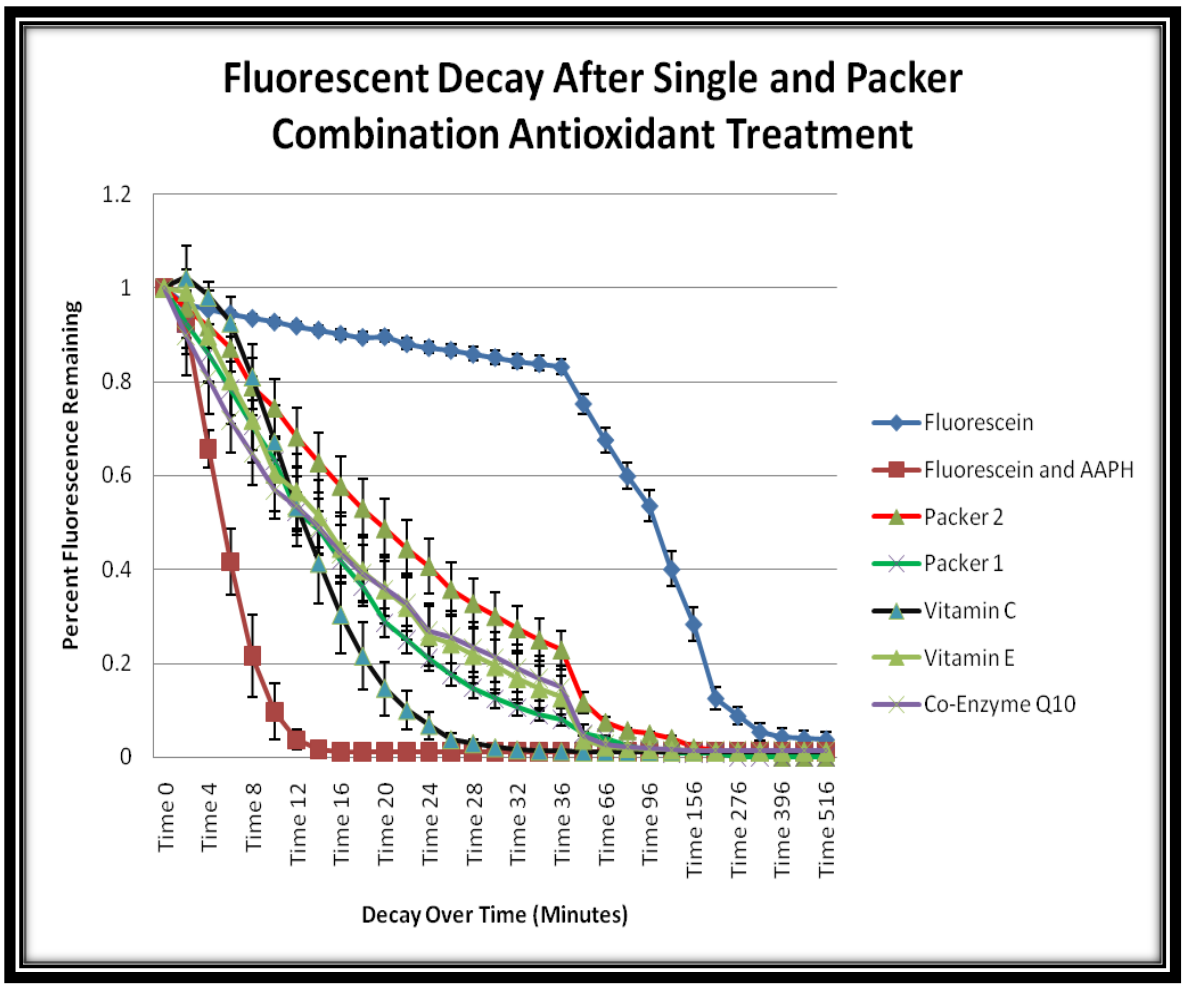


Figure 4A

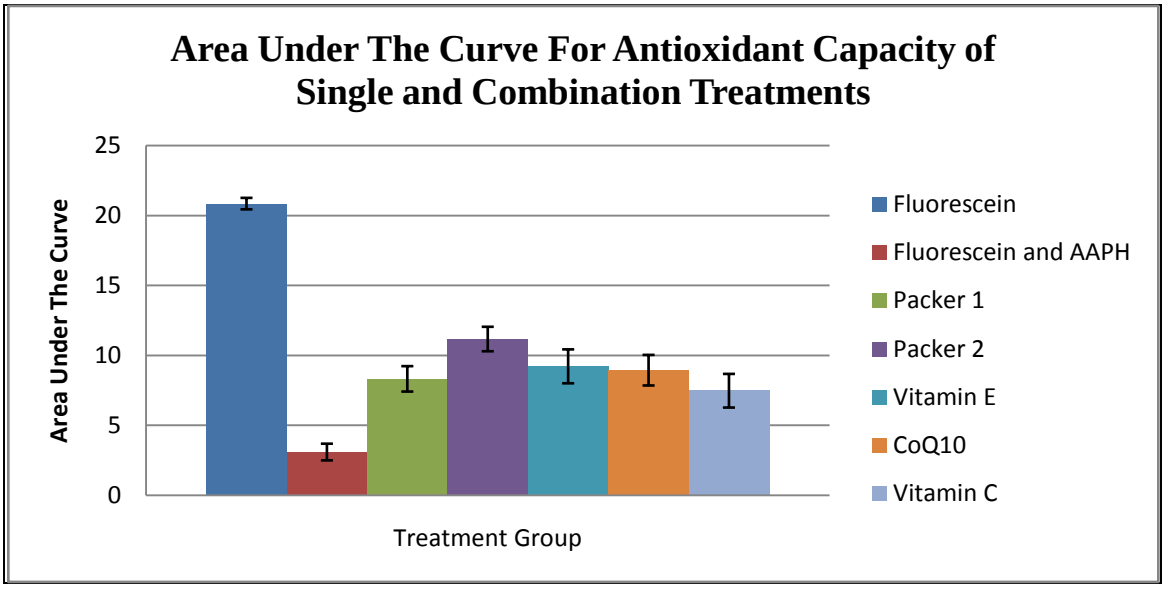


Figure 4B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
Packer 1 Provides More Antioxidant Protection Than Vitamin C	p<.02
Vitamin E Provides More Antioxidant Protection Than Packer 1	p<.02
Packer 2 Provides More Antioxidant Protection Than Vitamin E	p<.0001
Packer 2 Provides More Antioxidant Protection Than CoQ_{10}	p<.0001

Table 4

Figures 4A & B and Table 4. Graph comparing Packer combination to the poorest single antioxidant protection to fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples but Fluorescein contained AAPH. Fluorescein is the positive control. Fluorescein and AAPH are the negative control. Packer 1 is a combination of the 5 Packer antioxidants in the ratio that is listed in the Materials and Methods. Packer 2 is a combination of the 5 Packer antioxidants in even amounts. The results demonstrate that the Packer combination in the ratio recommended provides a low amount of protection in the ORAC assay, even worse than the two poorest fat soluble vitamins, although its protection is superior to vitamin C. The results also demonstrate that the Packer combination in equal amounts provides better protection than all the other samples.

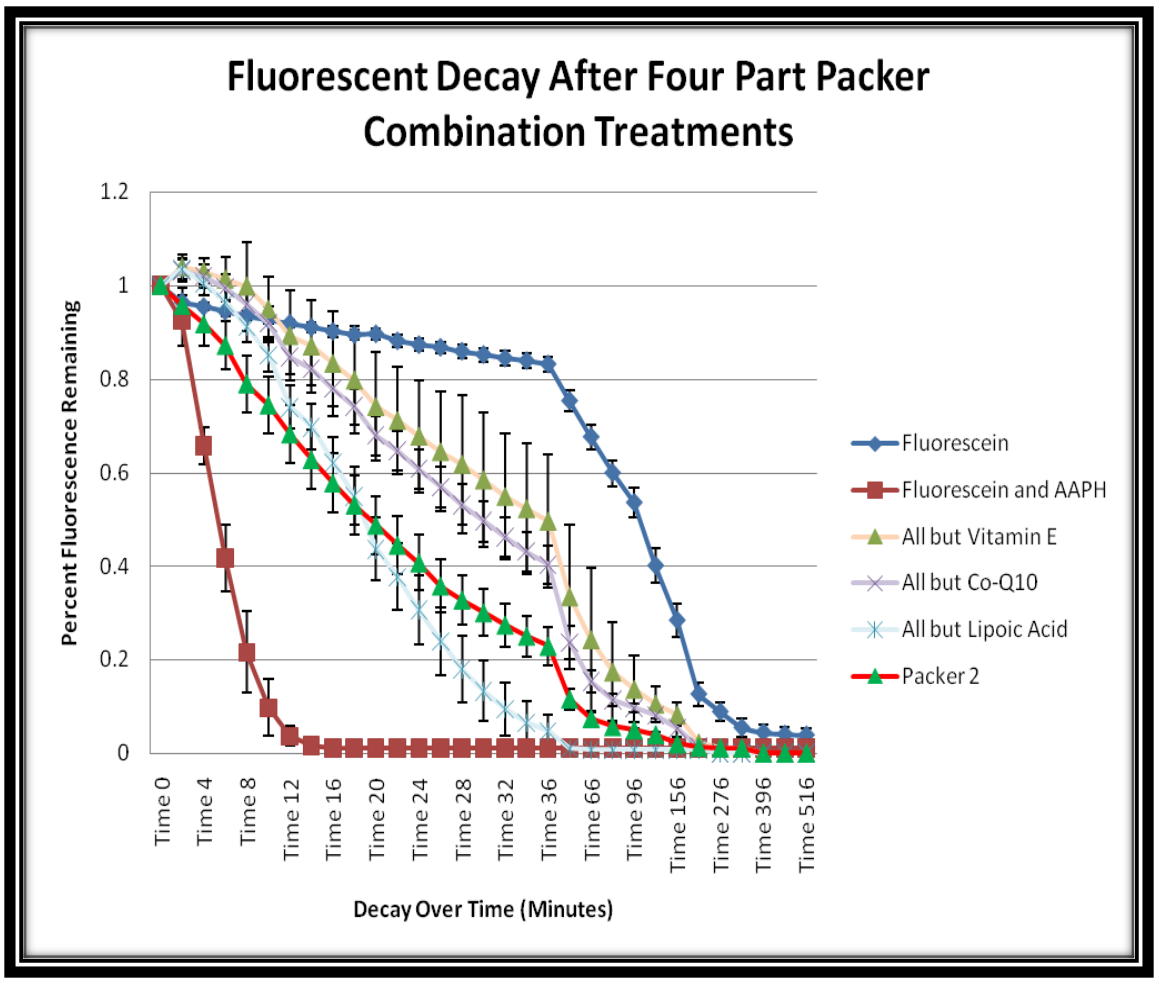


Figure 5A

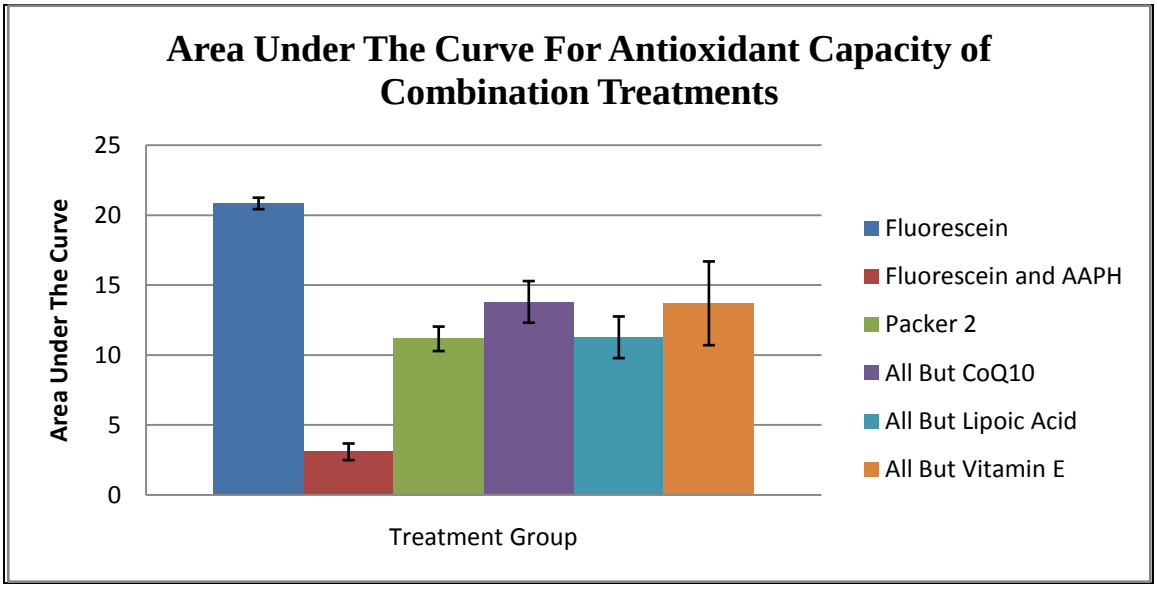


Figure 5B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
All but Vitamin E Combination Provides More Antioxidant Protection Than Packer 2	p<.0001
All but CoQ_{10} Combination Provides More Antioxidant Protection Than Packer 2	p<.0001
Packer 2 Provides More Antioxidant Protection Than All but Lipoic Acid Treatment	p<.001
No Difference Between All but Vitamin E and All but CoQ_{10} Antioxidant Protection	p<.2

Table 5

Figures 5A & B and Table 5. Graph showing Packer combination antioxidant protection missing one of the fat soluble Packer antioxidants to fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Packer 2 is a combination of the 5 Packer antioxidants in even amounts. The results demonstrate that removal of vitamin E or CoQ_{10} drastically improves the Packer 2 combination. Removal of Lipoic Acid improves the combination initially, but falls drastically below the Packer 2 combination after 20 minutes.

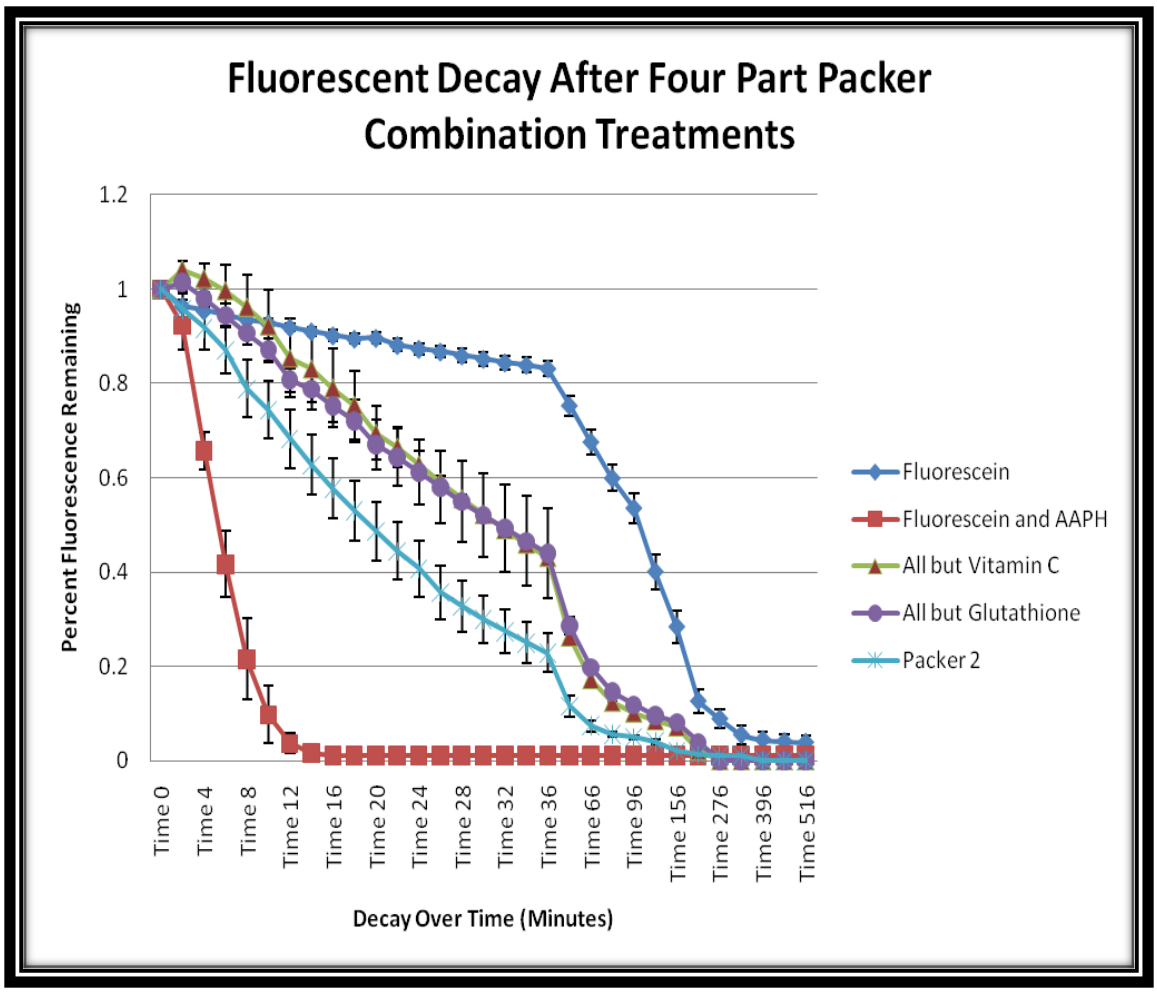


Figure 6A

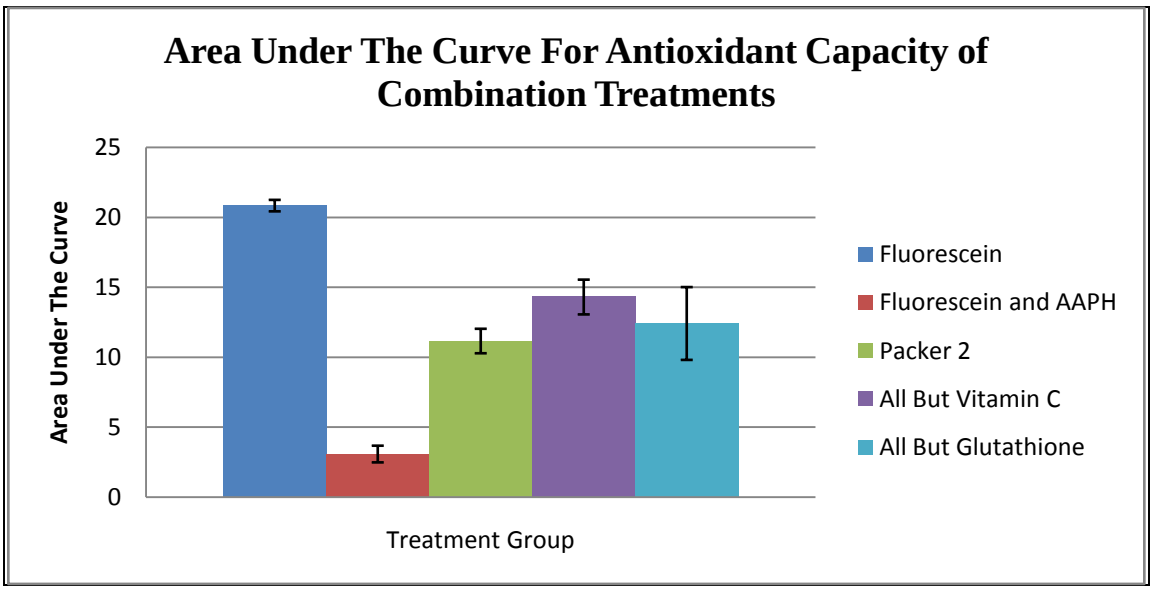


Figure 6B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
All but Glutathione Provides More Antioxidant Protection Than Packer 2	p<.0001
All but Vitamin C Provides More Antioxidant Protection Than Packer 2	p<.0001

Table 6

Figure 6. Graph showing Packer combination antioxidant protection missing one of the water soluble Packer antioxidants to fluorescein in the ORAC assay. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH are the negative control. The Packer 2 is a combination of the 5 Packer antioxidants in even amounts. The results demonstrate that the removal of Vitamin C and Glutathione results in very similar decay curves, much improving antioxidant protection compared to Packer 2.

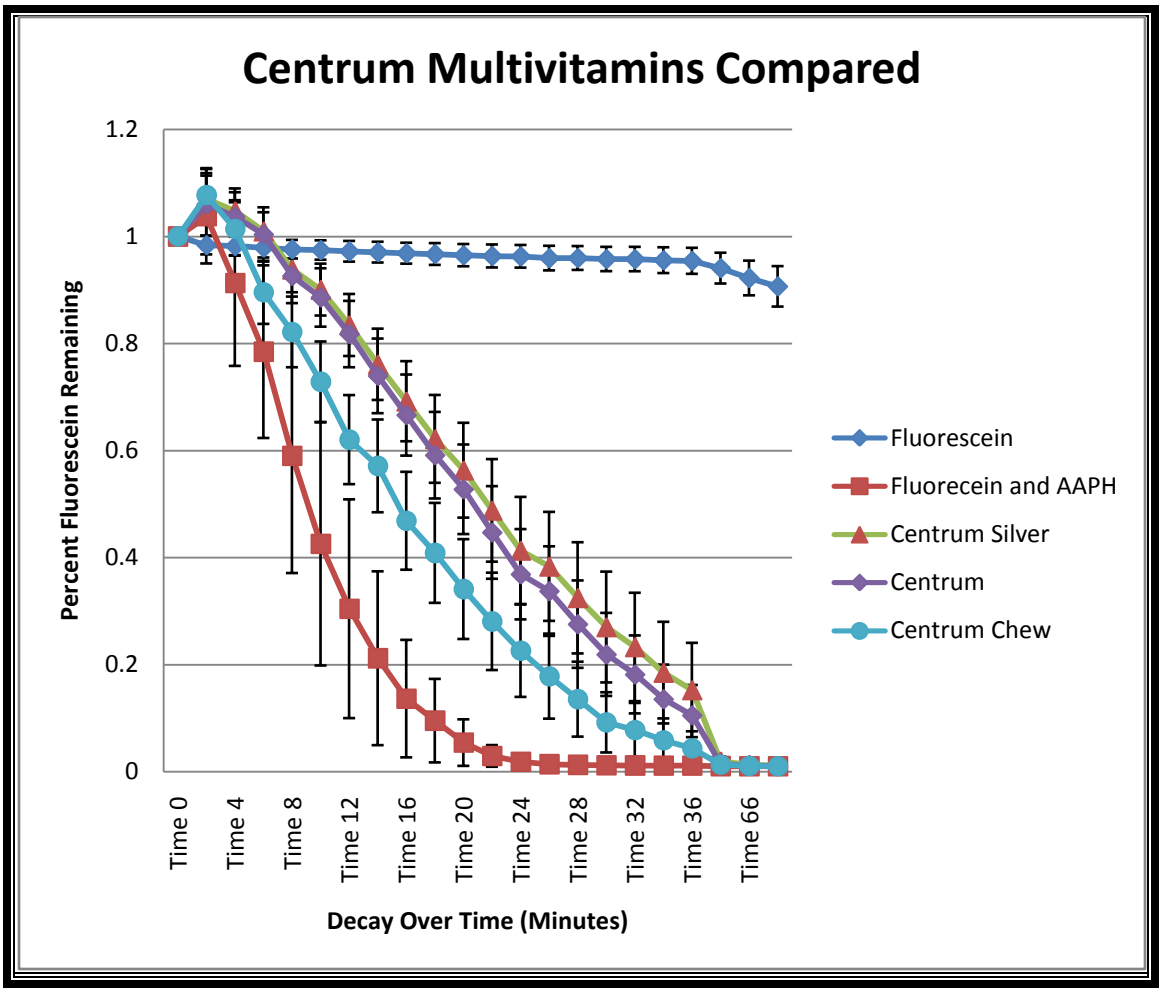


Figure 7A

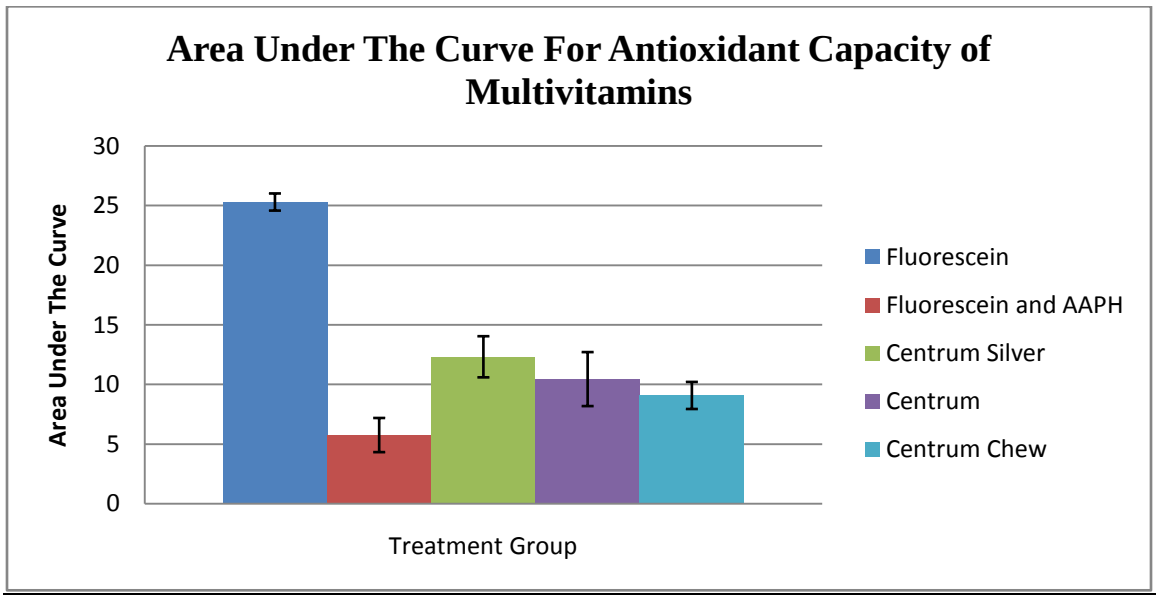


Figure 7B

Two-Sided p Value of Difference Between Area Under The Curve of Chart Values	
Centrum Silver Provides More Antioxidant Protection Than Centrum	p<.001
Centrum Provides More Antioxidant Protection Than Centrum Chew	p<.005

Table 7

Figures 7A & B and Table 7. Graph showing antioxidant capabilities of three different types of Centrum multivitamins. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Results demonstrate that Centrum Silver is a superior antioxidant than the other multivitamins of the same brand.

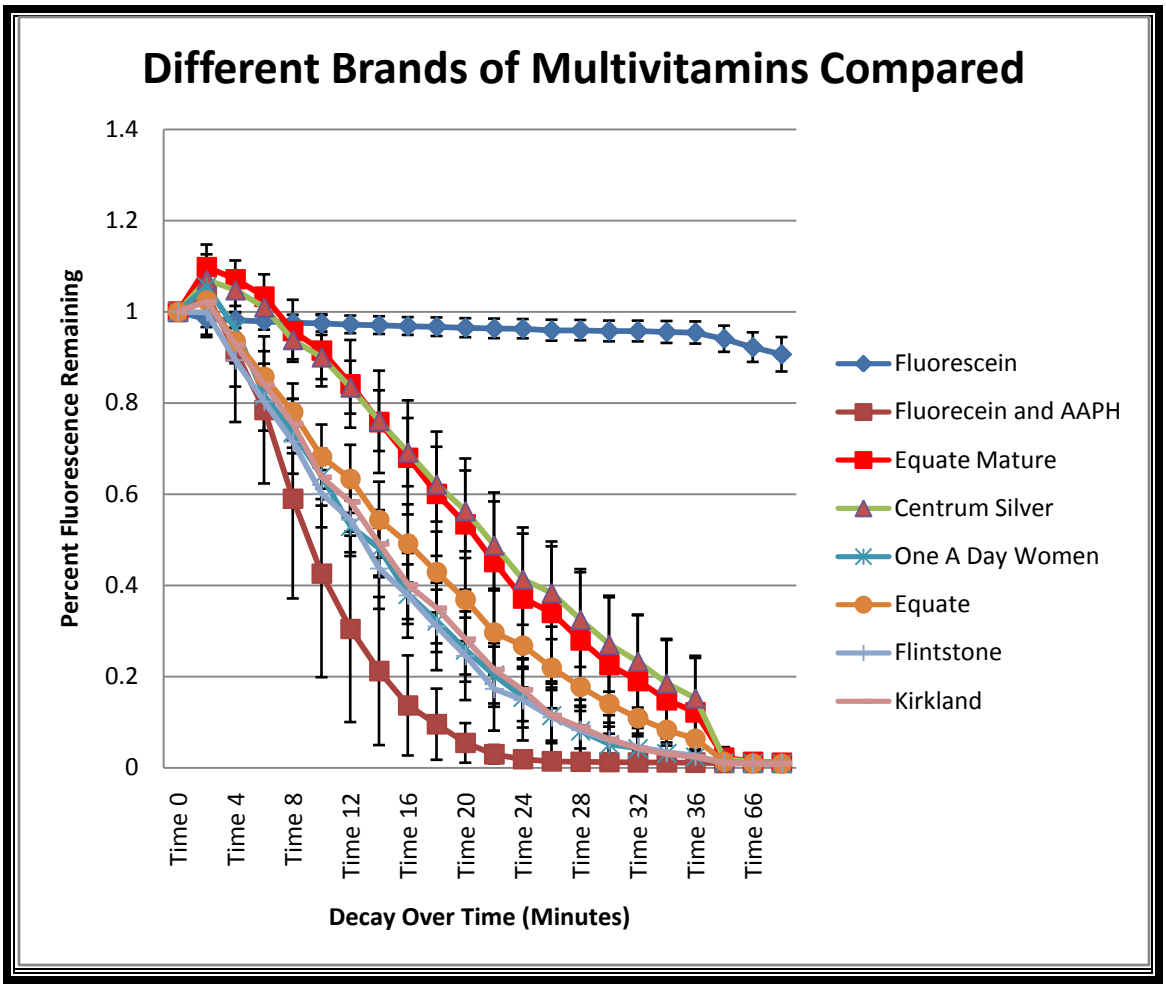


Figure 8A

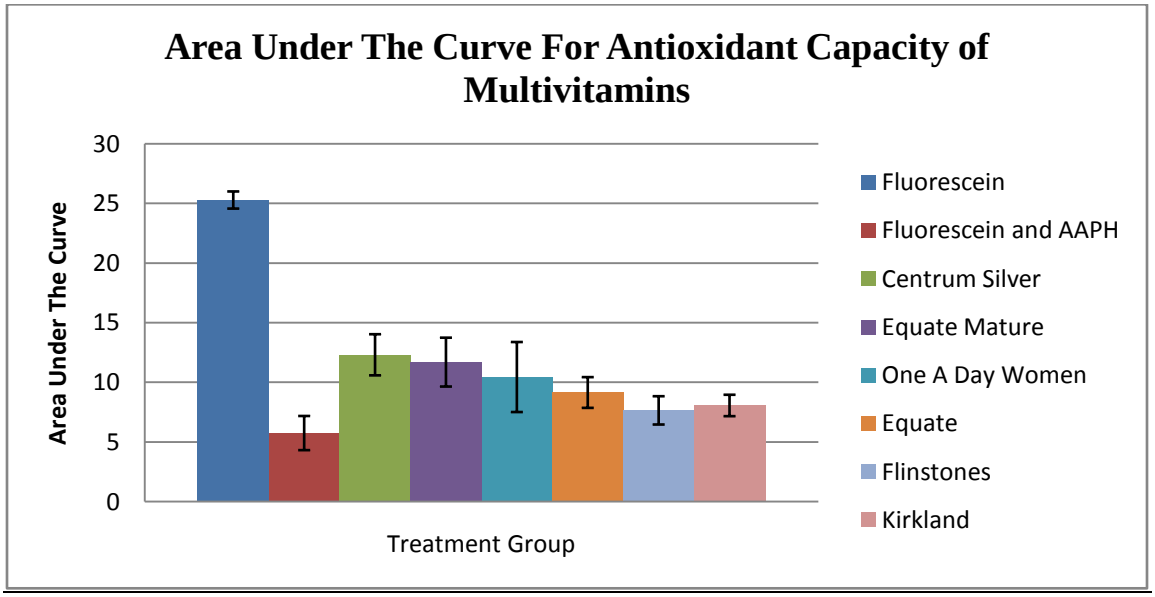


Figure 8B

No Statistically Significant Difference	
No Difference Between Kirkland and One A Day Women Antioxidant Protection	p<.2
No Difference Between Centrum Silver and Equate Mature Antioxidant Protection	p<.4
Statistically Significant Difference	
Kirkland Provides More Antioxidant Protection Than Flintstones	p<.01
One A Day Women Provides More Antioxidant Protection Than Flintstones	p<.001
Equate Mature Women Provides More Antioxidant Protection Than Equate	p<.001

Table 8

Figures 8A & B and Table 8. Graph showing antioxidant capabilities of different multivitamin brands. Each sample had 24 repeats. Antioxidant samples were at a 100 μM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Results demonstrate that the multivitamins geared towards the elderly are superior antioxidants than those for the general public.

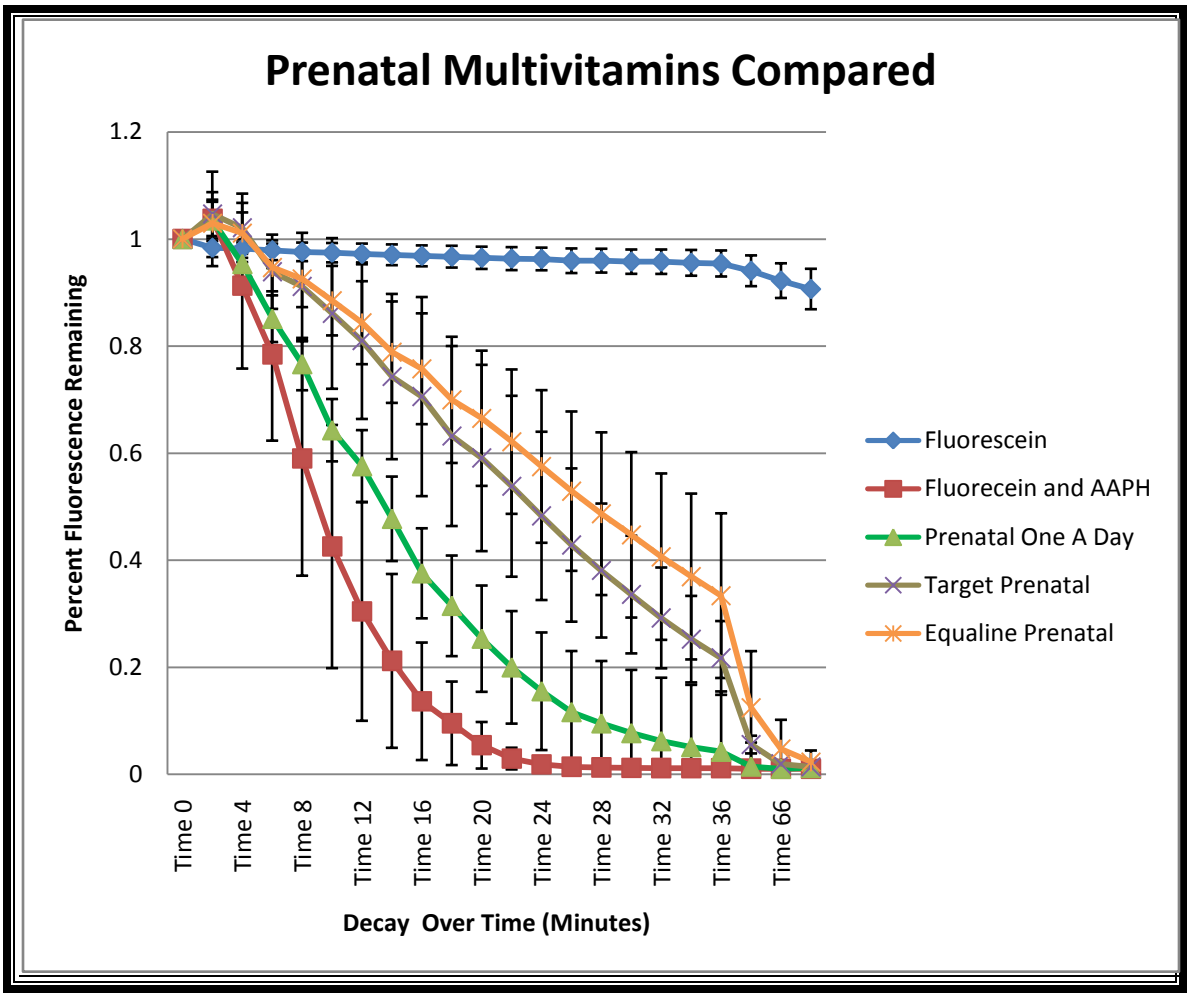


Figure 9A

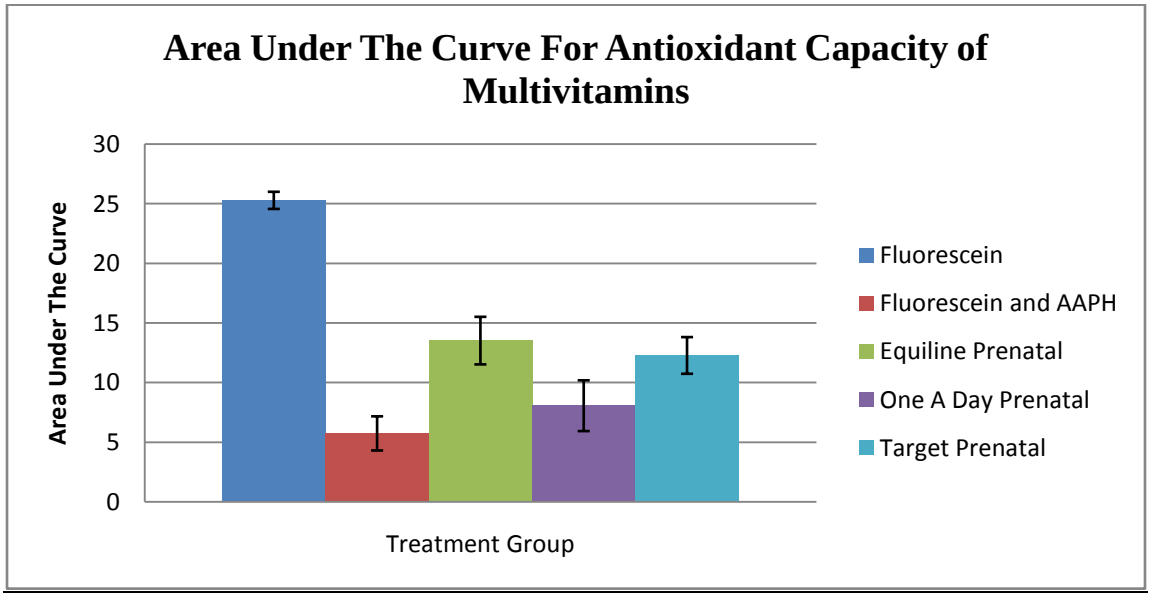


Figure 9B

Statistically Significant Differences	
Equaline Prenatal Provides More Antioxidant Protection Than Target Prenatal	p<.03
Target Prenatal Provides More Antioxidant Protection Than Prenatal One A Day	p<.0001
Equaline Prenatal Provides More Antioxidant Protection Than One A Day Prenatal	p<.0001

Table 9

Figures 9A & B and Table 9. Graph showing antioxidant capabilities of different prenatal multivitamin brands. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Results demonstrate that Prenatal One A Day is an inferior antioxidant than the other prenatals tested. Further, statistical analysis demonstrated that Equaline Prenatal is superior to Target Prenatal.

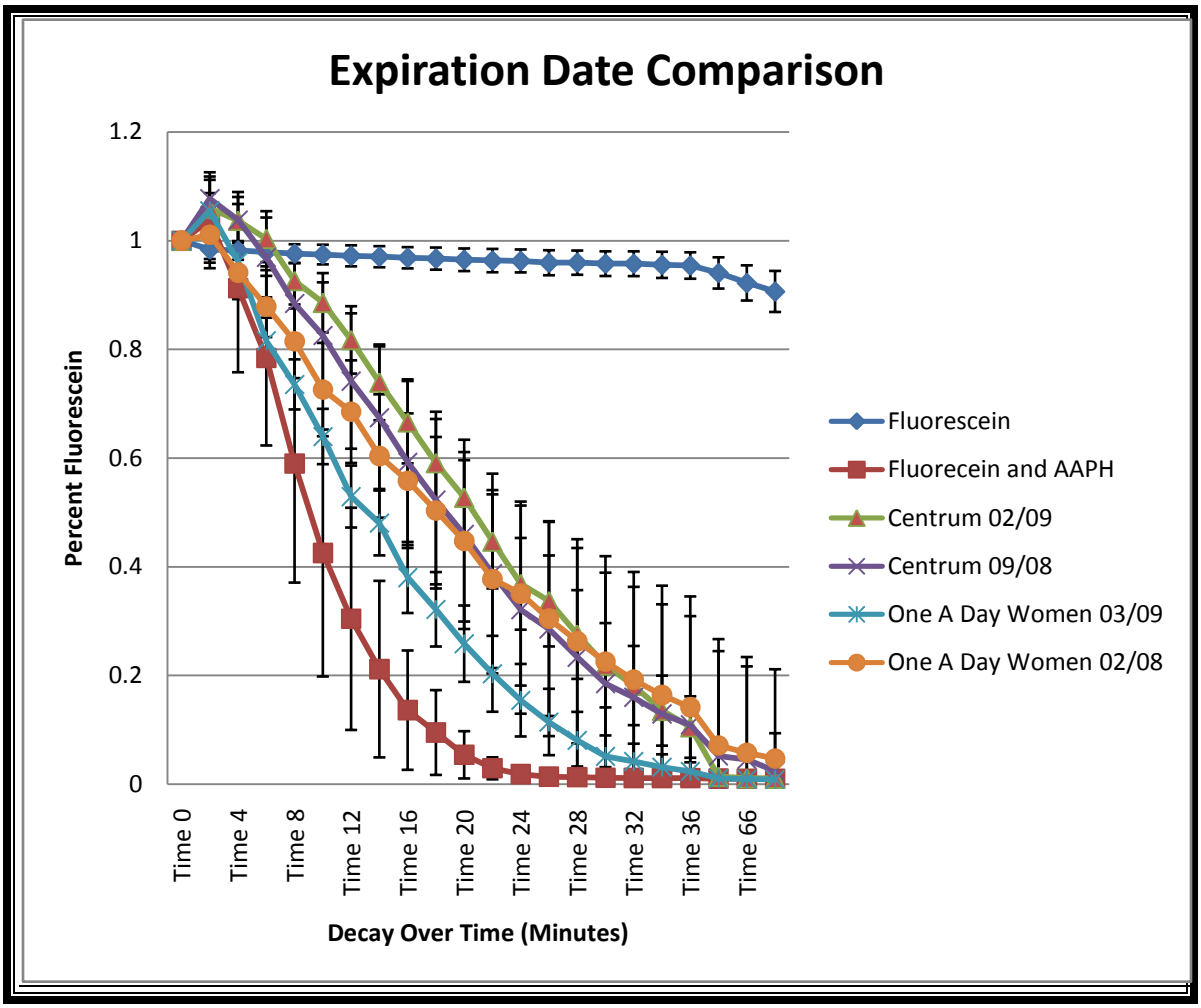


Figure 10A

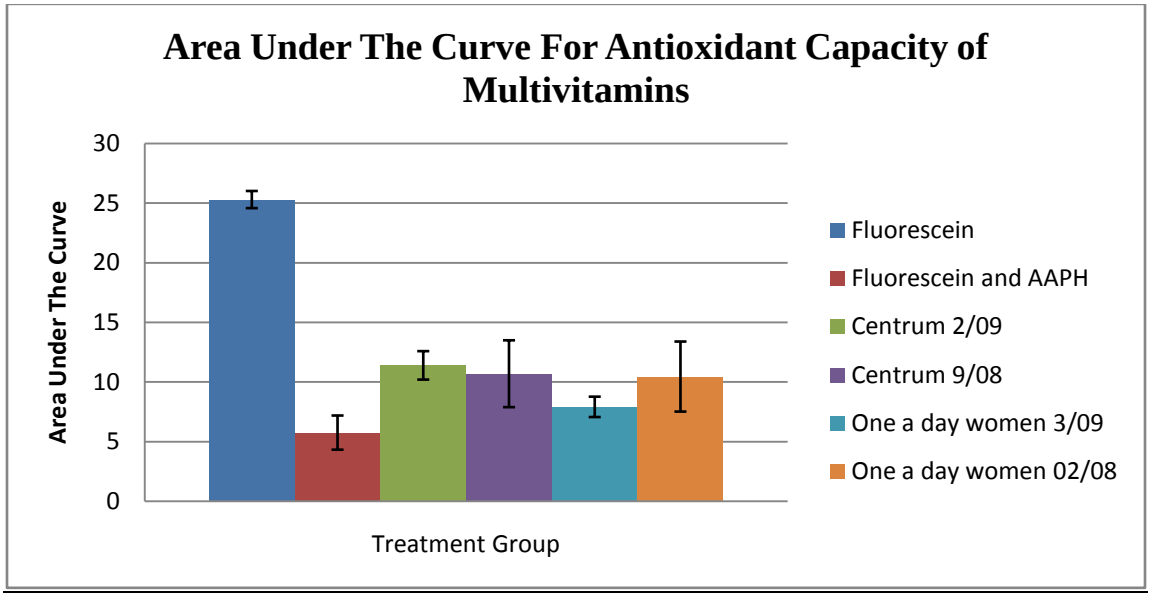


Figure 10B

Statistically Significant Difference	
Difference Between Centrum Antioxidant Protection	p<.03
Difference Between One A Day Women Antioxidant Protection	p<.0001

Table 10

Figures 10A & B and Table 10. Graph showing antioxidant capacities of the same multivitamins produced at different times. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. Statistical analysis shows that there is a statistically significant difference between the Centrum vitamins produced at different times. The newer of the two, Centrum with an expiration date of 02/09, is a superior antioxidant to Centrum with an expiration date of 09/08. There was a large statistical difference between the One A Day Women multivitamins. Surprisingly, the older multivitamins (One A Day Women 02/08) was a superior antioxidant to the One A Day Women 03/09 multivitamins.

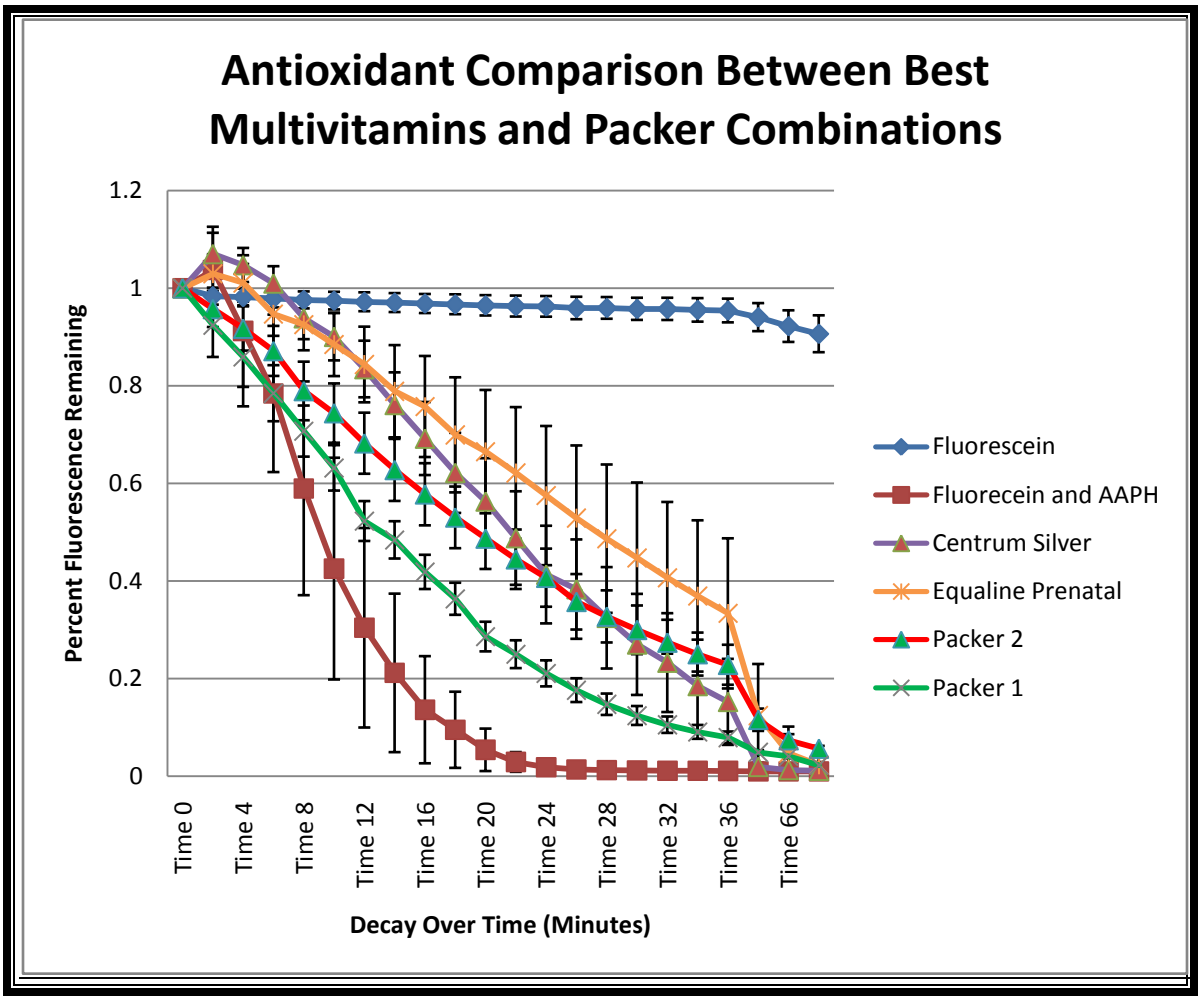


Figure 11A

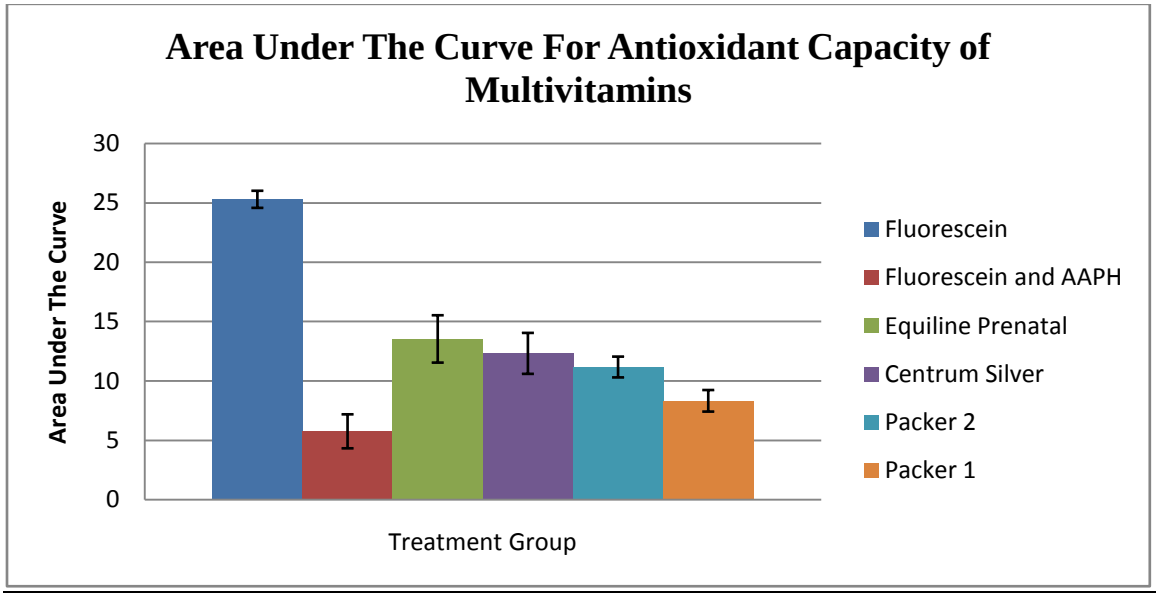


Figure 11B

Statistically Significant Differences	
Equaline Prenatal Provides More Antioxidant Protection Than Centrum Silver	p<.04
Centrum Silver Provides More Antioxidant Protection Than Packer 2	p<.005
Equaline Prenatal Provides More Antioxidant Protection Than Packer 2	p<.0001

Table 11

Figures 11A & B and Table 11. Graph showing antioxidant capacities of the best multivitamins versus the Packer combinations. Each sample had 24 repeats. Antioxidant samples were at a 100 µM concentration. All samples contained AAPH except Fluorescein. Fluorescein is the positive control. Fluorescein and AAPH is the negative control. The results demonstrate that Equaline Prenatal is the best antioxidant in terms of combination treatments. Centrum Silver was also found to be a superior antioxidant combination to that of the Packer combinations.

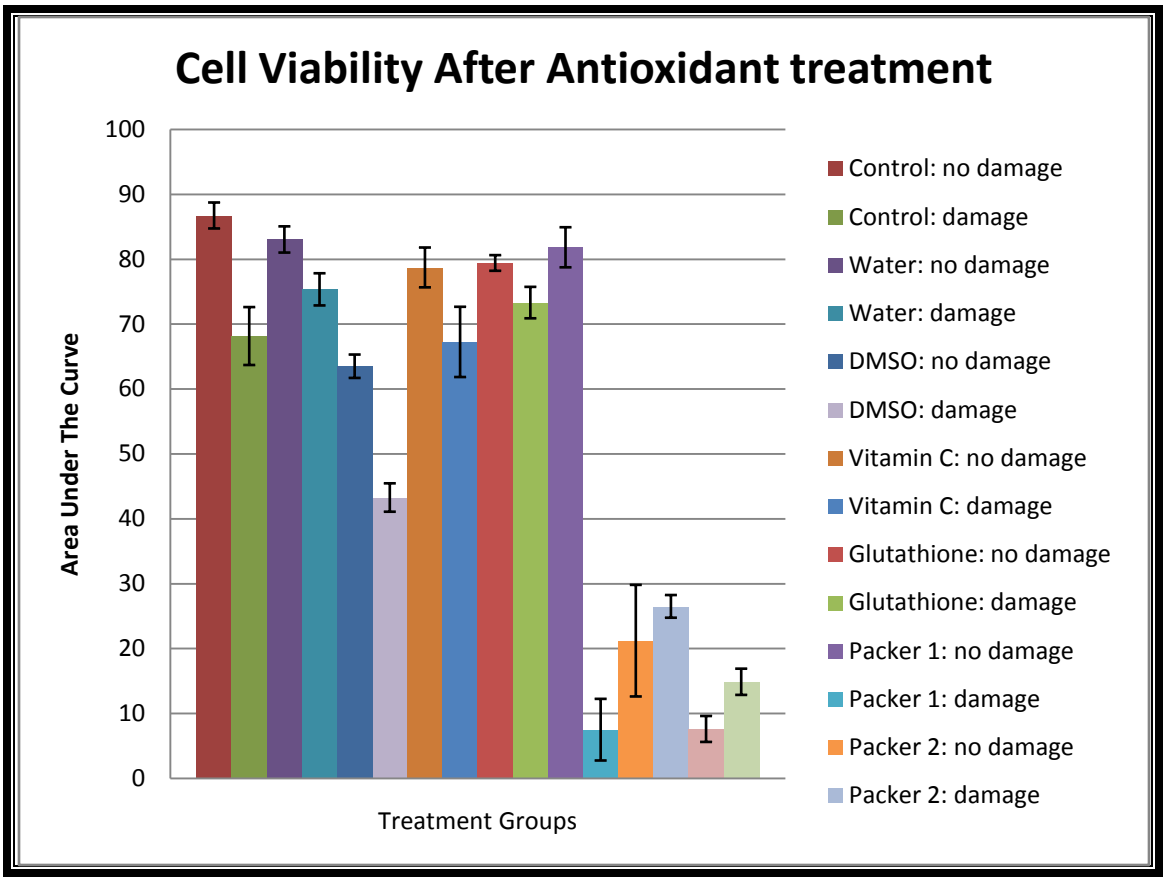


Figure 12

No Statistically Significant Difference	
No Difference Between Packer 2 Damaged and No Damage Group	p<.3
Statistically Significant Difference	
Water Damage Had A Higher Viability Than Control	p<.01
Control Had A Higher Viability Than DMSO No Damage	p<.0001
Control Had A Higher Viability Than DMSO Damage	p<.0001

Table 12

Figure 12 and Table 12. Graph showing antioxidant capacities of single water soluble antioxidants to multivitamin and packer combinations. Each sample had 4 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. Water and water soluble antioxidant samples had 1 ml of water added to 15 ml of media. DMSO and fat soluble samples had 1 ml of DMSO added to 15 ml of media. Packer and multivitamin samples had 500 µl of water and 500 µl of DMSO added to 15 ml of media. No damage means that cells were not damaged with hydrogen peroxide. Damage means that cells were damaged with hydrogen peroxide at a 1:10,000 H_2O_2 to water concentration for one hour before antioxidant treatment.The graph demonstrates that Packer combinations and multivitamins did not aid in cell recovery after hydrogen peroxide Treatment. Single water soluble antioxidant treatments had no

effect on cell viability. Of interesting note is the high viability of the Packer 1: no damage group compared to the damage group. Its viability is higher than just DMSO treatment alone, which is not consistant with the rest of the group.

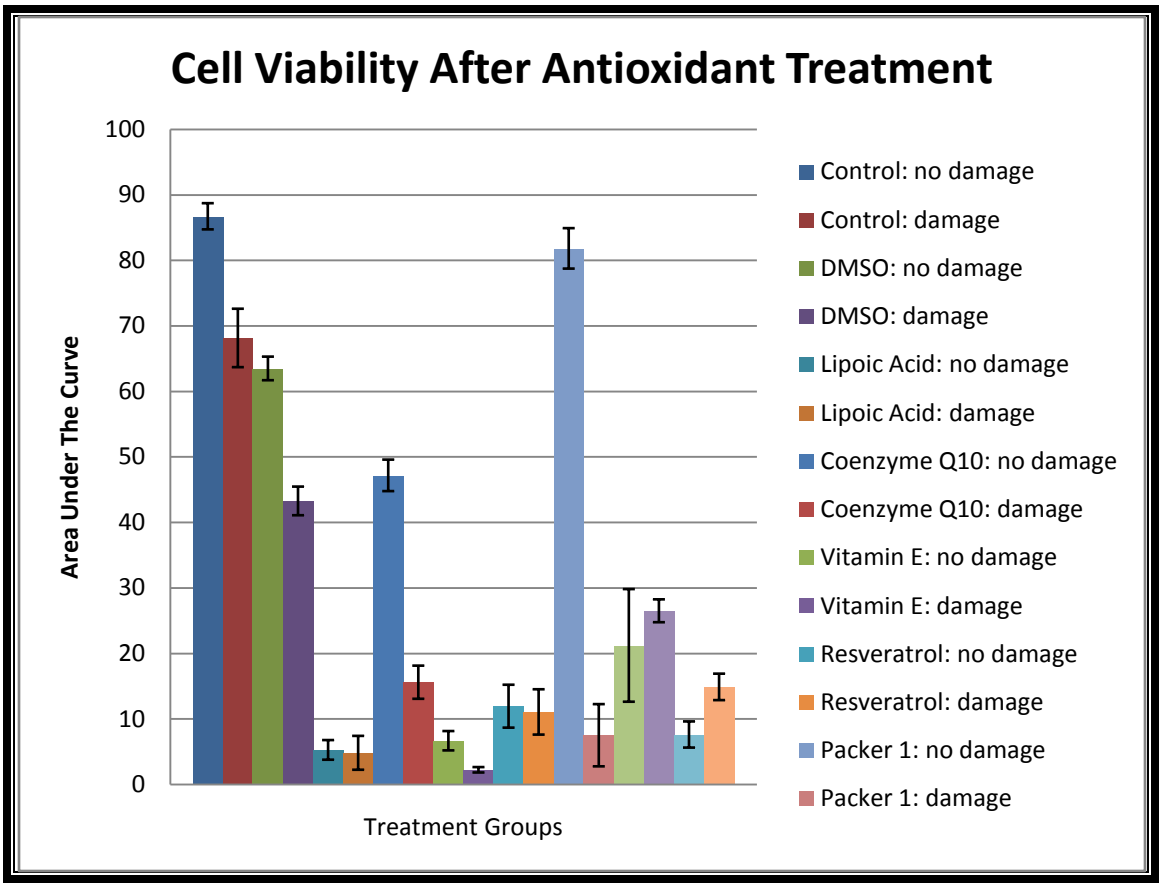


Figure 13

No Statistically Significant Difference	
No Difference Between Packer 2 Damaged and No Damage Group	p<.3
Statistically Significant Difference	
Water Damage Had A Higher Viability Than Control	p<.01
Control Had A Higher Viability Than DMSO No Damage	p<.0001
Control Had A Higher Viability Than DMSO Damage	p<.0001

Table 13

Figure 13. Graph showing antioxidant capacities of single fat soluble antioxidants to multivitamin and packer combinations. Each sample had 4 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. DMSO and fat soluble samples had 1 ml of DMSO added to 15 ml of media. Packer and multivitamin samples had 500 µl of water and 500 µl of DMSO added to 15 ml of media. No damage means that cells were not damaged with hydrogen peroxide. Damage means that cells were damaged with hydrogen peroxide at a 1:10,000 H_2O_2 to water concentration for one hour before antioxidant treatment.The graph demonstrates that the fat soluble vitamin solutions generally greatly decrease the cell viability of the samples. The exceptions to this of course are the undamaged lipoic acid and Packer 1 samples.

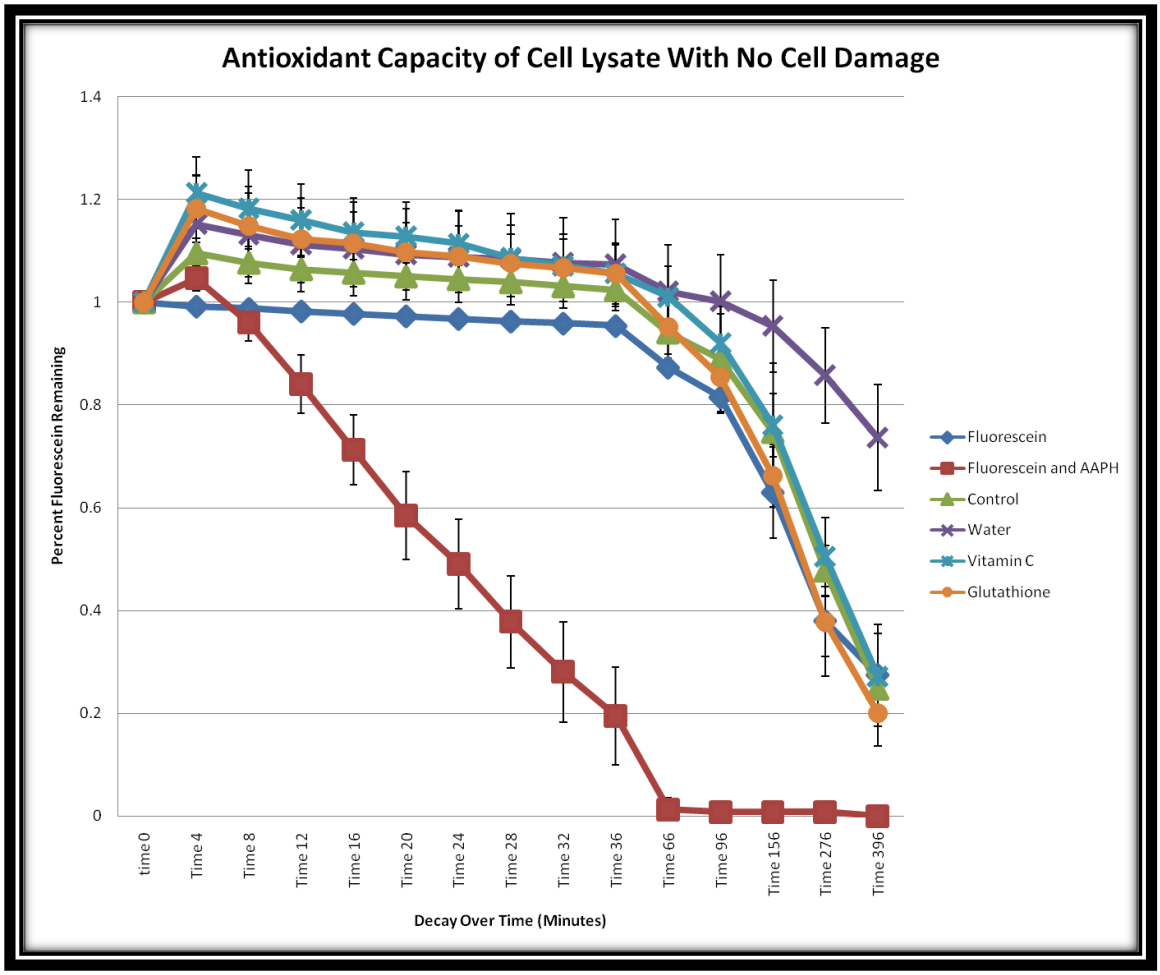


Figure 14A

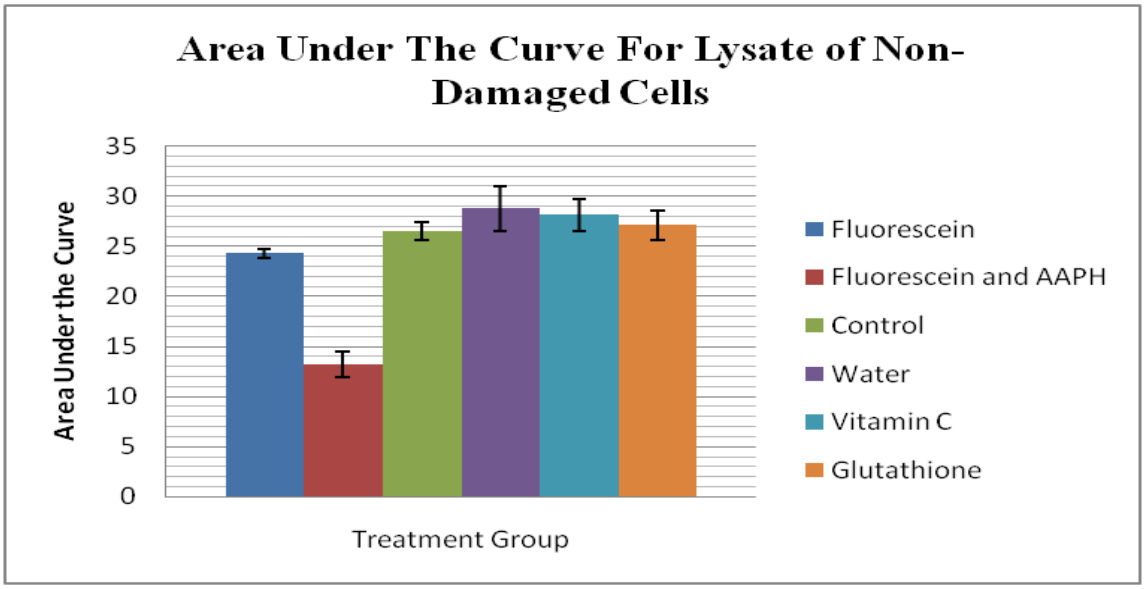


Figure 14B

No Statistically Significant Difference	
No Difference Between Protection Provided By Control and Glutathione	p<.2
Statistically Significant Difference	
Water Provided More Antioxidant Protection Than Control	p<.001
Vitamin C Provided More Antioxidant Protection Than Control	p<.002

Table 14

Figures 14A & B and Table 14. Graph showing antioxidant capacities of cells after 24 hours incubation with water soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. No damage means that cells were not damaged with hydrogen peroxide. The results demonstrate that the cell lysates of cells treated with anything, expecially water, increased the antioxidant capacity of the sample compared to the control. However, because the water sample had the most antioxidant capacity, the results suggest that the increase in antioxidant capacity is not due to antioxidants but instead the water in the antioxidant solutions. Water, or DMSO, could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 14B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

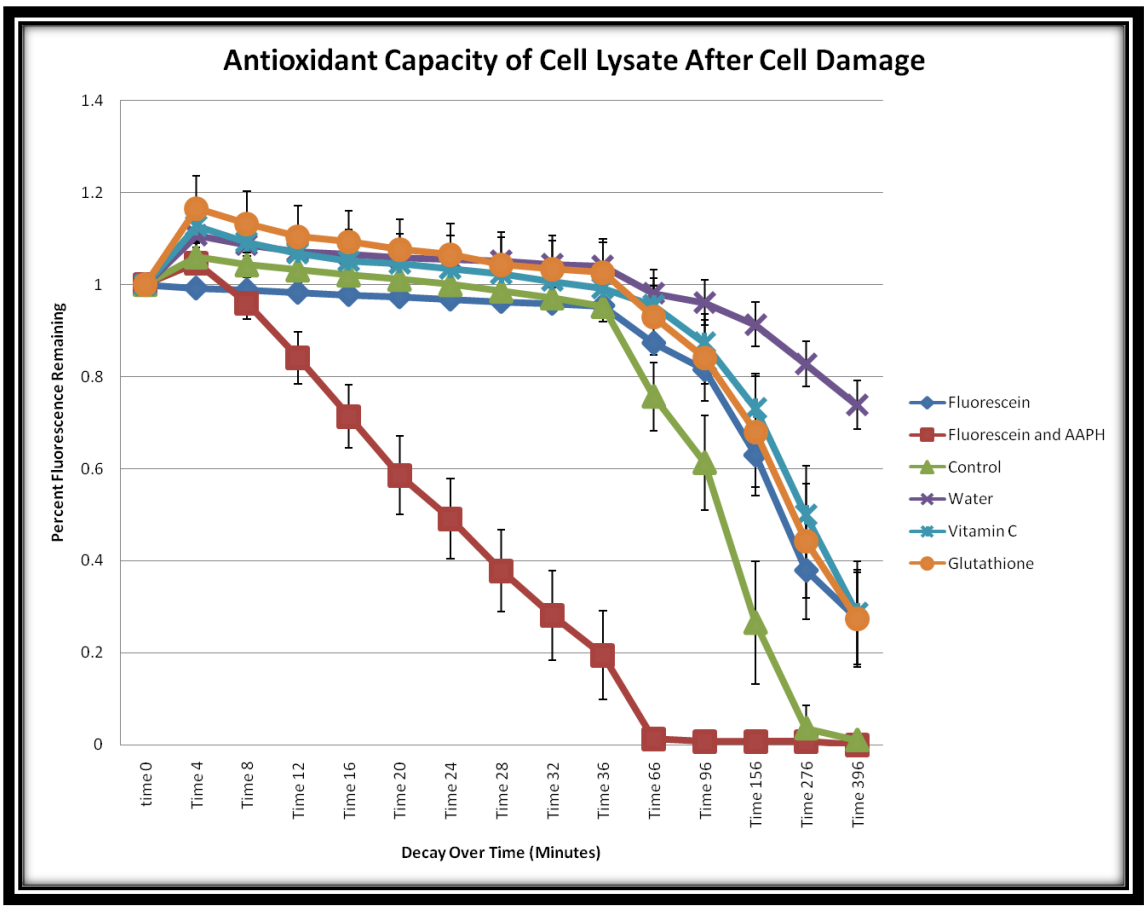


Table 15A

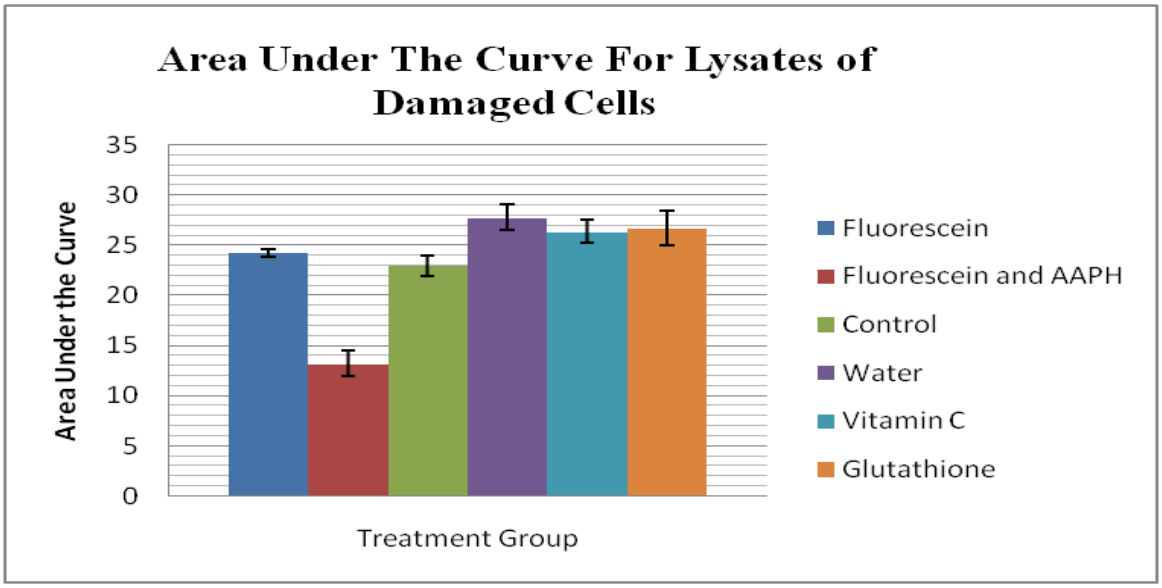


Figure 15B

Statistically Significant Difference	
Water Provided More Antioxidant Protection Than Control	p<.0001
Vitamin C Provided More Antioxidant Protection Than Control	p<.0001
Glutathione Provided More Antioxidant Protection Than Control	p<.0001

Table 15

Figures 15A & B and Table 15. Graph showing antioxidant capacities of cells after short Hydrogen Peroxide treatment and 24 hours incubation with water soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. Damage means that cells were damaged with hydrogen peroxide at a 1:10,000 H_2O_2 to water concentration for one hour before antioxidant treatment. The results demonstrate that the cell lysates of cells treated with anything, expecially water, increased the antioxidant capacity of the sample compared to the control. However, because the water sample had the most antioxidant capacity, the results suggest that the increase in antioxidant capacity is not due to antioxidants but instead the water in the antioxidant solutions. Water, or DMSO, could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 15B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

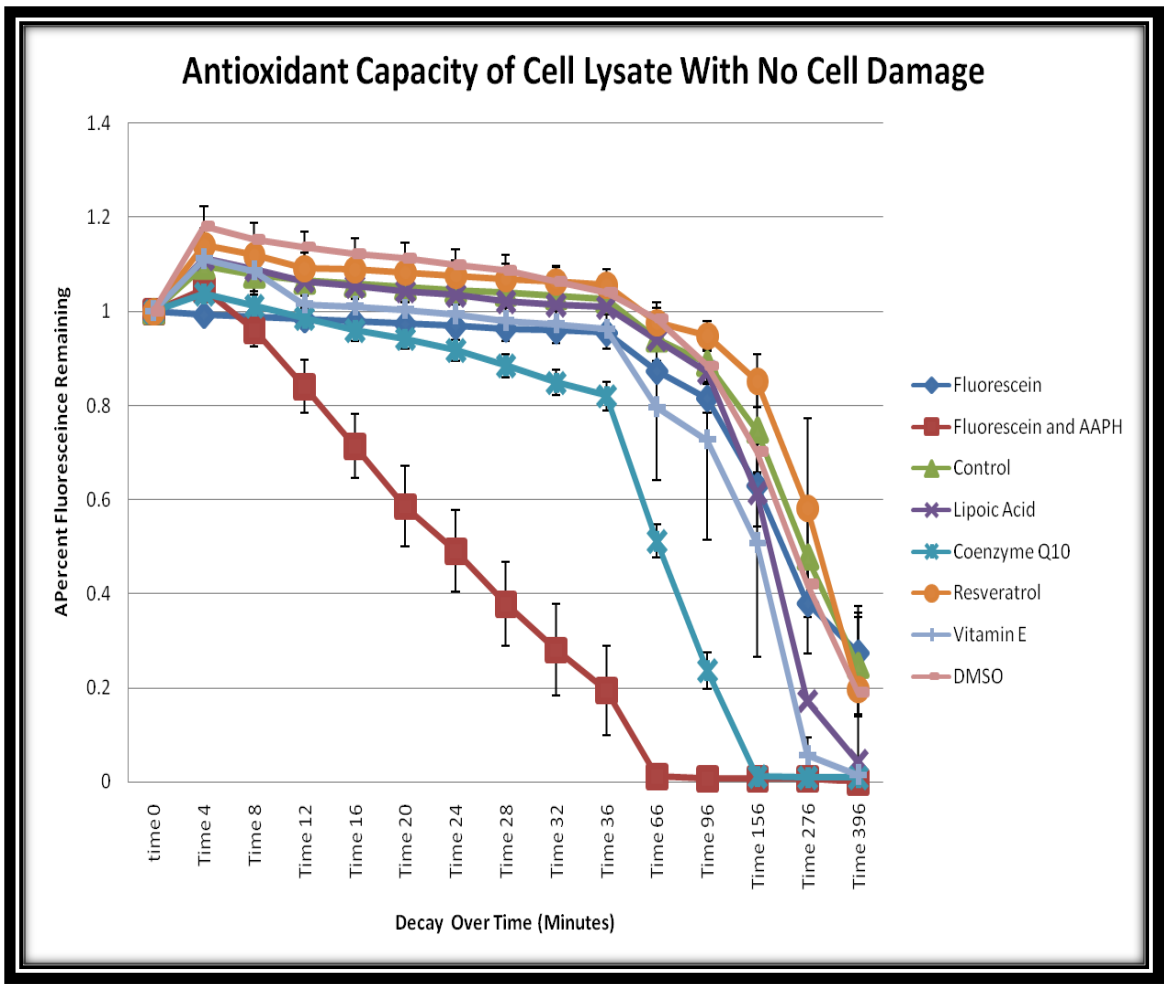


Figure 16A

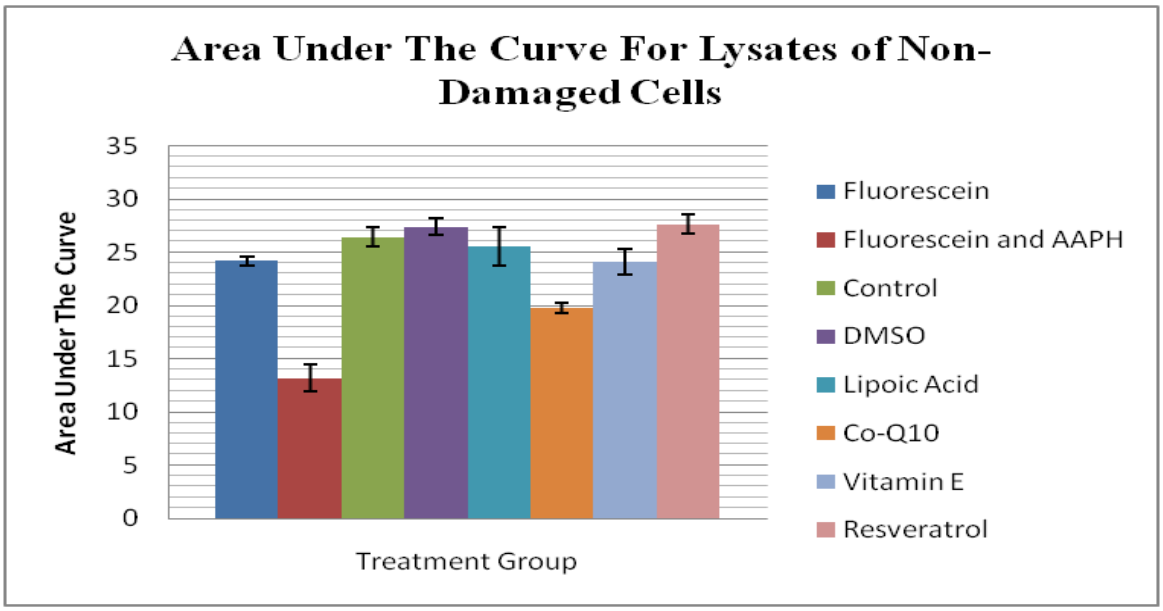


Figure 16B

No Statistically Significant Difference	
No Difference In Antioxidant Protection Provided By Control and Lipoic Acid	p<.1
Statistically Significant Difference	
DMSO Provided More Antioxidant Protection Than Control	p<.001
Control Provided More Antioxidant Protection Than CoQ_{10}	p<.0001
Control Provided More Antioxidant Protection Than Vitamin E	p<.001
Resveratrol Provided More Antioxidant Protection Than Control	p<.001

Table 16

Figures 16A & B and Table 16. Graph showing antioxidant capacities of cells after 24 hours incubation with fat soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. No damage means that cells were not damaged with hydrogen peroxide.The results demonstrate that CoQ_{10} did significantly worse than the other treatment groups, including plain DMSO. The high antioxidant capacity of DMSO alone suggests antioxidants did not have a significant effect on antioxidant capacity. DMSO could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 16B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

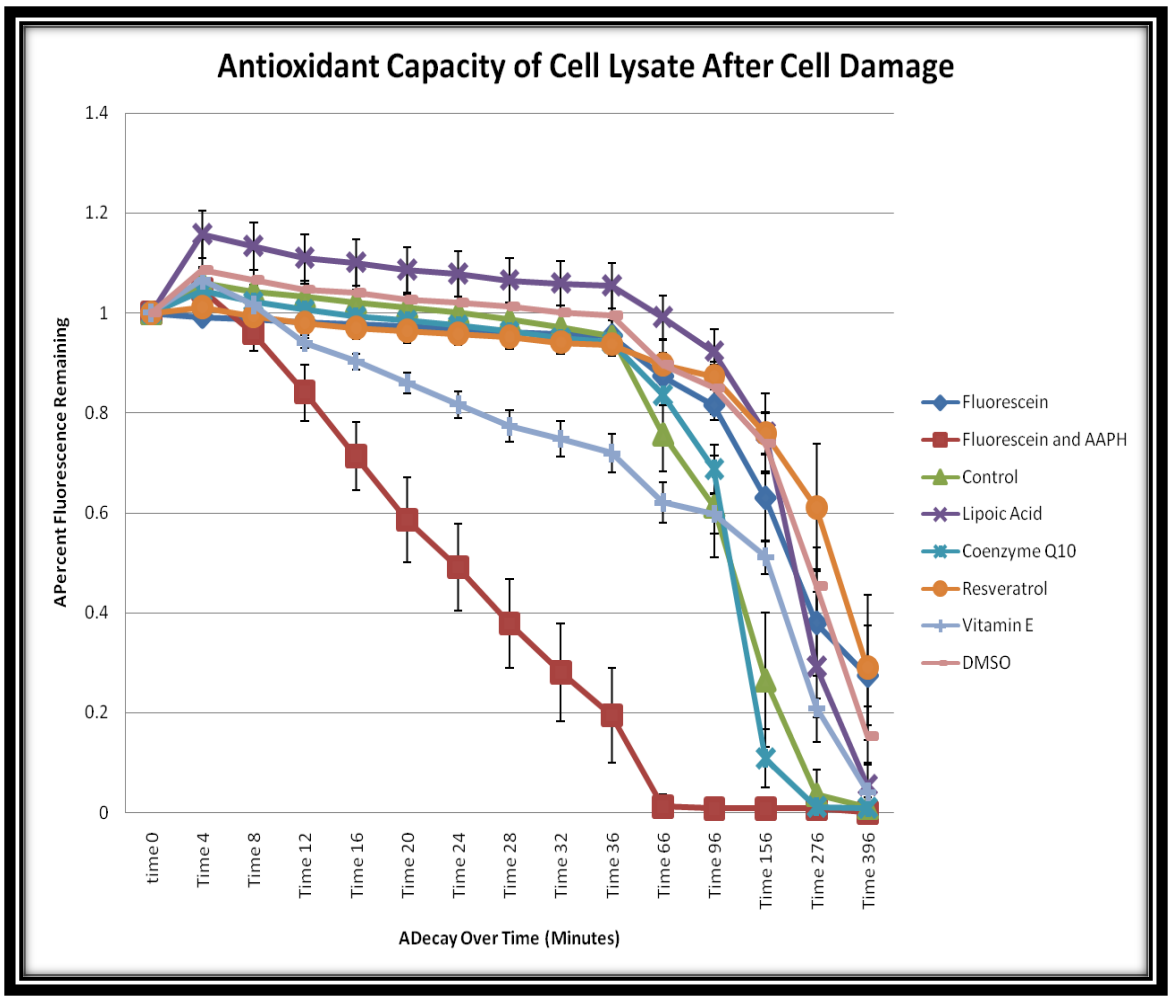


Figure 17A

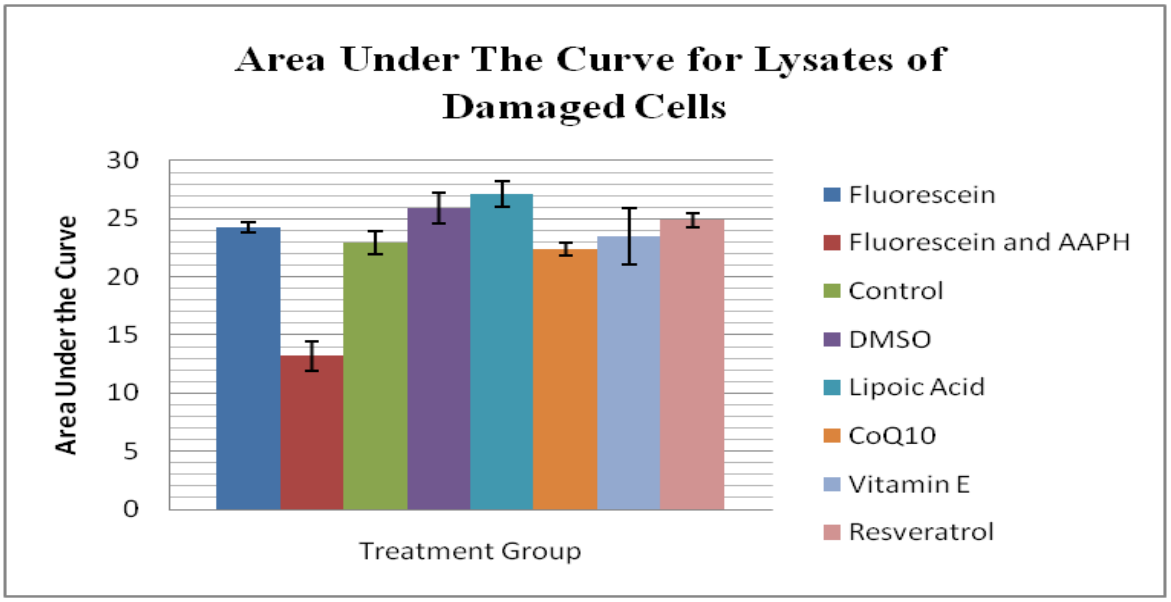


Figure 17B

No Statistically Significant Difference	
No Difference In Antioxidant Protection Provided By Control and CoQ_{10}	p<.1
No Difference In Antioxidant Protection Provided By Control and Vitamin E	p<.4
Statistically Significant Difference	
DMSO Provided More Antioxidant Protection Than Control	p<.0001
Lipoic Acid Provided More Antioxidant Protection Than Control	p<.0001
Resveratrol Provided More Antioxidant Protection Than Control	p<.0001

Table 17

Figures 17A & B and Table 17. Graph showing antioxidant capacities of cells after short Hydrogen Peroxide treatment and 24 hours incubation with water soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. Damage means that cells were damaged with hydrogen peroxide at a 1:10,000 H_2O_2 to water concentration for one hour before antioxidant treatment. The graph demonstrates that after hydrogen peroxide damage, Vitamin E had a lower degree of antioxidant protection and than Lipoic Acid protection improved. However, protection provided by DMSO was still very high suggesting that most of the antioxidant protection was provided by DMSO in the antioxidant solutions. DMSO could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 17B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

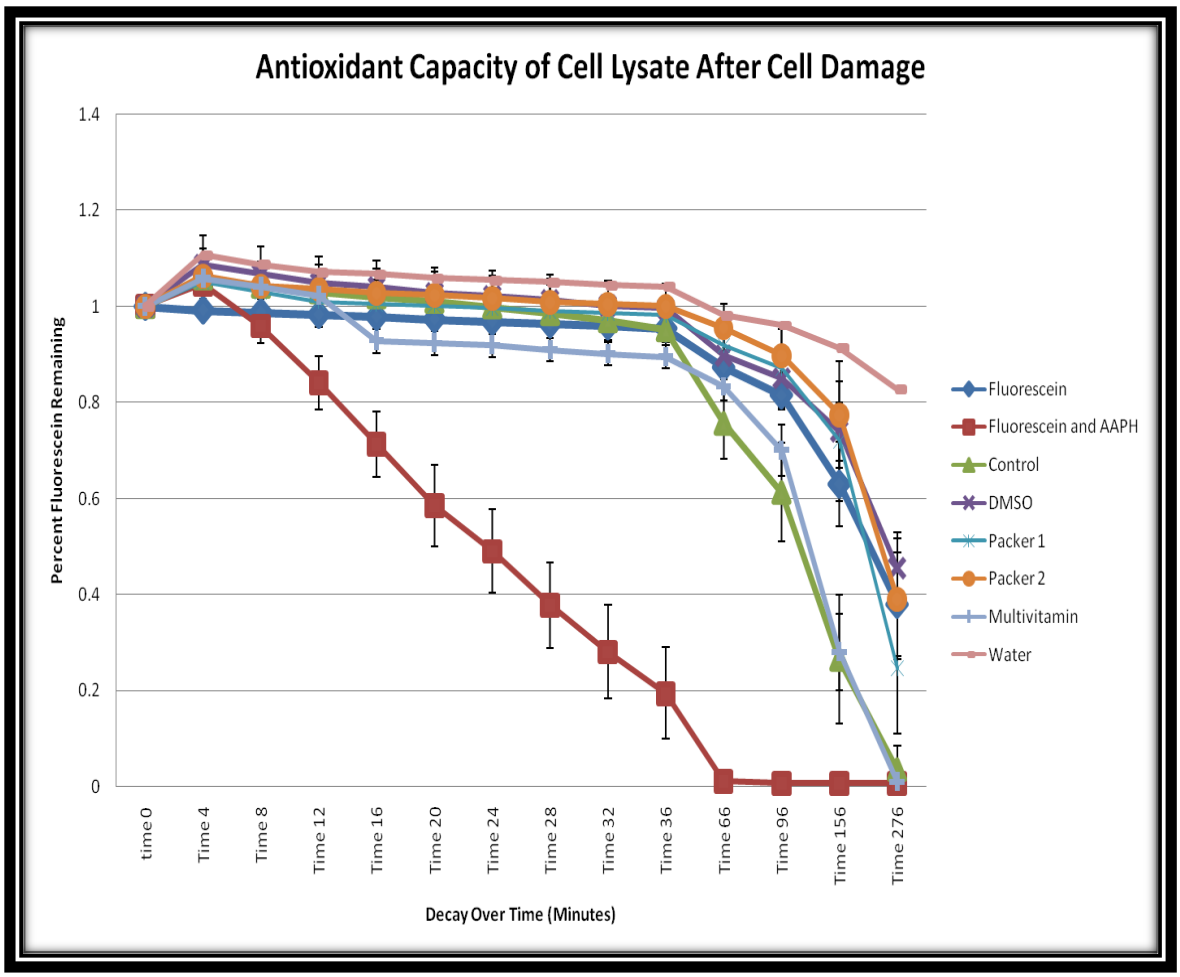


Figure 18A

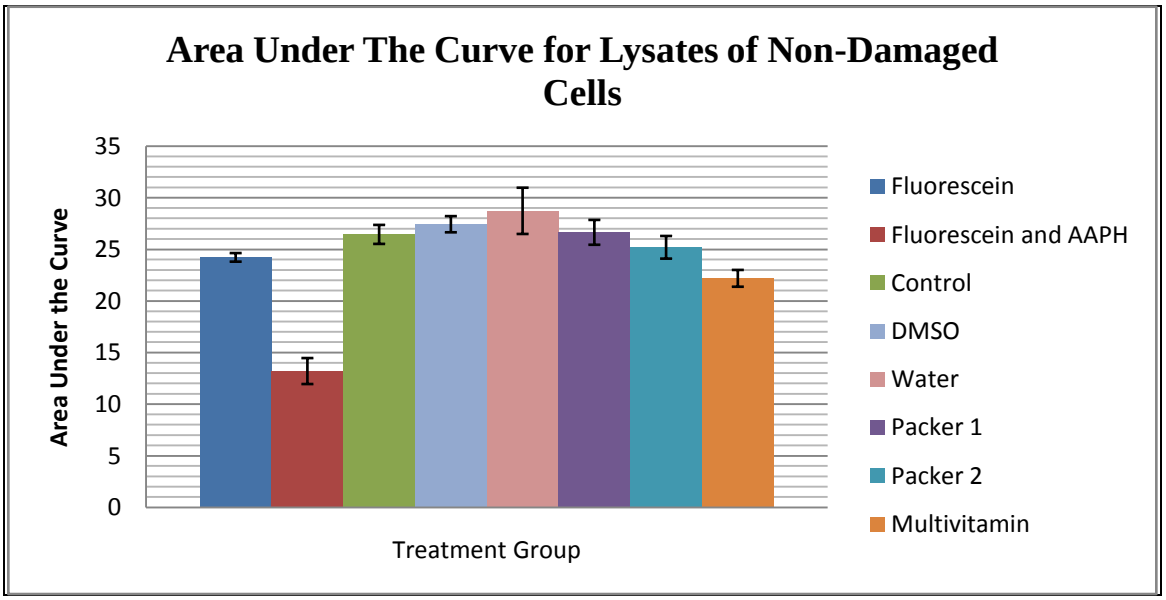


Figure 18B

Statistically Significant Difference	
Packer 1 Provided More Antioxidant Protection Than Control	p<.0001
Control Provided More Antioxidant Protection Than Packer 2	p<.005
Control Provided More Antioxidant Protection Than The Multivitamin	p<.0001

Table 18

Figures 18A & B and Table 18. Graph showing antioxidant capacities of cells after short Hydrogen Peroxide treatment and 24 hours incubation with water soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. No damage means that cells were not damaged with hydrogen peroxide. The result demonstrate that multivitamin treatment did most poorly. Water, or DMSO, could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 18B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

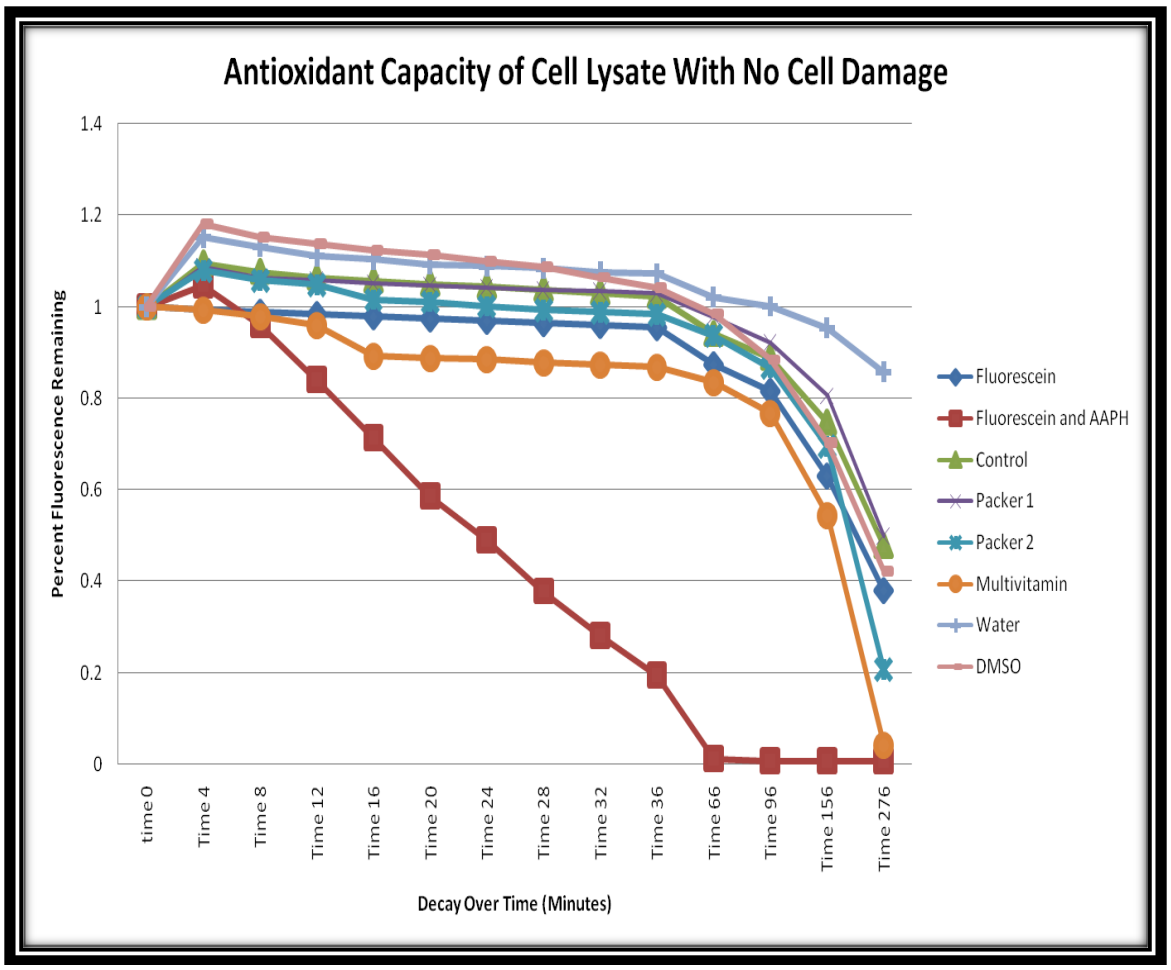


Figure 19A

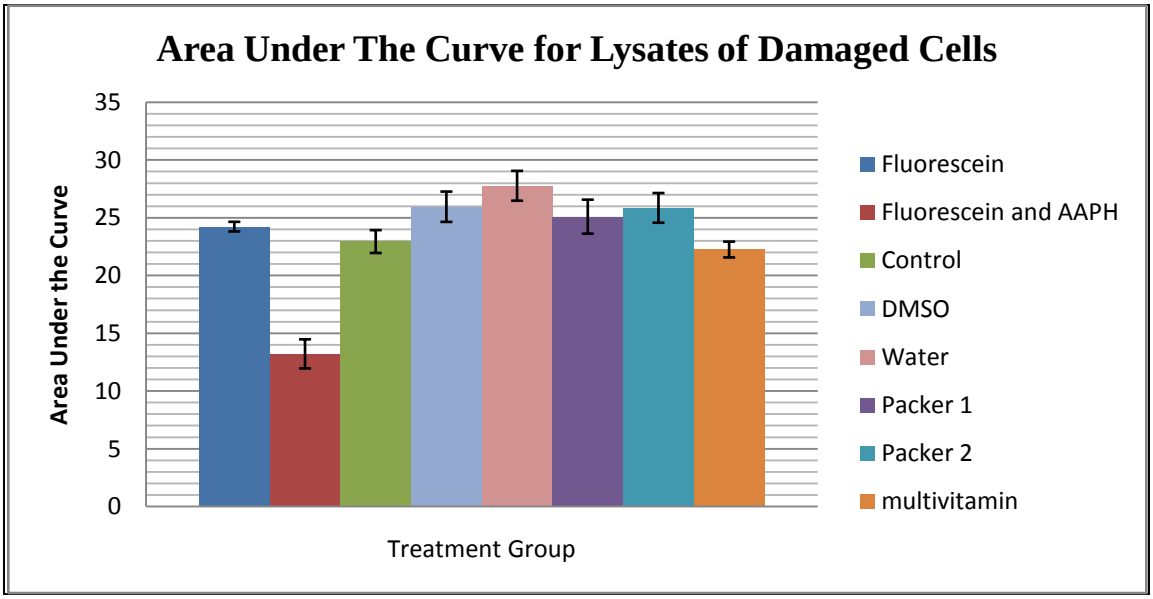


Figure 19B

Statistically Significant Difference	
Packer 1 Provided More Antioxidant Protection Than Control	p<.001
Packer 2 Provided More Antioxidant Protection Than Control	p<.0001
Control Provided More Antioxidant Protection Than The Multivitamin	p<.04

Table 19

Figures 19A & B and Table 19. Graph showing antioxidant capacities of cells after short Hydrogen Peroxide treatment and 24 hours incubation with water soluble antioxidants. Each sample had 24 repeats. Cells were incubated for 24 hours with antioxidant solutions in media at a 10 mM concentration. Control samples had no antioxidant treatment and were only incubated in media. Damage means that cells were damaged with Hydrogen Peroxide at a 1:10,000 H_2O_2 to water concentration for one hour before antioxidant treatment. The results demonstrate that multivitamin treatment still provided a lower level of antioxidant protection compared to the other antioxidant treatments. Water, or DMSO, could cause physiological stress on the cell and thus upregulate the cell's inate defenses. However, figure 19B demonstrates the small difference between the area under the curve of the water and antioxidant treatments, suggesting that these results are not statistically meaningful.

DISCUSSION

For this study, we wanted to examine Dr. Packer's theory of antioxidant networking to learn if the combination given in *The Antioxidant Miracle* may have a superior protective capacity compared to other single and combination treatments. Based on the research of other laboratories, such as Dr. Packer's, we expected to see that combination treatments would prove to be superior antioxidants because of the "recharging" abilities they would have on one another. We also theorized that multivitamins would prove to be the most superior antioxidants seeing as they contain many types of vitamins and minerals. Before discussing our conclusions, however, a reiteration of observations for each antioxidant and combination would be helpful in understanding our conclusions.

Our *in vitro* results for vitamin C alone suggest that it is an inferior antioxidant to all other single antioxidants (figure 2A) and Packer combinations (figure 4) tested. Our results also suggest that removal of vitamin C from the Packer 2 combination results in an improvement in antioxidant protection (figure 6A). Vitamin C did not greatly affect the viability of cells with which it was incubated for 24 hours.

However, the *in vivo* results of the cell lysates for vitamin C suggest that it did provide a higher level of antioxidant protection, in the lysate of the cells with which it was incubated, than the control sample lysates (figures 14A and 15A).

In vitro cell culture results for glutathione demonstrated that it was the best water soluble Packer antioxidant in terms of single antioxidant protection (figure 2A). Also shown is that the removal of glutathione from the four part combinations results in an improvement in antioxidant protection in the ORAC assay (figure 6A).

The *in vivo* results demonstrated that glutathione did not greatly affect the viability of the cells with which it was incubated (figure 12A). Further, glutathione appeared to provide a higher level of antioxidant protection for the cell lysate than the control samples (figure 14A and 15A).

The *in vitro* results for vitamin E show that there is no statistically significant difference between the protection provided by vitamin E and CoQ_{10}, which were the worst single fat soluble antioxidants (figure 1A). However, vitamin E did provide more protection than the Packer 1 combination (figure 4A). The removal of vitamin E drastically improves the Packer combination antioxidant protection (figure 5A).

The *in vivo* results demonstrated that the cells grown with vitamin E had a lower viability (figure 13A) and suggest that it did not provide a higher level of antioxidant protection than the control samples (figure 16A and 17A).

From the results we observe that CoQ_{10} was one of the worst single fat soluble antioxidants in the *in vitro* ORAC analysis (figure 1A). Our results demonstrate that removal of CoQ_{10} drastically improves the Packer combination in terms of antioxidant protection in the ORAC assay (figure 5A).

The *in vivo* results suggest that CoQ_{10} did not provide a higher level of antioxidant protection than the control samples (figure 16A and 17A).

In vitro results for lipoic acid showed that it was the best fat soluble Packer antioxidant in terms of single antioxidant protection (figure 1A). Interestingly, four part combinations decreased in terms of antioxidant capacity with the subtraction of every antioxidant but lipoic acid. Removal of lipoic acid improves the combination initially, but

falls drastically below the Packer combination antioxidant protection after 20 minutes (figure 5A).

Our *in vivo* results suggest that lipoic acid did not provide a higher level of antioxidant protection than the control samples (figure 16A) unless the cells were damaged (figure 17A).

Our *in vitro* results suggested that resveratrol was a superior antioxidant compared to any single or combination treatments tested, in that it provided a higher level of antioxidant protection initially, and remained very high throughout testing (fiugres 1A and 2A). In fact, the results of our *in vitro* study showed that resveratrol actually had higher fluorescent counts than our control of fluorescein with no AAPH. This occurance was not surprising to us, however. Our theory as to how resveratrol could raise the fluorescent counts is that it is not only able to protect the fluorescein from degradation by oxidation from AAPH, but also from oxidation by air that comes into contact with the samples. Thus control samples will be exposed to air and have no antioxidant protection, which could explain why the sample with resveratrol had higher fluorescent counts than the control.

In vivo results suggest that resveratrol did provide a higher level of antioxidant protection than the lysate control samples (figures 16A and 17A).

Now having reviewed single antioxidant treatment results, here follows the combination treatment reuslts.

For review, Packer 1 is a combination of the 5 Packer antioxidants in the ratio that is listed in *The Antioxidant Miracle*. In the combination there were low levels of glutathione, CoQ_{10}, and lipoic acid. It also contained high levels of vitamins C and E.

The results of the *in vitro* Packer 1 testing demonstrated that the Packer combination in the ratio recommended provides the least amount of protection in the ORAC assay compared to the Packer 2 combination, the fat soluble vitamins (figure 4A), and glutathione alone (figure 3A); however, it is a superior antioxidant compared to vitamin C alone (figure 3A).

The viability results of Packer 1 were surprising. For non-damaged cells the Packer 1 combination greatly enhanced the viability above the DMSO control. However, for damaged cells, the results were expected in that the viability was very low (figure 12A and 13A). The lysate results suggest that Packer 1 did not provide a higher level of antioxidant protection in non-damaged cells compared to the control samples (figure 18A). However, Packer 1 did provide a higher level of protection in damaged cells compared to the control cells (figure 19A).

For review, Packer 2 is a combination of the 5 Packer antioxidants in even amounts in solution. The Packer 2 combination, in comparison to Packer 1, had an increase in the amount of lipoic acid, and glutathione, and a decrease in the amounts of vitamin C and vitamin E, which might explain why it appeared to be a better antioxidant combination than Packer 1 in the *in vitro* analysis (figure 4A).

Lipoic acid and glutathione are the only single antioxidants that appeared to be superior in terms of antioxidant protection compared to the Packer 2 combination (figure 3A). CoQ_{10}, a relatively poor antioxidant in our results, was also increased in quantity for Packer 2, decreasing its antioxidant capacity. Vitamins E and C were also poor antioxidants, and the amounts of each were decreased in the Packer 2 combination, which might explain why it was a superior antioxidant.

The *in vivo* results suggest that Packer 2 did not provide a higher level of antioxidant protection than the control samples.

Our analysis of multivitamin solutions demonstrated multivitamins geared towards the elderly are superior antioxidants than those for the general public. Further, statistical analysis demonstrated that Equaline Prenatal was the most superior multivitamin tested in terms of antioxidant protection. Equaline was also a superior antioxidant combination to that of the Packer combinations in our study.

Our *in vivo* results demonstrated that the multivitamin Equaline provided less protection than any of the the other combination treatements and the control.

The complex nature the antioxidants and their combinations make the results surprising, and at times difficult to interpret. Further, our *in vitro* and *in vivo* analysis was limited in terms of literal interpretation, and thus value of analytical data. However, seeing as we had no true *in vivo* model in which to test antioxidant combinations, we felt our analysis models could, at a minimum, suggest antioxidant capacity trends and indicate in which direction we should take our research. Having acknowledged our analysis limitations, we would like to state some of the tentative conclusions which we have drawn.

Our *in vitro* analysis led us to conclude that the majority of the antioxidants retained activity for longer periods of time when tested alone, rather than in combination as Dr. Packard's theory has suggested. This tentative conclusion is in stark contrast to our original hypothesis in that we believed that antioxidants have the ability to recharge one another, which was not clearly shown in this study.

Our four part combination experiments also produced surprising results in that there was an improvement in the antioxidant protection provided in these samples compared to the control, except in the case of lipoic acid. This would tend to suggest that lipoic acid truly is unique compared to the other Packer antioxidants, possibly because of its ability to act as a central recharging antioxidant as Dr. Packer and others have suggested.

The basic analysis of single antioxidants did, however, produce some results which were anticipated. Vitamin C was the poorest antioxidant alone in our *in vitro* analysis, and its removal significantly improved the Packer combination. This result was not surprising because it is well accepted that vitamin C is a short term antioxidant which does not provide long term antioxidant protection (28)(29).

Continuing with single antioxidant analysis, resveratrol was found to be the best single antioxidant, providing equal or superior antioxidant protection compared to combinatorial treatments. This was not a surprising result seeing as resveratrol is one of the best antioxidants known at this time.

In our analysis of multivitamin protection, we have come to believe that multivitamins geared towards expectant mothers and the elderly are generally superior antioxidants than those intended for the general public. Further, we produced results that suggest that multivitamins are, at times, meaningfully statistically different in antioxidant capacity from one another depending on brand and expiration data. In terms of comparison, multivitamins were better antioxidants than the Packer 1 combination *in vitro*; however, *in vivo* they were poor antioxidants. These conflicting results leave us

unable to conjecture their actual antioxidant capacities *in vivo* compared to the Packer combinations.

From this preliminary study, it is clear that more antioxidant research must be done in order to grasp the separate roles and synergistic affects of the many antioxidants on the market today. Research should not only look into possible *in vitro* models that suggest antioxidant synergy and probable health benefits, but *in vivo* models that demonstrate synergy in the context of the human body. However, such trials are complicated, and the results can be misleading for many reasons. Firstly, results of such trials are confusing because all individuals have some level of antioxidant consumption from their diets and physiological production; therefore the effect of a supplement depends on the additional amount of a given antioxidant. Further, trial participants characteristically have good diets, so the results of a study finding no effect of a supplement might not apply to those with poorer diets (17).

Because *in vitro* models are difficult to interpret, and more prone to inaccuracy, and *in vivo* trials are expensive, long, and also prone to error, I feel that the best suggestion would be to use animal models in the study of antioxidants. *In vivo* animal models would have the added benefit of a biological setting with realistic comparisons to human physiological reactions. At the same time, results would not be prone to error in that specimen diet and genetics could be controlled. For example, the use of an animal model prone to any of the diseases associated with free radical damage would provide an ideal model in which to test different antioxidant combinations for synergy.

Although there is little doubt that antioxidants are a necessary component of good health, no one knows if supplementation is vital to optimum health and, if so, how much

should be taken (56). Antioxidant supplements were once thought to be harmless for the healthy general public; however, increasingly we are becoming aware of interactions and potential toxicity (57). Further, very little is known about the long term consequences of super doses of antioxidants. The body's defense mechanisms are carefully balanced to withstand a variety of insults. Taking substances lacking a complete understanding of all effects may unknowingly lead to a disruption in this balance. For this reason, antioxidant research is an important field of study.

Until more conclusive results are found concerning which antioxidants provide the best oxidative protection, a daily multivitamin that does not surpass the recommended daily allowance of its individual vitamins would be a wise decision for most individuals (17)(56).

CONCLUSION

- The majority of the antioxidants retained activity for a longer period of time when tested alone, rather than in combination.
- Four part combinations improved in antioxidant protection, except in the case of lipoic acid.
- Vitamin C was the poorest antioxidant alone *in vitro,* and its removal significantly improved the Packer combination.
- Resveratrol was found to be the best single antioxidant, providing equal or superior antioxidant protection compared to combinatorial treatments.
- Multivitamins geared towards expectant mothers and the elderly are generally superior antioxidants than those intended for the general public.
- Multivitamins were better antioxidants than the Packer 1 combination *in vitro*. However, *in vivo* multivitamins were poor antioxidants.

REFERENCES

1. **Pacher P, Beckman JS, Liaudet L.** 2007. Nitric oxide and peroxynitrite in health and disease. Physiol. Rev. 87 (1): 315–424.
2. **Sies H.** 1997. Oxidative stress: oxidants and antioxidants. Exp Physiol 82 (2): 291-295.
3. **Nakabeppu Y, Sakumi K, Sakamoto K, Tsuchimoto D, Tsuzuki T, Nakatsu Y.** 2006. Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. Biol Chem 387 (4): 373-379.
4. **Valko M, Izakovic M, Mazur M, Rhodes C, Telser J.** 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266 (1–2): 37–56.
5. **Stadtman E.** 1992. Protein oxidation and aging. Science 257 (5074): 1220–1224.
6. **Valko M, Leibfritz D, Moncol J, Cronin M, Mazur M, Telser J.** 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39 (1): 44–84.
7. **Raha S, Robinson B**. 2000. Mitochondria, oxygen free radicals, disease and aging. Trends Biochem Sci 25 (10): 502-508.
8. **Davies K.** 1995. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp 61: 1–31.
9. **Villena J, Henriquez M, Torres V, Moraga F, Díaz-Elizondo J, Arredondo C, Chiong M, Olea-Azar C, Stutzin A, Lavandero S, Quest AF**. 2008. Ceramide-induced formation of ROS and ATP depletion trigger necrosis in lymphoid cells. Free Radic Biol Med. 44 (6): 1146-60.
10. **Gomez-Cabrera MC, Martínez A, Santangelo G, Pallardó FV, Sastre J, Viña J**. 2006. Oxidative stress in marathon runners: interest of antioxidant supplementation. Br J Nutr. Suppl (1):S31-33.
11. **Gomez-Cabrera MC, Domenech E, Viña J**. 2007. Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. Free Radic Biol Med. 44 (2):126-131.
12. **Sachdev S, Davies KJ**. 2008. Production, detection, and adaptive responses to free radicals in exercise. Free Radic Biol Med. 44 (2):215-223.
13. **Vertuani S, Angusti A, Manfredini S.** 2004. The antioxidants and pro-antioxidants network. Curr Pharm Des 10 (14): 1677–1694.
14. **Chaudière J, Ferrari-Iliou R.** 1999. Intracellular antioxidants: from chemical to biochemical mechanisms. Food Chem Toxicol 37 (9–10): 949 –962.
15. **Sies H.** 1993. Strategies of antioxidant defense. Eur J Biochem 215 (2): 213 –219.
16. Dietary Reference Intakes: Vitamins The National Academies, 2001.
17. **Walter C Willett, M.D., Dr. P.H., and Meir J Stampfer, M.D., Dr. P.H**. 2001. What vitamins Should I Be Taking, Doctor? New England Journal of Medicine. (345): 1819-1824.
18. **North/South Ireland Food Consumption Survey Public Health Nutrition**. 2001. The efficacy and safety of nutritional supplement use in a representative sample of adults. Public Heal Nutr. (4): 1089-1097.

19. **Mayo Clinic**. 2007. Dietary supplements: Using vitamin and mineral supplements wisely. Retrieved May 18, 2008, from http://www.mayoclinic.com/health/supplements/NU00198.com
20. **RCSB Protein Data Bank**. 2004. Molecule of the Month. Catalase. 09-01.
21. **Chi Chiu Wang, Ching Yan Chu, Kai On Chu, Kwong Wai Choy, Kim Sun Khaw, Michael Scott Rogers' and Chi Pui Pang. 2004.** Trolox-Equivalent antioxidant capacity assay versus oxygen radical absorbance capacity assay in plasma. Clinical Chemistry. (50):952-954.
22. **Benzie IF, Strain JJ**. 1996. The ferric reducing ability of plasma FRAP as a measure of antioxidant power. Anal Biochem. 239 (1):70-76.
23. **Tomer DP, McLeman LD, Ohmine S, Scherer PM, Murray BK, O'Neill KL.** 2007. Comparison of the total oxyradical scavenging capacity and oxygen radical absorbance capacity antioxidant assays. Journal of Medicinal Food. 10 (2): 337-344.
24. **Stefania Gorbi, Francesco Regoli**. 2003. Total oxyradical scavenging capacity as an index of susceptibility to oxidative stress in marine organisms. Comments on Toxicology. 9 (5): 303 – 322.
25. **Cao G, Verdon CP, Wu AHB, Wang H, Prior RL**. 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clin Chem. (41):1738-1744.
26. **Cao G, Alessio H, Cutler R**. 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 14 (3): 303–311.
27. **Ou B, Hampsch-Woodill M, Prior R**. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agric Food Chem 49 (10): 4619–4626.
28. **Packer L., Coleman C.** 1999. The antioxidant miracle. John Wiley and Sons inc.
29. **Wilson JX**. 2005. Regulation of vitamin C transport. Annu. Rev. Nutr. (25): 105–25.
30. US Recommended Dietary Allowance. 2007. Retrieved May 28, 2008, from http://fnic.nal.usda.gov/nal_display/index.php
31. **Witschi A, Reddy S, Stofer B, Lauterburg BH**. 1992. The systemic availability of oral glutathione. Eur J Clin Pharmacol. 43(6):667-9.
32. **De Quay B, Malinverni R, Lauterburg BH**. 1992. Glutathione depletion in HIV-infected patients: role of cysteine deficiency and effect of oral N-acetylcysteine. AIDS. 6 (8):815-819 ,
33. **Pendyala L, Creaven PJ**. 1995. Pharmacokinetic and pharmacodynamic studies of N-acetlycysteine, a potential chemopreventative agent during phase I trial. Cancer Epidemiol Biomarkers Prev 4 (3):245-51.
34. **Traber MG and Packer L**. 1995. Vitamin E: Beyond antioxidant function. Am J Clin Nutr. (62):1501S-1509S.
35. **Meydani, M., R.A. Fielding, N. Fotouhi.** 1996. Vitamin E In: Sports Nutrition Vitamins and Trace Minerals. CRC Press. p. 119-131.
36. **Traber, M.G**. 1999. Vitamin E. In: Modern Nutrition in Health and Disease. Ninth Edition. p. 347-362.)

37. **Food and Nutrition Board, Institute of Medicine**. 2000. Vitamin E. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. National Academy Press. p.186-283.
38. **Ernster L, Dallner G**. 1995. Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta. (1271): 195-204.
39. **Dutton PL, Ohnishi T, Darrouzet E, Leonard, MA, Sharp RE, Cibney BR, Daldal F, Moser CC.** 2000. 4 Coenzyme Q oxidation reduction reactions in mitochondrial electron transport (pp 65-82) in Coenzyme Q: Molecular mechanisms in health and disease. p. 65-82.
40. **Okamoto, T**.1989. Interna.J.Vit.Nutr.Res. (59):288-292
41. **Aberg F**. 1992. Archives of Biochemistry and Biophysics. (295):230-234.
42. **Shindo, Y., Witt, E., Han, D., Epstein, W., and Packer, L**. 1994. Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin, Invest. Dermatol. (102):122-124.
43. **Kaikkonen J, Tuomainen TP, Nyyssonen K, Salonen JT**. 2002. Coenzyme Q10: absorption, antioxidative properties, determinants, and plasma levels. Free Radic Res. 36 (4):389-97.
44. **Walle, Faye Hsieh, Mark H. DeLegge, John E. Oatis, Jr., and U. Kristina Walle**. 2004. Drug metabolism and disposition fast forward. High absorption but very low bioavailability of oral resveratrol in humans. p. 495-586
45. **Bunin AIa, Filina AA, Erichev VP**. 1992. A glutathione deficiency in open-angle glaucoma and the approaches to its correction. Vestn Oftalmol. 108(4-6):13-15.
46. **Costagliola C, Iuliano G, Menzione M**. 1986. Effect of vitamin E on glutathione content in red blood cells, aqueous humor and lens of humans and other species. Exp Eye Res. 43(6): 905-14.
47. **Winkler BS, Orselli SM, Rex TS**. 1994. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic Biol Med. 17(4):333-349
48. **Stoyanavsky DA, Goldman R, Darrow RM**. 1995. Endogenous ascorbate regenerates vitamin E in the retina directly and in combination with exogenous dihydrolipoic acid. Curr Eye Res. 14 (3):181-189.
49. **Alton Meister**. 1994. Glutathione-Ascorbic Acid Antioxidant System in Animals, The Journal of Biological Chemistry. 269 (13): 9397-9400.
50. **Pulvertaft JV**. 1964. Cytology of Burkitt's tumour (African lymphoma). Lancet (1): 238-240.
51. **S. Matthijs Boekholdt, Marijn C. Meuwese, Nicholas E. Day, Robert Luben, Ailsa Welch, Nicholas J. Wareham and Kay-Tee Khaw.** 2006. Plasma concentrations of ascorbic acid and C-reactive protein, and risk of future coronary artery disease, in apparently healthy men and women: the EPIC-Norfolk prospective population study. British Journal of Nutrition. (96): 516–522
52. **Xiang Gao, Martin Antonio, Hai Lin, Bermudez Odilia I, Tucker Katherine L.** 2006. α-tocopherol intake and plasma concentration of hispanic and non-hispanic white elders is associated with dietary intake pattern. The Journal of nutrition. (136): pp. 2574-2579

53. **David A. Carlson; Anthony R. Smith, PhD; Sarah J. Fischer; Karyn L. Young; Lester Packer, PhD.** 2007. The Plasma Pharmacokinetics of R-(+)-Lipoic Acid Administered as Sodium R-(+)-Lipoate to Healthy Human Subjects. Alternative Medicine Review. (12): Pg 343-351
54. **Littarru, et al.,** 1991. Metabolic and diagnostic implications of human blood Q10 levels, in Biomedical and Clinical Aspects of Coenzyme Q. Elsevier North Holland. (4): pp. 167-178
55. **David W Essex, Mengru Li, Richard D Feinman and Anna Miller.** 2004. Platelet Surface Glutathione Reductase-Like Activity. Blood. (1): Pg 1-13
56. **Robert H. Fletcher MD, Kathleen M. Fairfield**. 2002. Vitamins for chronic disease prevention in adults, Clinical applications. JAMA. (287): 3127-3129.
57. **Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C**. 2008. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database of Systematic Reviews*. Issue 2.