Characterization of Proteins and Tissue Remodeling Components in Porcine Aqueous Humor

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(ABSTRACT)

Connective tissue remodeling is an important area of study in biomedical engineering with respect to cancer and wound healing. Tissue remodeling components may be involved in the pathogenesis of open-angle glaucoma. Risk factors for open angle glaucoma include increased intraocular pressure (IOP), male gender, and advanced age. In a 1963 study, the hormone relaxin decreased IOP in the human eye through a mechanism that may involve the up-regulation of tissue remodeling matrix metalloproteinases (MMPs). The effects of age and gender on MMP and protein activity in porcine aqueous humor were determined in this study to identify correlations existing between MMP activity and glaucoma risk factors. Gelatin zymography identified MMPs at 66 kD and approximately 105 kD. The concentration of the 66 kD band compared to human MMP-2 standard was 0.22 ± 0.06 ng/μl for the adult female (AF) samples and 0.28 ± 0.04 ng/μl for the juvenile samples. This difference in concentration was statistically significant ($p < 0.05$). The concentration of the protease migrating to 66 kD was statistically independent of gender. Casein zymograms identified two non-MMP proteinases at 51 kD and 80 kD. The average total protein concentration for all aqueous humor samples was 2.54 ± 0.89 mg/ml. The mean IgG, transferrin, and albumin concentrations for all aqueous humor samples was 11.4 ± 4.2 μg/ml, 17.11 ± 6.8 μg/ml, and 78.0 ± 26.3 μg/ml respectively. Results from these experiments establish baseline levels of MMP and protein activity, allowing for identification of potential changes caused by relaxin in tissue culture studies.

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Chapter 1 Introduction

Glaucoma, the second leading cause of blindness worldwide, is characterized by gradual visual field loss and degeneration of the optic nerve. [1] The major risk factor for glaucoma is an elevated intraocular pressure (IOP). Advanced age, family history, and race are other risk factors. [2,3] While several forms of glaucoma exist, the most prevalent is open angle glaucoma (OAG), which is characterized by IOPs over 21 mmHg and advanced age. [4] IOP is a function of aqueous humor production and drainage. Aqueous humor, produced by the ciliary body, bathes the anterior segment of the eye, providing nutrients and removing metabolic wastes. After entering the posterior chamber and then the pupil, aqueous humor flows into the anterior chamber of the eye (Figure 1.1). There are two drainage routes for the aqueous humor in the anterior chamber of the eye: 1) 85 % drains through the pressure dependent trabecular outflow route and 2) 15 % leaves through the pressure independent uveoscleral route. [5] The trabecular route involves drainage through the porous trabecular meshwork and then Schlemm's canal, while the uveoscleral route involves drainage through the interstitial spaces between ciliary smooth muscle cells. [6] Barriers to either drainage route increase IOP, which can lead to glaucoma. Thus, most current glaucoma medications are aimed at reducing IOP by reducing aqueous humor production or increasing outflow.

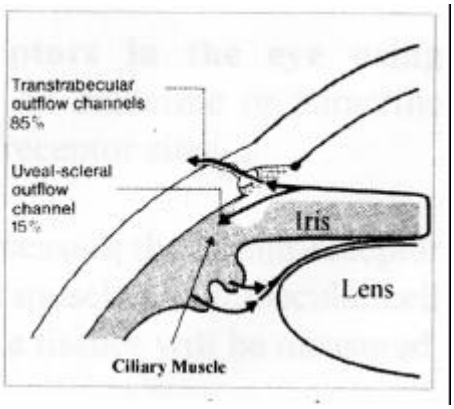

Figure 1.1. Flow of Aqueous Humor. [7]

Current glaucoma medication has many drawbacks such as harmful side effects and short-term efficacy. Prostaglandin analogues, primarily affecting the uveoscleral route, lower IOP by inducing the release of matrix metalloproteinases (MMPs) that degrade and remodel tissue between ciliary smooth muscle cells. [6,8,9] Unfortunately, burning and stinging in the eye are side effects of prostaglandin treatment. [10,11] Laser trabeculoplasty has been successful in decreasing IOP in some patients by opening drainage holes in the trabecular meshwork, but side effects such as scar tissue and short-term IOP increase have kept it from being the primary method used. [12,13] Filtration surgery decreases IOP by creating a hole in the sclera to allow fluid to escape the eye, but can cause cataract progression and hypotony (very low IOP). [14,15]

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade different extracellular matrix components. The MMPs are divided into three different subclasses based on their substrate specificities: collagenases (degrade types I-III collagen), gelatinases (degrade types IV, V, VII collagen, gelatin, fibronectin), and stromelysins (degrade wide variety). MMPs are secreted in a latent form containing a propeptide that is cleaved off producing an active form. MMP activation is inhibited *in vivo* by specific tissue inhibitors of MMPs (TIMPs) and α2-macroglobulin. Four major TIMPs have been identified as of 1999: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Table 1.1 lists the MMPs with their molecular weights and substrate specificities discovered as of January 2000.

In 1963, a study was carried out with relaxin, a pregnancy hormone, to test whether it affected IOP in a small group of patients. [16] In the study, after intramuscular injection of relaxin, male and female patients responded with lower IOPs, and showed an increased outflow facility of aqueous humor using tonography. Outflow facility is a measure of pressure dependent outflow. Further work on relaxin and IOP was abandoned largely due to the lack of available relaxin. The increase in outflow facility and decreased IOP indicates that relaxin is removing or remodeling tissues in the drainage routes. Relaxin remodels connective tissue during pregnancy, and affects MMP expression. [17,18] Since relaxin affects the outflow facility, it may be affecting the pressure dependent trabecular outflow route. Relaxin, due to its ability to lower IOP in patients,

may be a possible glaucoma therapeutic in the future. The way relaxin affects MMP expression is the first step to understanding its potential role in the eye.

Relaxin has been identified as a hormone that lowers IOP in the eye, but the mechanism by which it does so is unknown. The role the MMPs play in tissue remodeling after being up-regulated by relaxin is important in understanding how IOP is lowered. A major objective of this thesis is to determine the levels of different MMPs in the porcine aqueous humor. Pigs have been chosen as the animal model due to their availability and the quantity of aqueous humor (100-150 μl) that can be taken from a porcine eye. Pigs have also been used as tissue culture models for prostaglandin analogue studies. [19,20] Identifying MMPs in aqueous humor establishes baseline values that can be compared to changes occurring after relaxin introduction in cell culture models.

Table 1.1. Classification, molecular weights (MW), and extracellular matrix (ECM) substrate specificities of the matrix metalloproteinases (MMPs).

MMP	Class	MW (kD)	ECM Substrates
1	Collagenase	55 [21]	Collagens I, II, III, VII, X
2	Gelatinase	72 [21, 22]	Gelatins, elastin, aggrecan, collagens IV, V,VII, X, XI, fibronectin
3	Stromelysin	57 [21]	Proteoglycans, fibronectin, laminin, gelatins, collagens III, IV, V, IX, XI
7	Stromelysin	28 [21]	Proteoglycans, collagen IV, fibronectin, laminin, gelatins
8	Collagenase	75 [21]	Collagens I, II, III
9	Gelatinase	92 [22]	Gelatin, collagens IV, V; proteoglycan, elastin
10	Stromelysin	57 [21]	Proteoglycans, fibronectin, laminin, gelatins, collagens III, IV, V, IX; elastin
11	Stromelysin	51 [21]	Unknown
12	Stromelysin	57 [21]	Elastin, fibronectin
13	Collagenase	48 [23]	Collagens I, II, III
14	Stromelysin	60 [23]	Unknown
15	Stromelysin	62 [24]	Unknown
16	Stromelysin	62 [25]	Unknown
17	Stromelysin	64.3 [26]	Unknown
18	Collagenase	57 [27]	Unknown
19	Stromelysin	58 [23]	Unknown
20	Stromelysin	54 [28]	Unknown
25	Stromelysin	28 [29]	Unknown

Summary of Objectives

1. Identify the MMPs (and other tissue remodeling components) present in porcine aqueous humor. Using densitometry, MMPs and other components will be quantified by comparison to a standard. The characterization of these MMPs is important for future work as it establishes baseline values of MMPs in adult (female) and juvenile pigs. This has not been done previously for porcine aqueous humor. To understand the effects of relaxin on MMP activity, it is necessary to know the MMPs present in the aqueous humor before addition of the hormone.

2. Determine how age and gender affects MMP activity in aqueous humor. Adult female and juvenile female aqueous humor samples will be compared to determine the effects of age on MMP concentration in the aqueous humor. This is important since glaucoma generally affects people only after they have reached adulthood. Comparison of juvenile female and male aqueous humor samples will determine whether gender affects the MMP levels. Adult male porcine aqueous humor samples will not be used due to the difficulty of obtaining a sufficient sample number of adult male pigs. The effects of age and gender on MMP levels in porcine aqueous humor have not been explored previously.

3. Quantify levels of some blood serum proteins in aqueous humor using immunoassays. The quantity of the some of the serum proteins will be determined using immunoassays. The choice of serum protein to analyze will come from the molecular weight patterns of proteins shown by gel electrophoresis. The effects of age and gender on the serum protein concentration will be determined and compared to the trends noted in MMP concentration.

4. Determine whether age and gender affect protein composition. Total protein assays, gel electrophoresis, and immunoassays will be performed as stated above on adult female, juvenile female, and juvenile male aqueous humor samples to determine whether age or gender affect protein levels in porcine aqueous humor. The differences in protein composition in the right and left eyes will also be compared.

5. Quantify the total amount of protein in aqueous humor. A total protein assay will be used to quantify the concentration of protein in the aqueous humor.

6. Determine the protein composition of porcine aqueous humor. The protein composition of porcine aqueous humor will be analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Results from the total protein assay will determine the quantity of aqueous humor that needs to be loaded onto the gel. The gel electrophoresis and subsequent silver staining will identify the molecular weights of the major proteins found in aqueous humor.

Chapter 2 Review of Pertinent Literature

2.1 Glaucoma Pharmacology

Most of the current medication is aimed at reducing intraocular pressure (IOP) in the eye, since it is the major risk factor in the onset of glaucoma. The majority of the current glaucoma therapeutic options involve direct application of a drug into the eyes. There are five major classes of common monotherapeutic antiglaucoma medications [30,31]:

1) Beta-adrenergic receptor antagonists (β-blockers)
2) Parasympathomimetics
3) Carbonic anhydrase inhibitors (CAIs)
4) Prostaglandin analogues
5) Alpha-adrenergic and β_2-adrenergic agonists (sympathomimetics)

2.1.1 b-blockers

β-blockers are often the first therapy of choice in treating glaucoma. [30,31] Common β-blockers that are used are timolol and betaxolol. Timolol, a non-selective β-blocker, inhibits aqueous humor production [32] while betaxolol, a β_1-blocker, inhibits aqueous humor flow. [33] Neither of these drugs affects the outflow facility of aqueous humor. [34,35] Both of these drugs have shown the ability to decrease IOP levels in glaucoma patients. [36,37,38] There are side effects associated with both timolol and betaxolol. Burning, stinging, tear flow reduction, and hyperemia (redness from increased blood flow) of the conjuctiva are all side effects of timolol use. [39,40,41] The most frequent ocular side effects of betaxolol are stinging and burning in the eye. [30]

2.1.2 Parasympathomimetics

Parasympathomimetics can be classified as cholinergic agents or cholinesterase inhibitors. The cholinergic agents are known as miotics. Common parasympathomimetics are pilocarpine and demecarium. Pilocarpine, a miotic drug, is one of the oldest glaucoma medications, while demecarium is a cholinesterase inhibitor. Pilocarpine contracts the ciliary muscle; this action pulls the iris away from the trabecular meshwork and allows aqueous humor to flow out through the drainage channels,

lowering IOP. Pilocarpine has been shown to increase the outflow facility in monkeys,[42] rabbits,[43] and human eyes. [44] There are many adverse effects associated with pilocarpine use such as muscle spasms caused by ciliary muscle contractions, blurred vision, and headaches. [45] Severe side effects such as cataract formation are associated with cholinesterase inhibitors like demecarium, making their use much less frequent. [46]

2.1.3 Carbonic Anhydrase Inhibitors (CAIs)

Carbonic anhydrase inhibitors (CAIs) reduce IOP in the eye by decreasing the production of aqueous humor. CAIs were initially available in tablet form to be taken orally, but the systemic side effects proved to be too severe. [47] Topical CAIs such as dorzolamide and brinzolamide appear to have removed the major systemic side effects, but have only been available for five years, and therefore the long-term effects are not known. Stinging and burning were some of the ocular side effects noted after use of both drugs, with brinzolamide having slightly less discomfort. [48]

2.1.4 Prostaglandin Analogues

Prostaglandin analogues reduce IOP by increasing the outflow of aqueous humor through the uveoscleral route. [49] These analogues have been shown to alter the extracellular matrix adjacent to the ciliary muscle cells allowing greater uveoscleral outflow. [8] The prostaglandins alter the extracellular matrix by increasing the release of matrix metalloproteinases from ciliary smooth muscle cells.[50] The major prostaglandin analogue that has been available to glaucoma patients is Latanoprost. Side effects such as stinging, burning, and increased iris pigmentation were detected in patients. [10,11]

2.1.5 Sympathomimetics

Sympathomimetic drugs such as epinephrine, apraclonidine, and brimodine stimulate the adrenoreceptors in the eye. Epinephrine is nonselective to adrenergic receptors, and reduces IOP by increasing aqueous humor outflow through both the uveoscleral and trabecular routes. [51,52] Some of the many side effects associated with epinephrine are anorexia [53] and ocular allergic dermatitis. [54] Both apraclonidine and brimodine are selective to alpha-2-adrenergic receptors, and reduce IOP by decreasing

aqueous humor flow. [55] Brimodine removes some of the ocular allergic side effects of apraclonidine [56], but mild hyperaemia and cornea staining are side effects that are observed. [57]

2.1.6 Other Glaucoma Therapies

Combinational treatments that mix two or more different monotherapeutic drugs are also used when the effects from any single medication are insufficient. The goal in combinational therapy is to reduce IOP by an additional 15 %. [30] Drugs that act at different receptors, and have different modes of action are preferred in combination. [30] Some examples of therapies combine β-blockers and CAIs, or β-blockers with epinephrine. Argon laser trabeculoplasty (ALT) is used to treat glaucoma often after medications have failed. [30] IOP is lowered by placing tiny burns onto the trabecular meshwork, which creates drainage holes. For some patients ALT has shown to be successful in lowering IOP, and keeping the pressure down for several years. [58,59] For other patients, however, additional laser surgery was required, since IOP increased after a year. [58,59] Scarring of the trabecular meshwork by the laser burns may also cause a worsening of the glaucoma. [12] The most common side effect associated with laser therapy is a short-term rise in IOP occurring in the patient within the first 6 hours of the procedure. [13] Filtration surgery involves creating an incision in the sclera, which allows fluid to escape and be absorbed in another part of the eye. Filtration surgery has been successful in some patients in decreasing IOP but cataract progression and hypotony are reported side effects. [14,15]

2.2 Total protein content in aqueous humor

The total protein content in the aqueous humor of various animals was compared to determine a range of concentrations as a guide for the BCA assay. The total protein in the aqueous humor of four domestic animals (dog, cat, horse, cow) was determined using the phenol method to be 0.36 mg/ml, 0.44 mg/ml, 0.56 mg/ml, and 0.58 mg/ml for 12 dogs, 15 cats, 7 horses, and 6 cattle respectively. [60] The average total protein content of 68 young Rhesus monkey aqueous humor samples was found to be an average of 0.06 ±

0.02 mg/ml. [61] In this case, the Bradford colorimetric protein assay was used for total protein determination for the 10-20 μl samples. The phenol method is a modification of the Lowry protein assay, and uses the same $Cu^{2+} \rightarrow Cu^{+}$ reaction that is the basis of the BCA assay. [62]

Human aqueous human samples were taken following cataract surgery. Using the Bradford protein assay with bovine serum albumin (BSA) as a standard, the mean total protein concentration was 0.12 ± 0.02 mg/ml for 25 samples. [63] Human aqueous humor samples from 25 patients with open-angle glaucoma contained an average total protein concentration of 0.26 ± 0.09 mg/ml using the BCA assay with a BSA standard. [64] Eleven aqueous humor samples taken from patients undergoing cataract surgery were analyzed for total protein concentration using the Bradford assay. [65] Using BSA as a standard, the mean concentration was 0.22 ± 0.05 mg/ml. [65]

2.3 Aqueous Humor Protein Profiles

Protein profiles generated from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on various aqueous humor samples were compared to determine the protein homology of porcine aqueous humor to other species. Silver staining of rabbit aqueous humor following SDS-PAGE revealed nine major bands at 35, 31, 29, 27, 25, 23, 21, 15, 14 kD. [66] Human aqueous humor samples revealed twelve major polypeptide bands at 140, 80 (doublet), 67, 60 (doublet), 35, 27, 25, 17, 14.6, and 9 kD with silver staining. [63] Polypeptide bands at 160 kD and 140 kD were identified in human aqueous humor taken from patients with primary open angle glaucoma (POAG). [67] These bands were not present in aqueous humor taken from patients with cataracts. The proteins were analyzed using SDS-PAGE, and detected by silver staining. Protein analysis of monkey aqueous humor using one and two-dimensional gel electrophoresis identified polypeptides at 170, 130, 110, 80, 67, 60, 42, 34, 28, 25, 22, 16, and 14 kD. [61]

2.4 Specific Protein Detection in Aqueous Humor

To gain further insight into the protein homology of porcine aqueous humor to other species, specific proteins detected in aqueous humor of other species were compared. Western blots were used to probe for IgG, IgM, albumin, and transferrin in

rabbit aqueous humor samples. [66] Each of the proteins was detected with affinity-purified antibodies using a PVDF membrane. [66] In human aqueous humor, transferrin, albumin, ceruloplasmin, IgA, and IgG were identified by immunoassay. [68] Using crossed immunoelectrophoresis, human aqueous humor was probed for different serum proteins. [69] In human aqueous humor, there was 0.31-0.41 mg/dl of prealbumin, 5.4-6.5 mg/dl of albumin, and 1.3-1.7 mg/dl of transferrin. [69] Transferrin concentrations also were determined in human aqueous humor using radioimmunoassays. [70] In human patients without glaucoma, the mean transferrin concentration was 1.36 ± 0.66 mg/dl. [70] In human patients with primary and secondary glaucomas, the mean concentration was 2.07 ± 1.90 mg/dl and 2.79 ± 2.24 mg/dl. [70] IgG concentrations in human aqueous humor have been detected at approximately 3 mg/dl. [71]

On-line capillary electrophoresis-mass spectrometry was used to analyze the proteins in human aqueous humor. [72] Comparison of the measured average molecular weights of proteins to the SWISS-PROT database using TagIdent from ExPaSy identified β-2-microglobulin, apolipoprotein A1, and serum albumin. [72] The 14 kD protease inhibitor, cystatin, was identified using western blot immunodetection in monkey aqueous humor. [61]

An enzyme-linked immunosorbent assay (ELISA) was used to detect and quantify the levels of transforming growth-factor-β2 (TGF-β2) in human aqueous humor taken from 15 patients with POAG as well as 10 patients undergoing cataract surgery. The average TGF-β2 concentration in the aqueous humor from patients undergoing cataract surgery was 1.48 ± 0.68 ng/ml, and 2.70 ± 0.76 ng/ml in patients with POAG. The results were statistically significant. [73]

2.5 Zymographic Methodology

Zymography is an electrophoretic method used to measure proteolytic activity of MMPs. Zymograms are polyacrylamide gels permeated with substrate. The key steps in zymography are: 1) electrophoresis of the denatured MMP; 2) renaturing of the protease; 3) incubation of the zymogram as the protease digests substrate; 4) staining/destaining of the gel; 5) quantifying bands using densitometry. Gelatin zymograms are useful for detecting MMPs with strong gelatinase activity such as MMP-2 and MMP-9. Gelatinases

digest denatured collagen (gelatin) in addition to other substrates. MMPs with weak or no gelatinase activity are more suited for identification using casein zymograms. MMPs digesting proteoglycans and other matrix components can be detected on casein zymograms. Casein zymography, however, has a detection limit at least two orders of magnitude less than gelatin zymography. [74] MMPs are inactive after electrophoresis due to SDS denaturation, and must be renatured to be active. [75] A renaturing buffer (solution of Triton-X-100) removes the SDS, activating the MMPs. The MMPs are then placed into a developing buffer to allow the active protease to digest the zymogram substrate. As the digestion time with the developing buffer increases, detection of smaller quantities of enzyme is possible. [75] Within a certain range of MMP dilutions, there exists a linear relationship between MMP activity detected and concentration loaded. [75] At high concentrations of enzyme, increasing the digestion time shifts MMP activity detection outside of a linear relationship with the enzyme concentration loaded onto the gel. [75] The amount of time spent to stain the zymogram also affects the resolution. Overstaining the gels reduces assay sensitivity, and bands with low activity will not be detectable. [76] Gelatin zymograms stained for less than four hours showed greatest sensitivity. [76]

2.6 The MMP Family

The matrix metalloproteinases (MMPs) are a family of enzymes that digest specific components of the extracellular matrix. MMPs, which contain tightly bound zinc, are often divided into three subclasses based on substrate specificity:

1) Collagenases (ex. MMP-1 and MMP-8)
2) Gelatinases (ex. MMP-2 and MMP-9)
3) Stromelysins (all other MMPs)

All MMPs contain a propeptide lost during activation, and a catalytic domain containing zinc- binding ligands. MMPs are secreted in an inactive "zymogen" form, and are activated by cleavage of the propeptide. [22] In a zymogram, both the MMP pro and active form may be visible, with a 10 kD difference between the two. [75] The major inhibitors to MMPs are α_2-macroglobulin, and a family of tissue inhibitors of metalloproteinases

(TIMPs). TIMP-1 and TIMP-2 have been fully characterized and cloned. TIMP-1 is a 30-kD glycoprotein [77], while TIMP-2 is a 23-kD unglycosylated protein. [78]

Several models have been proposed on the interactions between the TIMPs and the MMPs. Many of the models involve the serine proteinases plasmin and plasminogen activators. Plasmin has been shown to activate MMPs in cell culture with connective tissue and tumor cells. [79] Plasmin is generated from plasminogen by plasminogen activators. [80] Regulation of plasmin generation is controlled by plasminogen activator inhibitors. [80] The ability of plasmin to activate the pro-MMPs is regulated by the TIMPs and α_2-macroglobulin. Thus, MMP activation appears to be tightly regulated process.

2.7 MMPs and their Inhibitors in Aqueous Humor

Aqueous humor, produced by the ciliary body, bathes the anterior segment of the eye, providing nutrients and removing metabolic wastes. Gelatin zymography on 11 human aqueous humor samples taken from patients undergoing cataract surgery revealed a major gelatinase band at 66 kD. [65] Minor bands at 125, 95, and 62 kD were detected too. All the bands were inhibited by treatment with 10 mM of specific MMP inhibitors 1,10-phenathroline and EDTA, indicating that the bands were metalloproteinases. [65] Casein zymography performed on the same aqueous humor samples revealed bands at 48 kD, 68 kD, and a band between 80-84 kD. Detection of protease bands on the casein zymogram occurred only after the incubation time used for proteolytic digestion was increased from 24 to 48 hours.

Bovine aqueous humor was analyzed for MMP content using gelatin zymography. [81] Two bands migrated near 92 kD corresponding to the MMP-9 family of enzymes, and two bands migrated near 66 kD corresponding to the MMP-2 family of enzymes. Several gelatinolytic bands were identified below 50 kD, and one was identified at approximately 100 kD. [81] All the gelatinolytic bands were inhibited with 1,10-phenathroline indicating metalloproteinase activity in all the bands.

A comparison of MMPs in human aqueous humor taken from patients with primary open-angle glaucoma (POAG) and patients undergoing cataract surgery was made using gelatin zymography. [82] A major band appeared at 66 kD for both sets of patients, although densitometry showed a higher density for patients with POAG.

Several proteinase inhibitors were identified using immunoblot assays. A major 53 kD band corresponding to α1-proteinase inhibitor was identified in human aqueous humor using western blot immunodetection. [65] An enzyme linked immunosorbent assay (ELISA) detected an average concentration of 32.2 ± 9.9 μg/ml of α1-proteinase inhibitor in all the human aqueous humor samples. [65] Alpha-2-macroglobulin, detected on western blots, was quantified using ELISAs. The average concentration of α-2-macroglobulin, an MMP inhibitor, was 3.2 ± 1.3 μg/ml. [65] Western blot analysis identified three immunoreactive bands migrating to molecular weights of 21 kD, 25 kD, and 28 kD in bovine aqueous humor. [81] TIMP-1 and TIMP-2 standards co-migrated to 28 kD and 21 kD with the aqueous humor bands, indicating that two of the detected bands were likely TIMPs. [81]

2.8 Relaxin

Relaxin is a peptide hormone synthesized mainly by the corpus luteum during pregnancy in several mammals. [83] Relaxin influences connective tissue remodeling in the uterus and cervix, and increases the secretion of collagenase and plasminogen activator (PA). [84] Collagenase and plasminogen activator are key enzymes involved in collagen synthesis. [84] Porcine relaxin is a 6.3 kD peptide consisting of an A and B chain covalently linked by disulfide bonds. [85] The mechanism of relaxin induced tissue remodeling is unclear, but relaxin affects the matrix metalloproteinases (MMPs) in addition to PA and collagenase. [18] Relaxin also increased levels of the tissue inhibitors of MMPs (TIMPs) in the uterix and cervix. TIMP-1 and TIMP-2 levels were enhanced in the uterix and cervix when treated with relaxin. [18] Relaxin, in 20 mg injections, lowered the IOP in the eyes of male and female patients. [16] Although the mechanism for the IOP decrease is unknown, tonographic studies showed that the pressure dependent outflow facility increased with relaxin administration. [16]

Chapter 3 Materials and Methods

Table 3.1 Buffer Recipes

Buffer	pH	Components
TBST	7.2-7.5	50 mM NaCl 25 mM Tris-HCl 0.05 % Tween-20
TBS-BSA	7.2	50 mM NaCl 25 mM Tris-HCl 0.1 % BSA
Coating Buffer	9.6	0.1 M $NaHCO_3$ 0.1 M NaCl
Tris-Glycine Running Buffer	7.2-7.4	12 mM Tris Base 96 mM Glycine
Tricine SDS Sample Reducing Buffer		450 mM Tris-HCl 12 % Glycerol 4 % SDS 0.0025 % Coomassie Blue G 0.0025 % Phenol Red 200 mM DTT
Blocking buffer (western blots)	7.2-7.5	0.5 % Casein in TBST

3.1 Aqueous Humor Extraction

Following anesthetization of pigs by injection of sodium pentathol, 100-150 µl of aqueous humor was extracted from each eye by veterinary ophthalmologists (Dr. Phil Pickett DVM and Dr. Ian Herring DVM). The samples were immediately frozen at -70°C. In some cases, samples were extracted after the pig had been electrocuted following the sodium pentathol injection. Removing samples after pig electrocution did not show any effect on the aqueous humor composition. Juvenile pigs were 3-4 weeks old, and adult pigs were one year olds. Samples were taken from each eye of every pig. Sixty adult female samples, twenty-two juvenile female samples, and ten juvenile samples were available as a result.

3.2 Bicinchonic Acid (BCA) Assay

The BCA assay was used to quantify the total amount of protein in the aqueous humor. The assay depends on the ability of copper (Cu^{2+} since $CuSO_4$ used) to bind to the peptide bonds of proteins to produce Cu^{+}. Bicinchonic acid is then reacted with Cu^{+} to

produce a strong purple color with intensity relative to the quantity of protein in the sample tested. The absorbance of the sample is measured on a spectrophotometer. The standard assay method [86] was followed. Briefly, aqueous humor samples were pipetted into 96 well Immulon 2 microtiter plates (Dynex Technologies; Chantilly, VA). Bovine serum albumin (BSA; Sigma; St. Louis, MO) was used as a standard. Five dilutions falling between 0.05 – 1.0 mg/ml were plated for the BSA standard, while three dilutions of each sample were made. Deionized water was used as the diluting liquid. A copper (II) sulfate solution was mixed 1:50 (v/v) with the BCA reagent (Sigma) to create a Standard Working Reagent (SWR). 25 µl of sample was then added to 0.5 ml of SWR and mixed using a vortex. 150 µl of this solution was then added to each well in the plate, and the plate was placed in an incubator at 60°C until a strong purple color appeared (usually 30 min – 1 hour). The absorbance for each well on the plate was then measured on a Microplate Reader EL-308 (Bio-Tek Instruments; Vermont, USA) at a wavelength of 562 nm.

3.3 SDS-Page Gel Electrophoresis

To identify the molecular weights of different proteins in the porcine aqueous humor, gel electrophoresis was performed on the aqueous humor samples. Precast fifteen well tricine gradient (10-20 %) polyacrylamide gels (Novex; Carlsbad, CA) were used. Aqueous humor samples were diluted 1:1 with tricine reducing sample buffer (Table 3.1). The mixture of sample buffer and aqueous humor was vortexed and then boiled at 95°C–100°C for 3–5 minutes. The sample was vortexed briefly, and then 10 µl of each sample was loaded into a well. Low range molecular weight markers (BioRad; Hercules, CA) were used to identify the molecular weights of the unknown protein bands of the aqueous humor. Porcine standards of IgG (Sigma), IgA (Intercell technologies; Vienna, Austria), relaxin, transferrin (Accurate Chemicals; Westbury, NY), and albumin (Sigma) were diluted 1:1 with sample buffer and loaded into separate wells also. Approximately 0.25 µg of relaxin, IgG, albumin, and IgA, and 0.44 µg of transferrin was loaded for each gel. Gels were run in a Novex X-Cell II system with a Novex Power Ease 500 system. A voltage of 125 V was applied to the gel until the dye "front" of the samples (from the

blue dye in the sample buffer) reached the bottom of the gel. Following electrophoresis, proteins were visualized by silver staining of the bands.

3.4 Western Blots

Western blots were used to detect the presence of TIMP-1 in the aqueous humor. SDS-gel electrophoresis of aqueous humor samples on 10-20 % tricine gels was performed with a prestained molecular weight marker (Gibco Brl; Rockville, MD) in one of the wells. The proteins from the gel were transferred overnight onto a Sequi-Blot polyvinylide fluoride (PVDF) membrane (BioRad) according to the western blot protocol described in the Invitrogen 2000 products catalog. The Novex Power Ease 500 system was set to 20V for overnight transfer. Following transfer of the proteins to the membrane, the membrane was placed in 200 ml of blocking buffer at 37°C for 1 hour. The primary antibody, anti-human-TIMP-1 raised in rabbit (carboxy end; Sigma) was then diluted 1:1000 in TBST. The blocking buffer was removed, and the membrane was placed in 20-25 ml of TBST/primary antibody for 1 hour at 37°C. A secondary antibody (anti-rabbit IgG w/horse radish peroxidase (HRP); Sigma) raised against the animal used to produce the primary antibody, and conjugated to HRP was then diluted 1:1000 in TBST. The membrane was washed three times for 5 minutes each in TBST prior to placing in 20-25 ml of secondary antibody/TBST, and incubating at 37°C for 1 hour. After incubation, the membrane was washed three times for five minutes each in TBST. DAB/metal concentrate (Pierce; Rockford, IL) was diluted 1:10 in stable peroxide substrate buffer (Pierce), and added to the membrane until a suitable color had been reached of the targeted protein bands. The DAB solution was disposed into a proper storage container, and the membrane was washed in deionized water for 5 minutes and air-dried.

3.5 Enzyme Linked Immunosorbent Assays (ELISAs)

Sandwich type ELISAs were used to quantify the amount of a specific protein in the aqueous humor. Protein standards of IgG (Sigma), albumin (Sigma), and transferrin (Accurate Chemicals) were used to construct standard curves. Antibody (Table 3) raised against the specific porcine protein tested was diluted to between 1:200 and 1:1000

(depending on the protein) in the coating buffer. 100 µl of this buffered "capture" antibody was added to each well in a 96 well Immulon 2 microtiter plate (Dynex Technologies; Chantilly, VA) and allowed to bind to the wells overnight at 4°C. The next day, the plate was washed out with TBST twice, and then filled with TBST and incubated at 37°C (to block the plate) for 1 hour. The plates were then washed 4-5 more times with TBST before 100 µl of sample (aqueous humor and standards) diluted in dilution buffer was added to each well. Triplicates of each sample were made. After adding the samples, the plate was covered and incubated at 37°C for 1 hour. The plate was then washed out 6 times with TBST. A second antibody conjugated to HRP, and raised against the protein being detected (Table 3.2), was then diluted 1:1000 in dilution buffer prior to adding to the plates at 100 µl/well. The plate was covered and incubated at 37°C for 1 hour before washing with the same wash/vacuum system 6 times with TBST. 100 µl of an o-phenylenediamine•2HCl (OPD) solution (Sigma) was then added to each well of the plate. When the wells of the standards changed color to a deep orange hue (5-15 minutes), 100 µl of 3N H_2SO_4 was added to each well to stop the colorimetric reaction that took place between the OPD reagent and the HRP of the antibody. The absorbance of each well was then measured at 490 nm using the Microplate Reader EL308 (Biotek Instruments; Winooski, VT). The dilution buffer was used as the blank. For the albumin ELISA, the dilution and blocking buffer of TBS-BSA and TBST was replaced with 1 % casein in TBS. Absorbance values were quantified by comparison to a standard curve. Standard curves were generated by plotting successive dilutions of the probed protein against the absorbance read by the spectrophotometer. Concentrations with relative standard deviations between each dilution of less than 25 % were used for analysis.

Table 3.2 Antibodies used for ELISAs

Detected Protein	Capture Antibody	Secondary Antibody
IgG	Rabbit antiserum w/IgG (Nordic Immun; Tilburg, Netherlands)	Anti-pig IgG whole molecule w/HRP (Sigma)
Transferrin (Trf)	Goat antiserum w/Trf (Nordic Immun)	Goat anti-pig Trf w/HRP (Bethyl Labs; Montgomery, TX)
Albumin	Goat anti-pig albumin (Bethyl Labs)	Goat anti-pig albumin w/HRP (Bethyl Labs)

3.6 Zymography

Zymography was used to visualize and quantify (with densitometry) the levels of MMPs and other proteases in the aqueous humor. Precast tris-glycine zymogram gels (Novex) containing either 10 % gelatin or 12 % casein were used to separate protease fragments electrophoretically. The method used to run the zymograms was similar to Oliver *et al.* (1997) [87] Aqueous humor samples were diluted 1:1 with non-reducing Tris-glycine sample buffer (Novex), and a total of 10 µl of the resulting well mixed sample was added to each well of the zymogram. Samples were not boiled prior to being added to the well. Human MMP-2 and MMP-9 standards (Calbiochem; La Jolla, CA) were added to wells as molecular weight markers. For some zymograms, prestained molecular weight markers (Gibco Brl) were added. Zymograms were run in the same Novex XCell II/Novex Power Ease 500 system as normal SDS-electrophoresis of tris-glycine gels. Gels were run at constant voltage of 125 V until the dye front moved to the bottom of the gel. The power was then turned off, and the gel was immersed in 50-100 ml of a zymogram renaturing buffer (Novex) and incubated at 37°C. The buffer was replaced after 30 min, 1.5 hours, and 2.5 hours. After three hours, the gel was transferred to 50-100 ml of a zymogram developing buffer (Novex) and incubated at 37°C for 24 or 48 hours (depending on the experiment). Developing buffer was replaced every 12 hours of an experiment. To test the effects of MMP inhibitors (and determine whether a given protease band was an MMP), 10 mM of 1,10 phenathroline (Sigma), a specific MMP inhibitor, was added to the renaturing buffer. The gel was placed into the

inhibitor/renaturing buffer at the same intervals as the non-inhibited samples as described before. In instances where different portions of the gel were subjected to inhibition/non-inhibition renaturing, gels were simply sliced with a knife at the appropriate lane and placed into the intended buffer. After developing, gels were stained according to instructions in the Colloidal Blue Staining Kit (Novex) for 3 hours (staining time only). Some gels were stained for 2 hours (staining time only) according to instructions for the Brilliant Blue G Colloidal concentrate (Sigma). The staining solutions bound to protein, and thus bound all the areas of the zymogram except where proteolytic digestion of protein took place. Thus, protease bands appeared white on a blue background.

3.7 Densitometry

The protease bands that appeared on the zymogram differed visually in brightness and width based on the quantity of protease present. To quantify the concentration of protease in the aqueous humor compared to a standard, densitometry was used. Zymograms were scanned on a Microtek Scanmaker X6EL in Adobe Photoshop 5.0. The gels were converted to black and white images (as *.tif files) and imported into Gelworks 1D Intermediate 4.01 (UVP products; Upland, CA). The densitometry program was able to calculate the volume of each protease band, after the band dimensions were defined by the user. The volume of a protease band is the area of the band times the intensity of each pixel in the band. It is thus a three dimensional calculation of the band. Dilutions of the human MMP standards were plotted as a volume versus concentration plot to create a standard curve. MMP bands migrating to the same molecular weight as the human MMP standard were quantified by comparison to the standard curve.

3.8 Statistics

Statistical calculations were limited to standard deviation and a students t-test. The t-test calculations were performed using the MS Excel t-test correlations for two-pair, heteroscedastic (unequal variance) samples. Correlations were deemed statistically significant for t-test p-values ≤ 0.05.

Chapter 4 Results

4.1 BCA Assay Results

The total protein concentration of different porcine aqueous humor samples was measured using the Bicinchonic Acid (BCA) standard assay. [21] Bovine serum albumin (BSA) was used as the standard. Sample data from the BCA assay are available in Appendix A. The average total protein concentration of all the aqueous humor samples was 2.54 ± 0.89 mg/ml. The adult female samples were compared to juvenile samples (male and female) to determine whether age affects the total protein concentration (Table 4.1). The mean concentration of 55 adult female samples was 2.77 ± 0.84 mg/ml. The mean concentration of 8 total (male and female) juvenile samples was 2.18 ± 0.60 mg/ml. A t-test between the adult and juvenile samples showed a low statistical relation between the two means ($p = 0.06$). Another comparison was made between the juvenile male and female samples to determine the effects of gender on the total protein in the aqueous humor (Table 4.2). The juvenile female mean concentration based on four samples was 2.11 ± 0.54 mg/ml. The juvenile male mean concentration based on four samples was 2.25 ± 0.74 mg/ml. The p-value from the t-test showed a low statistical relation ($p = 0.78$).

Table 4.1 Effects of age on total protein concentration. Comparison of total protein concentrations between adult female and juvenile samples. Fifty-five adult female samples and eight juvenile samples were used for the comparison.

Sample	Mean Concentration (mg/ml)
Adult Female	2.77 ± 0.84
Juvenile Male and Female	2.18 ± 0.60
p = 0.060	

Table 4.2 Effects of gender on total protein concentration. A comparison of total protein concentrations between juvenile male and female aqueous humor samples. Four juvenile female samples were compared to four juvenile male samples.

Sample	Mean Concentration (mg/ml)
Juvenile Female	2.11 ± 0.54
Juvenile Male	2.25 ± 0.74
p = 0.78	

4.2 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the adult female and juvenile male and female aqueous humor samples. Molecular weight protein standards (BioRad) were used to construct standard curves for each set of samples. Gels of adult female, juvenile female, and juvenile male samples are shown in Figures 4.1, 4.2, and 4.3 with the molecular weight markers and some protein standards. All of the samples were run with reducing buffer, and the protein bands were visualized by silver staining. There were 18 protein bands identified in the female adult, 10 bands in the juvenile female, and 16 bands in the juvenile male aqueous humor samples. Smeared or blurred bands were counted as individual bands. The proteins in the adult female samples ranged from approximately 9-142 kD. The proteins in the juvenile female samples ranged from approximately 14-128 kD. The proteins in the juvenile male samples ranged from approximately 9-151 kD.

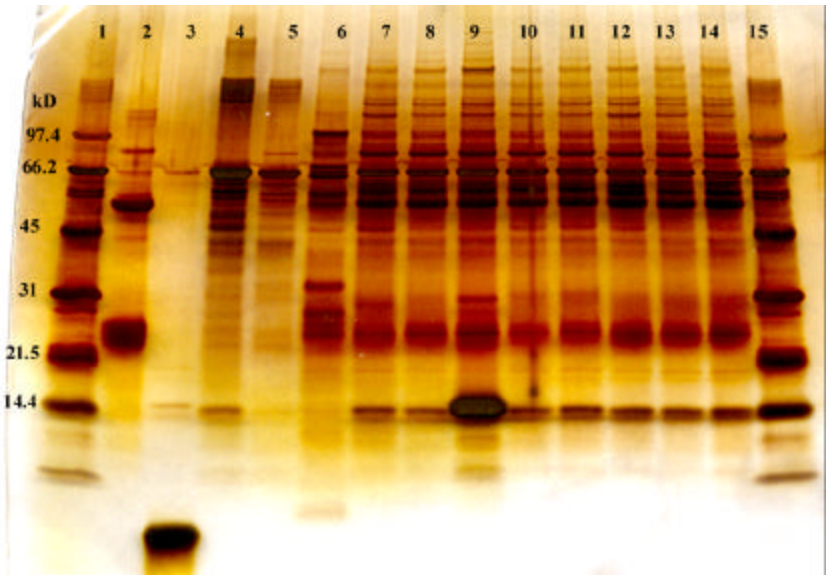

Figure 4.1. SDS-page gel electrophoresis on adult female aqueous humor samples with protein standards. Silver staining was used for visualization. All samples were run under reducing conditions. Lanes 1,15 are molecular weight markers. Lane 2: Swine IgG. Lane 3: Swine relaxin. Lane 4: Bovine serum albumin (BSA). Lane 5: Swine Albumin. Lane 6: Swine IgA. Lanes 7-14 are female adult aqueous humor samples.

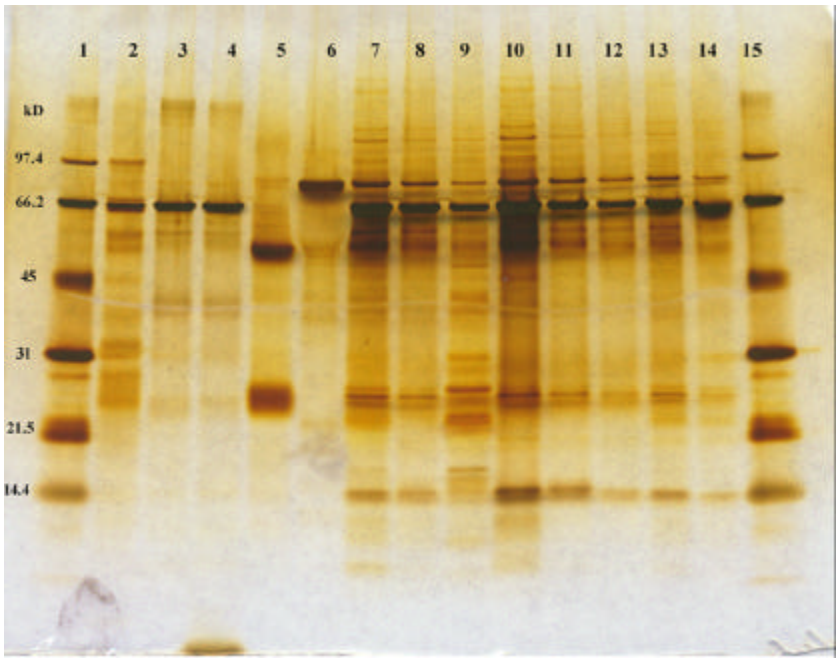

Figure 4.2. SDS-page gel electrophoresis on juvenile female aqueous humor samples with protein standards. Silver staining was used for visualization. All samples were run under reducing conditions. Lanes 1,15 are molecular weight markers. Lane 2: Swine IgA. Lane 3: Swine Albumin. Lane 4: Swine Relaxin. Lane 5: Swine IgG. Lane 6: Swine Transferrin. Lanes 7-14 are juvenile female aqueous humor samples.

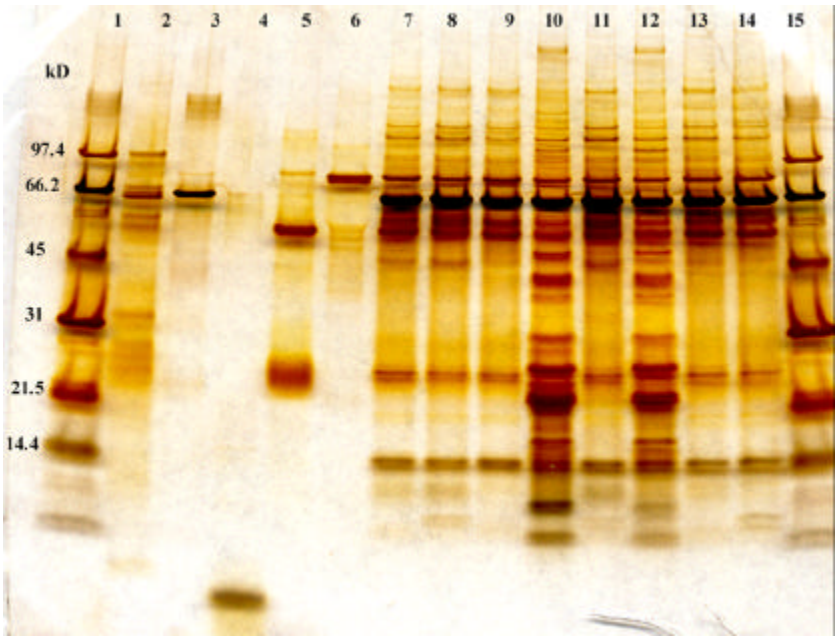

Figure 4.3 SDS-page gel electrophoresis on juvenile male aqueous humor samples with protein standards. Silver staining was used for visualization. All samples were run under reducing conditions. Lanes 1,15 are molecular weight markers. Lane 2: Swine IgA. Lane 3: Swine Albumin. Lane 4: Swine Relaxin. Lane 5: Swine IgG. Lane 6: Swine Transferrin. Lanes 7-14 are juvenile male aqueous humor samples.

4.3 ELISA Results

To identify the effects of age and gender on specific proteins, enzyme linked immunosorbent assays (ELISAs) were used to quantify the concentration of IgG, transferrin, and albumin in the aqueous humor samples. The ELISA data for all the samples are in Appendix B.

For all the aqueous humor samples, the mean IgG concentration was 11.40 ± 4.21 μg/ml. The effects of age on the IgG concentration were analyzed by comparing 10 adult female porcine aqueous humor samples and 6 juvenile (male and female) samples. The mean IgG concentration of the adult female samples was 13.65 ± 4.33 μg/ml, and 8.94 ± 1.70 μg/ml for the juvenile samples (Table 4.3). A t-test of the two sample populations showed that the difference in mean concentrations was significant ($p \leq 0.05$). The effects of gender on IgG concentration were examined by comparing three juvenile male samples to three juvenile female samples. The mean concentration of IgG in the juvenile female aqueous humor was 9.49 ± 0.53 μg/ml, and 8.39 ± 2.46 μg/ml for the juvenile male samples (Table 4.4). A t-test showed no statistical difference between the mean concentrations ($p = 0.52$).

For all the aqueous humor samples, the mean transferrin concentration was 17.11 ± 6.78 μg/ml. The effects of age on transferrin concentration in aqueous humor were analyzed by comparing 7 adult female porcine aqueous humor samples and 6 juvenile (male and female) samples. The mean transferrin concentration was 14.55 ± 8.47 μg/ml for the adult female, and 20.09 ± 2.05 μg/ml for the juvenile samples (Table 4.5). A t-test showed a low statistical difference between the mean concentrations ($p = 0.14$). The effects of gender on transferrin concentration were examined by comparing three juvenile male samples to three juvenile female samples. The mean concentration of transferrin in the juvenile female aqueous humor was 19.32 ± 2.88 μg/ml, and 20.86 ± 0.68 μg/ml for the juvenile male samples (Table 4.6). A t-test showed no statistical difference between the mean concentrations ($p = 0.46$).

Three adult female, two juvenile female, and three juvenile male samples were used for analysis of albumin concentration in aqueous humor. Gender, age, and eye effects were not used in analysis on albumin concentrations, due to the small sample

sizes. An average albumin concentration for all seven samples was 77.97 ± 26.32 μg/ml compared to a BSA standard in an ELISA.

Table 4.3. Effects of age on IgG concentration. Comparison of IgG concentrations between all adult female samples and all juvenile samples. Ten adult female samples and six juvenile samples were used for the comparison.

Sample	**Mean Concentration (mg/ml)**
Adult Female	13.65 ± 4.33
Juvenile Male and Female	8.94 ± 1.70
p = 0.009	

Table 4.4. Effects of gender on IgG concentration. A comparison of IgG concentrations between juvenile male and female aqueous humor samples. Three juvenile female samples were compared to three juvenile male samples.

Sample	**Mean Concentration (mg/ml)**
Juvenile Female	9.49 ± 0.53
Juvenile Male	8.39 ± 2.46
p = 0.52	

Table 4.5. Effects of age on transferrin concentration. Comparison of transferrin concentrations between all adult female samples and all juvenile samples. Seven adult female samples and six juvenile samples were used for the comparison.

Sample	**Mean Concentration (mg/ml)**
Adult Female	14.55 ± 8.47
Juvenile Male and Female	20.09 ± 2.05
p = 0.14	

Table 4.6. Effects of gender on transferrin concentration. A comparison of transferrin concentrations between juvenile male and female aqueous humor samples. Three juvenile female samples were compared to three juvenile male samples.

Sample	**Mean Concentration (μg/ml)**
Juvenile Female	19.32 ± 2.88
Juvenile Male	20.86 ± 0.68
p = 0.46	

4.4 Gelatin Zymography Results

Gelatin zymography was used to examine the levels of MMPs in the aqueous humor. Specifically, the concentrations of different matrix metalloproteinases (MMPs) in the aqueous humor were contrasted by age, gender, and left or right eye of the sample. A specific MMP inhibitor, 1,10-phenanthroline, was used to assess whether a given protease band was an MMP. Densitometry was used to quantify the bands seen on the zymogram. The MMP concentration in the aqueous humor sample was calculated by comparison to a MMP standard curve, assuming the molecular weights were equal.

Densitometry of the MMP bands migrating to the same molecular weight (MW) of MMP-2 standard was performed for the zymograms in Figures 4.3, 4.4, and 4.5. The volume of the bands was plotted against the protein of each dilution that was loaded. A line was fitted to the three points using linear regression for each zymogram. The volumes of the protease bands from the aqueous humor were then converted to protein using the equation from the fitted line for the standards.

Adult female (AF), juvenile female (JF), and juvenile male (JM) porcine aqueous humor samples were run on a 10 % gelatin zymogram with three dilutions of an MMP-2 standard. Zymograms are shown in Figures 4.4 and 4.5. The aqueous humor samples are different between those used in the zymograms for Figure 4.4 and those used in Figure 4.5. As seen in Figures 4.4 and 4.5, all the aqueous humor samples had protease bands appear at the same molecular weight as the human MMP-2 standard. A second band on the zymogram appeared for some of the aqueous humor bands at a higher molecular weight. These aqueous humor bands were a result of using between unconcentrated samples. Non-protease protein bands appeared for a juvenile female sample as seen in Figure 4.7 (lane 7). A dim and less distinct protease band appeared for the same sample (See Figure 4.4) at a much lower molecular weight. To determine whether the protease bands were MMPs, zymograms with porcine aqueous humor samples and MMP-2 standards were renatured in the presence of 10 mM 1,10 phenanthroline, a specific MMP inhibitor. The resulting zymogram is shown in Figure 4.6. Three dilutions of each human MMP standard, were run with three AF, JF, and JM aqueous humor samples to see whether the minor band was related to the MMP-9 family (Figure 4.7).

The data for the zymograms are given in Appendix C. The MMP-2 levels calculated for each aqueous humor band assumed that the protease band migrating to the same molecular weight as the MMP-2 standard was MMP-2. MMP-2 quantities were calculated for each zymogram sample based on the standard curve existing for the zymogram containing the sample under analysis.

The effects of age on MMP-2 concentration was compared by examining the MMP-2 levels in the adult and juvenile aqueous humor samples (Table 4.7). The mean MMP-2 concentration of the nine adult female samples was 0.22 ± 0.06 ng/µl, while the juvenile (both male and female combined) mean concentration from 18 samples was 0.28 ± 0.04 ng/µl. A t-test showed that the difference in mean concentrations was statistically significant ($p = 0.03$). The effects of gender on MMP-2 concentration in porcine aqueous humor were determined by comparing the juvenile male and female samples (Table 4.8). The mean concentration of the nine juvenile female samples was 0.28 ± 0.04 ng/µl, while the mean concentration of nine male samples was 0.28 ± 0.05 ng/µl. A t-test showed that the difference in mean concentrations was statistically insignificant ($p = 0.90$). To visualize the minor protease bands better, and to determine whether other MMPs existed in the aqueous humor, aqueous humor was concentrated six-fold by lyophilization and reconstitution in deionized water, and then added to the zymogram. Concentrated aqueous humor bands were also subjected to 10mM of 1,10 phenanthroline to test whether the bands represented MMPs. MMP-2 standard (1 ng loaded) and MMP-9 standard (550 pg loaded) were run with two AF samples, 1 JF sample, and 1 JM sample. Concentrated aqueous humor bands and MMP-2 standard (1 ng loaded) were also subjected to 10mM of 1,10 phenanthroline to test whether the bands represented MMPs. The human MMP-2 standard was run as a control to make sure 1,10 phenathroline activity was inhibitory to MMPs. The zymogram is shown in Figure 4.8. Thus, as seen in Figure 4.8, lanes 2-7 were developed in normal renaturing buffer, while lanes 8-10 were developed in renaturing buffer with 10 mM 1,10-phenanthroline. A prestained molecular weight marker is run in lane 1. Three major bands could be identified in the aqueous humor samples renatured without inhibitor, but only one band could be seen in the samples renatured with the inhibitor. Non-protease protein bands appeared as dark

bands in all of the aqueous humor samples. The number of non-protease protein bands was greater in the adult female samples than in the juvenile samples.

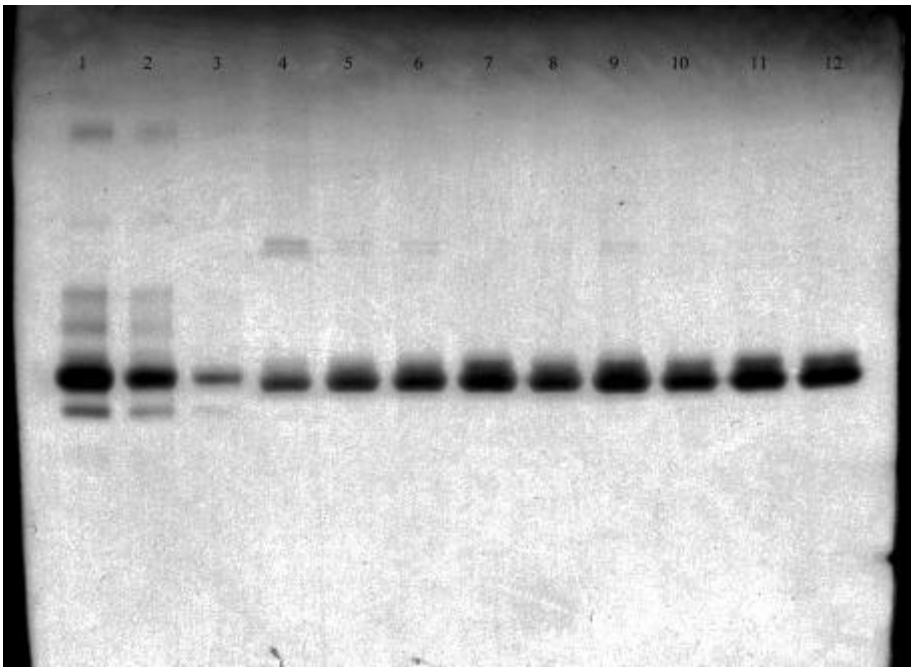

Figure 4.4. Gelatin zymogram of MMP-2 dilutions and aqueous humor samples. Zymograms are shown as negative images. Gels were kept in developing buffer for 48 hours. Lanes 1-3: MMP-2 standard, 2 ng, 1 ng, 0.5 ng loaded. Lanes 4,6: AF-left eye. Lane 5: AF-right eye. Lane 7: JF-left eye. Lanes 8-9: JF-right eye. Lanes 10,12: JM-left eye. Lane 11: JM-right eye.

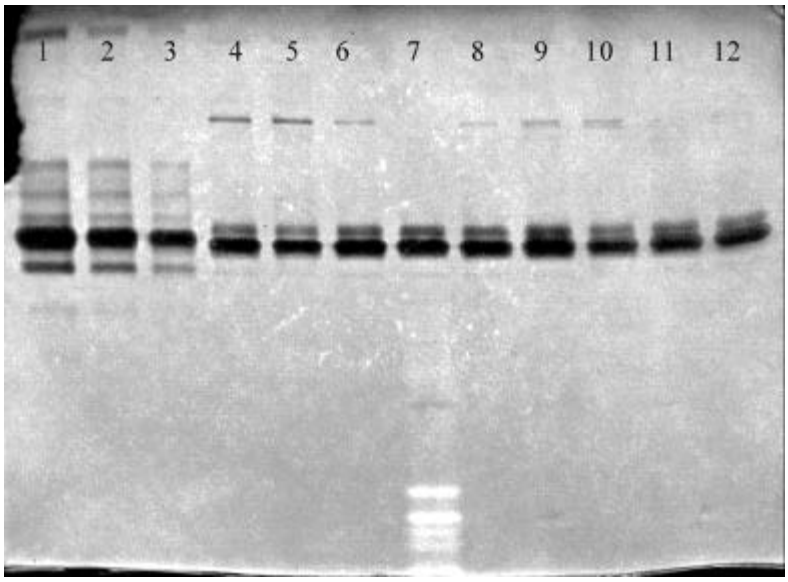

Figure 4.5. Gelatin zymogram of MMP-2 dilutions and aqueous humor samples. Zymograms are shown as negative images. Gels were left in developing buffer for 24 hours. Lanes 1-3: MMP-2 standard, 2 ng, 1 ng, 0.5 ng loaded. Lanes 4-5: AF-left eye. Lane 6: AF-right eye. Lanes 7-8: JF-left eye. Lane 9: JF-right eye. Lane 10: JM-left eye. Lanes 11-12: JM-right eye.

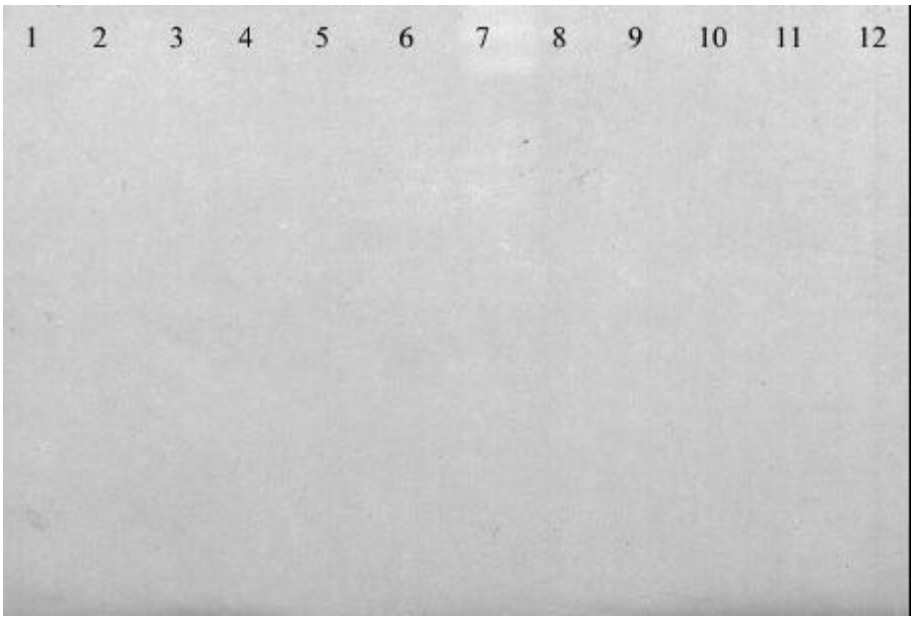

Figure 4.6. Gelatin zymogram renatured in the prescence of 10mM of MMP inhibitor 1,10 phenathroline. Zymogram of MMP-2 dilutions and aqueous humor samples. Gels were left in developing buffer for 48 hours. Lanes 1-3: MMP-2 standard, 2 ng, 1 ng, 0.5 ng loaded. Lanes 4,6: AF-right eye. Lane 5: AF-left eye. Lanes 7: JF-right eye. Lane 8,9: JF-left eye. Lane 10,12: JM-right eye. Lane 11: JM-left eye.

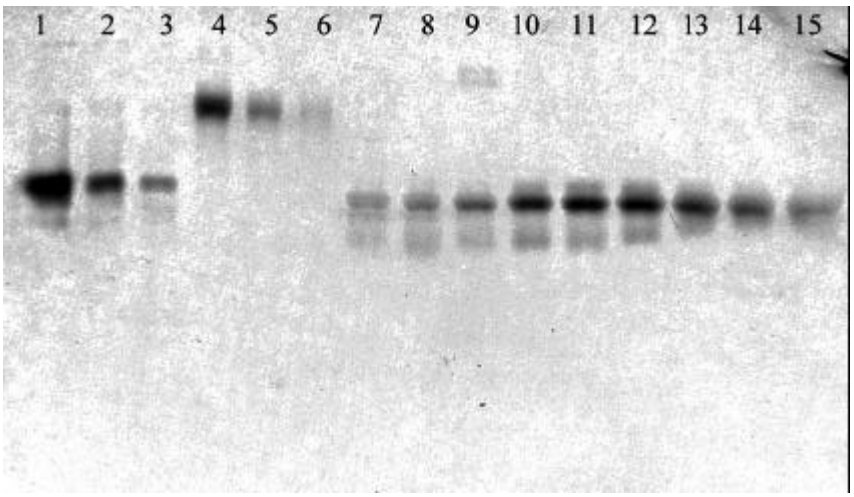

Figure 4.7. Gelatin zymogram of MMP-2, MMP-9 dilutions, and aqueous humor samples. Zymograms are shown as negative images. Gels were left in developing buffer for 24 hours. Lanes 1-3: MMP-2 standard, 2 ng, 1 ng, 0.5 ng loaded. Lanes 4-6: MMP-9 standard, 550 pg, 225 pg, 112.5 pg loaded. Lane 7: AF-left eye. Lane 8-9: AF-right eye. Lanes 10-11: JF-right eye. Lane 12: JF-left eye. Lane 13: JM-left eye. Lanes 14-15: JM-right eye.

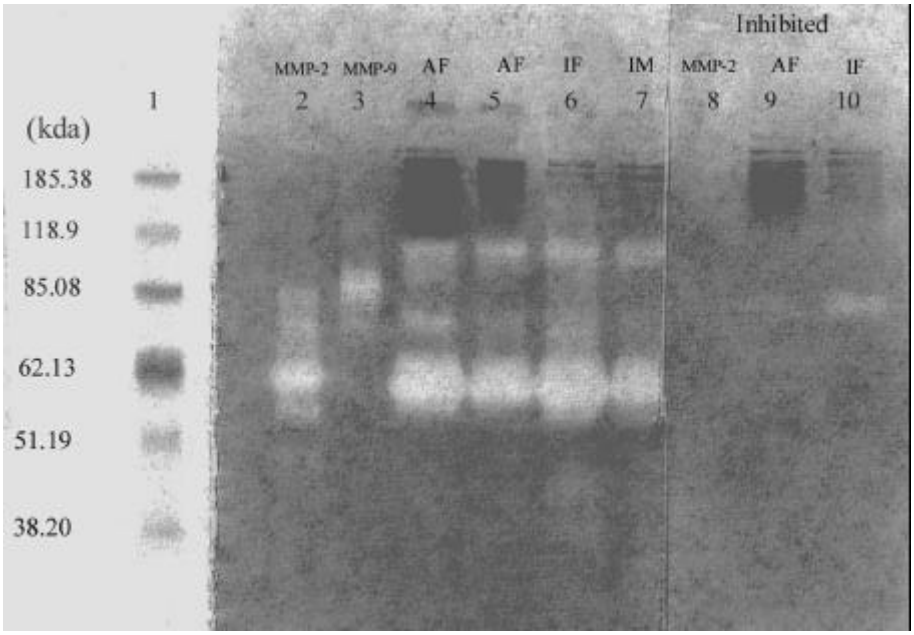

Figure 4.8. Gelatin zymogram with concentrated aqueous humor samples. Gels were left in developing buffer for 48 hours. Lane 1: Molecular weight marker. Lane 2: MMP-2 standard -1 ng loaded. Lane 3: MMP-9 standard-550 pg loaded. Lanes 4-5: AF-left eye. Lane 6: JF-right eye. Lane 7: JM-right eye. Lane 8: MMP-2 standard -1 ng loaded. Lane 9: AF-right eye. Lane 10: JF-left eye.

Table 4.7. Effects of age on MMP-2 concentration in aqueous humor. Comparison of MMP-2 concentrations between all adult female samples and all juvenile samples. Nine adult female samples and 18 juvenile samples were used for the comparison.

Sample	**Mean Concentration (ng/ml)**
Adult Female	0.22 ± 0.06
Juvenile Male and Female	0.28 ± 0.04
p = 0.03	

Table 4.8. Effects of gender on MMP-2 concentration in aqueous humor. A comparison of MMP-2 concentrations between juvenile male and juvenile female aqueous humor samples. Nine juvenile female samples were compared to 9 juvenile male samples.

Sample	**Mean Concentration (ng/ml)**
Juvenile Female	0.28 ± 0.04
Juvenile Male	0.28 ± 0.05
p = 0.90	

4.5 Casein Zymography Results

Casein zymography was used to detect casein-specific proteases that used casein as a substrate. MMPs digesting multiple substrates (both gelatin and casein) would be detected also. The first set of casein zymograms were run with just 5 µl of aqueous humor sample (diluted 1:1 with sample buffer to make 10 µl) and human MMP-2 and MMP-9 standards. Three adult female (AF), two juvenile female (JF), and two juvenile male (JM) aqueous humor samples were run. Two zymograms were set up with exactly the same volume of aqueous humor samples and concentration of human MMP standards. One of the zymograms (Figure 4.9) was renatured without the MMP inhibitor 1,10-phenanthroline, while the other (not shown) was renatured with it. As seen from Figure 4.9, no protease bands were detected.

Three aqueous humor samples (1 AF, 1 JF, 1 JM) were then concentrated 5-fold by lyophilization and reconstitution in deionized water. The samples were diluted with non-reducing sample buffer 1:1 and loaded onto the zymogram. A prestained molecular weight marker was run in the first lane of the zymogram to help identify the molecular weights of any protease bands. The resulting zymogram is shown in Figure 4.10. As seen in Figure 4.10, one major protease band appeared for all three samples at approximately 80 kD. The protease band was barely detectable in the juvenile male sample. Non-protease protein bands (dark bands) were detected for the juvenile female sample (lane 2) only.

To determine whether the protease bands in Figure 4.10 were MMP bands, concentrated (8.3-fold) aqueous humor samples were run on the casein zymogram and renatured with and without 10 mM 1,10-phenanthroline. MMP-2 and MMP-9 were loaded in higher concentrations (11.8 ng and 3.7 ng loaded respectively). Four aqueous humor samples (2 AF, 1 JF, 1 JM) and MMP standards were renatured without 1,10-phenanthroline (lanes 2-7 of Figure 4.10). A prestained molecular weight marker was run in lane 1. Two concentrated aqueous humor samples (1 AF and 1 JF) and 11.8 ng of MMP-2 standard were run in lanes 8-10 (Figure 4.11) and renatured with 10 mM 1,10 phenathroline. As seen in Figure 12, the band corresponding to MMP-2 standard did not appear when renatured with 1,10-phenathroline. The adult female aqueous humor samples not subjected to MMP inhibition by 1,10-phenanthroline (lanes 4-5 of Figure

4.11) showed two different protease bands at approximately 85 kD and 51 kD. The juvenile samples (lanes 6-7 of Figure 4.11), showed the 85 kD band only. The two aqueous humor samples subjected to MMP inhibition had protease bands appear at approximately 51 kD. A faint protease band appeared for both of these aqueous humor samples at approximately 85 kD, but the band was not detectable using the flatbed scanner. Thus, neither band detected on the casein zymogram was an MMP.

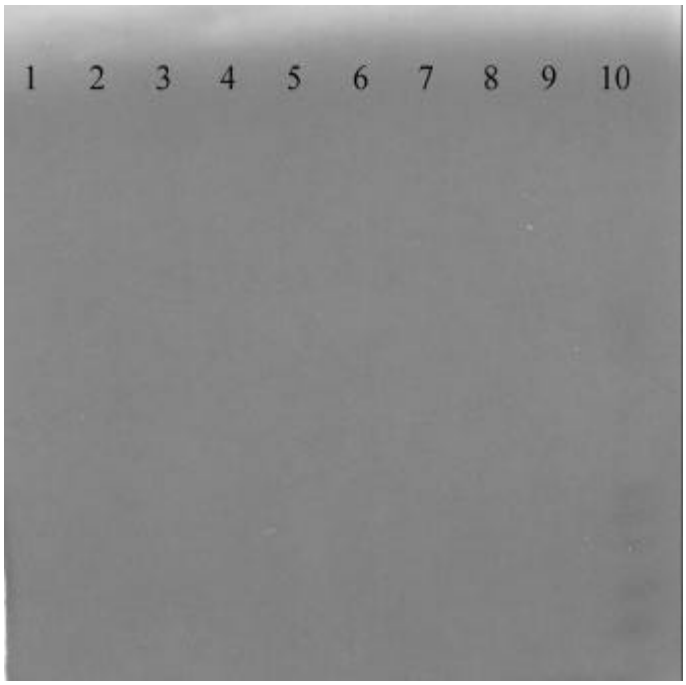

Figure 4.9. Casein zymogram with MMP-2 and MMP-9 standards, and aqueous humor samples. Gels were left in developing buffer for 48 hours. Lane 1: MMP-2 standard (2 ng loaded). Lane 2: MMP-9 standard (550 pg loaded). Lane 3: No sample. Lanes 4,6: AF-right eye. Lane 5: AF-left eye. Lane 7: JF-left eye. Lane 8: JF-right eye. Lane 9: JM-left eye. Lane 10: JM-right eye.

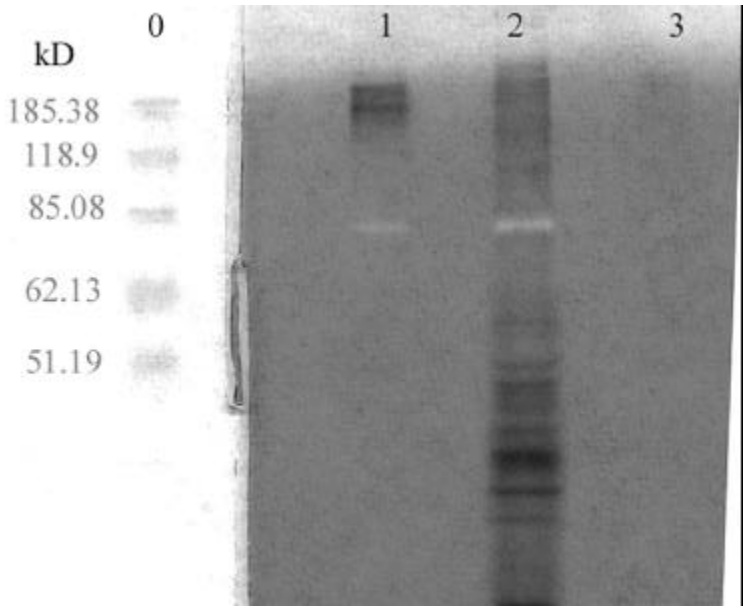

Figure 4.10. Casein zymogram with concentrated aqueous humor samples. Gels were left in developing buffer for 48 hours. Lane 0: Molecular weight marker. Lane 1: AF sample-left eye. Lane 2: JF sample-right eye. Lane 3: JM-left eye.

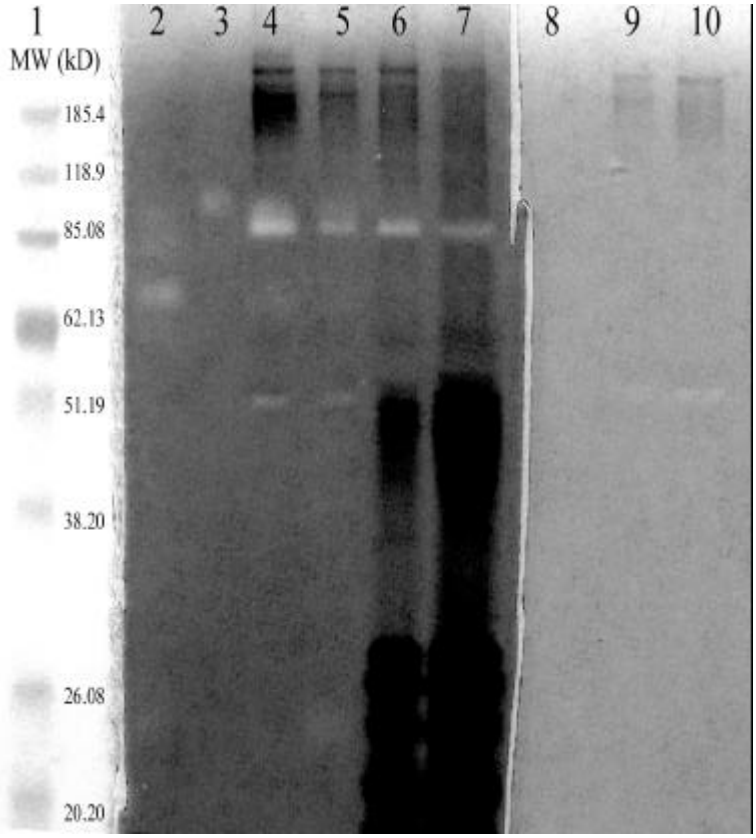

Figure 4.11. Casein zymogram with concentrated aqueous humor samples. Zymograms were placed in developing buffer for 48 hours following renaturation. Lane 1: Molecular weight (MW) marker. Lane 2: MMP-2 standard – 11.8 ng loaded. Lane 3: MMP-9 standard-3.7 ng loaded. Lanes 4-5:AF-left eye. Lane 6: JF-left eye. Lane 7: JM-right eye. Lane 8: MMP-2 standard-11.8 ng loaded. Lane 9: AF-left eye. Lane 10:JF-left eye. Lanes 8-10 were renatured with 10 mM of 1,10-phenanthroline.

4.6 TIMP-1 Detection

A western blot was used to try to detect TIMP-1, a tissue inhibitor to metalloproteinases. Aqueous humor samples were run electrophoretically on a 10-20 % tricine gel prior to transfer to a PVDF membrane. Thirty microliters of an adult female (AF), juvenile female (JF), and juvenile male (JM) aqueous humor sample was lyophilized and resuspended in 6 ml of deionized water prior to mixing with 6 ml of tricine reducing buffer. Ten microliters of sample was then loaded onto the gel. Lyophilization of aqueous humor samples was necessary as initial attempts to detect TIMP-1 in just 5 ml of aqueous humor failed to show any bands on the membrane. The western blot revealed three different immunoreactive bands for the AF, JF, and JM samples (Figure 4.12). The immunoreactive bands were found at approximately 62 kD, 85 kD, and 105 kD.

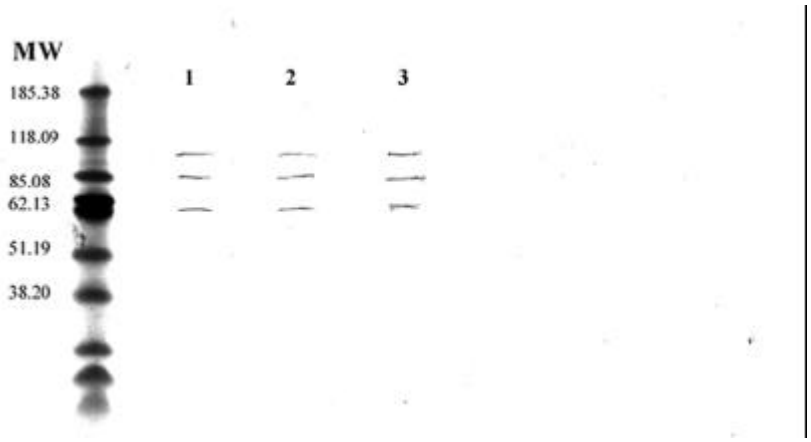

Figure 4.12. Western blot detection of TIMP-1 on concentrated aqueous humor samples. Following electrophoresis of lyophilized aqueous humor in reducing buffer on a 10-20 % tricine gel, the proteins were transferred onto a PVDF membrane. Molecular weight marker on the far left. Lane 1: AF; Lane 2: JF; Lane 3: JM

Chapter 5 Discussion

The primary objectives of this thesis were to quantify the matrix metalloproteinsases (MMPs) in porcine aqueous humor, determine the effects of age and gender on both MMP and serum protein concentrations in porcine aqueous humor. Pigs are suitable as animal models for studying glaucoma related research because their eyes are similar in size to humans, and contain a wedge-shaped mass of corneoscleral tissue comparable in size to human trabecular meshwork. [88] Pigs have also been used as tissue culture models for prostaglandin analogue glaucoma research. [19,20] Prostaglandin analogues lower intraocular pressure (IOP) by inducing the release of matrix metalloproteinases (MMPs) that degrade and remodel extracellular matrix (ECM) components between ciliary smooth muscle cells. [6,8,9] MMPs are therefore important in maintaining aqueous humor outflow channels. Future work with the results obtained in this thesis will be used to study the effects of relaxin on porcine trabecular cells in culture. Relaxin, a hormone, remodels connective tissue during pregnancy, and affects MMP expression in a number of tissues. [17,18]

Determining the effects of gender and age on MMP concentration in porcine aqueous humor is important in the study of glaucoma. Primary open-angle glaucoma, the most prevalent form of glaucoma, is characterized by high IOPs and advanced age. [4] In addition, adult males have a higher incidence of open-angle glaucoma than adult females. [89,90,91] Extracellular matrix (ECM) components in cells cultured from human trabecular meshwork were shown to increase with passage (age) in culture. [92] The IOP increase in primary open angle glaucoma may be caused by blockage to the aqueous humor outflow channels by accumulation of ECM components. Thus, the maintenance of these ECM components by MMPs in the trabecular meshwork may be a factor in the onset of glaucoma. Gender and age effects can be seen in relaxin production also. The majority of relaxin production is in females from the corpus luteum during pregnancy. [17] Relaxin is also produced during the reproductive cycle in females and in the testes and seminal fluid in males. [93]

The serum protein concentrations in porcine aqueous humor are quantified for comparison to MMP activity, and to determine if the blood-aqueous barrier remained

intact during euthanasia of the pigs. There were two methods of pig euthanasia: electrocution and lethal injection. The blood brain barrier may have been disrupted by the electrocution, necessitating a comparison of serum protein concentrations in aqueous humor samples between pigs killed by lethal injection and electrocution. A comparison of serum and total protein concentrations in porcine aqueous humor to human aqueous humor also shows whether a pig is a suitable animal model. The effects of gender and age on the serum and total protein concentration are carried out to see if the trends match those seen with the MMPs.

Zymography of aqueous humor samples from adult female, juvenile female, and juvenile male samples, and subsequent densitometry provided information on MMP activity in the eye. Three bands were identified in unconcentrated aqueous humor on gelatin zymograms. Two major gelatinolytic bands migrated to molecular weights of approximately 66 and 72 kD, and a minor band was seen at 105 kD (Figures 4.4 and 4.5). The gelatinolytic bands at 66 and 72 kD migrated to the same molecular weight as human MMP-2 standard. The 72 kD gelatinolytic band appears to be the pro-MMP form of MMP-2. MMPs are secreted in a latent form containing a propeptide that is cleaved off producing an active form. Thus, the 72 kD band would be the latent form of MMP-2. Concentrated aqueous humor samples (5-fold) run on the gelatin zymogram showed proteolytic activity at approximately 85 kD in addition to the 66 and 105 kD bands (Figure 4.8). The proteolytic bands at 66 and 105 kD were completely inhibited when exposed to the specific MMP inhibitor 1,10-phenathroline (Figure 4.6). The gelatinolytic band at 85 kD retained activity after exposure to 1,10-phenanthroline (Figure 4.8). Thus, the bands at 66 kD and 105 kD are MMPs, while the band at 85 kD is not. Casein zymograms run with a five-fold concentration of aqueous humor resulted in a detection of proteolytic activity at approximately 80 kD (Figure 4.10). Further concentration of aqueous humor samples (8.3-fold concentration) yielded a new proteolytic band at 51 kD in addition to the 80 kD band on a casein zymogram (Figure 4.11). The 80-kD band was partially inhibited when the zymogram was renatured in the presence of 1,10-phenanthroline (Figure 4.11). The 51-kD band, however, was not affected by the MMP inhibitor. A concentrated MMP standard was completely inhibited by the 1,10-

phenthroline on the same gel (Figure 4.11). Thus, neither caseinase bands appear to be MMPs.

The MMPs detected in porcine aqueous humor provide information on the extracellular matrix components likely remodeled in the trabecular meshwork and ciliary muscle. Elastin, proteoglycans (decorin, biglycan, syndecan, versican, perlecan), adhesive glycoproteins (fibronectin, laminin, vitronectin), and collagens (types I, III-VI, and VIII) are ECM components found in the trabecular meshwork. [94] Collagens (type I, III, and IV), adhesive glycoproteins (fibronectin, laminin), and hyaluron are ECM components found in the ciliary muscle. [95] MMP-2 digests gelatins, elastin, aggrecan, and collagens IV, V, VII, X, and XI. [22] The identity of the MMP bands at 105 kD is unclear since the molecular weight is higher than MMPs characterized as of January 2000 (Table 1.1). In human plasma, metalloproteinase activity has been detected at 130 and 225 kD. The 105 kD may be a fragment of the 130 kD band seen in human plasma, or may be a new metalloproteinase. The activity of the gelatinolytic band at 105 kD did not appear to vary with age or gender (Figures 4.5-4.7). The non-MMP proteolytic bands at 85 kD (gelatin zymogram), 80 kD (casein) and 51 kD (casein) may be serine proteinases analogous to those detected at 48 kD and between 80-84 kD on a casein zymogram in human aqueous humor. [65]

MMP activation is inhibited *in vivo* by binding to specific tissue inhibitors of MMPs (TIMPs) and α2-macroglobulin. Four major TIMPs have been identified as of 1999: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMP-1 binds to both MMP-9 and MMP-2, while TIMP-2 primarily inhibits MMP-2. [96] TIMP-1 has a binding affinity that is 2.4-fold higher for MMP-9 than MMP-2. [96] TIMP-1 expression can be increased using tumor promoters and growth factors, but TIMP-2 levels are constitutive. [97]

To determine whether TIMP-1 was detectable, western blots were used to probe for TIMP-1 in concentrated porcine aqueous humor samples. Human TIMP-1 migrates to 30 kD on a gel under reducing conditions, and has been detected previously in human and bovine aqueous humor using western blots. [98, 81] A western blot probing for human TIMP-1 on the porcine aqueous humor samples detected bands migrating to approximately 65, 85, and 105 kD in each adult female, juvenile female, and juvenile male aqueous humor sample (Figure 4.12). The bands are too high in molecular weight

to correspond to TIMP-1 (or TIMP-2) alone. The 85 kD band appears to be the major band, and the 65 and 105 kD may be protein fragments, or minor bands. TIMP-1 binds to both MMP-2 and MMP-9. Proteolytic bands corresponding to the molecular weight of MMP-9 (92 kD) did not appear in zymograms of porcine aqueous humor samples. The major gelatinolytic band at 66-72 kD in the aqueous humor samples appearing on the zymogram was believed to be MMP-2. A bound TIMP-1/MMP-2 complex would migrate to approximately 100 kD on a gel. This complexation could correspond to the band seen at approximately 105 kD. There could be proteins homologous in structure to TIMP-1 that would bind to the antibody. The antibody used for TIMP-1 detection was specific to the carboxy terminal end of human TIMP-1. Proteins would need to have a structure homologous to just the carboxy end of TIMP-1 to be detected by the antibody. There may have been aggregation of TIMP-1 and larger proteins that would have limited migration across the gel. Further work on the TIMP interactions in aqueous humor will need to take place before any conclusions can be drawn.

Observing the effects of age on MMP concentration in aqueous humor are important in determining if correlations between MMP activity and glaucoma exist. In the porcine model, age is a risk factor in the incidence of primary open angle glaucoma. Human MMP-2 standard and gelatinolytic bands from porcine aqueous humor migrated to 66-72 kD on zymograms. Inhibition of the gelatinolytic bands at 66-72 kD showed that they were MMPs. The MMP band in porcine aqueous humor migrating to 66 kD on the zymogram was assumed to be the active form of MMP-2, and densitometry was used to determine the concentration compared to human MMP-2 standard. There was a statistically significant difference ($p < 0.05$), between adult female and juvenile samples (Table 4.7). The mean MMP-2 concentration was 0.22 ± 0.06 ng/μl in adult female samples, and 0.28 ± 0.04 ng/μl in juvenile samples. Thus, MMP-2 activity in aqueous humor decreases with age.

MMP-2 is constitutively expressed in many cultured cell lines and skeletal muscle, and its expression is not affected by proteins enhancing MMP production. [99] This is in contrast to the other gelatinase, MMP-9, which is inducible in most cell lines by growth factors and hormones. [99,100] The changes in MMP-2 concentration with age seen in aqueous humor may be independent of variations in growth factors and hormones in

aqueous humor due to the constitutive nature of MMP-2. Thus, the difference in MMP-2 concentrations found in adult and juvenile aqueous humor samples is likely caused by aging factors instead of changes in MMP regulatory agents such as growth factors.

A decrease in MMP-2 activity with increasing age may result in an accumulation of extracellular matrix (ECM) components in the trabecular meshwork. Previous studies on age-related changes in porcine trabecular cells showed that several ECM components increased more than 5-fold in secondary and tertiary cultures subjected to 4-8 weeks of incubation following development of a primary culture. [92] Compared to primary cultures, tertiary cultures showed a 20.5-fold increase in type VI collagen, a 6-fold increase in thrombospondin, and a 5-fold increase in fibronectin. [92] In the study, antibodies specific to other ECM components were not used. Thus, only a few ECM components were studied. The shape of the trabecular cells changed between the primary and secondary cultures also, as revealed by phase-contrast microscopy. In the primary cultures, cells were flat and elongated, but cells increased in thickness and width in the secondary and tertiary cultures. [92] An earlier study of the human trabecular meshwork showed age related increases of 15 % (of total protein) for type I and and 3 % for type IV collagen. [101] MMP-2 digests gelatins, elastin, fibronectin, aggrecan, and collagens IV, V, VII, X, and XI. [21,22] The increases in fibronectin and type IV collagen detected in trabecular cells may correspond to the decrease in MMP-2 activity noted between the juvenile and adult female aqueous humor samples in this thesis. Thus, the higher MMP-2 activity noted in the juvenile aqueous humor compared to the adult samples may be attributable to fibronectin and type IV collagen decrease. An increase of fibronectin and type IV collagen with age because of decreased MMP-2 activity may block the porous trabecular meshwork, reducing aqueous humor outflow. The decreased outflow would then correspond to increased IOP, which can lead to glaucoma.

The factors controlling the increase in thrombospondin and type VI collagen in trabecular cell culture may be related to decreased proteolytic activity also. Recent studies have shown that thrombospondin interacts with MMP-2 via gelatin binding. [102] Type VI collagen has not been shown to be degradable by MMPs. Serine proteinases, however, were able to catabolize type VI collagen microfibrils isolated from bovine skin. [103] An earlier study identified bands migrating to 48 kD and 80-84 kD on a casein

zymogram as serine proteinases in human aqueous humor. Results from this thesis on porcine aqueous humor identified non-MMP activity at 85 kD on a gelatin zymogram, and non-MMP activity at 51 kD and 80 kD on a casein zymogram. If these bands are serine proteinases, then a correlation may exist between the decreased collagen VI in secondary and tertiary trabecular cultures and serine proteinases.

Establishing a connection between MMP activity and ECM remodeling in the eye has been attempted using drugs for the treatment of glaucoma. An earlier study with the prostaglandin glaucoma drug Latanoprost showed a correlation between MMP activity and ECM remodeling. [95] Treatment of cell cultures from human ciliary muscle with Latanoprost resulted in a reduction of fibronectin, laminin, hyaluronan, and collagens I, III, and IV, while MMP-2 and MMP-3 activity increased. Cell culture from the anterior part of the ciliary muscle showed a collagen IV and VI decrease after Latanoprost treatment. Relaxin has been proposed as a glaucoma drug due to its ECM remodeling capabilities and ability to lower IOP. [16] Relaxin can induce MMP-1, MMP-3, and MMP-9 expression in tissue, but does not appear to affect MMP-2 expression. [104,105] This correlates with the constitutive nature of MMP-2 in tissue. Elastin, proteoglycans, fibronectin, laminin, vitronectin, and collagen (types I, III-VI, and VIII) are ECM components found in the trabecular meshwork. [94] MMP-1 can digest collagen types I and III, and MMP-3 and MMP-9 can digest elastin, proteoglycans, fibronectin, laminin, and collagens III-V. Thus, only collagens VI and VIII are potentially not affected by relaxin treatment in the trabecular meshwork.

MMP-2 concentration in juvenile aqueous humor was statistically independent ($p = 0.90$) of gender (Table 4.8). The study of effects of gender on the MMP concentration ideally requires adult male samples. Adult human males have a higher incidence of open-angle glaucoma than adult females. The fact that juvenile samples do not have a statistically significant difference between sexes signifies that there may be specific changes occurring as males reach adulthood that make them more susceptible to acquiring open-angle glaucoma. A future study with adult male aqueous humor samples can provide more insight into gender affects.

Porcine aqueous humor appears to contain fewer proteolytic bands compared to other species. A study on human aqueous humor identified a major MMP band at 66 kD,

and minor MMP bands at 125, 95, and 62 kD on a gelatin zymogram. [65] A study on bovine aqueous humor detected two MMP bands at 92 kD, 2 MMP bands at 66 kD, one MMP band at 100 kD, and several MMP bands below 50 kD on gelatin zymograms. Appearance of MMP bands between 92-95 kD in human and bovine aqueous humor indicates probable MMP-9 activity. This activity was not seen in the porcine aqueous humor, as no protease bands migrated to the molecular weight of the human MMP-9 standard (Figure 4.6). MMP activity at 66 kD and between 100-125 kD appears in the porcine, human, and bovine species. Both human and bovine aqueous humor samples show MMP bands corresponding to MMP-9. MMP-9 and MMP-2 share similar substrate specificities. Elastin, fibronectin, proteoglycans, and collagens IV and V are ECM components found in the trabecular meshwork that are substrates of MMP-2 and MMP-9. MMP-9 is more likely than MMP-2 to digest proteoglycan, however. [21] In human aqueous humor, non-MMP caseinolytic activity was detected at 48 kD, 68 kD, and between 80-84 kD. The bands between 80-84 were brighter in comparison to the 48 kD and 68 kD bands. [65] The 48 kD band and the band between 80-84 kD match the bands on our casein zymograms. The caseinases were determined to be serine proteinases based on the proteolytic activity in the presence of different inhibitors. [65] Our results do show that the proteolytic bands are probably not MMPs based on activity after 1,10-phenanthroline exposure. The proteinase migrating to 80 kD appears to be more highly expressed in females than males based on the few samples analyzed. Future casein zymograms may need to focus on the differences between male and female samples.

Total protein assays were used to determine the validity of using pigs as an animal model in comparison to other animals. Age and gender affects on total protein concentration were also examined for comparison to serum protein and MMP concentrations. This comparison would show if age and gender trends were true for most proteins, or if age and gender had specific effects on certain proteins. BCA total protein assays on fifty-five adult female, four juvenile female, and four juvenile male aqueous humor samples determined total protein concentrations. The average total protein concentrations for all the porcine aqueous humor samples was 2.54 ± 0.89 mg/ml in comparison to BSA. The average protein concentration using the phenol method in aqueous humor for 12 dogs, 15 cats, 7 horses, and 6 cattle was 0.36 mg/ml, 0.44 mg/ml,

0.56 mg/ml, and 0.58 mg/ml respectively. [60] Thus, the total protein concentrations obtained for the porcine samples using the BCA assay appears to be higher than other farm animals. The method of total protein determination may play a role in the higher total protein concentrations for pigs. The phenol method (modified Lowry assay) was used to determine the total protein concentrations in aqueous humor of the dogs, cats, horses, and cattle, while the BCA assay was used to quantify the total protein concentration in pig aqueous humor. Human and monkey aqueous humor has less protein content by approximately a factor of ten. [61,63,64,65]

The effects of age on the total protein concentration were determined using the BCA assay with BSA as the standard to analyze 55 adult female samples and 8 juvenile samples. The average protein concentration was 2.77 ± 0.84 mg/ml in adult female aqueous humor, and 2.18 ± 1.00 mg/ml in the juvenile aqueous humor. A t-test on the two sample populations showed the difference in mean concentrations was not very significant ($p = 0.06$). A higher number of juvenile samples may make the difference in age to be statistically relevant. In a study with human aqueous humor samples ranging from the ages of 21 to 79 years, the protein concentration was found to significantly change with age. [106] The reason for an increased protein concentration with age is unclear. A decreasing aqueous humor formation rate with age was suggested to play a role in the increased protein concentration. A decreased rate of aqueous humor flow could allow proteins to concentrate near the ciliary processes, as the lower flow rate would be unable to force proteins in the aqueous through the interstitial spaces of ciliary muscle. The rate of aqueous humor formation decreases with increasing age [107,108], but at only 2.5 % per decade. [108] The low decrease in aqueous humor formation suggests an alternative theory may be necessary to explain the increase in protein concentration with age. The blood-aqueous barrier being less permeable with age is another possible theory to explain the increase of protein concentration with age. Fluorometric scans of the blood-aqueous barrier, however, show that the permeability is independent of age between 13-72 years. [109] Total protein concentrations between four juvenile male and four juvenile female porcine aqueous humor samples were compared using the BCA assay. The mean protein concentrations of 2.11 ± 0.54 mg/ml for juvenile female samples and 2.25 ± 0.74 for juvenile male samples were not statistically different ($p >$

0.7). An earlier study on human aqueous humor also found no significant difference in protein concentrations between males and females. [110]

The concentration of different serum proteins was examined to see whether the blood brain barrier of the pigs had been broken from the different methods of euthanasia. A comparison of the serum protein concentrations found in the porcine aqueous humor to other species would show whether the blood brain barrier was disrupted. There were two methods of pig euthanasia: lethal injection and electrocution. The serum protein concentrations in the aqueous humor did not vary statistically between pigs euthanized in the two ways. ELISAs were used to measure for IgG, transferrin, and albumin in porcine aqueous humor. The mean IgG concentration of 1.14 ± 4.21 mg/dl found for all of the porcine aqueous humor samples is lower than human aqueous IgG concentration of 3 mg/dl [71] found in literature. The mean transferrin concentration of 1.71 ± 0.68 mg/dl for all of the aqueous humor samples is similar to non-glaucoma human aqueous transferrin concentrations of 1.3-1.7 mg/dl. [69, 70] A mean albumin concentration of 7.80 ± 2.63 mg/dl for all of the porcine aqueous humor samples is close to the human aqueous albumin concentrations of 5.4-6.5 mg/dl. [69] Thus, the serum protein concentrations in pigs appear to be similar to humans, and the blood brain barrier appears to be intact. The similarity in serum protein concentrations in aqueous humor between pigs and humans indicates that pigs are good animal models of the human eye.

The effects of age and gender on the IgG and transferrin concentrations in aqueous humor were compared to see if serum protein concentration trends were similar to MMP concentration trends. Neither the IgG and transferrin concentrations differed statistically based on gender in aqueous humor ($p > 0.05$). This is in accord with previous studies on protein concentrations in serum. In two separate tests, no significant differences in IgG, IgA, and IgM concentrations were found between male and female subjects using a radial immunodiffusion technique. [111, 112] ELISA data showed a statistically significant correlation between age and IgG concentration ($p < 0.01$), but transferrin concentration was independent of age ($p > 0.1$). Previous experimentation on serum proteins offers contrasting evidence on the effects of age on specific protein concentrations. Albumin and transferrin proteins increase in concentration with age in studies using crossed immunoelectrophoresis in human aqueous humor. [113] The effects

of age on serum levels of different immunoglobulins differ based on the technique used for analysis. Future work may determine whether there is a correlation between the age differences detected with MMP-2 activity in aqueous humor and serum protein concentration.

SDS-PAGE was used to determine the protein composition of adult female and juvenile porcine aqueous humor samples. Silver staining of gels run with reducing buffer revealed 18 protein bands in the adult female (Figure 4.1), 10 bands in the juvenile female (Figure 4.2), and 16 bands in the juvenile male (Figure 4.3) aqueous humor. The aqueous humor proteins ranged in molecular weight from approximately 9–142 kD in the adult female samples, 14-128 kD in the juvenile female samples, and 9-151 kD in the juvenile male samples. This is consistent with results taken from literature. A silver stain of human aqueous humor samples revealed 12 major polypeptide bands ranging from 9 – 140 kD. [63] The protein profiles for human and pig aqueous humor are therefore similar.

In the adult female porcine samples, proteins migrated to molecular weights corresponding to molecular weight markers of lysozyme, carbonic anhydrase, pig albumin, phosphorylase b, pig IgG, and pig IgA. Proteins migrated to similar molecular weights in the juvenile samples (Figures 4.2 and 4.3). A polypeptide band migrated to the molecular weight corresponding to a transferrin standard in the juvenile samples. The gel containing adult female aqueous humor did not contain a transferrin standard, but a protein band migrated to a similar molecular weight as the transferrin standard in the other gels (Figure 4.1). Transferrin, IgG, and albumin have been detected in the aqueous humor of other species. [66, 68] Proteins migrating to molecular weights at approximately 17 kD in human aqueous humor were identified as basic fibroblast growth factor. [63] Proteins migrating to 14 kD in monkey aqueous humor were identified as the protease inhibitor cystatin using western blot immunodetection. [61] Polypeptide bands were detected near 14 and 17 kD in all the porcine aqueous humor samples analyzed. In a previous study, western blot analysis of human aqueous humor detected the 53 kD α1-proteinase inhibitor which had a concentration approximately 15 % of the total protein. [65] Since this proteinase inhibitor is found at high concentrations in the aqueous humor, the 56 kD polypeptide band appearing in all the gels may be α1-proteinase inhibitor. The

study performed in 1963 showed that relaxin decreased IOP after intramuscular injection. This indicated that there may be pre-existing relaxin receptors in the eye, and therefore low concentrations of relaxin in the aqueous humor. A protein migrating to a molecular near to relaxin was not seen in any of the gels (Figures 4.1, 4.2, 4.3). Thus, it appears that relaxin is either absent in aqueous humor, or is at lower than nanogram per milliliter concentrations.

Chapter 6 Conclusions & Engineering Significance

Risk factors for open angle glaucoma, the most prevalent form of glaucoma, include an increased intraocular pressure (IOP), and advanced age. In addition, adult males have a higher incidence of open-angle glaucoma than adult females. IOP, a function of aqueous humor production and outflow in the eye increases due to aqueous humor overproduction or a blockage to outflow. Accumulation of extracellular matrix (ECM) components at the porous trabecular meshwork can cause an outflow block. Matrix metalloproteinases digest and remodel ECM components, and can potentially open an ECM caused outflow block. The correlation between glaucoma and age indicates that there are changes occurring in the eye with time that are conducive to IOP increases. Our experimentation focused on determining how age and gender affected MMP activity in aqueous humor, to see whether there was a correlation between glaucoma risk factors and MMP activity. Pigs were the animal model due to their availability and the quantity of aqueous humor (100-150 μl) that can be taken from a porcine eye. Pigs have also been used as tissue culture models for prostaglandin analogue studies. [19,20] Total and selected serum protein concentrations in aqueous humor were quantified to determine if the blood brain barrier had been disrupted during euthanasia, and to see whether protein concentrations of pigs and humans were similar. Pigs were euthanized by lethal injection or electrocution. Similarities in protein concentrations would validate the pig as an animal model for studying glaucoma. The effects of age and gender on total protein, IgG, and transferrin concentrations in aqueous humor were analyzed to see if the trends observed in MMP activity existed for other proteins.

Gelatin zymography on aqueous humor revealed major gelatinolytic bands at approximately 66-72 kD, and a minor band at approximately 105-110 kD. The bands at 66-72 kD were identified as the inactive and latent forms of MMP-2 by comparison to a human MMP-2 standard. Densitometry on the gelatinolytic band migrating to 66 kD showed there was a statistically significant decrease ($p < 0.05$) in the activity of the band in adult aqueous humor compared to juvenile samples. All observed gelatinolytic bands (66-72 kD, 105-110 kD) were inhibited by a specific MMP inhibitor. The bands, therefore, contain MMP activity. Casein zymography on aqueous humor bands showed

two bands with proteolytic activity at 51 kD and approximately 80 kD. Neither caseinase was inhibited in the presence of an MMP inhibitor. The caseinase at 80 kD was expressed in greater concentrations in female than male aqueous humor.

The average total protein concentration for all the porcine aqueous humor samples was 2.54 ± 0.89 mg/ml. There was a small statistical difference ($p=0.06$) in total protein concentration between adult and juvenile porcine aqueous humor. Total protein concentrations were not significantly different between juvenile male and female samples. The average IgG, transferrin, and albumin concentrations detected in the porcine aqueous humor samples were 1.14 ± 0.42 mg/dl, 1.71 ± 0.68 mg/dl, and 7.80 ± 2.63 mg/dl respectively. Transferrin concentration in porcine aqueous humor was statistically independent of gender and age. IgG concentration was statistically independent of gender. IgG concentrations were significantly higher ($p \leq 0.05$) in adult than juvenile aqueous humor. The serum protein and total protein concentrations were not statistically different between pigs euthanized by lethal injection and electrocution.

Tissue engineering often focuses on the repair and replacement of nonfunctioning organs or structural components. Future research in tissue engineering will need to account for the factors regulating ECM turnover during tissue growth and repair. MMPs have been implicated as tissue remodeling components in bone growth [114], wound healing [100], and tumor metastasis [114]. Our experimental work gives insight into MMP activity in porcine aqueous humor. The decrease in MMP-2 concentration in porcine aqueous humor with age may correlate with why age is a major risk factor in the onset of open-angle glaucoma. Relaxin is a hormone that remodels ECM components during pregnancy. An earlier study showed relaxin introduction into the eye lowered IOP, but the mechanism remains unknown. A hypothetical mechanism is given in Figure 6.1. An increased IOP resulting from ECM components collecting at the aqueous humor outflow pathways requires relaxin treatment. Relaxin crosses the cell membrane after binding to extracellular receptors, carrying a gene sequence corresponding to a protein function to the cell nucleus. The gene sequence is translated to a protein function signaling pro-MMP synthesis and ECM synthesis. From the protein signal inside the cell, pro-MMP and ECM secretion take place outside of the cell. The propeptide is cleaved off activating the MMP, and the

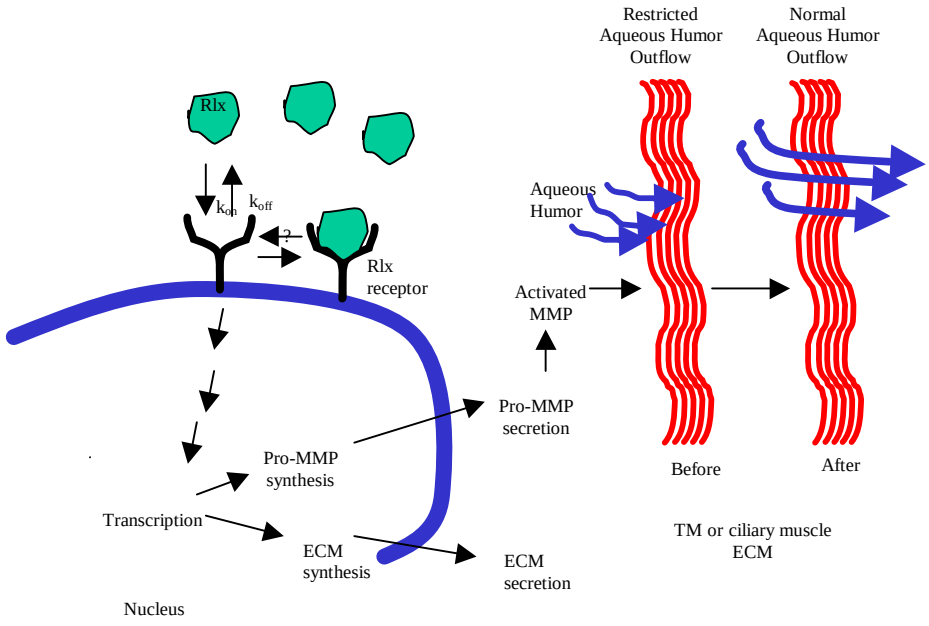

Figure 6.1. Hypothetical mechanism of relaxin in lowering IOP in the eye

MMP remodels excess ECM components collecting at the trabecular meshwork or the ciliary muscle. The MMP digestion of ECM components clears the outflow block, allowing aqueous humor to pass through the trabecular meshwork or ciliary muscle, lowering IOP.

Future experimentation in our lab will focus on the effects of the relaxin hormone on trabecular meshwork cells in culture. The results from this thesis provide a benchmark that can be compared to results taken after relaxin dosage in cell culture. That will help elucidate the mechanism used by relaxin to remodel connective tissue, and eventually determine whether relaxin is a potential therapeutic for open-angle glaucoma.

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Appendix A: BCA Data

Table A.1. BCA data of adult female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
O-197 L	4.15	4.34	3.86	4.12	0.24	5.87
O-200 L	4.47	4.98	4.77	4.74	0.26	5.41
O-197 R	2.59	2.79	2.26	2.55	0.27	10.51
O-200 R	2.41	2.17	2.34	2.31	0.12	5.33
O-344 L	1.33	2.56	1.08	1.66	0.80	48.12
O-344 R	1.91	2.08	1.08	1.69	0.54	31.84
W-27 L	1.89	0.85	1.37	1.37	0.52	38.05
P-32 R	1.33	0.67	1.37	1.12	0.39	35.05
P-35 R	1.96	1.27	1.50	1.58	0.35	22.21
P-32 L	1.17	1.15	1.05	1.12	0.06	5.70
O-07 R	1.13	1.05	1.32	1.17	0.14	11.86
W-27 R	2.41	2.35	2.00	2.25	0.22	9.87
O-07 L	1.14	1.21	1.17	1.17	0.04	3.17
P-35 L	1.73	1.55	0.97	1.41	0.40	28.09
P-40 R	2.47	2.17	2.46	2.37	0.17	7.27
P-40 L	2.26	2.38	2.20	2.28	0.09	3.92
O-344 L	2.89	2.37	3.34	2.87	0.49	16.92
W-27 L	3.21	2.94	3.95	3.36	0.52	15.49
P-32 R	1.71	1.79	2.03	1.84	0.16	8.91
P-35 R	2.03	2.50	2.30	2.28	0.24	10.43
P-35 L	2.57	2.22	2.60	2.46	0.21	8.63
W-37 L	3.03	3.83	2.89	3.25	0.51	15.60
O-18 L	2.67	2.90	3.20	2.92	0.27	9.21
W-37 R	3.58	4.08	3.99	3.88	0.27	6.87
P-30 R	3.43	2.42	3.01	2.95	0.51	17.25
O-18 R	2.97	2.47	2.25	2.56	0.37	14.44
P-39 L	4.64	4.60	4.85	4.70	0.14	2.91
O-8 R		2.58	2.99	2.79	0.29	10.40
O-8 L	2.59	2.14	2.17	2.30	0.25	10.91
O-2 L	2.29	1.87	2.09	2.08	0.21	10.03
O-2 R	2.31	2.34	2.38	2.34	0.03	1.46
O-8 R	2.83	2.52	2.92	2.76	0.21	7.56
W-32 R	2.52	1.90	2.15	2.19	0.32	14.40
P-30 L	3.09	2.49	2.27	2.62	0.42	16.16
W-32 L	2.74	2.30	2.55	2.53	0.22	8.57
W-44 R	2.81	2.74	2.46	2.67	0.19	7.00
W-44 L	2.56	1.91	2.53	2.33	0.37	15.80
O-12 R	2.36	2.05	2.29	2.23	0.16	7.33
O-12 L	3.25	2.99	3.27	3.17	0.15	4.84
P-46 R	1.85	1.24	0.98	1.36	0.45	32.79
P-46 L	1.59	1.02	0.88	1.16	0.37	32.24
W-39 R	3.40	3.14	2.56	3.03	0.43	14.17
W-39 L	2.11	1.31	0.96	1.46	0.59	40.18
W-47 R	2.34	1.78	1.70	1.94	0.35	17.85
W-47 L	1.74	1.20	1.21	1.38	0.31	22.51
O-16 R	2.40	1.96	1.86	2.07	0.29	14.04

O-16 L	4.13	3.28	3.80	3.74	0.42	11.35
P-11 L	3.17	3.04	4.15	3.45	0.61	17.57
P-11 R	3.13	2.99	6.09	3.06	0.10	3.19
P-14 R	2.77	2.32	2.07	2.39	0.35	14.84
P-14 L	3.01	2.77	2.34	2.71	0.34	12.62
P-04 R	2.82	2.79	3.09	2.90	0.17	5.82
P-04 L	2.87	2.52	3.06	2.82	0.27	9.68
P-03 L	2.77	2.48	2.32	2.52	0.23	9.03
P-03 R	4.07	3.79	4.13	4.00	0.18	4.54
O-197 L	3.65	4.72	5.05	4.47	0.73	16.43
O-197 R	3.14	3.61	3.74	3.50	0.32	9.07
O-200 L	3.16	3.27	3.96	3.47	0.43	12.49
P-39 R	2.73	2.84	3.18	2.92	0.23	8.03
W-44 L	3.15	3.30	3.47	3.31	0.16	4.78
W-39 L	3.65	3.84	4.12	3.87	0.24	6.08
P-46 R	2.99	3.36	3.05	3.13	0.20	6.43
P-11 R	3.02	3.26	3.78	3.35	0.39	11.55
P-11 L	1.18	1.26	1.32	1.25	0.07	5.86

Table A.2. BCA data on juvenile female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
12 R	2.95	2.85	2.64	2.81	0.16	5.62
15 L	2.08	1.79	1.72	1.86	0.19	10.20
9 R	1.55	1.59	1.50	1.55	0.05	2.92
9 L	2.19	2.16	2.34	2.23	0.10	4.32

Table A.3. BCA data on juvenile male samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
IM-5 L	1.67	1.68	1.60	1.65	0.04	2.52
IM-1 R	1.77	1.90	1.78	1.82	0.07	4.01
IM-1 L	2.13	2.36	2.21	2.23	0.12	5.35
IM-3 R	3.34	3.20	3.33	3.29	0.08	2.39

Appendix B: ELISA data

Table B.1. IgG concentration in adult female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Conc. #4 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
O-12 R	8.71	15.52	9.06	15.72	12.25	3.89	31.78
O-12 L		14.42	10.01	18.98	14.47	4.49	31.01
O-344 L		12.63	14.42	19.61	15.55	3.63	23.32
O-344 R		19.06	18.43	20.35	19.28	0.98	5.08
P-39 L		9.38	10.30	10.35	10.01	0.55	5.45
P-39 R		18.34	17.92	15.81	17.36	1.36	7.81
W-44 L		8.85	9.13	7.84	8.61	0.68	7.85
W-44 R		13.64	16.57	10.69	13.63	2.94	21.56
O-344 L	10.53	10.66	11.67	12.12	11.25	0.77	6.89
O-344 R	16.86	25.31	22.12	18.09	20.60	3.86	18.76
W-44 L	8.79	9.22	10.18	8.33	9.13	0.79	8.64
W-44 R	9.54	12.59	11.14		11.09	1.53	13.76

Table B.2. IgG concentration in juvenile female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
7 L	10.35	10.92	8.59	9.95	1.21	12.20
14 L	9.79	9.21	7.76	8.92	1.05	11.72
9 R	8.97	10.06	9.79	9.61	0.57	5.91

Table B.3. IgG concentration in juvenile male samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
5 R	8.38	12.74	10.76		10.63	2.18
4 R	6.37	10.48	9.54		8.80	2.15
5 L	5.47	6.23	5.58		5.76	0.41

Table B.4. Transferrin concentration in adult female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Conc. #4 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
O-8 R	13.36	22.05	36.60	38.03	27.51	11.88	43.18
O-8 L	12.94	9.12	7.46	10.79	10.08	2.34	23.24
O-07 R	21.28	28.25	35.74	36.82	30.52	7.24	23.73
O-07 L	12.83	10.71	10.05	12.34	11.48	1.32	11.48
O-12 R	13.57	14.42	18.84	20.44	16.82	3.34	19.89
O-12 L	10.51	9.42	9.71	12.17	10.45	1.23	11.81
O-344 L	7.85	9.91	13.61	14.80	11.54	3.22	27.92
O-344 R		33.86	33.96	11.63	26.48	12.87	48.58
P-39 L	4.74	3.39	4.11		4.08	0.68	16.55
P-39 R	33.25	28.24	7.00		22.83	13.94	61.05
W-44 L	2.92	1.26	5.94	3.10	3.30	1.94	58.74
W-44 R	18.97	19.60	16.66	*5.78*	18.41	1.55	8.42

Table B.5. Transferrin concentration in juvenile female samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
7 L		24.34	20.34	22.34	2.83	12.66
14 L	16.89	17.61	15.29	16.60	1.19	7.15
9 R	18.19	21.1	17.75	19.01	1.82	9.57

Table B.6. Transferrin concentration in juvenile male samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
5 R		24.58	16.13	20.36	5.98	29.35
4 R		21.45	19.72	20.59	1.22	5.94
5 L	18.34	21.8	21.46	21.63	1.91	8.82

Table B.7. Albumin concentration in aqueous humor samples

Sample	Conc. #1 (mg/ml)	Conc. #2 (mg/ml)	Conc. #3 (mg/ml)	Conc. #4 (mg/ml)	Avg. Conc. (mg/ml)	Standard Deviation	RSD (%)
P-08 L	83.12	102.62	95.60		93.78	9.88	10.53
P-04 R	79.16	80.67			79.91	1.07	1.34
W-27 L	37.36	33.15	58.42	37.15	41.52	11.43	27.53
P-08 L	83.12	102.62	95.60		93.78	9.88	10.53
14 L	103.34	123.55	128.42		118.44	13.30	11.23
13 R	89.25	126.89	94.82		103.65	20.31	19.60
1 R	48.38	64.76	80.63		64.59	16.12	24.96
5 L	46.34	64.22	55.41	36.10	50.52	12.07	23.88
3 R	46.82	77.67	89.52	71.42	71.36	18.00	25.22

Appendix C: Zymography data

Table C.1. Densitometry of adult female samples in comparison to human MMP-2 standard

Sample	MMP-2 Concentration (ng/㎖)
P-03 L	1.01
O-8 L	0.64
O-07 L	1.19
W-44 L	0.88
W-32 L	0.66
P-03 R	1.00
P-39 R	1.06
P-35 R	1.12
P-11 R	1.18

Table C.2. Densitometry concentrations of juvenile female samples in comparison to human MMP-2 standard

Sample	MMP-2 Concentration (ng/ml)
16 R	1.30
13 R	1.35
13 L	1.45
16 L	1.25
11 R	1.18
14 R	1.35
11 L	0.97
6 L	1.03
14 R	1.20

Table C.3. Densitometry concentrations of juvenile male samples in comparison to human MMP-2 standard

Sample	MMP-2 Concentration (ng/ml)
1 L	1.43
1 R	1.41
3 R	1.04
5 L	1.22
2 R	1.20
1 L	1.28
5 L	0.97
4 R	1.17
1 R	1.19

Vita

Jayanth Sankrit Chandran was born in Raleigh, North Carolina on December 6th, 1976. Jayanth graduated from Lake Braddock secondary school in 1994, and attended the University of Virginia from 1994-1998. He received his Bachelor of Science degree in chemical engineering in 1998, and has been attending Virginia Polytechnic Institute and State University from 1998 to the present. In the future, Jayanth intends to work in the field of biological science, with intentions on pursuing a PhD. Jayanth currently lives in Blacksburg.