Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in people over the age of 65. It is a condition that worsens with age. AMD causes the macula of the eye to degenerate, leaving dim spots in the center of vision. Over a long period of time, sometimes a number of years from onset, it may cause complete blindness. More than two million Americans age 50 and older have advanced AMD. As of now, there is no cure. Wet AMD involves the abnormal neovascularization, or growth, of blood vessels which rupture and cause vision loss. Several treatment options exist for wet AMD, primarily aimed at sealing off the leaking blood vessels, or preventing anomalous blood vessels from growing back. Lasers, light sensitive drugs, and anti-angiogenic therapies are used for these treatments (NEI NIH, 2009).

Studies regarding AMD and the role of genetic factors are fairly recent, having only begun in the past 10 years. In 2005, Edwards, Ritter, and Abel found that certain polymorphisms correlate with AMD. A study in 2007, by Edwards, Ritter, and Abel, tested the role of genetic factors in AMD, and its results supported the findings of the 2005 study; there is indeed a connection between AMD and genetics. In the same year, these genetic factors were narrowed down to mitochondrial DNA (Jones, Manwaring, and Wang, 2007). Furthermore, Canter, Olson, and Spenser found that specific mitochondrial DNA (mtDNA) polymorphisms, or mutations, were interdependently associated with AMD (2008). Udar (2009, 2010) further confirmed the findings of these studies, and the importance of mitochondria in eye disorders was recognized (Jarret, Lewin, Boulton, 2010). Recently, studies have looked further into the connection between AMD and mtDNA. For example, in a study conducted using human donor eyes (Lin, 2011) discovered that mtDNA damage and repair was associated with aging, and more

specifically, AMD . Oxidative stress has also been found to correlate with aging and AMD (Beatty, et al., 2000). Most recently, it has been shown that beta5 integrin knockout mice have oxidative damage (Nandrot, 2004; Yu, et al, 2012). These mice develop oxidative stress in the retinal pigment epithelium (RPE) of the eye, causing them to have age-related blindness.

Although mitochondrial defects and oxidative damage have both been associated with AMD in human patients, no studies as of yet have been conducted to determine whether or not they are interdependent, and if mitochondrial defects and oxidative damage *cause* AMD. By determining the relationship between the two, the scientific community will be one step closer to finding a cure, or at least a treatment, for AMD.

This study sought to determine whether or not oxidative stress can cause a decline in mitochondrial function. Tissues previously harvested postmortem from beta5 integrin knockout mice were analyzed in this study. These mice had developed oxidative stress in the RPE layer of the eye with age, causing age-related blindness. Their eye tissues were compared to tissues obtained from age-matched control wildtype mice. This experiment sought to confirm (or reject) the hypothesis that oxidative stress causes the mitochondrial function in mouse eyes to decline.

Materials and Methods

Eyeballs were extracted from 5 young beta5 integrin knockout mice, 5 young wildtype mice, 4 old knockout mice, and 4 old wildtype mice. The young mice were 3 months old and the old mice were 1 year old. Eyes were placed in a buffer on ice. Excess fats and tissues were removed from the eye through dissection, with the aid of a microscope. The retinal pigment epithelium (RPE) and retina were then obtained through dissection. Eppendorf tubes were

weighed before and after placing eyes inside to obtain the weight of samples. All types of eyes were kept in separate tubes.

A “DNAeasy Blood & Tissue Kit” manufactured by Qiagen was used to isolate the DNA from the samples. First, the RPE and retina were mashed in the separate Eppendorf tubes. Then, 180µl of ATL buffer, a tissue lysis buffer for use in purification of nucleic acids, was added to each tube. Using a Gilson pipette, 20µl of Protinase K was added. The pipette tip was used to mix the solution. Next, each tube was put into a centrifuge, in order to move all particles to the bottom of the tube. The tubes were then placed in an incubator at 56 degrees Celsius for an hour, and mixed with the centrifuge every 15 minutes. This procedure caused the tissues to be completely lysed, or broken down, in 1–3 hours. Thirty ml of pure ethanol was added to the AW2 buffer, and 25ml pure ethanol was added to the AW1 buffer.

A 2ml collection tube was added to each of the Eppendorf tubes. They were then placed in the DNAeasy centrifuge, and centrifuged at 8000 rpm for 1 minute. Five hundred µl of AW1 buffer was added to each tube, and centrifuged again at 8000 rpm for 1 minute. Flow through was discarded. The same procedure was performed with AW2 buffer and then centrifuged at 14,000 rpm for 3 minutes. Flow through was discarded, AE buffer added, and centrifuged at 8000 rpm for 1 minute.

Four µl of dye was added to each Eppendorf tube containing the DNA mixtures, in preparation for the gel electrophoresis. This was done in order to separate and analyze the DNA . An agarose gel solution was used for the gel electrophoresis. A Gilson pipette was used to put DNA and dye mixtures into each gel well in the chamber. Ladder substances of 100pb and 1kb were used in the first and last wells, to identify the approximate size of DNA run on the gel. The

chamber was connected to an electrical current to separate the DNA. Then, the gel was removed from the chamber and placed in a mixture of buffer and SYBR Green solution, to stain the nucleic acids, so that they were more visible to the naked human eye. The Typhoon Trio Variable Mode Imager was used to scan the gel and measure fluorescence. Bands were quantified using Image Quant TL Quantification of Intensity Software.

Polymerase chain reaction (PCR) tubes were prepared to amplify DNA. A PCR was prepared with the primers for the forward and reverse DNA sequences for the following: (1) mtDNA long fragments, (2) mtDNA short fragments, (3) nuclear DNA (nDNA) long fragments, (4) nDNA short fragments, (5) mtDNA long and short fragments, and (6) nDNA long and short fragments. The following master mix was created:

	x25	**x6**
5x buffer	5µl	30µl
10 mM DNT	0.8µl	4.8µl
25 mM $Mg(OAc)_2$	1.5µl	9µl
Forward primer	1µl	6µl
Reverse Primer	1µl	6µl
taq	1µl	6µl
DNA	2µl	12µl
ddH2O	12.7µl	76.2µl

Table 1: The first column indicates the various components of the master mix. The second column indicated how many µl of each is needed for 25 PCR tubes of the mixture, and the third column is how much was used per tube in this experiment.

This master mix was divided into the 6 PCR tubes, and the aforementioned primers were placed in their respective tubes. A non-diluted concentration of DNA was prepared for this procedure, and 2µl of DNA were pipetted into each tube. 90ng of DNA were used. The same master mix with a 1:10 diluted concentration of DNA was prepared for a second PCR, this time using 18ng of DNA. The Peltier Thermal Cycler DNA Engine Dyad was used to perform the PCR. Nucleic acid concentrations were measured using the thermoscientific nanodrop 1000 spectrophotometer, using 1µl of sample for each reading.

An ELISA Fluorescence Assay was used to detect oxidative damage on nucleotides. The wells in the tray were washed with buffer five times. Two hundred µl of Ellman's Reagent were added to each well (50ml ddH20 and 250 dtn vial of Ellman's Reagent). The antibody count was measured using a Fluorescence Absorbence Cuvette. The baseline measurement was recorded. The tray was placed in the dark on a rocker shaker, and then measured every 30 minutes for 240 minutes, or until a plateau formed.

Quantifications from knockout and wildtype mice were averaged to determine mean values +/- standard deviation. Statistical significance of differences between the samples was determined by comparing knockout to wildtype values using an unpaired Student's t-test, carried out with Microsoft Excel. A difference was considered significant if $p<0.05$, that is if the probability of rejecting the null-hypothesis of no difference between samples exceeded 5%.

Results

The ELISA assay results are shown in the following figures.

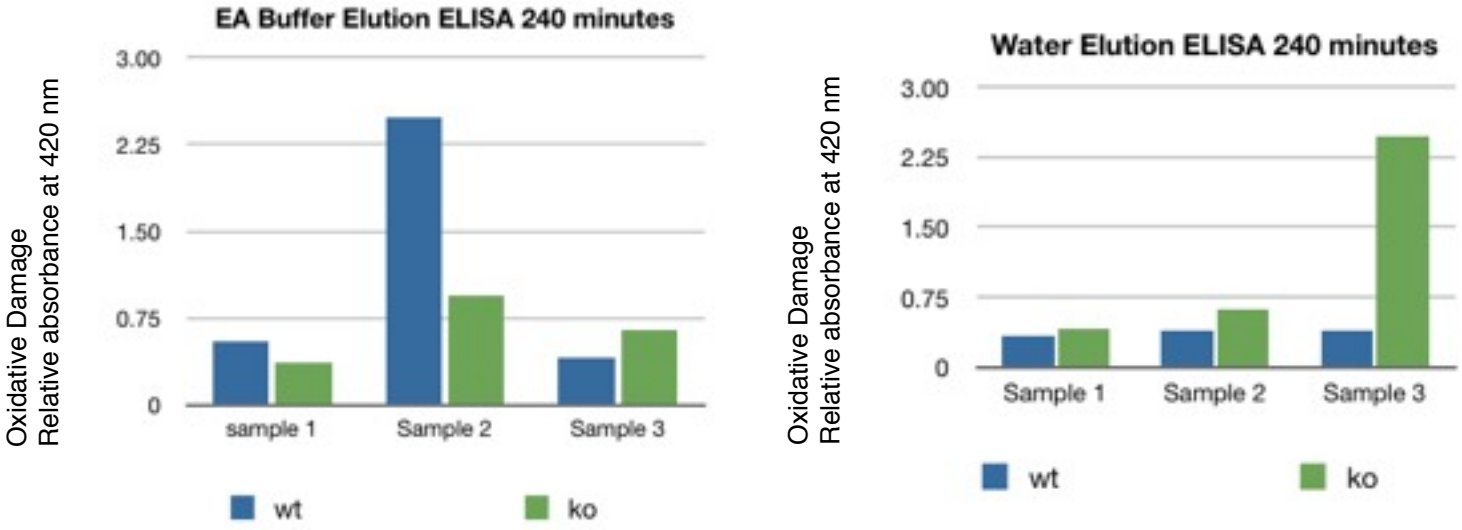

Figure 1

The ELISA assay shown in Fig. 1 indicates the amount of oxidative damage in the different samples: the higher the number, the higher the amount of oxidative damage. Therefore, there was more oxidative damage in beta5 integrin knockout mice than in the control, age-matched wildtype mice. This supports the hypothesis that oxidative damage causes a decline in the function of mitochondrial organelles. The values from the Water Elution ELISA Assay at 240 minutes were compared and found to have a p-value of 1.208, so they were not statistically significant. The values from the EA Buffer Elution ELISA Assay at 240 minutes were compared and found to have a p-value of 1.872. Therefore, neither of the results were statistically significant.

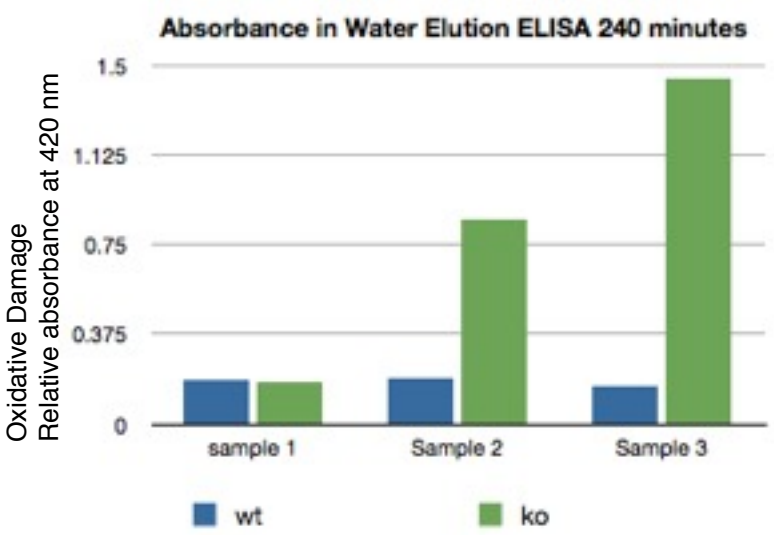

Figure 2

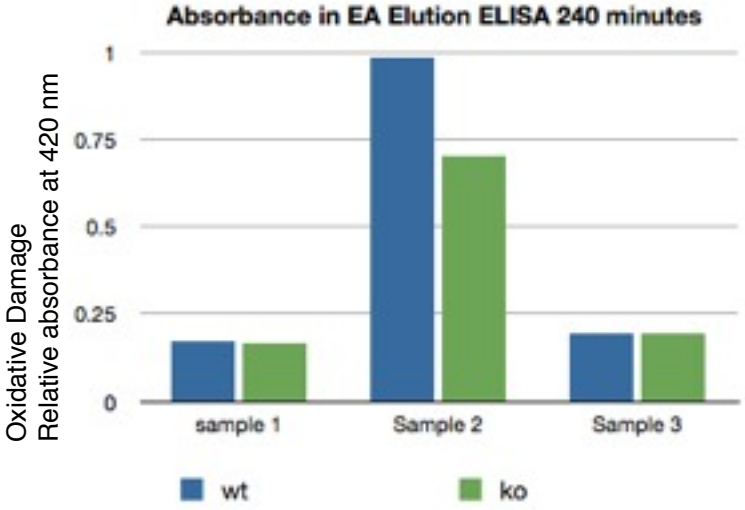

Figure 3

The ELISA assay shown in figures 2 and 3 displays similar results to those shown in figure 1. Similarly, it indicates the amount of oxidative damage present in the different samples. Figure 1 indicates the raw data, while figures 2 and 3 indicate the absorbance per µg of DNA. Therefore, figure 2 gives evidence that there was more oxidative damage in the beta5 integrin knockout mice than in the control mice, which supports the hypothesis. However, figure 3 does not support the hypothesis because the values of oxidative stress absorbance in wildtype mice are higher than in the knockout mice. The results from the samples in figures 2 and 3 were compared in an unpaired Student's t-test, and found to have a p-value of $p>0.25$, which is not statistically significant. The experiment found evidence that there was more oxidative damage in the beta5 integrin knockout mice than in the control, age-matched wildtype mice, but the evidence in these very small samples falls short of the traditional 5% and 1% levels. Using a t-test, the t-value was calculated to equal 0.375. A p-value of 0.25 corresponds to a t-value of 0.727, so this shows that the results from figures 2 and 3 had an even greater p-value than 0.25, and were thus also not statistically significant.

Long PCR results are shown in the gel electrophoresis scans in Fig. 4 and Fig. 5.

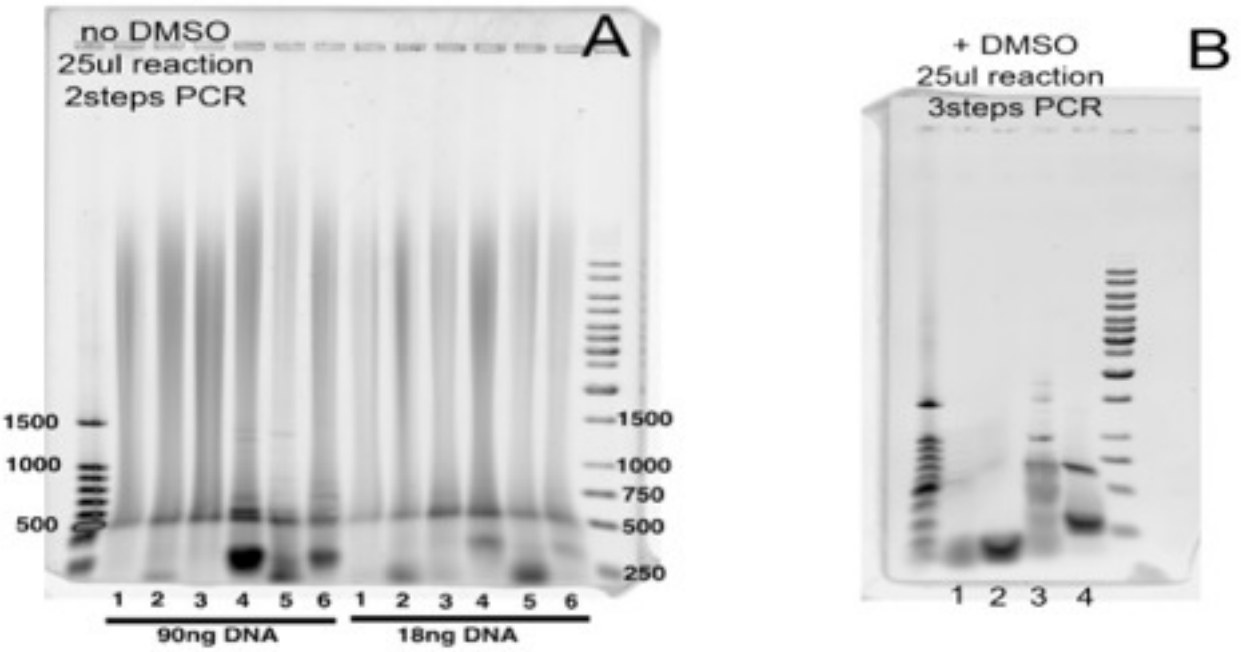

Figure 4: Gel electrophoresis scans. (a) First trial of long-short PCR products using 2-step PCR. 1= Mitochondrial DNA Long, 2= mtDNA Short, 3=Nuclear DNA Long, 4= nDNA Short, 5=mtDNA Long and Short, 6= nDNA Long and Short. Two different wt sample DNA concentrations were tested. (b) Second trial of long-short PCR products using 3-step PCR. 1= mtDNA Long, 2= mtDNA short, 3=nDNA Long, 4= nDNA Short.

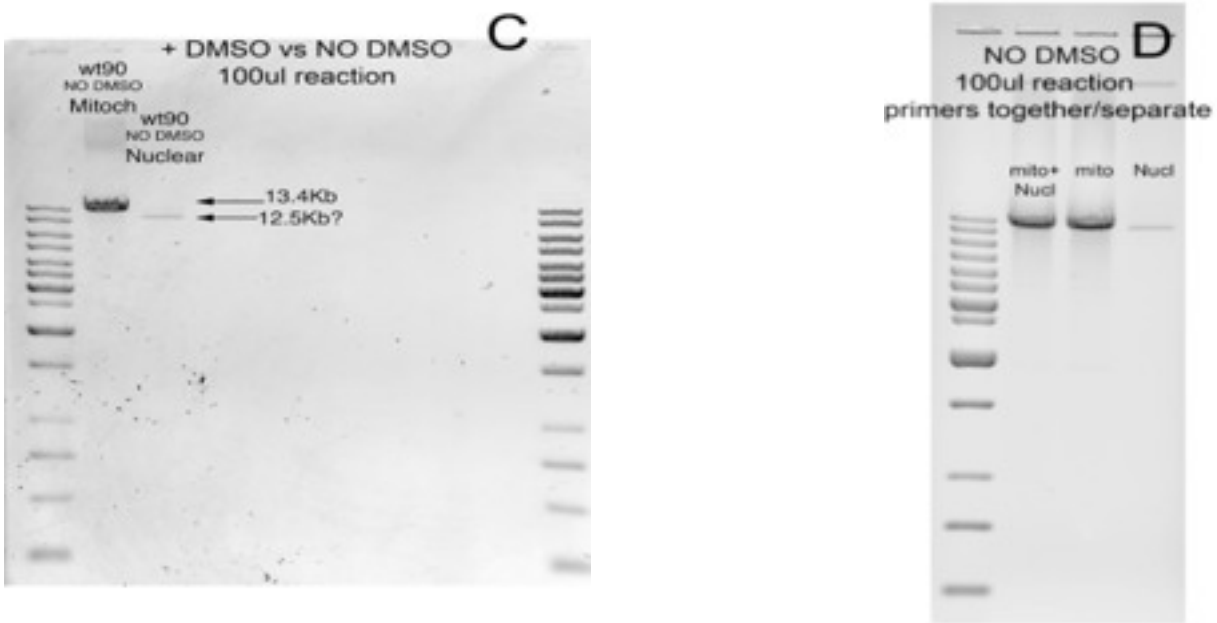

Figure 5: Additional gel electrophoresis scans. (c) Nuclear and mitochondrial primers tested in the Long PCR protocol established in **C**. They work separately. (d) Used conditions as described in dos Santos et al. (2011). DMSO vs. no DMSO, 90ng vs. 180ng. The products are visible in the 90ng reaction in no DMSO.

The results in Figs. 4 and 5 compare the mitochondrial DNA to nuclear DNA. The mitochondrial DNA bands (bands 2, 4 and 5 in Fig. 4a) are seen to be much darker than the nuclear DNA bands (bands 3 and 6 in Fig. 4a). This indicates that mitochondrial DNA production is influenced by oxidative stress. More mitochondrial organelles are produced when they do not function as well as other organelles. In other words, to compensate for the decreased function of mitochondria, more mitochondria are produced. In these mice, more mitochondrial DNA was present than nuclear DNA, suggesting that the function of mitochondrial organelles declines when oxidative stress is present. Figures 1 and 2 show that there was a higher amount of mitochondrial damage in the beta5 integrin knockout mice than in the control mice.

Discussion and Conclusion

As a result of this study, the theory that oxidative stress may cause mitochondrial dysfunction was supported. However, the results that were obtained were not statistically significant. This could be primarily due to the fact that one of the samples in each experiment had extremely different values when compared to the rest of the samples. In Figure 1, the Sample 3 knockout mouse DNA had a value of more than 2.25, while the rest of the samples had values under 0.75. This is the same in Figure 2, where the Sample 2 wildtype mouse DNA had a value of more than 2.25, while the other samples had values under 0.75. These outliers could be the reason that the results are not statistically significant. The reason for the extreme values could be in part due to human error when dissecting the mouse eyes and extracting DNA.

It was planned to measure mitochondrial fragmentation using long range PCR. The fragmented mitochondria wouldn't have shown up, only the complete pieces would have. Therefore, this would have indicated how many mitochondria were fragmented. However, the

long-range PCR was run under incorrect conditions, so fragmentation of mtDNA was not able to be measured. The number of fragmented mtDNA would have shown that damage and fragmentation caused the mitochondria to not function properly. This would result in energy depletion.

Although the results obtained in this study are promising, the sample size of 18 mice was very small, and this study was a pilot study. In order to obtain a more definitive answer, the same experiment should be repeated with a larger sample size of possibly one hundred mouse eyes. If the same results were obtained from a study done with a larger sample size, the results could most likely be more significant and credible. This is because the results from this study were already indicative of oxidative stress resulting from mitochondrial mutations, but since the sample size was so small, one can not be sure that these results were completely accurate. Similarly, the results from figures 2 and 3 were not statistically significant, but they might have statistically significant evidence with a larger sample size.

Once similar results were obtained from a larger sample size study, the scientific community could have the ability to look into preventing and reducing oxidative damage. This could help prevent age-related blindness and AMD. If the oxidative stress is reduced, then as a result, the mitochondrial damage would also be reduced. Less mitochondrial damage would mean more functioning mitochondria that could provide energy to the retina. The retina requires so much energy that fully-functioning mitochondria are vital for good eyesight. If there are fewer people with dysfunctional mitochondria caused by oxidative stress, then there could also be fewer people with age-related blindness and AMD. Methods such as taking antioxidant vitamins and wearing sunglasses with UV protection have been shown to be helpful in preventing and

reducing the amount of oxidative damage a person has, but finding a way to cure it would be ideal.

Recently, an eye drop that targets the membrane attack complex has been noted for its power in treatment of age-related macular degeneration. There are higher levels of the membrane attack complex in people who have age-related macular degeneration. This new eye drop interferes with both membrane attack complex formation and the neovascularization of abnormal blood vessels, which is another symptom of age-related macular degeneration that causes vision loss. Additionally, gene therapy with vascular endothelial growth-factor-binding (VEFG) protein has shown safety, efficacy, and biological activity, with a low retreatment rate. A telescopic eye implant has also had promising results.

Hopefully through continued experiments regarding the above treatments, and through advancements in scientific knowledge, the scientific community will soon find a cure for oxidative damage, and thus, cure AMD.