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Time dependent degradation of vitreous gel under enzymatic reaction: Polymeric network role in fluid properties

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Abstract

The viscoelastic behavior of vitreous gel is due to the presence of biopolymers in its structure. Fluid properties of the vitreous is mainly the result of interactions between the characteristics of collagen type II and Hyaluronic Acid networks. Having a better understanding of the structure of each component and their changes during aging and various diseases such as diabetes can lead to better monitoring and treatment options. We study the effects of collagenase type II on 44 samples of porcine vitreous using an in situ rheological experiment in comparison with 18 eyes in a control group injected with Phosphate Buffered Saline Solution. We analyze the behavior of each component over time in both groups. We focus on the changes of viscosity and elasticity of the collagen network within the vitreous. The results of the

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analysis in this study show that the changes in the fluid properties of the vitreous after collagenase injection is driven by the structural alterations of the collagen network. Creep compliance values of the collagen network are significantly higher in the first group compared to the control group one hour and twenty-four hours after the injection. In contrast, creep compliance of the HA network shows no statistically significant change one hour after the injection in both groups. The results of the reported analysis of individual components in this study support the previous findings on the alterations within the vitreous structure in its entirety.
Keywords: Biopolymers, Enzymatic reaction, Rheology, Viscoelasticity, Vitreous humor

1. Introduction

Vitreous humor is a clear gel that occupies approximately two thirds of the volume of the eye globe. Studying the structure of the vitreous gel (Sebag, 1989c), its roles in the functions of the eye (Sebag, 1989a) and in the pathology of vitreoretinal diseases (Sebag, 1989b), were brought to attention in the late 1900s. Prior to which it was thought vitreous does not play an important role within the eye globe (Foulds, 1987). It is now clear that the vitreous has many roles in protecting the structure and providing the means for the functions of the eye (Foulds, 2014). In addition, vitreous humor supports the lens (Jin et al., 2019) and regulates the intraocular oxygen (Holekamp et al., 2014). The vitreous gel is attached to the retina which results in a direct connection between the changes in its network and the pathology of many vitreoretinal diseases (Sebag, 2008; Sharif-Kashani et al.,

2011).

The network of the vitreous gel has two main biopolymers, collagen and hyaluronic acid (HA) (Sebag, 1989c; Yadav et al., 2015). Collagen has high tensile strength and HA provides a network to support the collagen fibrils within the vitreous (Friess, 1998; Lee et al., 2001; Yadav et al., 2015). It is hypothesized that alterations in the structure of the vitreous gel are due to the characteristic variations of its components. Therefore, a more detailed study of each one of the two networks is helpful to understand the behavior of the vitreous gel in its entirety. A better understanding of the vitreous structure and its properties can lead to improvement of the current intraocular drug delivery methods (Huang et al., 2018; Jin et al., 2019) and surgical treatment options, creation of a better engineered substitute (Morozova et al., 2016), and development of new methods of treatments. Several studies use the rheological properties of the vitreous humor to develop a better substitute with similar viscoelastic behavior(Swindle et al., 2008; Thakur et al., 2020).

Degeneration of the vitreous network leads to many retinal conditions. This degradation process can be due to many reasons such as aging and diseases (Skeie et al., 2015). The structure of the vitreous gel changes over time which causes a separation of the liquid part in the vitreous (i.e. water and HA) from the collagen fibers in the form of bundles (Sebag, 2008). This process is one form of the liquefaction of the vitreous. Liquefaction of the vitreous gel can alter vitreoretinal interface and lead to posterior vitreous detachment (PVD), vitreomacular traction (VMT) syndromes (Patronas et al., 2009), and eventually cause more serious diseases such as retinal tear (RT) or retinal detachment (RD) (Los et al., 2003). Diseases such as diabetes can

also change the vitreous structure in different ways and lead to retinopathy (Fong et al., 2004). These conditions can be treated by Pars Plana Vitrectomy (PPV), a surgical procedure to remove the vitreous humor and replace it with a fluid as a substitute (Los et al., 2003). Recently an enzymatic intravitreal injection, Jetrea (Ocriplasmin, Thrombogenics, Inc), has been found to induce pharmacologic vitreolysis by means of proteolysis of the vitreoretinal connections (Haller et al., 2015). In addition, studies introduced intravitreal injection of certain gases to exert mechanical forces and remove the adhesion of the vitreous gel to the retina (McDonald et al., 1994; Rodrigues et al., 2013). Further investigations are essential to fully characterize and improve the effects of the mentioned treatments on the vitreous gel.

Understanding the fluid properties of a polymeric network can shed light on the characteristics of its structure. Rheology is an indirect method to measure the properties of fluid. Prior investigations show that the vitreous humor is a viscoelastic gel (Nickerson et al., 2008; Sharif-Kashani et al., 2011; Silva et al., 2017). Similar to the molecular structure of the vitreous, its viscoelastic properties are mainly due to the presence of two main components; collagen and HA network. The viscoelastic properties of each component within the vitreous gel were studied previously using shear rheological methods (Sharif-Kashani et al., 2011; Tram and Swindle-Reilly, 2018). In addition, the enzymatic degradation of the vitreous gel was studied using the same method where the vitreous gel was dissected (Filas et al., 2014; Huang et al., 2018). It should be noted that microrheology can provide access without the need for dissection (Lee et al., 1992, 1994; Watts et al., 2013). However, because only the localized characteristics are reported, accuracy

of the technique needs improvement (Waigh, 2016). Furthermore, it was shown that using *in situ* rheological experiments, we can measure the effects of enzymes on the fluid properties of the vitreous gel over time without any major alterations to the structure of the eye globe (Rangchian et al., 2019). The described technique was previously evaluated using known Newtonian and Non-Newtonian fluids (Connelly et al., 2016). Using this method, it is possible to further investigate the changes in the structure of the vitreous humor and relate it to each one of the separate components. We analyzed the data from the eyes in two groups of injections over time to quantify the alterations in the properties of each network individually (i.e. collagen and HA). This is the first *in situ* study to analyze the characteristics of the two main components of the vitreous gel over time. We investigated the changes in the creep compliance and retardation time of each component as well as the steady state viscosity of the vitreous gel. We would like to emphasize the reported values in this study are an indication of the fluid properties of the individual components of the vitreous gel and the main purpose is to show the variations over time and due to the enzymatic degradation as opposed to the exact values of the properties.

2. Materials and methods

Fresh porcine eyes were harvested and shipped on dry ice, and sent within the same day by Sierra Medical Supplies (Whittier, CA, USA). In total we tested 44 eyes in the first group and 18 eyes in the control group. We followed the same protocol used in the previous publication (Rangchian et al., 2019) and here we provide a summary. A small triangular incision was made on

the pars plana of the eye to access the vitreous gel. This incision provided an access to the vitreous for testing and injections. A stress-controlled rheometer (TA instruments, AR 2000) was used with a 0.87mm diameter cylindrical probe. The rheological procedure used in this study is creep flow, one of the commonly used methods to understand flow characteristics of fluids. In this test a constant torque/shear (τ) is applied and the caused deformation (γ) is recorded. Creep compliance (J), which is an indicator of the elasticity of the fluid, is derived from the deformation using $J(t)=\gamma(t)/\tau$. After the incision was made, we secured the eye on a 3D printed cube (ABS polymer) with a half sphere cut out and a rough surface to avoid extra movements during the experiment. The probe was fully inserted into the eye to run the test close to the center of the vitreous cavity. We applied a torque of 1 μNm with zero normal force over 6 minutes on each eye. This first test was performed before any injections and we refer to it as the pre-injection result (T_0). Immediately after the pre-injection test, the eye was injected with 50μL of collagenase type II (group 1) or the same volume of PBS in group 2. Collagenase was chosen to look at the differences on the collagen network more than any other biopolymeric networks within the vitreous. We recorded the time of the injection and repeated the test one hour (T_1) after the injection to capture the changes during a short period of time and to monitor the initial conditions. The scleral opening was protected during the first hour by inserting a small capped plastic vial on top of the opening to reduce the alteration of the vitreous. Subsequent to this test, eyes were individually stored at 4 °C in a container filled with PBS to minimize evaporation of the water content of the vitreous gel. Eyes were brought back to the room temperature (25°C)

15 minutes before the last experiment to prevent alterations of the results due to temperature changes. The last test was 24 hours after the injection (T_{24}) to observe changes over a longer period of time. We were limited to repeat the test beyond 24 hours as vitreous would naturally degrade. The schematic steps of the experiment are shown in Figure 1.

The creep curves in this study are highly nonlinear with many local minima, hence we used Python non-linear regression model to improve the accuracy of the fittings. Non-linear regression model is an optimization technique to solve highly nonlinear problems using the least square minimization approach. We used the viscoelastic discrete spectra model with two Voigt-Kelvin elements in series (Ferry, 1980) to analyze the fluid characteristics of the elements present in the vitreous humor network (Sharif-Kashani et al., 2011).

To perform statistical analyses, R-studio was used which is an integrated development environment used for statistical computing. Mixed ANOVA analyses are used in all of the statistical calculations. In these analyses the injection types are the between subjects (collagenase type II or PBS) and the three time points that the experiments are repeated are the within subjects factor (Statistics, 2015). The significant differences reported by the ANOVA analyses are further investigated using paired or unpaired t-tests (depending on the repeated measure or between subjects analyses) adjusted by Bonferroni correction. These results are also compared to the Welch's t-test. The range of p values are reported as the p values are not exactly the same. However, both tests were in agreement on the existence of the significant differences.

3. Results

3.1. Fitting parameters

The viscoelastic discrete spectra model (Ferry, 1980; Sharif-Kashani et al., 2011) is used (Equation 1) to analyze the behavior of the vitreous gel as a result of the interaction of its two components.

$$J(t) = \sum_{k} J_k(1 - r^{(-t/t_k)}) + t/\eta_m \tag{1}$$

There are 5 parameters in this equation. Viscosity of the vitreous gel (η_m) in the steady state region (i.e. the linear segment of the creep curve). Each one of the components within the network of the vitreous gel (i.e. collagen and HA) has two parameters related to their fluid properties, creep compliance (J_k) and retardation time (t_k). The changes in creep compliance (J_k) and retardation time (t_k) values of each component lead to changes in the fluid properties of the vitreous gel.

3.2. Creep curve fitting

The result of each experiment for one eye is a creep curve. As explained earlier, the experiment is repeated at three time points (T_0, T_1, T_{24}), producing three curves for each eye. Samples of all three curves for one eye from each group are shown in Figure 2 a & b. Viscosity of the gel at the steady state was calculated for each one of the eyes using the slope of the linear segment of the creep curve between t=240 s and t=300 s. Using the calculated viscosity, we modeled the entire creep curve to find the rest of the parameters for each experiment. The fit of the pre-injection curve provided the values for J_1, t_1, J_2, and t_2. The calculated value of t_2 (i.e. retardation time of HA

network) from the first fit (T_0) was kept constant for T_1 and T_{24} modeling. The hypothesis is that the effect of time is the same in both groups on the retardation time of the HA network, hence the value of t_2 should not change significantly between the two groups.

3.3. Collagen parameters

The statistical results are provided in this section comparing collagen parameters between and within the two groups over time. At each time point the average value for elasticity of the collagen network is compared between the groups and p values of unpaired t-tests are provided (Figure 3). In addition, statistical comparison of each group over time (paired t-test) is shown in Table 1. The same analysis was done on the retardation time of the collagen network (Figure 4 and Table 2).

3.4. HA parameters

The same properties, creep compliance J_2 and retardation time t_2, can be calculated for the HA network. Average values of the creep compliance of the HA network is shown in Figure 5 as well as the p values from unpaired t-test analyses between the two groups at each time point. P values from the paired test for each group over time is reported in Table 3. The effects of collagenase and PBS injections on the retardation time of the HA network are minimal (Filas et al., 2014). Due to the high non-linearity of the data and fittings, we hypothesized that the changes of t_2 values over time are not significant. This hypothesis was validated by calculating the t_2 values at T_1 and T_{24} using the previously fitted parameters.

4. Discussion

Vitreous gel has a complicated fluid structure which is due to its polymeric network and molecular structure (Meyer and Palmer, 1934; Sharif-Kashani et al., 2011). HA network and collagen fibrils are the main components of this structure with distinguishable fluid characteristics. Aging can alter the structure of the vitreous gel where it becomes more liquid-like. This happens mainly as a result of the cross linking of collagen fibrils (Swann, 1987) that may lead to a pulling force on the retina at the vitreoretinal interface. This pulling can cause a full PVD but in some cases the vitreous gel remains partially attached to the retina and causes point forces at certain locations which could result in RT or RD. If a patient becomes symptomatic, PPV surgery can help removing the force (Rodrigues et al., 2013; Hikichi et al., 1995; McDonald et al., 1994; Writing et al., 2010). As PPV is an invasive course of treatment, there are many studies that focus on alternative options such as pharmacologic vitrectomy, and pharmacologic or gas induced vitreolysis (Rodrigues et al., 2013; Soman and Banerjee, 2003; Shah and Trese, 2016).

There have been many rheological studies to characterize the effects of the aging on the structure of the vitreous. Comparison of the creep results on ovine eyes from three different ages, showed a decrease in both loss and storage modulus with age which is due to the breakdown of the collagen network (Colter et al., 2015). A rheological study on the dissected human vitreous reported a decrease in the viscoelasticity of the vitreous humor which is related to the liquefaction of the eye (Schulz et al., 2019). Another study on the human vitreous at different ages showed higher stiffness and viscosity

for the solid phase of the older vitreous and lower viscosity for the liquid phase of the older vitreous gel (Tram and Swindle-Reilly, 2018); however, the discrete retardation analysis did not show significant correlation, which could be due to the dissection of the vitreous as well as the low number of samples tested. These findings are in contradiction with the studies that measure the vitreous properties in its entirety. Our findings in group 1 are in agreement with the aging analysis of the vitreous as one gel; we assume that the similarity is due to the injection of the collagenase which breaks the bonds in the collagen network.

The molecular structure of the vitreous gel and the interaction of its networks of biopolymers are very important in understanding of the pathology of many vitreoretinal diseases. It can also provide information about the possible approaches to slow down the degenerative aging process or to treat the resulting conditions. To be able to invent a substitute for the vitreous gel with better quality, one should be fully aware of its properties and roles (Berkowitz et al., 1991; Soman and Banerjee, 2003; Swindle et al., 2008; Januschowski et al., 2019; Thakur et al., 2020). Previous studies could identify the composition of the vitreous network (Swann, 1987; Meyer and Palmer, 1934; Sebag and Balazs, 1989) but due to the fragile structure of the vitreous gel, reported direct measurements require dissection and are limited (Nickerson and Kornfield, 2005; Nickerson et al., 2008; Filas et al., 2014; Silva et al., 2017; Tram and Swindle-Reilly, 2018).

Each one of the networks of biopolymers in the vitreous has its own properties (Sharif-Kashani et al., 2011; Tram and Swindle-Reilly, 2018) and changes in one of the components can lead to alterations in the vitreous

characteristics. Microrheological experiments show that the probe can be trapped in one of the components and lead to localized information. For instance if the probe is located in the fibrils of collagen network the results would drastically differ compared to when the probe is in the liquid pockets within the vitreous (Watts et al., 2013). Therefore, better understanding of the properties and structure of the main components on the macro scale are essential to investigate the changes in the characteristics of vitreous. In addition, exploring the behaviour of mentioned components after enzymatic degradation can provide better constraints for the design of new treatments for vitreolysis. There have been no reported *in situ* rheological studies of the vitreous network over time as with the normal shear rheological setup of the experiments, the structure of the eye globe is disturbed. Hence, after the first run of the test, the eye must be discarded. There are reported less invasive microrheological methods both *in vivo* and *ex vivo* however, the spatial size limitation of the experiment and localized characteristics constraint still exist (Pokki et al., 2015).

It is important to quantify the time variations on vitreous humor. The ability to repeat the experiment allows us to characterize the degradation due to time and/or different enzymes on the rheological properties of the vitreous. There are reported studies on rheological measurement of the dissected vitreous properties over time. Silva and co-workers reported the rheological properties of the dissected gel and liquid parts of the vitreous individually over time (Silva et al., 2017). In our *in situ* experimental setup, we can repeat the test over time (Rangchian et al., 2019). We measured the viscosity and creep compliance of the vitreous and their changes due to the injection

of collagenase over time. In the present study we modeled the individual components of the vitreous and their changes as a result of collagenase injection in comparison with a control group. The results reported here are in agreement with the previous studies that used the viscoelastic discrete spectra model to analyze porcine and human vitreous to show two distinguished components (Sharif-Kashani et al., 2011; Tram and Swindle-Reilly, 2018).

The slope of the creep curve in the steady state for group 1 is the smallest for the pre-injection curve with an average value of 6×10^{-5} [1/Pa.s]. One and 24 hours after the injection the slope increases, average values are 9×10^{-5} and 2×10^{-4} [1/Pa.s] respectively. Group 2 has approximately the same slope for all three time intervals, the average varying from 4×10^{-5} to 5×10^{-5} [1/Pa.s].

The average values of elasticity of collagen network (J_1) does not differ significantly between the two groups before the injection with an average of 0.16 for group 1 and 0.14 Pa^{-1} of group 2. However, results from the experiments at T_1 and T_{24} in group 1 are significantly higher than the one in group 2, p values are reported in Figure 2. The average values for J_1 in group 1 are 0.21 Pa^{-1} at T_1 and 0.58 Pa^{-1} at T_{24} compared to 0.11 Pa^{-1} and 0.14 Pa^{-1} for group 2. Moreover, J_1 increases noticeably over time in group 1, whereas in group 2 the increase is not significant (Table 1). Creep compliance is directly related to the inverse of elasticity. Therefore, the results suggest that elasticity of the collagen network has significantly decreased in group 1. This could be explained by having less bonds present in the network of collagen due to the enzymatic effect of the collagenase. The presence of less bonds in the network of a biopolymer makes it easier to elastically deform

the structure. This result supports the hypothesized changes on the collagen network and confirms that the change in the elasticity of the vitreous is due to the alterations of the collagen component of the gel.

It is noteworthy to mention that there are no statistically significant differences between the average values of the retardation time of the collagen network, t_1, between the two groups at T_0, with values of 7.48 s and 6.62 s for group 1 and group 2 respectively, and T_1 with an average value of 6.69 s for group 1 and 5.88 s for group 2. Whereas $t_1 = 9.61$ s is significantly higher in group 1 compared to $t_1 = (6.19$ $s)$ for group 2 at T_{24} (Figure 4). The paired t-test results of the comparison of t_1 values for each group are reported in Table 2. It is worth mentioning that in group 1 the t_1 value increases over time; however, the increase from T_0 to T_1 is not statistically significant. The average value of t_1 at T_{24} is noticeably higher than the other two time points in group 1. The increase in the retardation time suggests that there is a change from solid-like behaviour to more liquid-like behaviour in the collagen network. This change can be due to the presence of less bonds in the collagen networks. This data validates the previous findings on the changes of the vitreous network as one gel (Rangchian et al., 2019).

There are no statistically significant differences observed for t_1 values in group 2, but the value increases over time. This could have been the result of unavoidable evaporation of the water content of the gel. These results show that we can map the effects of various active and placebo injections on the specific parameters of the vitreous gel.

The average value of elasticity of HA network (J_2) does not differ significantly between the two groups at T_0 with 0.024 Pa^{-1} for group 1 compared

to 0.018 Pa^{-1} for group 2 and T_1, average values of 0.030 Pa^{-1} and 0.012 Pa^{-1} for group 1 and group 2 respectively. However, results from the experiment at T_{24} in group 1, 0.37 Pa^{-1}, is significantly higher than the one in group 2, 0.014 Pa^{-1}, p values of the unpaired t-tests are reported in Figure 5. Moreover, J_2 at T_{24} is higher compared to the other two time points in group 1, whereas in group 2 the increase is not significant (Table 3). Therefore, the results suggest that elasticity of the HA network has decreased 24 hours after the injection of collagenase. This could be due to unavoidable alteration on the HA network after the degradation of the enzyme in addition to the effect of time on the bonds in its network.

As it was mentioned earlier, PPV is an invasive surgery with longer time for the rehabilitation of the vision (Johnson et al., 2015). Enzymatic vitreolysis can induce PVD to remove the traction force of the vitreous on the retina. However, discovering the right enzymatic injection can be difficult. Jetrea (Ocriplasmin, Thrombogenics) is the first intravitreal drug injection that was approved by FDA in 2012. Ocriplasmin is a recombinant protease that has activity against fibronectin and laminin in the vitreoretinal interface (Kuppermann, 2012; Khan and Haller, 2016). While this method has many advantages compared to PPV, it is limited to some patients with specific conditions. In addition, there are many side effects associated with the Jetrea injection such as lens instability and macular hole enlargement (Johnson et al., 2015), temporary disturbances or loss of vision (Fahim et al., 2014), and acute visual loss due to the ellipsoid zone changes (Reiss et al., 2015; Johnson et al., 2015).

Effects of Jetrea should be more extensively studied as a treatment op-

tion, as its roles as a protease may not be limited to the vitreoretinal interface and modify the properties of the vitreous gel (Beebe, 2015) and cause the aforementioned side effects. This study was only on collagenase injection which is primarily active against collagen, but the described method can be used to further investigate the potential effects of an enzyme such as Jetrea on separate components of the vitreous. This can potentially help patients with diabetic retinopathy or abnormal vitreoretinal adherence to have an induced PVD which removes the need for surgical intervention.

We investigated the changes on the components of the vitreous structure due to an active enzyme injection on the specific components of the network over time. Effects of time can be more precisely modeled by adding more time intervals to the experimental procedure (i.e. add time points between 1 hour and 24 hours) and to predict the changes at other time points without the experimental procedure. In future, our method can help finding and evaluating possible enzymatic treatments and to predict the effects of other potential injections over time and their possible roles in the treatment of some vitreoretinal conditions.

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Conflict of interest

The authors have no affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript.

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Tables

Table 1

Mean $[Pa^{-1}]$	**0**	**1 hour**	**24 hour**
Group 1	0.16±0.01	0.21±0.02	0.58±0.05
Group 2	0.14±0.02	0.11±0.01	0.14±0.02
P values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	<0.05	<0.05	<0.05
Group 2	>0.05	>0.05	>0.05

Table 2

Mean $[s]$	**0**	**1 hour**	**24 hour**
Group 1	7.48±0.2	6.69±0.5	9.61±0.6
Group 2	6.62±0.5	5.89±0.4	6.19±0.4
P values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	>0.05	<0.05	<0.05
Group 2	>0.05	>0.05	>0.05

Table 3

Mean $[Pa^{-1}]$	**0**	**1 hour**	**24 hour**
Group 1	0.024±0.001	0.030±0.01	0.366±0.1
Group 2	0.018±0.003	0.012±0.001	0.014±0.002
P values	**0 vs 1 hour**	**0 vs 24 hour**	**1 vs 24 hour**
Group 1	>0.05	<0.05	<0.05
Group 2	>0.05	>0.05	>0.05