Title

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Journal

Investigative Ophthalmology & Visual Science, 56(5)

ISSN

0146-0404

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Publication Date

2015-05-04

DOI

10.1167/iovs.14-15840

Peer reviewed

Advanced Glycation End-Product Accumulation Reduces Vitreous Permeability

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Submitted: October 9, 2014
Accepted: March 31, 2015

Citation: Lee O-T, Good SD, Lamy R, Kudisch M, Stewart JM. Advanced glycation end-product accumulation reduces vitreous permeability. *Invest Ophthalmol Vis Sci*. 2015;56:2892–2897. DOI:10.1167/iovs.14-15840

Purpose. To evaluate the effect of nonenzymatic cross-linking (glycation) upon the permeability of the vitreous to small- and large-solute diffusion.

Methods. Vitreous from freshly excised porcine eyes was treated for 30 minutes with control or 0.01%, 0.1%, or 1% methylglyoxal (MG) solution. The efficacy of the glycation regimen was verified by measuring nonenzymatic cross-link density by fluorescence in the vitreous samples. Resistance to collagenase digestion as well as N^{ε}-(carboxyethyl) lysine (CEL) content were also measured. The permeability coefficient for fluorescein and fluorescein isothiocyanate (FITC)-IgG diffusion through 3 mL of the vitreous samples was determined by using a custom permeability tester.

Results. Vitreous cross-linking with MG treatment was confirmed by increased fluorescence, increased CEL concentration, and increased resistance to collagenase digestion. Vitreous glycation resulted in a statistically significant decrease in the permeability coefficient for fluorescein diffusion when either 0.1% or 1% MG solution was used ($5.36 \pm 5.24 \times 10^{-5}$ cm s^{-1}, $P = 0.04$; and $4.03 \pm 2.1 \times 10^{-5}$ cm s^{-1}, $P = 0.001$; respectively, compared with control, $9.77 \pm 5.45 \times 10^{-5}$ cm s^{-1}). The permeability coefficient for diffusion of FITC-IgG between control ($9.9 \pm 6.37 \times 10^{-5}$ cm s^{-1}) and treatment groups was statistically significant at all MG concentrations (0.01% MG: $3.95 \pm 3.44 \times 10^{-5}$ cm s^{-1}, $P = 0.003$; 0.1% MG: $4.27 \pm 1.32 \times 10^{-5}$ cm s^{-1}, $P = 0.004$; and 0.1% MG: $3.72 \pm 2.49 \times 10^{-5}$ cm s^{-1}, $P = 0.001$).

Conclusions. Advanced glycation end-product (AGE) accumulation reduces vitreous permeability when glycation is performed in ex vivo porcine vitreous. The permeability change was more pronounced for the larger solute, suggesting a lower threshold for AGE-induced permeability changes to impact the movement of proteins through the vitreous when compared with smaller molecules.

Keywords: vitreous humor, glycation, permeability, diffusion

The vitreous humor is the viscous and transparent gel-like material that fills the space between the lens and retina of the eye. Although it is composed mainly of water, a network of collagen, hyaluronan, proteoglycans, and glycoproteins maintains its structural form. These components undergo chemical modifications over time, particularly with the accumulation of nonenzymatic cross-links, which occur in the course of normal aging and to a much greater degree in diabetes as advanced glycation end-products (AGEs) form.[1] The process begins with the natural breakdown of sugars, as reactive intermediate species such as ketones condense with the amine side chains of lysine or hydroxylysine in collagen molecules, in what is known as the Maillard reaction, the same chemical process responsible for browning of foods.[2,3] This occurs to a much greater degree in diabetes than in normal aging, owing to the high levels of circulating sugars in the body.[4] As a result of these processes, AGE levels are elevated 20-fold in the vitreous in diabetic patients.[5]

We previously have reported that in sclera and cornea, the accumulation of nonenzymatic cross-links causes a decrease in tissue permeability to both solute diffusion and fluid flow.[6,7] The clinical consequence of these permeability changes in the ocular coat are presently unknown but could be expected to impact diffusion of nutrients and protein signaling molecules across natural barriers. We speculated that similar effects could occur in the vitreous, where matrix changes leading to alterations in permeability could presumably have an even greater impact upon the trajectory of ocular disease, because of the sensitive microenvironment within the eye adjacent to the retina. If, for example, diffusion of proteins through the vitreous is impeded in diabetes owing to AGE-induced chemical changes, this could lead to retention of pathologic cytokines in the vitreous cavity, with the potential to accelerate retinopathy.

We undertook the current study to model the effects of AGE accumulation in the vitreous as a first step toward understanding whether measurable changes in solute diffusion through the vitreous might occur in diabetes. We hypothesized that increased glycation would result in decreased solute diffusion through the vitreous. In addition, we speculated that the impact on permeability might be size-dependent, with larger molecules impeded even more than slow ones in their movement through vitreous. To test this hypothesis, we introduced exogenous glycation to vitreous in vitro and used a custom two-chamber diffusion system to quantify vitreous permeability to fluorescein (a small molecule) and FITC-IgG (representative of larger molecules).

Methods

Tissue Harvest and Treatment

A total of 180 freshly excised porcine eyes (age range, 6–8 months; Sierra for Medical Science, Whittier, CA, USA) were used in these experiments. All eyes were refrigerated and processed within 48 hours of death. The anterior segment was excised with a circumferential incision behind the limbus and the lens removed with forceps to expose the vitreous body. Using a sterile spatula, we removed the whole vitreous and placed it in a Petri dish. Adherent iris, ciliary body, and choroid were carefully separated from the vitreous with scissors. The vitreous body was then washed with Dulbecco phosphate-buffered saline (PBS) before incubation.

Samples were treated at 37°C in a 5% CO_2 atmosphere for 30 minutes in PBS containing 1% penicillin–streptomycin and protease inhibitor cocktail tablets (10213200; Roche, Mannheim, Germany) before their use in experiments. Selected samples also received treatment with 0.01%, 0.10%, or 1.00% methylglyoxal (MG) (Sigma-Aldrich Corp., St. Louis, MO, USA), a Maillard intermediate.[8] This corresponds to 0.139, 1.39, and 139 mM solutions, respectively. Control vitreous was treated in the incubation solution only.

Vitreous Fluorescence Following MG Treatment

Methylglyoxal was dialyzed out of each vitreous sample for 24 hours at 4°C by using dialysis tubing (SnakeSkin Dialysis Tubing; Thermo Scientific, Rockford, IL, USA) to prevent the reading of contaminating fluorescence by free methylglyoxal. After dialysis, vitreous was removed and vitrectomized at 1200 cpm by using an Intrector portable vitrectomy system (Insight Instruments, Stuart, FL, USA). Afterward, 100-µL aliquots of vitrectomized samples were removed to a black ELISA plate, and fluorescence was measured in a fluorescence spectrophotometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). Fluorescence was read at excitation and emission wavelengths of 370 and 440 nm, respectively, obtained from the literature.[9]

N^{ε}-(Carboxyethyl) Lysine Assay

In this study we did not undertake an exhaustive characterization of the specific AGEs generated in the vitreous after MG treatment, as the cascade of reactions that occurs following MG exposure has been well-described elsewhere.[10,11] However, to confirm that MG treatment of porcine vitreous in our study was behaving in a manner similar to what has been previously reported, we selected one of the principal MG-associated AGEs, N^{ε}-(carboxyethyl) lysine (CEL), and quantified its levels after treatment with control or MG solution. Vitreous samples were treated at 37°C in a 5% CO_2 atmosphere for 30 minutes in PBS containing 1% penicillin–streptomycin and protease inhibitor cocktail tablets (10213200; Roche) before their use in experiments. Selected samples also received treatment with 1.00% MG (Sigma-Aldrich Corp.). Control vitreous was treated in the incubation solution only. Following incubation, vitreous was removed and vitrectomized at 2500 cpm by using a 25+ gauge probe and an Alcon Accurus Surgical System (Alcon Laboratories, Inc., Fort Worth, TX, USA). The CEL concentration was measured in treated and untreated samples by using an OxiSelect N^{ε}-(carboxyethyl) lysine (CEL) Competitive ELISA Kit (STA-813; Cell Biolabs, Inc., San Diego, CA, USA). Absorbance of the ELISA plate was read at 450 nm with a spectrophotometer (SpectraMax M5).

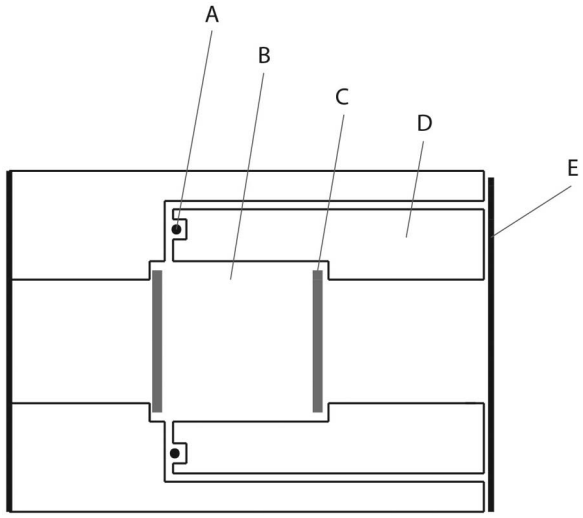

Figure 1. Diffusion apparatus used for testing vitreous permeability. (**A**) O-ring. (**B**) Vitreous sample. (**C**) Aerator insert. (**D**) Union housing. (**E**) Wax film seal.

Assessment of Vitreous Cross-Linking by Resistance to Digestion

For further confirmation of cross-linking in the vitreous following MG exposure, the resistance of the vitreous gel phase to digestion by collagenase was used as a proxy for the extent of cross-linking present. This was quantified by measuring the percentage decrease in mass of the gel phase after exposure to collagenase.[12] For each eye, the entire vitreous body was weighed and the gel portion of the vitreous was removed with forceps and transferred to a container for treatment. Samples were then treated at 37°C for 30 minutes in PBS containing 1% penicillin–streptomycin, with or without 1.00% MG. The vitreous was recovered from the solution with forceps and weighed. It was then submerged in a 22-unit/mL solution of Collagenase Type II, lyophilized (Life Technologies, Carlsbad, CA, USA) for 24 hours. The vitreous was then removed from the collagenase solution with forceps and was weighed, and the percentage of gel phase by mass was calculated for each sample before and after collagenase digestion. The difference between these values was calculated for each sample, and Student's *t*-test (one-tailed, unpaired) was used for statistical analysis of the data.

In Vitro Diffusion Apparatus

A modified version of our previously described diffusion assay[6,7] was used for these experiments (Fig. 1). In vitro diffusion studies were conducted with housing constructed from polyvinyl chloride unions (model No. 164-634HC; American Valve, Greensboro, NC, USA). A 3-mL containment chamber for the vitreous samples was made by sealing the fitting connections of the union joints with a water aerator insert (model No. 37.0083.98; Neoperl, Waterbury, CT, USA) at both ends. A small amount of cyanoacrylate was applied to the entire boundary to prevent leakage. Vitreous body, after control or MG solution treatment as described above, was blotted dry and 3 mL of each sample was placed in the containment chamber. This assembly was then mounted between the two reservoir chambers, and the union housing was threaded together to provide the compression necessary to prevent leakage around the specimen. The open ends of the union were sealed (Parafilm; Pechiney Plastic Packaging,

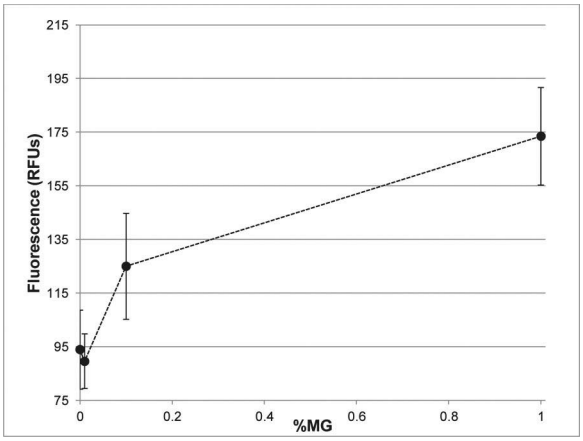

FIGURE 2. Plot of vitreous nonenzymatic cross-link content, as indicated by fluorescence spectroscopy, versus MG treatment concentration ($n = 6$ for each group; *error bars* denote standard deviation).

Chicago, IL, USA) after both chambers were filled with PBS and visually inspected for leakage. The apparatus was incubated at 37°C in a 5% CO_2 atmosphere for 1 hour to restore normal hydration and temperature.

At the end of the hydration period, the media in both reservoirs were decanted. In one side, 36 mL fresh PBS at 37°C was used to fill the chamber to the brim. At the other end, an equal volume of 10 μg/mL fluorescein (F6377; Sigma-Aldrich Corp.) or 10 μg/mL FITC-IgG (F9636; Sigma-Aldrich Corp.) heated to 37°C was added. The apparatus was returned to the tissue incubator and placed onto a slowly rotating dial in a rotary hybridization oven (model 2000; Robbins Scientific Corp., Sunnyvale, CA, USA) to ensure that the contents of each compartment were evenly distributed at all times. (Stability of the FITC-IgG complex during this slow mixing process is assumed from previously reported experimental use of this agent under conditions of more aggressive agitation.[13]) The chamber was removed from the incubator and shaken briefly with a mixer (Vortex Genie 2; Fisher Scientific Co., Santa Clara, CA, USA) before samples measuring 100 μL were removed from each reservoir at 30-minute intervals for 4 hours and stored in the dark at −80°C.

Diffusion from the donor chamber to the receiver chamber was characterized by means of a permeability constant, P (cm/s). The apparent permeability coefficient, P, was calculated with the following equation:

$$P = \frac{dQ}{dt}\left(\frac{36}{60*A*C}\right),$$

where dQ/dt is the steady state rate of appearance of the sample molecule in the receiver chamber (μg/mL/min), 36 mL accounts for the volume of the fluid in the chamber, 1/60 is the conversion factor from minutes to seconds, A is the cross-sectional area of the specimen exposed to the solution (cm^2), and C is the initial drug donor concentration (μg/mL). Fluorescence was measured at room temperature with a fluorescence spectrophotometer (SpectraMax M5). Excitation and emission wavelengths were 492 and 520 nm, respectively, for both compounds. Standard curves of fluorescence versus concentration were obtained by serial dilution of the compounds in PBS. Concentrations of samples were determined by linear regression analysis within the linear portion of the standard curve. The values of steady state flux were estimated from the linear portion of the cumulative amount of drug permeated (μg/mL) versus time (minutes) profile in each case. All permeation studies were carried out in duplicate, and the results were expressed as mean ± SD. Treatment groups were compared to controls by using Student's t-test.

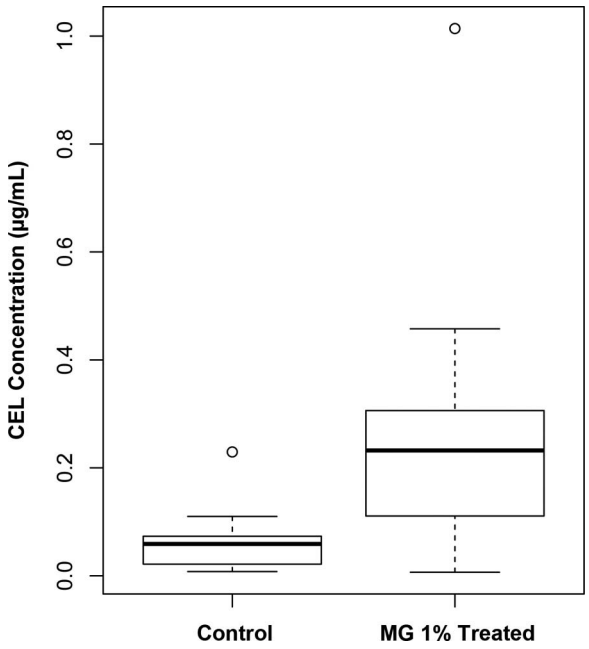

FIGURE 3. *Boxplot* showing statistically significant difference in CEL concentration in porcine vitreous following treatment with control versus 1% MG solution. *Central bars* are group means; *whiskers* show standard deviation. *Box boundaries* are first and third quartiles. *Open circles* are outliers; ($n = 18$, each group).

RESULTS

Nonenzymatic Cross-link Content Resulting From MG Treatment

The efficacy of the glycation regimen was verified by measuring nonenzymatic cross-link density by fluorescence in vitreous samples exposed to control and MG (0.01%, 0.1%, and 1%) treatment conditions but not used for permeability studies ($n = 6$ for all groups). The results showed a statistically significant increase in fluorescence with increased MG concentrations of 0.1% ($P = 0.002$) and 1% ($P = 9.33 \times 10^{-6}$), whereas 0.01% MG ($P = 0.5$) was not statistically different from control (Fig. 2).

Additionally, levels of the specific AGE CEL were shown to be increased in a statistically significant manner when comparing 1% MG-treated vitreous and control vitreous ($P = 0.005$; Student's t-test, paired, $n = 18$ each group; Fig. 3).

As further confirmation that vitreous collagen cross-linking occurred after MG treatment, MG-treated vitreous was more resistant to enzymatic digestion by collagenase than control-treated vitreous ($P = 4.278 \times 10^{-6}$; Student's t-test, paired, n = 10 each group; Fig. 4).

Small-Molecule (Fluorescein) Diffusion Through Vitreous

Fifty-three porcine eyes ($n = 17$ for the control group and $n = 12$ each for the 0.01%, 0.1%, and 1% MG groups) were used to study the effect of exogenous glycation upon small-molecule diffusion through vitreous. The studies showed that the permeability coefficient for fluorescein diffusion through porcine vitreous diminished with increasing MG concentration

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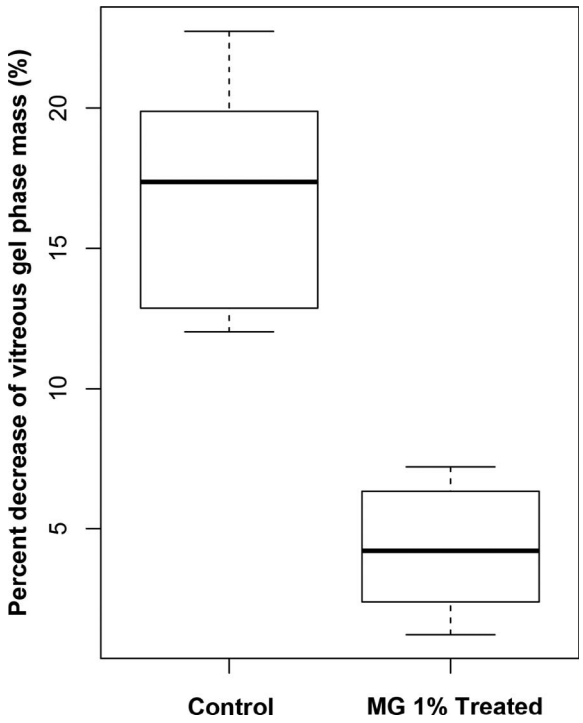

FIGURE 4. *Boxplot* showing statistically significant difference between control and 1% MG-treated porcine vitreous in percentage decrease of gel phase vitreous mass after collagenase exposure. *Bars* are group means; *whiskers* show standard deviation. *Box boundaries* are first and third quartiles. *Open circles* are outliers; ($n = 10$, each group).

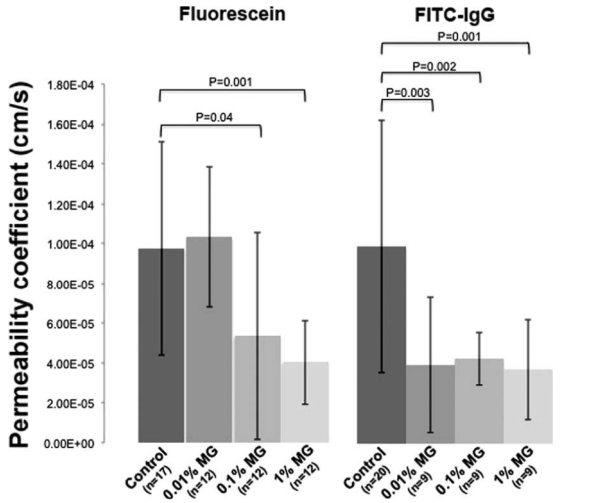

FIGURE 5. Vitreous permeability to fluorescein and FITC-IgG, representative small and large molecules, respectively. Methylglyoxal treatment impaired diffusion of FITC-IgG more than that of fluorescein (*error bars* denote standard deviation).

(Fig. 5). The difference in permeability coefficient between the control ($9.77 \pm 5.45 \times 10^{-5}$ cm s^{-1}) and treatment groups was statistically significant when glycation was performed with the higher MG concentrations of 0.1% or 1% ($5.36 \pm 5.24 \times 10^{-5}$ cm s^{-1}, $P = 0.04$, and $4.03 \pm 2.1 \times 10^{-5}$ cm s^{-1}, $P = 0.001$, respectively). At 0.01% MG, the permeability coefficient ($1.03 \pm 0.34 \times 10^{-5}$ cm s^{-1}) was not statistically significantly different from that of controls.

Large-Molecule (FITC-IgG) Diffusion Through Vitreous

Forty-seven porcine eyes ($n = 20$ for the control group and $n = 9$ each for the 0.01%, 0.1%, and 1% MG groups) were used to study the effect of exogenous glycation upon vitreous permeability to a larger molecule. As with fluorescein, the studies showed that the permeability coefficient for diffusion of FITC-IgG through porcine vitreous diminished with increasing MG concentration (Fig. 5). The difference between control ($9.9 \pm 6.37 \times 10^{-5}$ cm s^{-1}) and treatment groups was statistically significant at all MG concentrations (for 0.01% MG: $3.95 \pm 3.44 \times 10^{-5}$ cm s^{-1}, $P = 0.003$; for 0.1% MG: $4.27 \pm 1.32 \times 10^{-5}$ cm s^{-1}, $P = 0.004$; and for 0.1% MG: $3.72 \pm 2.49 \times 10^{-5}$ cm s^{-1}, $P = 0.001$).

DISCUSSION

Our objective in this study was to evaluate the effect of nonenzymatic cross-linking (glycation) upon the permeability of the vitreous to solute diffusion. The results confirmed our hypothesis that AGE accumulation impairs vitreous permeability. Reduced diffusion was seen for both a small and a large molecule. Of note, the permeability change was more pronounced for the larger solute, suggesting a lower threshold for AGE-induced permeability changes to impact the movement of protein signals through the vitreous when compared to smaller molecules such as nutrients.

The results of this study may be explained by considering the gelatinous vitreous as a porous medium as outlined in the theory of Bear and Bachmat,[14] in which permeability is determined by porosity, conductance through the medium (channel size), and tortuosity through the medium (pathway complexity). Nonenzymatic cross-links impose constraints on collagen fibrils, which would impact these factors and result in reduced solute movement through the medium. This conceptual framework is consistent with our finding that permeability was more reduced for a larger solute (i.e., less cross-linking was needed to achieve a statistically significant reduction in permeability); since both the small and the large molecule must navigate the same network through the medium, it will be more difficult for a larger solute to make its way through.

Our findings may have important implications for the pathogenesis of diseases such as diabetic retinopathy, in which vitreous AGEs are greatly increased over normal levels.[5] Levels of cytokines, such as vascular endothelial growth factor, interleukin-8, monocyte chemotactic protein-1, and others, are increased in the vitreous in diabetic retinopathy.[15,16] Reduced movement of these through the vitreous could drive a cycle of cytokine buildup in the vitreous cavity, as they are released into the vitreous but do not exit as easily. Higher overall levels of cytokines in the vitreous could accelerate or prolong secondary retinal consequences such as vascular leakage and retinal edema. Also, cells resident in the vitreous, such as hyalocytes,[17] could be affected by a reduction in vitreous permeability, perhaps through impaired access to trophic factors or by increased cellular cytokine activation from secondarily elevated cytokine levels. Additional studies would be needed to confirm any possible impacts upon disease state resulting from alterations in vitreous permeability.

As the human eye ages, the vitreous separates into a gel and a liquid phase, and posterior vitreous detachment forms.[18,19] Since the experiments performed in this study used gel vitreous from ex vivo porcine eyes, our model is representative of the permeability changes induced by cross-linking in the gel portion

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of human vitreous. Exposure of the liquid portion of human vitreous to AGEs may have little or no impact upon permeability properties in that phase, since the collagenous constituents are far less organized and thus less subject to the steric hindrance that results when cross-links form and likely determine the permeability changes seen in gel vitreous. This is consistent with prior findings that cross-linking affects solute diffusion more in cornea, where collagen is highly ordered, than in sclera, where it is arranged more randomly; this is because in a more highly ordered medium, solute diffusion is more acutely impacted by the effect upon conductance through the medium, as occurs when pore size is reduced,[20,21] rather than by the effect upon path tortuosity, the property that is predominantly affected in a more isotropic structure.[22] Also consistent with this rationale is the prior report that clearance of fluorescein and FITC-dextran occurs more rapidly from liquefied vitreous than from gel vitreous in an animal model.[12] We believe that the findings presented herein represent behavior in a substantial portion of the vitreous cavity in diabetic patients, particularly those with more advanced disease[23]; diabetics often have anomalous posterior vitreous detachment, with vitreoschisis posteriorly[24] and gel vitreous elsewhere. In the same fashion, it remains unclear at this time what the net effect upon permeability throughout the vitreous overall would be due to AGE accumulation in a nondiabetic aging eye, with a substantial liquid vitreous component—but assuming a reduction in permeability of the gel phase and a negligible effect, if any, upon the liquid phase, we speculate that an overall reduction in vitreous permeability occurs. It is also unclear whether the permeability reduction in the gel vitreous phase due to age-related AGE accumulation would be less significant than modeled in our experiments, owing to the more disorganized collagen fiber arrangement in aged vitreous[18,19]; one could reason that this might result in a less dense network through which solutes must navigate, even with the increase in matrix constraints imposed by the presence of cross-links.

A limitation of this study was that it used a model of AGE accumulation in normal porcine vitreous to mimic a disease state. It cannot account for other changes in the vitreous that might take place in diabetes, such as cytokine-induced modifications, which could independently alter permeability in an unknown fashion. Future studies are needed that compare normal and diabetic vitreous in order to achieve a more realistic assessment of the permeability changes occurring in a high-AGE condition such as diabetes. We also concede that it is difficult to mimic accurately the levels of AGE accumulation that occur in vivo when performing a one-time treatment in vitro. At the same time, the concentration of MG in plasma has been difficult to determine, with the literature demonstrating a range of reported values up to 0.4 mM.[25,26] This has been attributed to the high reactivity of this species, making it difficult to assay.[27] As such, although the concentrations of MG in the vitreous treatment solutions exceeded the reported plasma levels, the conditions in this experiment likely model the in vivo situation reasonably well, since exposure to MG and other glycating agents in vivo takes place on a chronic basis, with continuous accumulation of irreversible cross-links.

In conclusion, this study demonstrated that AGE accumulation reduces vitreous permeability when glycation is performed in ex vivo porcine vitreous. The findings suggest that previously unrecognized changes may occur in diabetic vitreous, and to a lesser degree in aged nondiabetic vitreous, that impede the diffusion of molecules. If validated in subsequent studies, these findings could have implications for the pathogenesis of disease states such as diabetic retinopathy, by increasing the retention of cytokines in the vitreous, or even for the behavior of drugs in the vitreous and their distribution throughout the eye. Additional studies are underway to further elucidate these changes and their consequences for ocular disease and its treatment.

Acknowledgments

Supported by That Man May See, Inc. (San Francisco, CA, USA), Research to Prevent Blindness (New York, NY, USA), UCSF Diabetes Research Center (National Institutes of Health [NIH] Grant P30 DK063720), and NIH-National Eye Institute Grant EY002162 - Core Grant for Vision Research.

Disclosure: **O.-T. Lee**, None; **S.D. Good**, None; **R. Lamy**, None; **M. Kudisch**, None; **J.M. Stewart**, None

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