

Comparison of the Preventative Effects of Vitamins C and E in the Formation of Cataracts in Murine Lenses

**By
Karla E Atcheson
April, 2018**



**Allegheny College
Department of Biology**

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**A Senior Comprehensive Project in Partial Fulfillment of the
Requirements for a Bachelor of Science Degree from Allegheny College**

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

Pledge

Approval by the Senior Thesis Committee:

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Table of Contents

Acknowledgments.....1

Abstract.....3

Introduction.....4

Methods.....10

Results.....17

Discussion.....20

References.....26

Appendices.....29

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Abstract

Age-related cataracts are the cause of nearly 50% of all cases of blindness worldwide and about half of all visual impairment cases in the USA. Research has shown that the consumption of antioxidants may help reduce the severity of the cataract formed in the lens. The aim of this study was to compare the preventative effects of vitamins C and E in the formation of cataracts in murine lenses. Whether vitamins C and E have a synergistic effect was also tested. Wild-type mice were given weekly vitamin injections for four weeks. At the end of the treatment period, mice were sacrificed, and both of their lenses were collected. The formation of a cataract was induced using H_2O_2 , and the protein carbonyl concentration within the lens was analyzed. The vitamin C group had the lowest mean carbonyl concentration of all of the treatment groups (439 ± 155 nmol/mg), while the control group had the highest (933 ± 1117 nmol/mg). The cataracts tended to be less severe in those two groups than those from the vitamin E and the combination group which suggests that vitamin C may be a more effective antioxidant in the lens. This study provides insight into possible treatments can be prescribed to help protect the lens from the formation of a cataract.

Introduction

The eye is responsible for detecting light and enabling sight. The lens is located inside the eye and is important in the process focusing the light in the eye. The primary role of the lens is to refract light onto the retina; it must remain transparent. It sits posterior to the cornea between the vitreous and aqueous humors which both provide nourishment for the lens (Figure 1). The lens itself is made up of protein fibers called crystallins. The lens grows continuously, and old cells are not destroyed but instead are compressed towards the center of the lens as new cells form around them (Taylor, 1995).

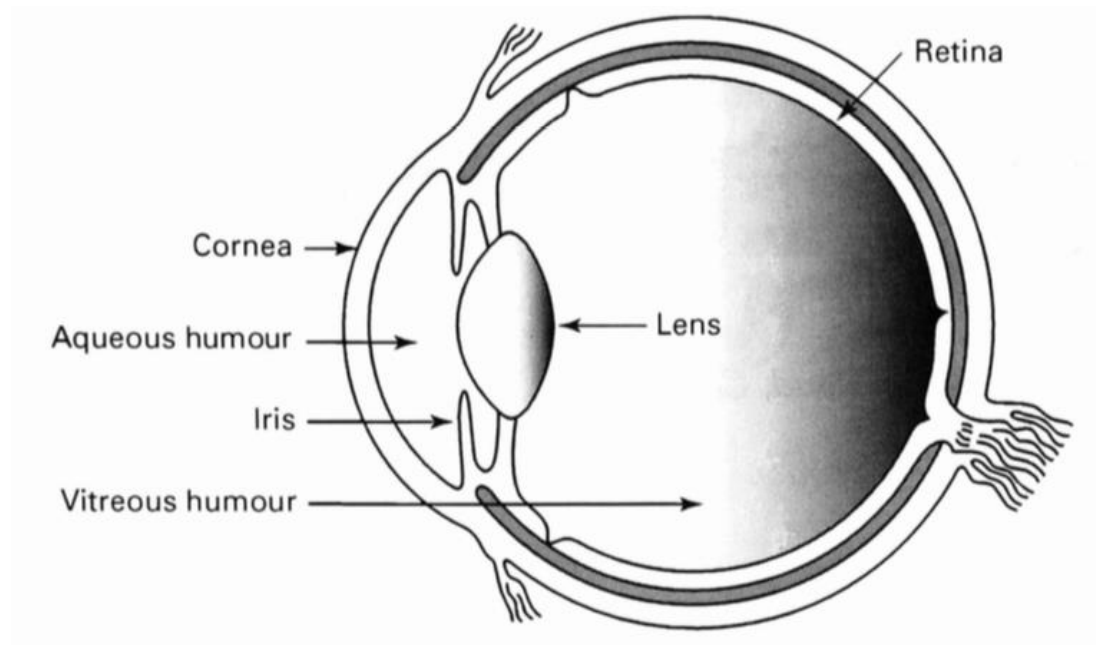


Figure 1. Cross section of the eye (van der Pols, 1999)

The system in the eye for lens growth is unique in which epithelial cells on the surface of the lens differentiate into new fiber cells. These new cells are laid over the existing cells which are then compressed towards the center of the lens. This process continues throughout life, and the lens grows larger with the oldest cells packed in the nuclear or

central region of the lens (Augusteyn, 2007). As the lens ages, it loses its flexibility and increases in size (Taylor, 1995). In the lens, normal protein repair mechanisms do not exist because there is no DNA or RNA in the cells. This leaves the lens vulnerable to damage from harmful substances that may be present in the aqueous or vitreous humors. The lens is maintained by the epithelium and a thin layer of developing fiber cells to protect the transparency of the lens (Spector, 1995).

A cataract is an opacification or cloudiness of the lens that prevents light from being refracted onto the retina (Spector, 1995). Cataracts form due to the crystallin proteins within the lens unfolding and denaturing which leads to protein aggregation or clumping. As aggregation continues uncontrolled, the proteins eventually cause scattering of light (Horwitz, 2003). Age-related cataracts originate in one of three regions of the lens: the nucleus, cortex, or posterior pole. These sites result in the formation of a nuclear, cortical or posterior subcapsular cataracts (PSC), respectively. Nuclear cataracts occur in the lens nucleus which is composed of fiber cells that have been present since birth. Cortical cataracts occur in mature fiber cells in the outer half of the lens in cells that were formed after birth. Posterior subcapsular cataracts form in fiber cells that failed to differentiate properly; PSCs are the least common type of cataract (Beebe et al., 2010).

Age-related cataracts that form in the lens of the eye are responsible for nearly 50% of all cases of blindness in the world and about half of all visual impairment cases in the USA (Beebe et al., 2010). Cataract surgery is an effective treatment for blindness caused by cataracts and is the most expensive and common ocular surgery in the USA, costing approximately \$3 billion annually (Beebe et al., 2010). In the USA, there are more than 1 million cataract operations performed every year, and it is the most frequently performed

surgery in people ages 65 and over (Spector, 1995). It has been proposed that if the development of cataracts could be delayed by 10 years, then the cost of cataract surgery would be diminished by 50% (Wynn & Wynn, 1996).

The primary risk factor for the formation of cataracts is increasing age, while other risk factors include diabetes mellitus, smoking, alcohol use, dehydration, hyperoxia, low socioeconomic status, nutritional deficiencies, and female gender (van der Pols, 1999). The prevalence of cataracts is greater in people who live in areas with a higher intensity and duration of sunlight. It is also widely accepted that oxidative damage through exposure to UV light is one of the initiating events in the formation of cataracts (van der Pols, 1999).

Reactive oxygen species are the result of cellular metabolism and exist in the body in low concentrations. At higher concentrations, they can cause damage to various cell components including DNA, lipids and proteins. When there is a shift in oxidant and antioxidant levels favoring oxidants, it is known as oxidative stress. Oxidative stress contributes to many conditions including cancer, neurological disorders, hypertension, chronic obstructive pulmonary disease, and asthma (Birben et al., 2012). One of the major endogenous oxidants is hydrogen peroxide (H_2O_2) which is derived from superoxide. Studies have shown that high levels of H_2O_2 are generated in the lens with photochemically-induced cataracts (van der Pols, 1999). Oxidative stress in the lens has been shown to cause the formation of protein carbonyls and increased levels of carbonyls corresponds with cataract formation (Boscia et al., 2000).

There is evidence to suggest that H_2O_2 is one of the major oxidants that contribute to cataract formation. In one study, lenses from patients with cataracts were found to have elevated H_2O_2 levels ranging from two- to seven-fold higher than the normal range (Spector,

1995). When the H_2O_2 level was measured in the aqueous humor, it was elevated as much as 30-fold greater than normal (Spector, 1995).

Various antioxidants in the body can counteract the effects of oxidants, and they can be divided into two categories: enzymatic and non-enzymatic. For the context of this study, non-enzymatic antioxidants will be the primary focus. Examples of non-enzymatic antioxidants include vitamins A, C, E, beta-carotene and glutathione. In the study, the two primary antioxidants will be vitamins C (ascorbic acid) and E (alpha-tocopherol) (Birben et al., 2012).

Vitamin C is water-soluble and provides protection by scavenging oxygen radicals and neutralizes vitamin E radicals (Birben et al., 2012). Vitamin C helps with the detoxification of H_2O_2 by maintaining low concentrations (1-2 mM) in the aqueous humor (van der Pols, 1999). Vitamin E is lipid-soluble and is the primary defense mechanism against oxidant-induced membrane injury. Vitamin E is present in the lens in very low concentrations and in several *in vitro* experiments, it was suggested to have a protective role against cataracts through the protection of membrane lipids against peroxidation (Ohta et al. 1996). Other studies have suggested that higher levels of plasma Vitamin E have a protective effect (Knekt et al. 1992; Leske et al. 1996). Vitamin E also triggers apoptosis of cancer cells and inhibits free radical formation (Birben et al., 2012).

Of all the molecules present in the aqueous humor, vitamin C is present in concentrations ten to twenty times greater than those in the plasma, which indicates that vitamin C is actively transported into the eye (Wei et al., 2016). A relationship between vitamin C and cataracts has been established based on the observed decrease in lens vitamin C levels with increasing age and cataract severity. Using this information, it has been

suggested that vitamin C can prevent or delay experimentally-induced cataracts (Devamanoharan et al., 1991; Reddy et al., 1998; Spector et al., 1998; Wei et al., 2016). Reddy et al. used an *in vivo* experiment to show that high levels of vitamin C in the lens and aqueous humor are more protected against UV-induced damage in the lens epithelium (1998). This study used intraperitoneal injections of vitamin C and the cells exhibited reduced levels of DNA damage. Another study showed that cataract formation that was induced by selenite in rats could be prevented by intraperitoneal administration of vitamin C (Devamanoharan et al, 1991). In contrast, Spector et al. showed that vitamin C is a major contributor to H₂O₂ production in the aqueous humor (1998). According to most studies, vitamin C seems to have a protective effect and helps reduce damage caused by oxidants.

Vitamin E exerts an antioxidant action by interrupting the chain reaction of lipid peroxidation which has a membrane-stabilizing action. Lipid peroxidation is a process that involves formation of lipid radicals, rearrangement of double bonds in lipids and the destruction of lipid membranes. This is the result of the production of breakdown products such as alcohols, ketones, aldehydes and ethers (Buege & Aust, 2004). It has been found that lenses with cataracts result in an altered distribution of vitamin E within the lens. Vitamin E levels are typically higher within the region of the lens affected by a cataract (Ohta, 1996). Vitamin E intake has been shown to decrease occurrence of lens opacities, and there have been several experimental models to test the effects of vitamin E in cataract prevention (Ayala & Soderberg, 2004). Creighton et al. showed that vitamin E reduced the formation of cataracts in a galactose-induced cataract model (1985). Ohta et al. showed the beneficial effects of vitamin E in the steroid cataract model (1996). In mice fed a diet deficient in vitamin E, Varma et al. showed that protein oxidation developed in their murine lenses

(1994). These studies all have similar conclusions; vitamin E shows protective effects against the formation of cataracts in various models of cataract formation.

For this study, the comparison of the preventative effects of vitamins C and E in the formation of cataracts in murine lenses *in vivo* were compared. The effectiveness of each vitamin was analyzed, and the study tested whether the vitamins synergize. It was hypothesized that vitamin C would be more effective than vitamin E at preventing the formation of a cataract since vitamin C not only neutralizes reactive oxygen species but also converts vitamin E radicals back to vitamin E. When comparing the individual vitamin treatments and the combination treatment, it was hypothesized that the combination group would more effectively prevent the formation of cataracts than the individual treatments.

Methods

Animals

Sixteen C57 or B6 wild type mice (*Mus musculus*) born between March 10-20, 2017 were obtained from the Allegheny mouse colony. Mice were fed regular chow (LabDiet 5P00 Prolab RMH 3000) and had free access to water with 12:12 dark:light cycle beginning at 7 AM. The mice were maintained for 6 weeks for the purposes of the experiment.

Treatment Groups

To analyze the effects of vitamins C and E in the prevention of cataracts, mice were randomly assigned to three experimental groups and a control group containing 4 mice each as shown in Table 1. All groups received weekly intraperitoneal injections for 4 weeks. A disposable 1 mL tuberculin syringe with a 26-gauge needle was used for each injection. The control (CTL) group received an injection containing 0.9% saline solution. There was a Vitamin C (C), Vitamin E (E) and a combination of Vitamin C and E (CE) group. The doses for the vitamin groups were obtained from a study done by Acharya et al. which determined the effectiveness of vitamins C and E in the protection of spermatogenesis from the effects of cadmium exposure. The dose of vitamin C was 10 mg/kg body weight (BW) dissolved in 10 mL/kg of deionized (DI) water. For a mouse weighing 25 g, the volume was about 25 μ L. The dose of vitamin E was 10x the vitamin C dose at 100 mg/kg BW dissolved in 10 mL/kg of olive oil (Acharya, et al., 2007). Since vitamins C and E are not soluble in the same solution, it was necessary to administer the substances to the combination group as two separate injections, one 10 mg/kg vitamin C injection in 5 mL/kg of DI water and one 100 mg/kg vitamin E injection in 5 mL/kg of olive oil. The vitamin C was Spring Valley C supplement with 500 mg per tablet (Lot# 000604). The Vitamin E was Spring Valley d-alpha

supplement with 1000 IU per soft gel (Lot# 000116). Both vitamins were purchased at Walmart.

Table 1. Treatment groups and injection contents

Treatment Group	Injection Contents
Control (CTL)	10 mL/kg of 0.9% saline
Vitamin C (C)	10 mg/kg Vitamin C in 10 mL/kg of DI water
Vitamin E (E)	100 mg/kg Vitamin E in 10 mL/kg of olive oil
Vitamins C and E (CE)	10 mg/kg Vitamin C in 5 mL/kg of DI water and 100 mg/kg Vitamin E in 5 mL/kg of olive oil

Administration of Vitamins

To administer the solutions, an isoflurane chamber was used to briefly anesthetize the mice. Each mouse was placed under 5% isoflurane for approximately 90 seconds before they were removed from the chamber and injected with their treatment. This was necessary due to their aggressive behavior while awake.

Induction of Oxidative Stress

A week following their last injections, the mice were euthanized using carbon dioxide inhalation for approximately 2 minutes and their eyes were removed. Lenses were dissected using dissection scissors, tweezers, and a Leica dissection microscope. The lenses were rinsed with DI water before being placed into a 1 mL microcentrifuge tube wrapped in aluminum foil containing 1 mM hydrogen peroxide. They were left for 24 hours.

The appropriate concentration of hydrogen peroxide was determined in a pilot study using three mouse lenses and varying concentrations of hydrogen peroxide. The control lens was

placed in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, and the other lenses were placed in either 1 mM or 10 mM H_2O_2 for 24 hours. The lenses were removed from the hydrogen peroxide, rinsed with DI water and compared under a dissection microscope. Based on the appearance of the lens on a grid background after H_2O_2 exposure, a 1 mM concentration of hydrogen peroxide was sufficient to induce a cataract in the lens (Figure 2).

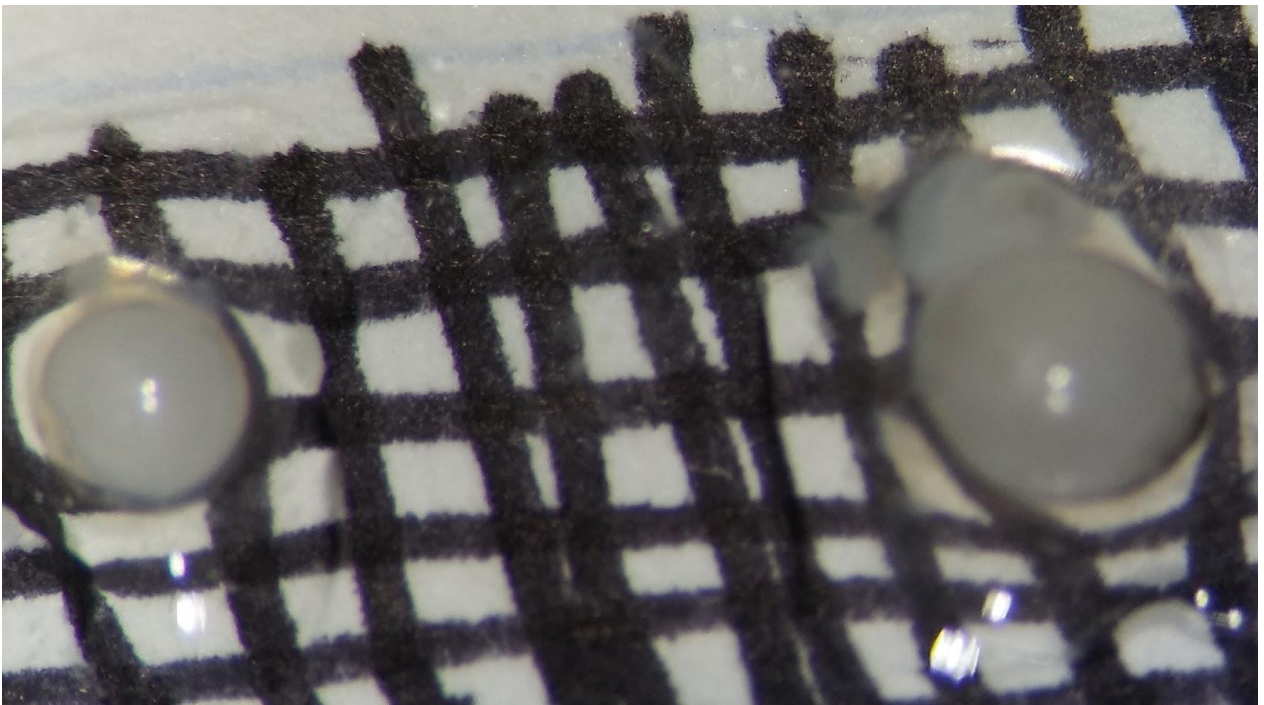


Figure 2. Lens treated with 1 mM H_2O_2 on left. Lens treated with 10 mM H_2O_2 on right.

Analysis of Oxidative Stress

After oxidative stress had been induced via 24-hour H_2O_2 exposure, the lenses were rinsed with DI water and placed in a 0.1 mL centrifuge tube with 0.08 mL HEPES buffer. Each lens was stored at 5 °C for two months before being analyzed using a Protein Carbonyl Content Assay Kit (Catalog# MAK094, Lot# 3M18K08300) from Sigma-Aldrich.

Prior to the start of the Protein Carbonyl Content Assay Kit (Catalog# MAK094, Lot# 3M18K08300) from Sigma-Aldrich, 10 mL of acetone was transferred to a 50 mL centrifuge

tube and placed in a -20 °C freezer. Each sample was transferred to a 1 mL centrifuge tube, homogenized using a handheld pellet homogenizer in HEPES buffer and diluted to a protein concentration of approximately 10 mg/mL. The protein concentration was estimated by using a study analyzing lens mass versus mouse age (Shupe et al., 2005). The lens mass was estimated by observation and then the appropriate volume of HEPES buffer was added to dilute the sample.

There are two parts to the carbonyl content assay and the first is to conduct the 2,4-Dinitrophenylhydrazine (DNPH) reaction. First, 100 μ L of DNPH solution was added to each sample, vortexed and incubated for 10 minutes at room temperature. Next, 30 μ L of the 100% trichloroacetic acid (TCA) solution was added to each sample, vortexed, and incubated on ice for 5 minutes. After the incubation period, samples were centrifuged at 13,000g for 2 minutes using a Thermo Legend Micro 17 centrifuge, and the supernatant was removed and discarded without disturbing the pellet. Then, 500 μ L of ice-cold acetone was added to each pellet, and the samples were placed in a sonication bath (Ultrasonic Cleaning Bath, Model CPX3800H, Branson Ultrasonics Corp.) for 30 seconds or until the pellet dissolved into solution. The samples were incubated at -20° C for 5 minutes and then centrifuged at room temperature at 13,000g for 2 minutes. The supernatant was removed from the pellet and discarded. Finally, 200 μ L of 6 M guanidine solution was added to the pellet and sonicated briefly. Samples were incubated in a 60 °C water bath for 30 minutes and centrifuged briefly to pellet any insoluble material. Samples were cooled to room temperature, and 100 μ L of each sample was transferred to a microplate well in a Thermo Scientific Pierce 96-Well Plates (Product No. 15041). The absorbance of the assay samples and blank wells was

measured at 375 nm using a BioTek Synergy HTX multi-mode plate reader (Model# S1LFA).

Bicinchoninic Acid (BCA) Assay

Twenty-five μL of each sample were transferred to another set of 1 mL centrifuge tubes, and protein concentration was measured using the Pierce Bicinchoninic Acid Protein assay kit from Thermo Scientific (Catalog# 23227, Lot# TA260208). Next, 200 μL of the Working Reagent (WR) was added to each centrifuge tube and vortexed. The samples were then incubated at 37 $^{\circ}\text{C}$ for 30 minutes. Samples were cooled to room temperature, and 200 μL of each sample was transferred to a microplate well. The absorbance was measured at 562 nm with a plate reader. A protein standard curve was generated using bovine serum albumin (BSA) provided in the Pierce Bicinchoninic Acid Protein Assay kit. The concentrations of BSA and the volume of DI water for diluting the solutions are found in Table 1 (Appendix A). Figure 3 shows the 11 solutions of the prepared standard curve in a 96-well plate. A new standard curve was made each time the assay was run.

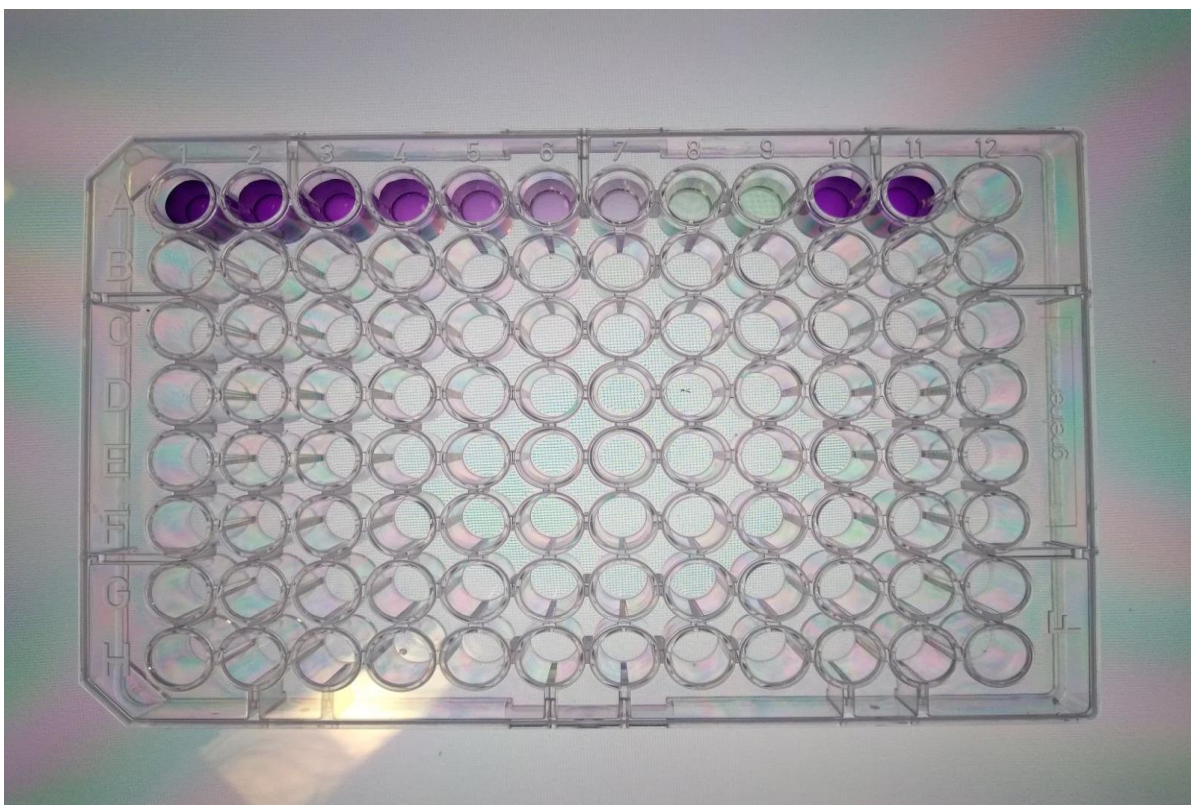


Figure 3. A 96-well plate was used to measure absorbance values of samples from the BCA assay. Wells A1-A9 are the standard curve created using BSA. Wells A10-A11 are unknown samples.

Data Analysis

The average 375 nm absorbance of the blank wells were subtracted from every carbonyl assay sample well, to calculate the corrected absorbance values. The amount of carbonyl in each sample well was calculated using the equation $C \text{ (nmol/well)} = (A_{375}/6.364) \times 100$. The final carbonyl concentration, in the form of nmol of carbonyl per mg of protein was determined using the equation $CP = \text{nmol carbonyl/mg protein} = (C/P) \times 1,000 \times D$.

The average 562 nm of the blank wells were subtracted from every standard and unknown sample well to calculate the corrected absorbance values. A protein concentration standard curve was created by plotting the standard BSA concentration versus absorbance value and fitting a linear regression line to the data. The concentrations of the unknown samples were determined by inputting the absorbance of the unknowns into the linear

regression equation of the standard curve. The two lens carbonyl concentrations from each mouse were averaged to produce one carbonyl concentration for each mouse. A one-way analysis of variance (ANOVA) was performed to determine if there were statistical differences for the mean protein carbonyl concentrations between treatment groups.

Results

All of the lenses developed a cataract after exposure to H_2O_2 meaning that none of the treatments were able to prevent the formation of a cataract. Carbonyl concentrations of the lenses were measured and used to indicate the severity of the cataract that formed in comparison to those of other lenses. A lens with low carbonyl concentration than other lenses means that it has a less severe cataract. The greater the lens carbonyl concentration, the more severe the cataract that formed. An average was calculated per mouse using the carbonyl concentrations of its two lenses (Figure 4).

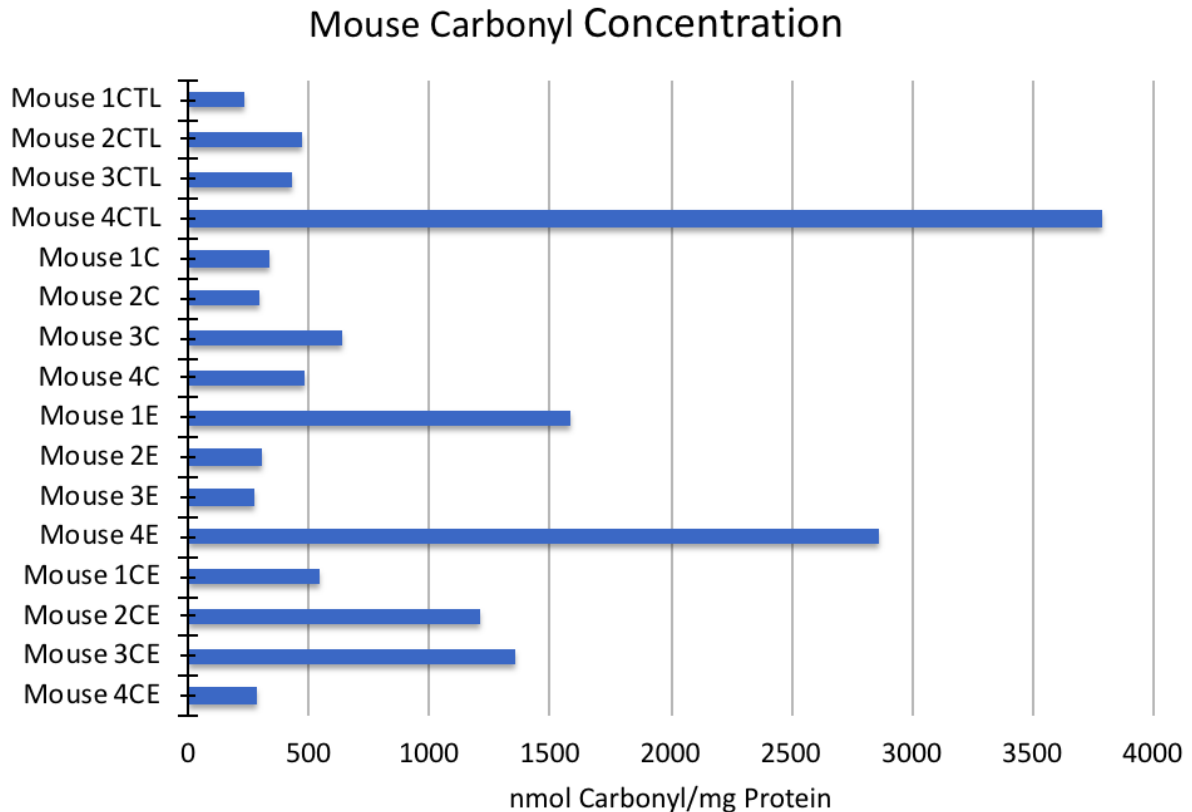


Figure 4. Mean carbonyl concentration for each mouse excluding the two outliers. (Mouse 4CTL n=1, Mouse 4E n=1)

After calculating the carbonyl concentrations of each lens, there were two lenses that appeared to be outliers. Their carbonyl concentrations were more than two standard

deviations away from the mean of their groups. Mouse lens 7CTL had a carbonyl concentration of 4979 nmol/mg protein. The mean of the control group including the outlier was 1230 ± 1716 nmol/mg. The second outlier was mouse lens 8E which had a carbonyl concentration of 5459 nmol/mg protein. The mean of the vitamin E group including the outlier was 1255 ± 1905 nmol/mg. Both samples fall outside the 2-standard deviation range and both were excluded from the group data.

The averaged mouse carbonyl concentrations were between 260 and 2700 nmol/mg (Figure 4) while the individual lens carbonyl concentrations ranged between 100 and 2800 nmol/mg (Figure 5).

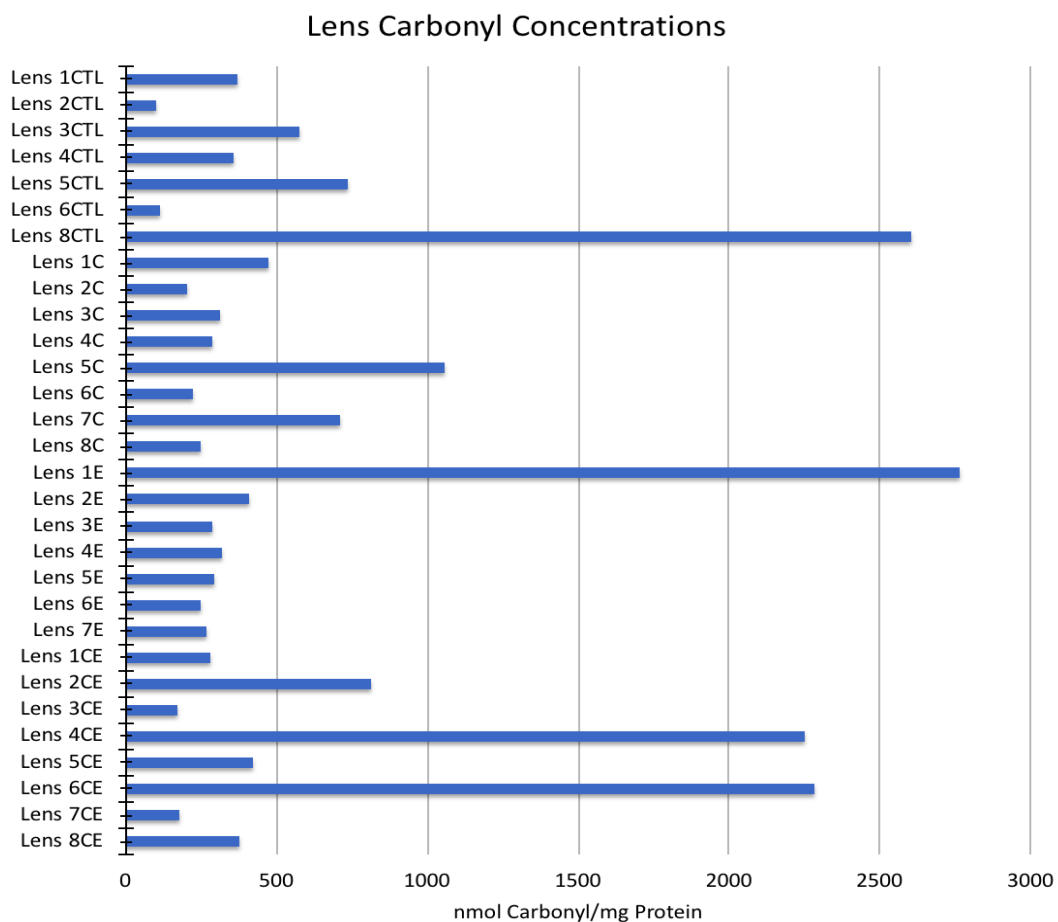


Figure 5. The measured carbonyl concentrations for each lens except lenses 7CTL and 8E.

The control (CTL) group had the largest mean carbonyl concentration 933 ± 1117 nmol/mg. The combination (CE) group followed closely with a mean carbonyl concentration of 848 ± 517 nmol/mg. The vitamin E (E) and vitamin C (C) groups had mean carbonyl concentrations of 606 ± 654 and 439 ± 155 nmol/mg. There were no significant differences between the treatment groups ($F = 0.4139$, $p = 0.7461$) (Figure 6).

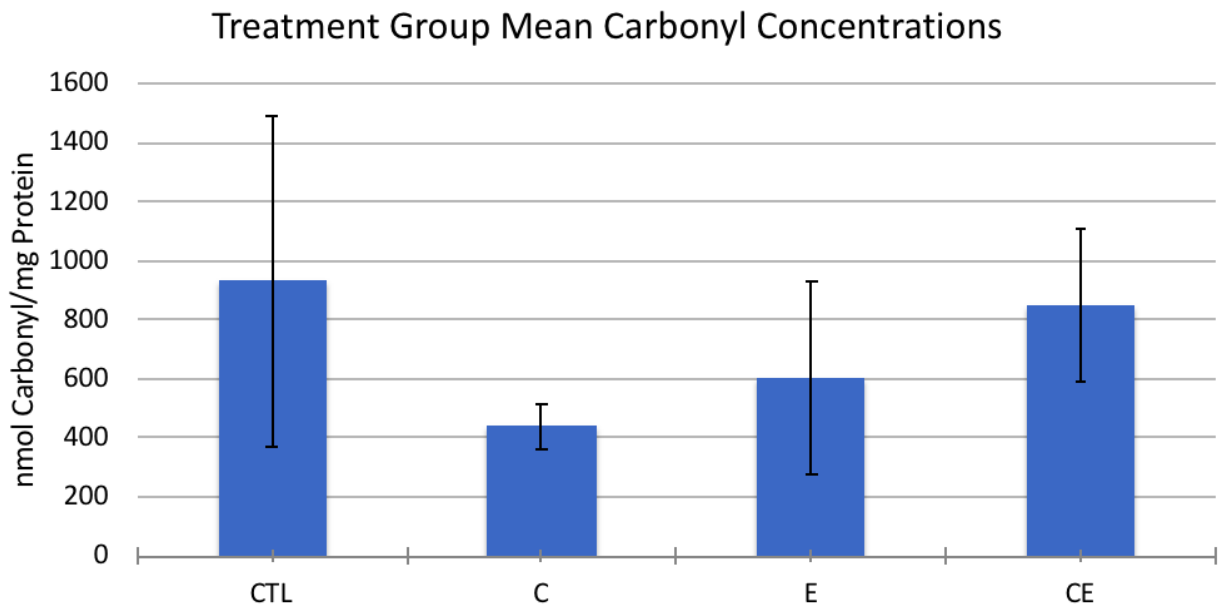


Figure 6. Mean and standard error carbonyl concentrations for each treatment group excluding the two outliers. (CTL n=7, C n=8, E n=7, CE n=8)

Discussion

The goal of this research was to compare the preventative effects of vitamins C and E in the formation of cataracts in murine lenses. The severity of the cataracts was compared using protein carbonyl concentrations in the lenses. This project was inspired by a previous Allegheny senior project that studied cataract prevention in bovine lenses using vitamin E and astaxanthin (Schuver, 2017) and a Junior Seminar independent project studying the preventative effects of vitamin C in bovine lenses. My project aimed to compare the preventative effects of vitamins C and E *in vivo* using wild-type mice. I hypothesized that vitamin C would be more effective than vitamin E and that the combination of the two vitamins would be most effective in preventing the formation of cataracts. The vitamin C group had the lowest mean carbonyl concentration. The results between the four groups were not statistically significant.

The mean and standard deviation carbonyl concentrations of the treatment groups are as follows: Combination (CE) $\bar{x} = 848 \pm 237$ nmol/mg, Control (CTL) $\bar{x} = 695 \pm 250$ nmol/mg, Vitamin E (E) $\bar{x} = 655 \pm 57$ nmol/mg, Vitamin C (C) $\bar{x} = 439 \pm 182$ nmol/mg. I expected the vitamin C to have the lowest mean as the literature suggested that vitamin C would have a more protective role than vitamin E since it neutralizes vitamin E radicals (Birben et al., 2012). I predicted the combination group to have a larger protective effect than the individual groups. The data do not support this prediction as the combination group had the highest carbonyl concentration. This is not consistent with the rest of the data in which vitamin E still had a lower mean than the control group meaning that the combination group still should have had a mean smaller than the control group.

A meta-analysis of the association of vitamin C with the risk of age-related cataract was done to analyze and summarize the results from various studies in humans. Results show that there is an inverse relationship between high vitamin C intake and reduced risk of cataract (Wei et al., 2016). This is primarily because vitamin C is effective in settings with low partial pressures of oxygen, which is the condition within the lens. It is also found in many fruits and vegetables and is not associated with any harmful side effects, even when ingested in high doses (Wei et al., 2016).

The aqueous humor is important to maintaining lens health as it provides essential nutrients such as vitamin C. One study showed that diurnal guinea pigs have higher levels of vitamin C in their aqueous humor (Reddy et al., 1998). Therefore, their lenses had more protection from UVB radiation than the lenses of nocturnal rats. When the rats were injected intraperitoneally with vitamin C, there was less damage to the lens caused by the UVB radiation (Reddy et al., 1998). A similar study was performed in which rats were administered selenite which produces the formation of H_2O_2 which normally causes cataracts to form. However, the intraperitoneal injection of vitamin C helped to prevent the formation of a cataract (Devamanoharan et al., 1991). Although my results were not statistically significant, taken together, these studies suggest that vitamin C does show a tendency to protect the lens from the formation of a cataract.

Studies have shown that the vitamin E is not present in the eye at similar levels to that of vitamin C (Ohta et al. 1996). Vitamin E protects the lens from lipid peroxidation of the epithelial cells whose primary function is to continue synthesizing proteins and allowing the lens to continuously grow (Augusteyn, 2007). This suggests that vitamin E plays a lesser role in the prevention of cataracts than that of vitamin C. Vitamin E also is not actively

transported into the aqueous humor and the lens in the same way the vitamin C is indicating that its role is not as important in the protection of the lens.

There were two outlier samples, one lens from the control group and one lens from the vitamin E group. Both lenses came from male C57 mice born on March 10, 2017. The mice were two of the heaviest (31.7g and 29.0 g) that I was given. For mouse 4CTL, both lenses had carbonyl concentrations much higher than the group mean with one lens being more than 2 standard deviations from the mean and the other being about 1 standard deviation from the mean. Mouse 4E was different. One lens was more than two standard deviations from the mean, while the other lens had a carbonyl concentration that was less than the mean of its group. There are a number of factors that could have led to these values, including ineffective treatment, difference in lens size, confounding factor of being male, C57 and large. The primary inconsistency that is difficult to explain is why mouse 4E had one lens that fell within normal range for the treatment group and another lens with carbonyl concentrations two standard deviations outside the mean.

During dissection, I noticed that occasionally a lens would not be perfectly round and had small bumps or other irregularities. Since this was the case for perhaps 4 or 5 of the lenses, it was not concerning as I also saw this in bovine lenses. This leads me to think that these irregularities may have contributed to the outlier carbonyl concentrations. If this experiment were to be replicated, I would suggest taking a photo of each lens after it was dissected or to make note of any special characteristics or irregularities of the lens. This would provide more information and perhaps a relationship between lens irregularities and high carbonyl concentration values.

Limitations

A limitation I had in my study occurred during the administration of the intraperitoneal injections, specifically the vitamin E injections of the combination group. I gave two injections for the combination group, one on either side of the abdomen, and I always gave the vitamin C injection first, followed by the vitamin E injection. I noticed that sometimes in the smaller mice, the vitamin E solution would escape the abdomen at the injection site. To address this issue, I injected the solution at a slower rate, kept the needle in the abdomen longer to allow the solution to diffuse inside the intraperitoneal cavity, and sometimes this technique would fix the problem. I noticed that when the mice woke from the anesthesia, they often would groom the injection site and ingest the solution which means the delivery was altered.

A possible explanation as to why the combination group seemed to be the least effective treatment could be due to an excessive amount of vitamin E in the eye. Studies have shown that high doses of vitamin E are harmful and do not help protect the lens from cataract (Selin et al., 2013). The administration of both vitamin C and E might have caused excess vitamin E levels since vitamin C could have neutralized pre-existing vitamin E radicals (Birben et al., 2012). Further investigation could be done on this topic in which varying concentrations of vitamin E in combination with a constant dose of vitamin C could be tested to determine which concentration of the two vitamins effectively prevents the formation of a cataract.

The measurement of protein carbonyl concentration in the lens has been shown to be an effective indicator of the formation of cataracts (Boscia et al., 2000). The lens is a tissue made up almost entirely of crystallin proteins and the oxidation of these proteins plays a significant role in the formation of cataracts (Taylor, 1995). There are other forms of

oxidative stress that stem from lipid peroxidation, although carbonyl concentration measurement is an effective form of measuring the severity of a cataract, it is only one form of oxidative stress in the lens. This study is limited in that it only addressed one product of oxidative stress, protein carbonyl formation.

Future Research

A future experiment could use oral administration of vitamins such as dissolving the vitamins in the water provided to the mice or providing food rich in a particular vitamin. This would more accurately create an environment similar to that of human vitamin consumption, although it would be costly. This would also result in daily vitamin consumption which might be more effective for vitamin E since it is not actively transported in the aqueous humor of the eye like vitamin C. A longer treatment period would be possible with this experimental design allowing for the mice to be exposed to the vitamins for a longer time period than 4 weeks.

Various studies have proven with statistical significance that vitamin C does have a protective effect on the formation of cataracts (Devamanoharan et al., 1991; Reddy et al., 1998; Spector et al., 1998; Wei et al., 2016). Future experiments could use varying dosages of vitamin C to determine which concentration is more effective in preventing the formation of cataracts. Based on the previous studies mentioned, it is unclear what dose of vitamin C is effective in mice as these studies have been done on guinea pigs, rats and humans.

Conclusion

Various non-enzymatic antioxidants have been shown to prevent the formation of cataracts, but there are few studies that compare the effectiveness of antioxidants. This experiment analyzed and compared the preventative effects of vitamins C and E in the

formation of cataracts in murine lenses. It was hypothesized that vitamin C would be more effective than vitamin E. There was an additional hypothesis that the combination of the two vitamins would be the most effective in preventing the formation of cataracts. The results do not support either hypothesis. Although there is presently a solution that cures blindness caused by cataracts, this study aimed to find a preventative solution that can be used globally. Antioxidants such as vitamin C and E are found in many foods and other resources available to populations in developing countries where surgical replacement of lenses is not an option. Finding an effective preventative treatment for the formation of cataracts would help minimize the number of people left blind by this condition and reduce surgical costs.

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Appendix A

Dilution Scheme for Standard Microplate Procedure (Working Range = 20-2,000 $\mu\text{g/mL}$)

Vial	Volume of DI Water (μL)	Volume and Source of BSA (μL)	Final BSA Concentration ($\mu\text{g/mL}$)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

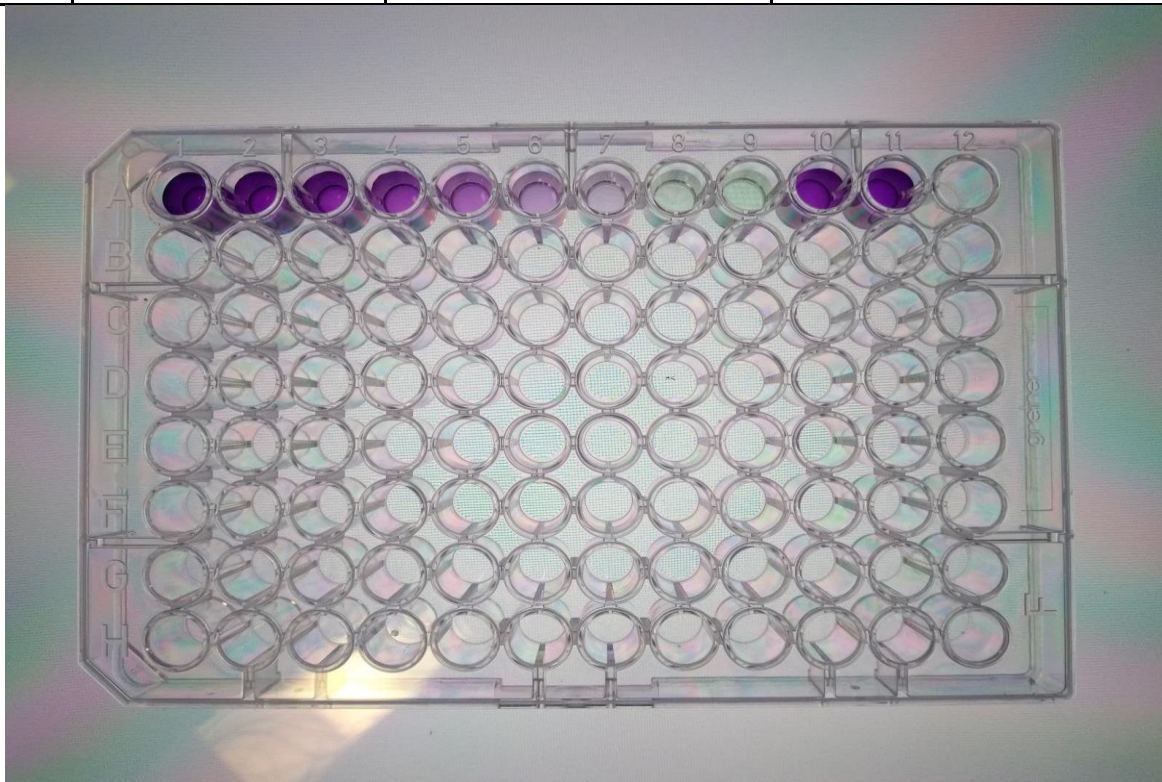


Figure 3. 96-well plate used to measure absorbance values of samples. Samples in the image above are from the BCA assay. Wells A1-A9 are the standard curve created using BSA. A10-A11 are unknown samples.

Appendix B

Preparation of the BCA Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:
 $(\# \text{ standards} + \# \text{ unknowns}) \times (\text{volume of WR per sample}) = \text{total volume}$
Note: 200 μ L of WR reagent is required for each sample
2. Prepare WR by mixing 50 parts of BCA Reagent's A with 1 part BCA Reagent B (50:1, Reagent A:B).

Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

Appendix C

Treatment Group Mouse Information

	DOB	Sex	Strain	Week 1 BW (g)	Week 2 BW (g)	Week 3 BW (g)	Week 4 BW (g)
Control Group							
Mouse 1CTL	3/20/17	M	B6	29.2	28.3	29.1	29.1
Mouse 2CTL	3/10/17	F	C57	26.4	28.5	27.5	26.9
Mouse 3CTL	3/20/17	F	B6	22.5	22.2	21.8	22.7
Mouse 4CTL	3/10/17	M	C57	29.0	26.0	26.1	25.7
Vitamin C Group							
Mouse 1C	3/20/17	M	B6	30.4	30.4	30.5	30.3
Mouse 2C	3/15/17	F	C57	23.3	24.5	25.1	26.7
Mouse 3C	3/20/17	F	B6	19.9	19.7	20.0	19.4
Mouse 4C	3/10/17	M	C57	32.9	30.2	30.9	30.1
Vitamin E Group							
Mouse 1E	3/20/17	F	B6	23.1	22.9	24.2	24.1

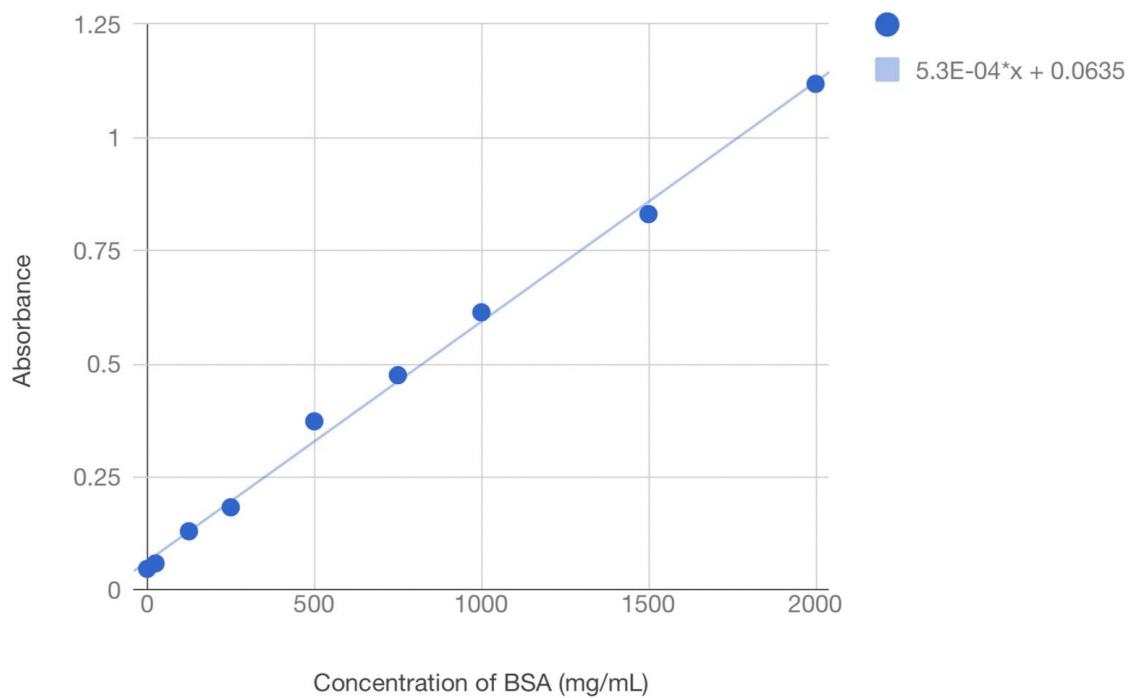
Mouse 2E	3/10/17	F	C57	25.1	24.7	25.8	25.3
Mouse 3E	3/15/17	F	C57	22.9	23.0	23.0	23.2
Mouse 4E	3/10/17	M	C57	31.7	29.9	29.8	29.6
Combination Group							
Mouse 1CE	3/10/17	F	C57	23.9	24.8 g	25.1 g	25.8 g
Mouse 2CE	3/15/17	F	C57	25.2	24.9 g	25.1 g	25.2 g
Mouse 3CE	3/15/17	F	C57	22.5	23.2 g	23.1 g	23.6 g
Mouse 4CE	3/10/17	M	C57	32.2	30.1 g	29.7 g	29.8 g

Appendix D

Group 1 Standard BSA Curve

BSA Concentrations (mg/mL)	Raw Absorbance (562 nm)	Corrected Absorbance
0	1.179	0.047
25	0.891	0.059
125	0.674	0.130
250	0.535	0.183
500	0.433	0.373
750	0.243	0.475
1000	0.19	0.614
1500	0.119	0.831
2000	0.107	1.119

Group 1 BSA Standard Curve

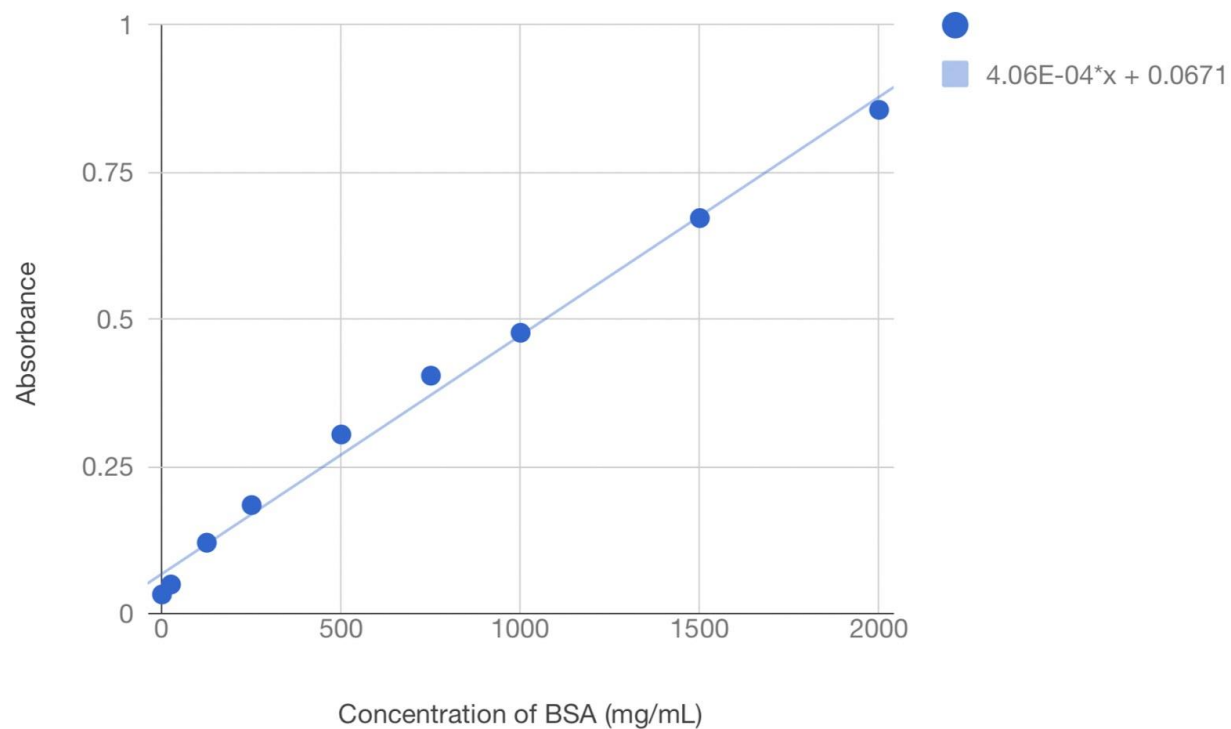


Appendix E

Group 2 Standard BSA Curve

BSA Concentrations (mg/mL)	Raw Absorbance (562 nm)	Corrected Absorbance (
0	0.915	0.032
25	0.731	0.049
125	0.536	0.12
250	0.463	0.184
500	0.363	0.304
750	0.243	0.404
1000	0.179	0.477
1500	0.108	0.672
2000	0.091	0.856

Group 2 BSA Standard Curve



Appendix F

Absorbance Values

Treatment Group	Lens	Raw Absorbance (562 nm)	Corrected Absorbance (562 nm)	Raw Absorbance (375 nm)	Corrected Absorbance
Control (CTL)	1CTL	2.101	2.041	0.164	0.099
	2CTL	1.29	1.230	0.091	0.026
	3CTL	1.56	1.500	0.212	0.147
	4CTL	1.814	1.754	0.157	0.092
	5CTL	0.931	0.872	0.252	0.188
	6CTL	1.701	1.642	0.094	0.030
	7CTL	1.017	0.958	1.332	1.268
	8CTL	1.034	0.975	0.727	0.663

Vitamin C (C)	1C	2.642	2.582	0.186	0.121
	2C	1.796	1.736	0.117	0.052
	3C	2.113	2.053	0.145	0.080
	4C	2.442	2.382	0.138	0.073
	5C	0.93	0.871	0.334	0.270
	6C	1.129	1.07	0.121	0.057
	7C	1.071	1.012	0.245	0.181
	8C	1.367	1.308	0.128	0.064
Vitamin E (E)	1E	2.151	2.091	0.769	0.704
	2E	1.488	1.428	0.17	0.105
	3E	1.911	1.851	0.138	0.073
	4E	2.34	2.280	0.146	0.081
	5E	1.219	1.16	0.14	0.076
	6E	0.976	0.917	0.128	0.064
	7E	1.041	0.982	0.132	0.068
	8E	0.762	0.703	1.454	1.390
Combination (CE)	1CE	2.124	2.064	0.137	0.072
	2CE	2.484	2.424	0.272	0.207
	3CE	1.618	1.558	0.11	0.045
	4CE	2.245	2.185	0.638	0.573
	5CE	1.043	0.984	0.172	0.108
	6CE	0.958	0.899	0.646	0.582
	7CE	0.803	0.744	0.11	0.046
	8CE	1.09	1.031	0.161	0.097

Appendix G

Protein Concentration

Treatment Group	Lens	Protein Concentration (mg/mL)	Amount of Protein in Sample Wells (μg)
Control (CTL)	1CTL	3730.96	746.19
	2CTL	2200.77	440.15
	3CTL	2710.20	542.04
	4CTL	3189.45	637.89
	5CTL	1982.51	396.50
	6CTL	3879.06	775.81
	7CTL	2194.33	438.87
	8CTL	2236.21	447.24
Vitamin C (C)	1C	4754.71	950.34
	2C	3155.49	631.10
	3C	3753.60	750.72
	4C	4374.35	874.87
	5C	1980.05	396.01
	6C	2470.20	494.04
	7C	2327.34	465.47
	8C	3056.40	611.28
Vitamin E (E)	1E	3825.30	765.06
	2E	2574.35	514.87
	3E	3372.47	674.49
	4E	4181.90	836.38
	5E	2691.87	538.37
	6E	2093.35	418.67

Combination (CE)	7E	2253.45	450.69
	8E	1566.26	313.25
	1CE	3774.35	754.87
	2CE	4453.60	890.72
	3CE	2819.64	563.93
	4CE	4002.66	800.53
	5CE	2258.37	451.67
	6CE	2049.01	409.80
	7CE	1667.24	333.45
	8CE	2374.14	474.83

Appendix H

Formula used to calculate amount of carbonyl per sample well

$$C \text{ (nmol/well)} = A_{375}/6.364 \times 100$$

C = amount of carbonyl per sample well

A₃₇₅ = absorbance value from spectrophotometer

6.364 = millimeter extinction coefficient x 0.2893 cm pathlength for enclosed 96 well plate

100 = total volume (V) in the well (uL)

Formula used to calculate carbonyl concentration (nmol/mg protein)

$$CP = \text{nmol carbonyl/mg protein} = (C/P) \times 1,000 \times D$$

C = amount of carbonyl per sample (nmol/well)

P = amount of protein from the carbonyl well x 20 = $\mu\text{g/well}$

D = dilution or concentration of sample

1,000 = conversion factor (μg to mg)

Amount of Carbonyl per Sample Well (nmol/well)

Treatment Group	Lens	Amount of Carbonyl per Sample Well (nmol/well)	Carbonyl Concentration (nmol/mg protein)	Mean $\pm$ Std Dev (nmol/mg protein)	Std Error
Control (CTL)	1CTL	1.485	371.27	933.14 $\pm$ 1117.53	558.79
	2CTL	0.402	100.39		
	3CTL	2.303	575.72		

	4CTL	1.439	359.66		
	5CTL	2.947	736.71		
	6CTL	0.464	116.03		
	7CTL	19.92	4979.3 (excluded from data analysis)		
	8CTL	10.41	2602.7		
Vitamin C (C)	1C	1.894	473.58	439.18 ± 155.00	77.50
	2C	0.810	202.53		
	3C	1.250	312.52		
	4C	1.140	285.02		
	5C	4.235	1058.8		
	6C	0.888	222.10		
	7C	2.837	709.21		
	8C	0.998	249.60		
Vitamin E (E)	1E	11.06	2763.8	606.62 ± 653.94	326.97
	2E	1.643	410.73		
	3E	1.140	285.02		
	4E	1.266	316.45		
	5E	1.187	296.74		
	6E	0.998	249.60		
	7E	1.061	265.31		
	8E	21.83	5458.6 (excluded from data analysis)		
Combination (CE)	1CE	1.124	281.10	847.72 ± 517.30	258.65
	2CE	3.246	811.42		

	3CE	0.700	175.03		
	4CE	8.997	2249.2		
	5CE	1.690	422.44		
	6CE	9.138	2284.5		
	7CE	0.716	178.89		
	8CE	1.512	379.23		

Mouse Carbonyl Concentrations

	Carbonyl Concentration Mean (nmol/mg protein, n=2)
Mouse 1CTL	235.83
Mouse 2CTL	467.69
Mouse 3CTL	426.37
Mouse 4CTL	2602.7 (n=1)
Mouse 1C	338.06
Mouse 2C	298.77
Mouse 3C	640.47
Mouse 4C	479.41
Mouse 1E	1587.3
Mouse 2E	300.74
Mouse 3E	273.17
Mouse 4E	265.31 (n=1)
Mouse 1CE	546.26
Mouse 2CE	1212.1
Mouse 3CE	1353.4
Mouse 4CE	279.06

Appendix I

ANOVA Statistics

ANOVA Table	(Type II Tests)		
	df	F	p-value
Treatment	3	0.4139	0.7461