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ABSTRACT

Although cataracts are the most prevalent cause of blindness in the world, the only effective treatment currently available is surgical lens replacement, an invasive and expensive procedure.[1] The probability of developing cataracts is a function of increasing age. Thus, as an increased proportion of people live longer, the need for a less invasive, more accessible treatment, including prevention, becomes more important.

Understanding the mechanism of cataract formation through the use of basic research approaches can potentially lead to alternative treatment. The ICR/f rat in this investigation is a spontaneous, hereditary model of cataract disease and is one of the few effective animal models available for the study of senile cataractogenesis. Because of mutations in chromosomes 8 and 15, all ICR/f rats will develop cataracts at approximately 75 to 80 days of age.[1]

The goal of this study is to examine changes that occur in the proteins in the eye lens of these rats with aging. The hypothesis is that changes in the solubility of lens proteins occur with aging and these alterations are associated with the process of cataract development.

The soluble protein in the lens ocular tissue collected from ICR/f rats of varying ages were analyzed by SDS-PAGE gels in order to characterize them by molecular weight. A method was developed to study the remaining insoluble lens protein fraction in conjunction with the soluble extractions.

The results from this investigation suggest that the once soluble lens proteins appear to become insoluble during the aging process. As the proteins precipitate out of solution in the lens, the refractive index decreases and the lens becomes opaque, the characteristic feature of cataracts. This method provides biochemical information about cataract development and can be used to evaluate the efficacy of potential cataract treatments.

Keywords: cataracts, aging, lens, vision, protein

INTRODUCTION

Although cataracts are the most prevalent cause of blindness in the world, the only effective treatment currently available is surgical lens replacement, an invasive and expensive procedure.[1] As with any other surgery, the risk of complications such as lens capsular tear or endophthalmitis, although rare, can accompany a cataract surgery.[2] In addition, a lack of trained professionals to complete this procedure or even the lack of the infrastructure necessary to accommodate for surgery can prevent patients from regaining their vision in third-world countries. The probability of developing cataracts is a function of increasing age. Hence, as an increased proportion of people live longer, the need for a less invasive, more accessible treatment, including prevention, becomes more important.

Understanding the mechanism of cataract formation through the use of basic research approaches can potentially lead to alternative treatment. The goal of this study is to examine changes that occur in the proteins in the eye lens of these rats with aging. The hypothesis is that changes in the solubility of lens proteins occur with aging and these alterations are associated with the process of cataract development.

Lens Structure

The lens is not innervated, lacks a blood supply, and does not come into contact with other tissues.[3] Instead, the aqueous humor provides the lens with nutrients and removes waste products through ion channels.[2] The lens is located between the cornea and retina and consists of structures that enhance its transparency and refractive index.[3] An example of these structures is the lens fiber, an anucleated, elongated epithelial cell. Lens fibers scatter little to no light because they have few organelles which allows for the transparency of the lens. However, they do not undergo apoptosis. [3] The eye lens continues to grow with age as fiber cells are constantly produced. Of the proteins present in the fiber cells, 90% of them are crystallin proteins, which will be discussed in detail in a later section. However, differentiated fiber cells cannot synthesize proteins, so the nucleus contains proteins largely from the period of embryogenesis.[4]

Filaments are also present in the lens. Some serve structural purposes but the unique beaded filament is somehow involved with maintaining lens transparency. This special filament may control distribution or orientation of the crystallin proteins to facilitate lens transparency.[3]

Energy and Metabolism

The lens capsule surrounds the eye lens and is essential to the metabolism of the lens. The lens capsule is a semi-elastic basement membrane and serves as a barrier between the lens and other tissues. Nutrients and other essential molecules pass through the lens capsule, but larger entities such as bacteria and viruses are inhibited from reaching the lens. Most of the metabolic activity occurs in epithelium which is attached to the lens capsule.[2]

Glycolysis is the main form of energy production because

very little oxygen is available to the lens.[5] The oxygen that is available is present in the aqueous and vitreous humors around the lens. However, the aerobic respiration pathway yields plenty of ATP in addition to the ATP produced through the less efficient but more common anaerobic pathway in the lens.[3]

Lens Protein

Crystallins are synthesized along with the lens fibers.[3] Most proteins in the ocular lens are low molecular weight, crystallin proteins.[6] Crystallin proteins are common to all vertebrates and are highly homologous in mammals.[4] The more water-soluble crystallin proteins present, the greater the refractive index of the lens.[3] A refractive index gradient is present in the lens since most of the protein is concentrated in the nucleus, the center of the lens. In contrast, little to no protein is present in the periphery, or cortex, of the lens.[2] Hence, the transparency of the lens depends on the fragile balance of the fractions of water and soluble protein; a deviation in concentration of either factor can impact the clarity of the lens.[3]

Furthermore, the high refractive index remains consistent throughout the lens because of the even distribution of lens protein. Transparency is achieved because of the close packing of the soluble and small crystallin proteins. Larger proteins tend to be less water-soluble; thus they cause more light to scatter, compromising lens transparency. Although the exact structure of the crystallin proteins is not known, the protein is believed to possess a tight spherical shape. The filaments in the cytoskeleton are believed to assist in the maintenance of the packing and distribution of the proteins.[3]

Of the three types of crystallins present in the lens, α-crystallins, the most common lens protein (40%), is the largest. The β-crystallin is moderate in size and γ-crystallins have the lowest molecular weight.[6] The molecular weight of a substance is the mass of the substance present in one mole and is generally expressed in Daltons (Da). Although the monomeric weight is 20 kDa, α-crystallin is usually found in complexes with itself, and the molecular weights of these complexes are most commonly within 300 to 1,000 kDa. However, the molecular weight of the native structure varies because of environmental factors; thus, the exact molecular weight is very difficult to accurately measure.[4] Methods have been developed to maximize the accuracy of these measurements, but the molecular weight of the native structure is not relevant or necessary for this investigation since the denatured protein was studied.

Since α-crystallins are the most abundant crystallin protein in the lens, the introduction will focus on elaborating on the types and known functions of this protein. αA and αB crystallins are the two subclasses of α-crystallins, and in humans, they have a 57% amino acid sequence homology. In mammals, these subclasses are generally present in a 3:1 ratio of αA to αB-crystallins. αA is found primarily in the eye lens and can act as a molecular chaperone. In contrast, αB is commonly found throughout the body and is classified to be a ubiquitous chaperone protein.[4]

Yet, the function of alpha crystallin is not fully understood, especially in tissues outside the lens. Prior to 1985, the αA-crystallin was believed to be exclusive to lenticular tissue, but αA-crystallins have been found in the spleen and thymus. αB-crystallins are found throughout the body. Increased amounts of αB-crystallins have been linked to many neurological diseases. Two genes code for the two classes of α-crystallins. The αA gene is on chromosome 21 and the αB gene is on chromosome 11 in humans.[7]

The lens cannot synthesize protein, so the proteins contained in the lens last a lifetime. However, the long lifespan of the lens proteins leaves them vulnerable to chemical and physical modifications.[5] These modifications and their relationship to cataracts are addressed in the following section.

Cataracts and PTMS

Older lens proteins are more susceptible to PTMs (post-translational modifications) such as deamination and aggregation. Deamidation is the most common cause of insolubilization in proteins. PTMs of the crystallins have been linked to nuclear hardening which leads to low antioxidant concentrations.[2] The role of antioxidants in cataract formation will be elaborated upon in the remainder of this section. Older proteins can denature or unfold. As mentioned previously, α-crystallins are small heat shock proteins that can act as a chaperone protein which maintains the solubility of any damaged proteins in the lens. But with age, α-crystallins are not as effective, so proteins begin to aggregate causing cloudiness in the eye. The increased protein aggregation can compromise the transparency of the lens leading to cataracts.[4]

In addition to aggregation, proteins can fragment with age. Full length proteins are common in the lens of younger rats, but protein fragments are ubiquitous in older rats.[8] Mutations in the alpha-crystallin protein have also been linked to cataract formation in humans and animals.[4] Currently, the opacification of the lens cannot be reversed; the lens can only be replaced with a prosthetic lens.[1]

Additionally, environmental factors can influence the transparency of the lens. Increasing exposure to ionizing and UV radiation can hasten the onset of cataract disease. Ultraviolet radiation leads to the production of reactive oxygen species (ROS). ROS cause protein denaturation which increases the risk for cataracts.[1] The antioxidants ascorbic acid and glutathione (GSH) along with the UV filter 3-hydroxykynurenine are naturally produced in the epithelium to protect the lens. GSH is most prevalent in the lens and helps prevent post-translational modifications of the

crystallin proteins. As the concentration of GSH in the lens nucleus decreases, the rate of PTMs increases. However, antioxidants in the lens rely on water-soluble transport. The rates of transport have been shown to decline with age which can hasten the onset of cataracts due to the lack of protection from oxidation.[2]

Thus, antioxidants are currently being researched as a potential therapeutic solution to cataract formation.[1] However, others factors have been linked to cataracts as well. Elevated calcium and sodium levels have been noted in naturally occurring cataracts, and increasing these levels have induced cataracts in experimental lenses.[3] An increased level of calcium is thought to facilitate the degradation of crystallins through the protease calpain.[2]

Furthermore, the loss of the compound lanosterol has been linked to the onset of cataracts in patients who lack the enzyme Lanosterol Synthase (LSS) which catalyzes the reaction that yields lanosterol. Lanosterol is an amphipathic intermediate in the *de novo* synthesis of cholesterol and is able to solubilize insoluble proteins. In a recent study, cataractous rabbit lenses were incubated in lanosterol resulting in clearer lenses. Further investigations are being conducted to evaluate lanosterol as a possible cataract treatment.[9]

Animal Models

Hereditary animal models without congenital onset are the closest representation of senile cataractogenesis, the focus of this study. The inheritance of cataracts in these rats is not fully understood. Inducible rats are often used to study the risk of cataract onset for conditions not directly associated with aging. For example, the inducible rats can be exposed to chemicals such as galactose and streptozotocin to study the effects of cataract formation in diabetics.[1]

The ICR/f rat in this investigation is a spontaneous, hereditary model of cataract disease and is one of the few effective animal models available for the study of senile cataractogenesis. Because of mutations in chromosomes 8 and 15, all ICR/f rats will develop cataracts at approximately 75 to 80 days of age. The rapid lens discoloration is believed to be caused by increased oxidation of the lens during early development in the rats. No statistically significant differences in cataract development have been noted between males and females of the same age under identical conditions.[10]

However, these rats develop cataracts much earlier in their life span, relative to senile cataractogenesis in humans, but no animal model is a perfect representation of human senile cataracts.[10,2] Moreover, the lens shape of rodents and humans vary because human lenses accommodate, but rodent lenses do not accommodate. In species that can accommodate, the lens adopts a flattened shape. In contrast, species that cannot accommodate have spherically shaped lenses.[3, 11] Despite some of the differences between human and rodent lenses, the ICR/f rat is a good model for this study. Rodents are a common model for this type of research. All of the ICR/f rats develop cataracts in a similar time frame which allows for a controlled study comparing lenses from rats of different ages. This particular strain was used in this investigation because the ICR/f rat colony has been maintained by this facility for several years. Thus, the care, breeding, and tissue collection protocols for these animals are not only standardized but are specific and effective for this colony. The formation of cataracts from the initial stages to the latter stages cannot be studied effectively with human lenses due to the paucity of human cataractous lenses.[2] Lenses from each stage of cataract development in the ICR/f rats were procured and analyzed.

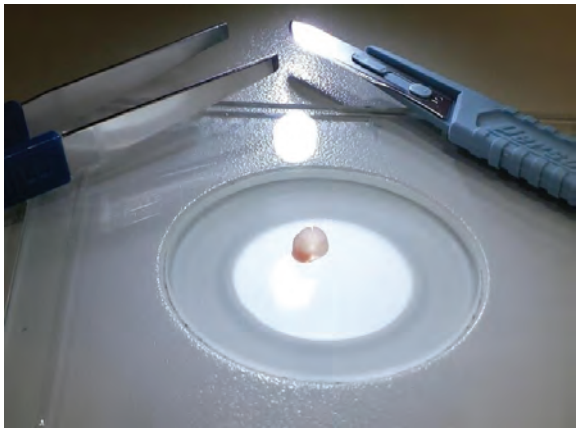

Figure 1| An eye from an ICR/f rat. The lens is isolated prior to experimentation (See "Experimental" section for more information.

EXPERIMENTAL

Homogenization and Extraction

The rat eyes were extracted prior to the start of the study immediately following euthanasia and were stored at -80 °C. Since only the lens was needed, each lens was removed from the eyeball using a scalpel and forceps under a microscope. The lens resides in a capsule within the eye. Each lens was then pulverized in the presence of liquid nitrogen using a glass homogenizer. The pulverized material was then transferred into a centrifugation tube.

Four aqueous extractions resulted from this process. An aliquot of 200 µL of Tris-HCl buffer (50 mM, pH 7.4) was added to the homogenized lens and the solution was centrifuged for 5 minutes at 16,000 rpm. Then, the supernatant was removed and 200 µL of Tris-HCl buffer was added again to the precipitate with vigorous mixing. The samples were centrifuged under the same condition once again. In order to solubilize non-aqueous proteins, the last two extractions were conducted using 6 M tris-urea solution (200 µL per extraction). The urea-protein solution was stored on ice for 15 minutes prior to centrifugation. These four supernates were stored at

-80 °C and were further studied in protein analyses. A solid "pellet" consisting of water-insoluble protein remained and was set aside.

Bradford Assay

A 20% Bradford Solution was prepared using BioRad Bradford Reagent. Five BSA Standards of 0, 4, 8, 12, and 16 µg mL^{-1} were analyzed in the spectrophotometer at 595 nm. The absorbance resulting from these standards were used to construct a linear calibration curve. The four extractions from each of the lens were all analyzed through the Bradford Assay. Each absorbance was converted to a corresponding protein concentration using the calibration curve. The results from the assay provide an estimate of the concentration of protein (µg mL^{-1}) present in each of the samples which was used to determine the amount of sample loaded into protein gels for further analysis.

The Bradford Assay was conducted for several lenses aged 40 days and 166 days. Eleven lenses were analyzed. The average lens protein concentration (µg mL^{-1}) for the two age groups is shown in the charts below.

Table 1| Average protein concentration for younger lenses (40 days old). The table below includes the average protein concentration values for the 4 extractions of 40 day lenses determined by interpolation into the BSA standard curve.

40 day Lens Extraction	Average Protein Concentration (µg mL^{-1})
Extraction 1 (Tris)	116.5
Extraction 2 (Tris)	10.63
Extraction 3 (Tris-urea)	4.97
Extraction 4 (Tris-urea)	3.75
Average Total Protein Concentration: 135.86 µg mL^{-1}	

Table 2| Average protein concentration for older lenses (166 days old). The table below also includes the average protein concentration values for the 4 extractions of 166 day lenses determined by interpolation into the BSA standard curve.

166 day Lens Extraction	Average Protein Concentration (µg mL^{-1})
Extraction 1 (Tris)	22.94
Extraction 2 (Tris)	11.47
Extraction 3 (Tris-urea)	9.15
Extraction 4 (Tris-urea)	5.58
Average Total Protein Concentration: 49.14 µg mL^{-1}	

When the results for older and younger lenses were compared, the total protein concentration is approximately two-fold higher in the younger rats than the older rats. The statistical significance of the data was evaluated (see "Statistical Analysis" subsection below). Based on the results from the two-tailed t-test ($\alpha = 0.05$), there is a statistically significant difference between the total protein concentrations of younger and older lenses.

According to the Bradford Assay, the older lenses contain significantly less lens protein. Protein cannot simply disappear. This "missing" protein had to go somewhere and was most likely present in the insoluble extraction. After lens homogenization, a white, solid pellet remains at the bottom of the test tube – this is the insoluble fraction. In the figure below, the vials containing the insoluble pellets for an older lens from a rat of 166 days and a younger lens from a rat of 40 days are shown below. A large pellet is prominent for the older lens, but the insoluble pellet is extremely difficult to see for the younger lens.

Therefore, it is possible that a larger fraction of the total lens protein precipitates out of solution as the cataract develops with age. One of the Bradford Assay's limitations is that all samples have to be in solution, so the concentration of the insoluble protein could not be measured by this assay or most other protein quantification methods.

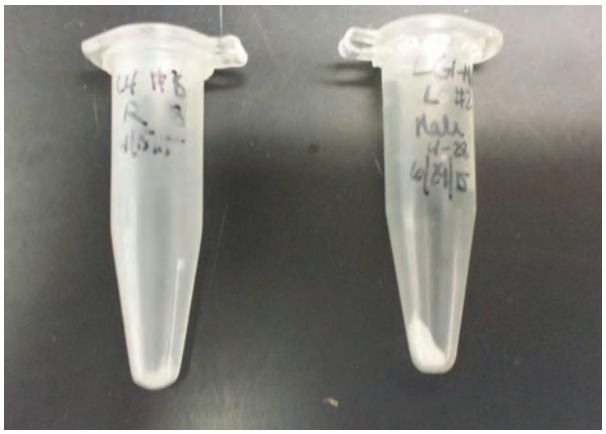

Figure 2| Vials containing the insoluble pellets for an older lens from a rat of 166 days and a younger lens from a rat of 40 days. A large pellet is prominent for the older lens, but the insoluble pellet is extremely difficult to see for the younger lens.

Statistical Analysis

A Grubbs' Outlier test was conducted to identify outliers in the data set. An F-test determined that a t-test assuming equal variances should be completed as opposed to a t-test assuming unequal variances. A two-tailed t-test ($\alpha = 0.05$) was then completed. Microsoft Excel was used for all tests and calculations.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Since the Bradford Assay could not be used, the auxiliary hypothesis was tested by analyzing the insoluble and soluble protein extractions through SDS-PAGE. Several trials were initially run to develop a procedure that allowed for the insoluble protein to be analyzed as the solid pellet had never been analyzed. Although the entire insoluble pellet did not go into solution, the solubilized proteins from the pellet were suitable for analysis by SDS-PAGE. The process of determining an effective protocol is further described at the end of this section.

Separating Gel

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels of 1.5 mm thickness were poured by hand. Tris-HCl (1.5 M, pH 8.8), 30% acrylamide/0.8% bisacrylamide, deionized water, TEMED, and ammonium persulfate were used to prepare the resolving gel. All of the lens samples were analyzed in 12.5% acrylamide gels unless otherwise noted. Other acrylamide percentages were tested with the lens protein samples, and 12.5% acrylamide gels yielded the best separation.

Once the unpolymerized gel was loaded, water saturated isobutanol (50% isobutanol and 50% water) was pipetted over the gel to flatten the gel, prevent evaporation and desiccation of the gel, and remove any air pockets present in the gel. Polymerization of the gel required a minimum of 30 minutes at room temperature. For overnight storage of the gel, the apparatus was wrapped in plastic saran wrap and stored at 4 °C.

Stacking Gel and Sample Preparation

After the separating gel polymerized, the stacking gel and samples were prepared. A solution of BioRad SDS Laemmli Buffer and mercaptoethanol was prepared and mixed with each of the samples. These solutions were then boiled in a hot water bath for 5 minutes.

A 3.9% acrylamide stacking gel was prepared and loaded over the separating gel once the isobutanol was removed. The stacking gel is composed of Tris-HCl (0.5 M, pH 6.8), 30% acrylamide/0.8% bisacrylamide, deionized water, TEMED, and ammonium persulfate. The volume of each solution varied based on the number of gels. A comb was placed between the plates as the stacking gel was loaded to create wells to load the sample after polymerization.

After the 30 to 45 minutes had elapsed for polymerization of the stacking gel, the casting set-up was dismantled and the gels were prepared for the next step. The apparatus was then placed in a buffer tank and the samples were loaded with a micropipette into the wells. The tank was filled with BioRad Tris-Gly-SDS running buffer. The lid was connected to the electrodes and the gel was run for approximately 45 to 60 minutes at 200 V depending on the gel.

Once the gels were removed from the electrodes and glass plates, they were placed in a 40% methanol and 7% acetic acid fixing solution. The trays were then washed with water before staining with approximately 20 mL of Invitrogen stain. The gels were de-stained with 7% acetic acid.

Procedure Development

A procedure was developed for analysis of the insoluble fractions. SDS was included in the sample preparation prior to gel loading to solubilize the insoluble proteins in the presence of heat. When insoluble proteins are heated in SDS, some of the insoluble proteins can solubilize. The amount of protein that becomes soluble depends on the nature of the proteins in the sample. Different volumes of the insoluble fraction were loaded on to the gel to determine the best loading volume. An example of one of the gels used to evaluate the solubilization method is shown below. This gel contains a lens from a 166 day old female.

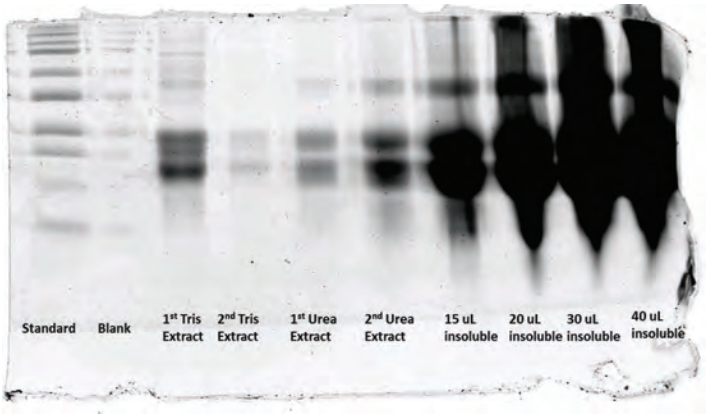

Figure 3| Loading volume test. The gel above contains a lens from a 166 day old female. Different volumes of the insoluble fraction were loaded on to the gel to determine the best loading volume.

This gel shows that the procedure used to prepare the insoluble fraction solubilized a sufficient portion of the insoluble protein analysis by SDS-PAGE. Volumes of 15, 20, 30, and 40 microliters of the insoluble fractions were loaded. All of these volumes resulted in overloaded lanes, so the succeeding gels were loaded with a lower volume of insoluble protein. Another gel with the lens from a 166 day old female was analyzed and is shown below.

Band separation in this gel is clearer, so three corresponding groups of bands can be seen for all of the extractions. Similar loading volumes were used for all subsequent gels.

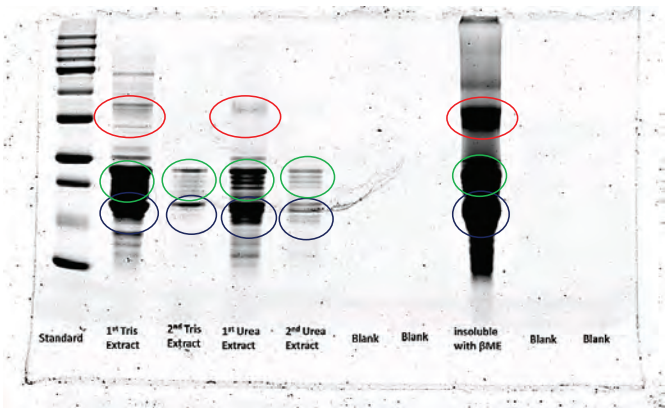

Figure 4| Gel with protein from a 166 day female. The gel above contains lens protein from another 166 day old female but this gel was run after a general procedure had been established. The band groups containing similar sized proteins are circled in matching colors for each of the extractions.

During sample preparation, β-Mercaptoethanol (BME) is usually mixed with the Laemmli Sample Buffer, a reagent that is added to all samples loaded onto the gel. BME breaks disulfide bonds between protein chains unfolding the proteins further. In order to gain a greater understanding of the nature of the proteins present in the insoluble fraction, the insoluble fractions from young and old lenses were analyzed without BME ("SDS Only") and compared with the same sample treated with BME (see Figure 5 in the Results section).

RESULTS AND DISCUSSION

The results of the Bradford Assay comparing the average total lens protein concentrations of relatively younger and older rats suggest there is a statistically significant difference in the amount of protein in the lenses of the two age groups. A two-tailed t-test ($\alpha = 0.05$) was conducted to evaluate the assay results. The p-value was calculated to be 0.021. These results prompted the study of the solid pellet that remains after the lenses are homogenized. (See Experimental section for more information.) A procedure to study the protein in the insoluble fractions through SDS-PAGE was developed. The results from the resulting experiments are shown below.

As described in the Experimental section, the band separation is more discernible in the gel presented in Figure 4. Three corresponding groups of bands are visible in each of the lanes.

These groups are marked in red, green, and blue circles. The 2nd Tris buffer and Tris-urea buffer extractions do not contain as much protein as the other extractions since most of the proteins soluble in each of the buffers were extracted in the first extraction. Thus, the bands are fainter for the second extractions, and the highest molecular weight band (red circle) is extremely faint and not visible for these two extractions.

The results from the gel suggest that the proteins present in the insoluble fraction are the same proteins in the other aqueous extractions since they all have bands in the same molecular weight region of the gel. The main difference among the extractions is the intensity of the bands. Some of the extractions contain more protein. For example, the darkest bands are from the insoluble extraction, so the most protein in the 166 day old female's lens is concentrated in the insoluble fraction. The 1st Tris extraction contains less protein than the insoluble fraction but more protein than the other extractions. The following lists the data in descending order (highest protein content to least): the insoluble fraction, 1st Tris buffer extraction, 1st Tris-urea buffer extraction, 2nd Tris buffer extraction, and 2nd Tris-urea buffer extraction.

Furthermore, the group marked by the green circle contains bands in the 20 kDa range. α-crystallin proteins, the most common protein in the eye lens, has a molecular weight of 20 kDa[6], so these bands on the gel are most likely to be α-crystallin. Since the insoluble fraction contains bands in the same region, it is possible that the insoluble extraction contains α-crystallin proteins that have become insoluble. α-crystallin is usually soluble, so its presence in the insoluble fraction is unexpected (see Introduction).

To better understand the proteins in the insoluble fraction, the insoluble fractions from young and old lenses were analyzed without BME ("SDS Only") and compared with the same sample treated with BME (see Figure 5). Insoluble protein from a young rat (40 days) and an old rat (166 days) were analyzed to study the potential differences correlated with age.

The bands resulting from the samples treated with BME are generally of lower molecular weight than the samples treated with SDS only in the Sample Buffer because the BME further fragments the proteins by breaking the disulfide bonds. The samples treated with only the SDS in the Laemmli Sample Buffer are unfolded by the SDS, but they are not as fragmented. Therefore, the insoluble proteins unexposed to BME provide more information about the structure of the proteins as there are more variations in size and an increased number of high molecular weight proteins.

Although most bands are still present in the 20 kDa region (green circle), the samples not treated with BME show extensive "streaking" in the lane indicating that a variety of proteins with different molecular weights are present in the sample. Since the lens cannot synthesize proteins, these unidentified proteins have to be proteins already present in the lens. α-crystallin proteins are the most common protein in the lens, so these proteins could potentially be α-crystallin polymers of varying sizes. However, this assertion cannot be confirmed by SDS-PAGE. Mass spectrometry (MS) could provide more information about the insoluble protein.

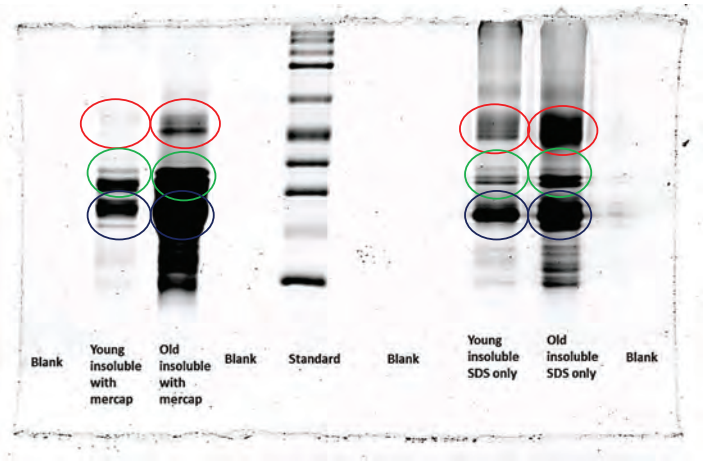

Figure 5| Insoluble protein gel analysis (with and without β-Mercaptoethanol or BME). To better understand the proteins in the insoluble fraction, the insoluble fractions from young and old lenses were analyzed without BME ("SDS Only") and compared with the same sample treated with BME. The "young" lens is from a rat of 40 days of age and the "old" lens is from a rat of 166 days of age.

In addition to the differences between the lens protein in the presence of BME and the protein in the absence of BME, disparities in the concentration of the insoluble protein are apparent when the young and old lenses are compared. The younger lens appears to have less insoluble protein. At 40 days of age, the rats do not typically have visible cataracts, but rats that are 166 days old have a fully developed, stage 4 cataract. The increased precipitation of lens protein in older rats may be correlated to the phenotypic appearance of the clouding in the lens. α-crystallins may polymerize in response to a signal or change within in the lens that occurs in these rats as they age.

Consequently, this hypothesis based on the SDS-PAGE and Bradford Assay results was tested by running SDS-PAGE gels for lenses of varying ages:

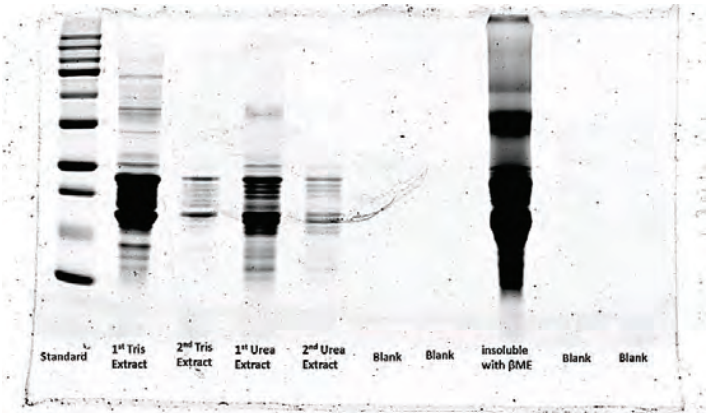

Figure 6| Gel with 166 day female. The aqueous and insoluble fractions of a lens from a 166 day old female were analyzed by SDS-PAGE. This gel is the same gel as the one in Figure 4 and is juxtaposed with the other gels for convenience.

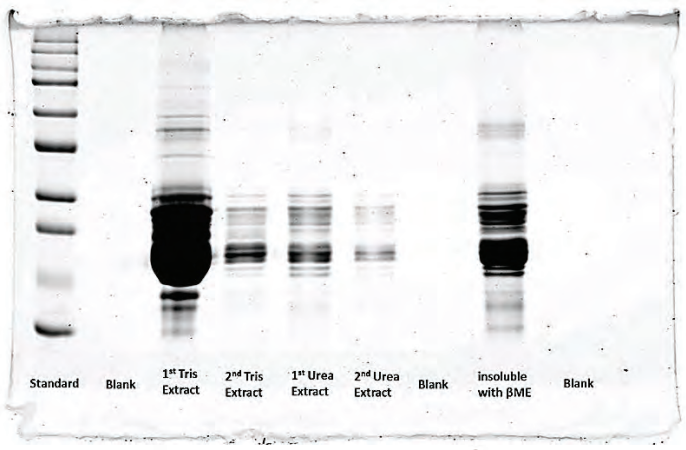

Figure 7| Gel with 66 day female lens. The aqueous and insoluble fractions of a lens from a 66 day old female were analyzed by SDS-PAGE.

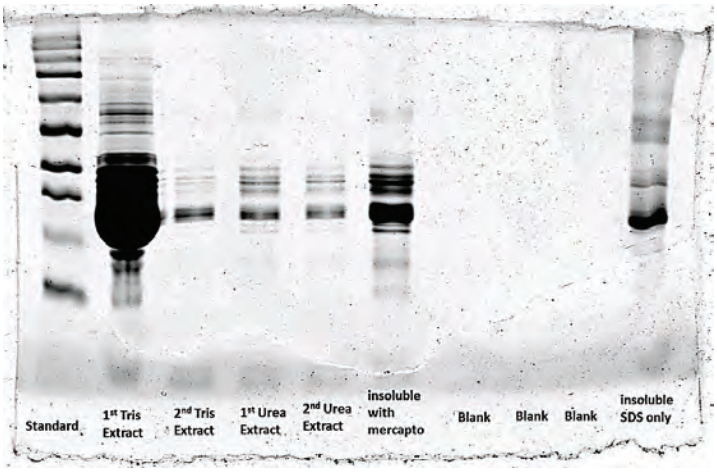

Figure 8| Gel with 46 day female lens. The aqueous and insoluble fractions of a lens from a 46 day old female were analyzed by SDS-PAGE.

Three lenses for each age group were analyzed. The loading volume and all other experimental aspects were controlled; the only difference among the gels is the age of the lens analyzed. A gel from one of three trials for each age is shown in Figures 6–8 above. Most of the protein in the youngest lens (46 days old, Figure 8) is concentrated in the first aqueous extraction suggesting that the majority of the proteins are soluble. A much smaller proportion of the protein is present in the insoluble fraction, as expected, since a 46 day old rat generally does not exhibit visible lens clouding. Similar to the 46 day old rat lens, the 66 day old rat lens contains predominantly soluble protein but the band for the 1st Tris buffer extraction is not as intense as that of the 46 day lens. However, the insoluble extraction appears to be more intense for the 66 day lens.

In contrast, the 166 day old lens (Figure 6) has a strikingly lower amount of protein in the aqueous extractions. The 1st Tris buffer extraction band is intense but not nearly as dark as those of the younger lenses. The decrease in protein in

the aqueous extractions is compensated by the increase in protein in the insoluble extraction. The band for the insoluble extraction of the 166 day lens is as dark as the band for the 1st aqueous extraction of the 46 day lens.

For easier comparison of older and younger lenses, the following gel juxtaposes the major lens extractions for a 166 day and 40 day old rat:

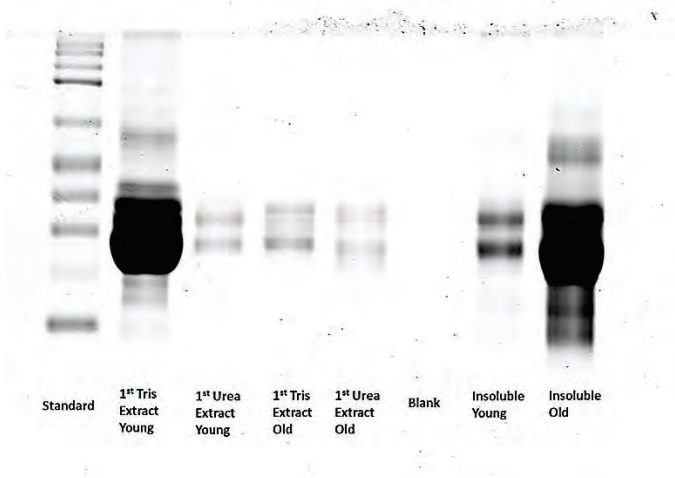

Figure 9| "Summary Gel." The gel above juxtaposes the protein from a young and old lens (40 days and 166 days of age, respectively). Qualitatively, the 1st Tris buffer extraction for the young lens and the insoluble extraction for the old lens have similar concentrations. Similarly, the insoluble extraction for the young lens and the 1st Tris buffer extraction for the old lens have similar concentrations.

As seen with Figures 6–9, the 1st Tris buffer extraction for the young lens and the insoluble extraction for the old lens have similar concentrations. Similarly, the insoluble extraction for the young lens and the 1st Tris buffer extraction for the old lens have similar concentrations. Because the 166 day lens belonged to an older rat, the cataract was fully developed (Stage 4), so more of the proteins in the lens were insoluble. All of the lanes appear to have protein bands in the three regions highlighted in Figures 4 and 5 suggesting that all of the lens extractions contain the same proteins but in different forms and varying distribution during cataract development. The results seem to indicate that once soluble lens proteins are prompted to become insoluble during the aging process. As the proteins precipitate out of solution in the lens, the refractive index decreases and the lens becomes opaque, the characteristic feature of cataracts.

In addition to the time points compared above, two older lenses were compared. A 187 day male lens was analyzed and compared with the 166 day female lens from Figure 6. A 166 day old male or a 187 day old female was not available, so the lenses from two different genders had to be compared.

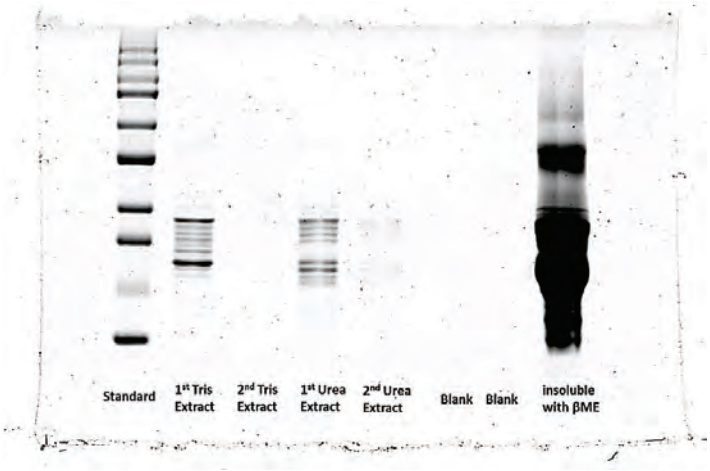

Figure 10| Gel containing lens protein from a 187 day old male. The aqueous and insoluble fractions of a lens from a 187 day old male were analyzed by SDS-PAGE.

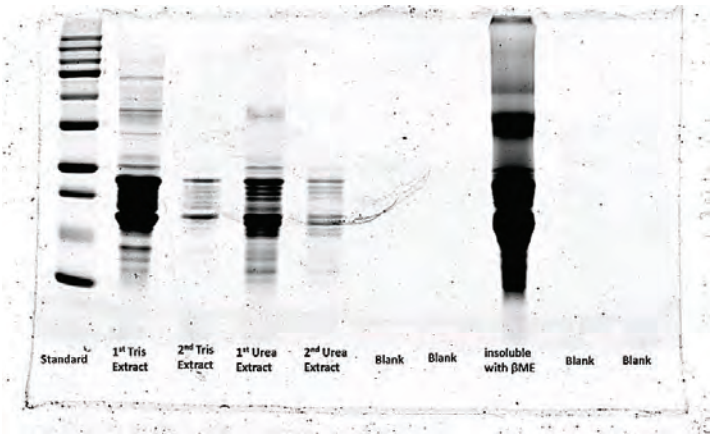

Figure 11| Gel containing 166 day female lens. The aqueous and insoluble fractions of a lens from a 166 day old female were analyzed by SDS-PAGE. This gel is the same gel as the ones in Figures 4 and 6 and is juxtaposed with the gel containing the 187 day male lens for convenience.

Based on Figures 10 and 11, protein aggregation continues to occur with age, even when the cataract is considered to have reached the latest stage in the clinical classification scheme. More protein is present in the insoluble fraction for the 187 day old since the band is much darker than that of the 166 day old rat. The aqueous fraction bands are extremely faint for the 187 day lens.

Sex-based Differences and Experimental Limitations

Although the SDS-PAGE results seem promising, a major limitation is that only three time points were studied (46, 66, and 166 day old rats). Ideally, the lenses from rats of 30 days to 80 days should be studied for a total of six time points. An increased number of trials would also improve the accuracy of the results. In this investigation, three trials were conducted for each of the time points, but for the 66 day lenses, two of the rats were female and one was male. There was not sufficient time to analyze a third 66 day old female rat.

Based on the lenses that were studied, a difference between male and female lenses of the same age was not observed. However, only one male lens with a comparable female lens was studied (both lenses were from 66 day old animals). The resulting gels are shown in Figures 12 and 13.

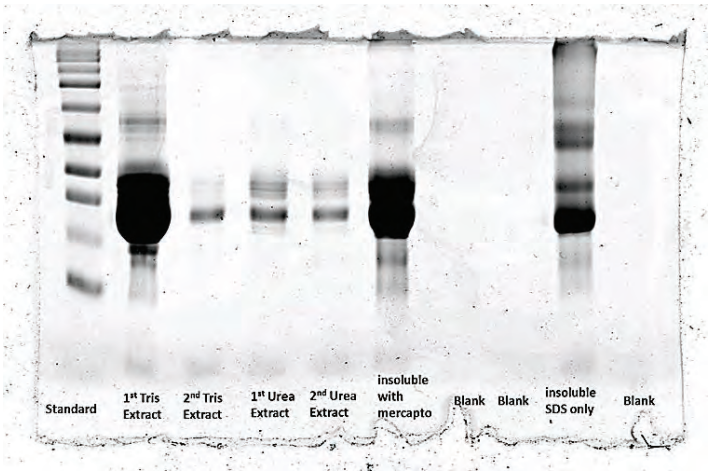

Figure 12| Gender comparison, male lens. The aqueous and insoluble fractions of a lens from a 66 day old male were analyzed by SDS-PAGE.

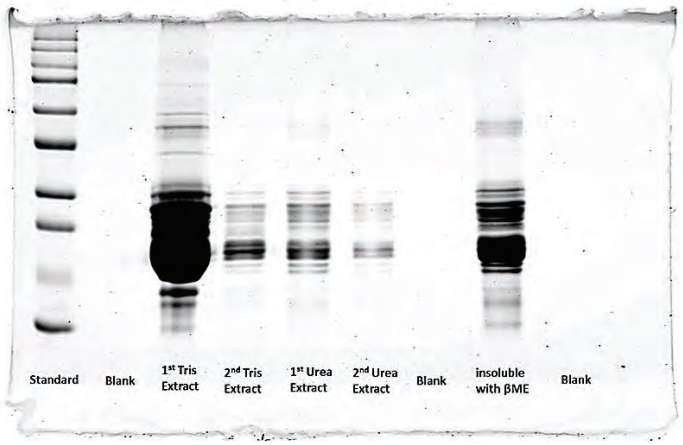

Figure 13| Gender comparison, female lens. The aqueous and insoluble fractions of a lens from a 66 day old female were analyzed by SDS-PAGE. This gel is the same gel as the one in Figure 7 and is juxtaposed with the gel with the 66 day male lens for convenience.

A difference between the two genders is not apparent, but only one male lens was analyzed. More male lenses at this time point and at other time points should be analyzed in order to determine if a sex-based difference in lens protein distribution exists. Nevertheless, some variation can be seen between the two gels. This variation is also present among gels of the same age and even two different gels containing lens protein from the same lens. Thus, comparing lenses of similar ages is difficult because the differences in the bands are too minute. Qualitative analysis of gels from lenses of significantly different ages is easier because the disparities in the bands are very noticeable and cannot be explained by variation inherent to the gels. Therefore, quantitative analysis would be a likely solution to this limitation.

Future Studies

As mentioned previously, MS can be used to identify and quantify the proteins present in each of the aqueous extractions. MS will be able to verify the protein identifications that were tentatively suggested based on current literature and the results from the SDS-PAGE gels. An untargeted mass spectrometry analysis could also reveal the entire protein composition of the insoluble and aqueous lens fractions.

Furthermore, densitometry is a method that can quantify relative protein content of the aqueous and insoluble fractions by analyzing the intensities of the corresponding bands on the gel. Quantitative analysis would help distinguish normal variation from actual differences in the bands of two gels. In addition, this method would also allow for the comparison and validation of data by statistical analyses such as the t-test.

Dark bands are convenient for the qualitative analysis whereas lighter bands are necessary for quantitative analysis by densitometry. In the gels shown in this paper, some of the lanes are often overloaded, usually the insoluble fraction or 1st Tris buffer extraction depending on the age of the lens. The loading volume could have been reduced to prevent overloading, but because of the disproportionate protein distribution among the extractions, the already faint bands would no longer be visible with a decrease in loading volume. Hence, qualitative analysis would not be possible for these gels. However, for densitometry, a lower loading volume would be required because the instrument measures the intensity of the bands and cannot distinguish between very high intensity readings. Although some bands will be too faint to see with the naked eye, the instrument would still be able to detect those bands.

On the other hand, the same protein concentration could be loaded onto the gel to ensure all the bands are visible, but the protein concentration cannot be determined for the insoluble fraction. Also, the purpose of this experiment was to compare relative protein content among the aqueous and insoluble fractions within in the same lens in addition to comparing the results of different lenses. For these reasons, a consistent loading volume for all the protein samples was selected.

A two-dimensional gel analysis in which the SDS-PAGE gel and the isoelectric strip could be run at once to separate the proteins based on molecular weight and isoelectric point could also be conducted. The 2-D gel will provide an additional perspective to the study.

Although no control group was used due to a limitation in resources in this investigation, white Wistar rat lenses should also be analyzed at identical time points. The control group would indicate the natural change in lens protein composition that occurs with aging. The addition of the control group would result in a more thorough analysis and, therefore, will provide a better understanding of the unique changes in the lens that leads to cataracts.

Applications in the Medical Field

To further understand cataract development, the phenotypic appearance of cataracts can be related to the results from the biochemical approach this technique provides. The rat's lenses can be analyzed by slit-lamp prior to ocular tissue extraction. Furthermore, this technique can be used to evaluate the effectiveness of a potential cataract treatment in addition to following the progression of cataracts with age. Untreated cataractous lenses can be compared to treated cataractous lenses by comparing the relative protein contents of the aqueous and insoluble fractions.

CONCLUSION

A difference in total protein concentration between a young and an old lens prompted the study of the previously unanalyzed insoluble fraction. When 46, 66, and 166 day female lenses were compared by SDS-PAGE, once soluble lens proteins appear to become insoluble during the aging process. As the proteins precipitate out of solution in the lens, the refractive index decreases and the lens becomes opaque, the characteristic feature of cataracts. Furthermore, based on the comparison of a 187 day lens and a 166 day lens, the aqueous lens protein continues to precipitate even when the cataract is considered to have reached the latest stage in the clinical classification scheme.

No difference could be distinguished between male and female rats of the same age, but this conclusion is based on a statistically insignificant number of trials. More trials could not be conducted in this investigation because of constraints in time and resources. Nevertheless, current literature suggests that there is no sex-based difference in cataract formation.[10]

However, this study did not address the composition of the proteins in each of the fractions made from the lenses at different ages. Besides 2-D gel electrophoresis, further studies should use mass spectrometry to identify and quantify the protein in the different lens fractions.

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