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Effects of Collagenase type II on Vitreous Humor, an *in situ* Rheological Study

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Abstract

The purpose of this study is to quantify the impact of enzyme activity on the vitreous humor structure over time to understand the mechanical characteristics of the vitreous humor gel. Changes in the mechanical behavior of the vitreous occur due to many reasons including aging, which may lead to many vitreoretinal diseases. The degeneration process of the vitreous has been studied; however, *in situ* experimental procedures to validate the existing hypotheses are limited. We examined thirty-eight porcine eyes using *in situ* rheological creep tests to measure the mechanical properties of the vitreous humor of the eyes prior to, 1 and 24 hours after the intravitreal injection. Eyes in one group were injected with collagenase type II solution and eyes in the control group were injected with Phosphate Buffered Saline solution with calcium and magnesium chloride. Prior to the injection, viscosity and creep compliance intercept values between both groups were not statistically different. At 1 hour and 24 hours after the injection, vitreous properties in the eyes from the first group showed a statistically significant increase in the J intercept values (representing the inverse of elasticity) compared to the control group. In addition, 1 and 24 hours after the injection, vitreous viscosity was lower in the eyes from the first group than in the eyes from the control group. These findings are a foundation for future studies on the effectiveness of intravitreal drugs that modify the mechanical properties of the vitreous humor.

1. Introduction

Vitreous is the gel-like fluid inside the ocular globe of the eye. Vitreous is important in various physiological processes such as embryological development, optical transmission and mechanical support [1–3]. Viscoelastic characteristics of the vitreous gel has a significant role in many of its functions in the eye [4]. For example, the gel provides an excellent protection for the ocular tissues [2, 5-7], and maintains the lens transparency by providing a gradient of Oxygen concentration (i.e. lower concentration towards the center of the vitreous) [8–12]. Many vitreoretinal diseases are related to the changes in the viscoelastic properties of the vitreous. Therefore, changes in the network structure of the vitreous (e.g. age related degeneration) or removal of it (i.e. vitrectomy) [13–15] can cause oxidative and structural damages to the center of the lens resulting in opacification [10].

In the study of the movement and structure of the vitreous, Hildling emphasized the behavior of the vitreous during both the acceleration and the deceleration phases of saccadic movements; vitreous movement lags behind the eye wall, resulting in markedly reduced acceleration [16]. It is suggested that the adhesion of the vitreous internal structure to the sclera [16] and its viscoelastic properties [5, 17] cause the lag in the movement. Movement of the eye can also provide information about the significant compartment variations in the vitreous network [18]. Pathogenesis of many vitreoretinal diseases are related to the changes in the fluid properties of the vitreous. Therefore, many authors study the vitreous dynamic by focusing on the movement of the vitreous *in-vivo* through various imaging techniques [18–20], where the velocity field of the vitreous was used to model the viscosity and elasticity of the vitreous gel.

The macromolecular structure of the vitreous is nearly 99.9% water and salts (by weight percent, wt%), and is less than 0.1 wt% heterotypic collagen fibrils [5], [21]. The network of heterotypic collagen fibers (II, V/XI and IX) and proteoglycans are embedded within a network of

hyaluronan [21, 22]. A number of recent studies have suggested that the viscoelastic features of the vitreous are associated with its macromolecular structure [5]. Similar to many viscoelastic biomaterial and gels [23], vitreous humor dissipates and stores energy under applied stress. The exact relationship between vitreous macromolecular structure and its mechanical properties remains unknown [7].

Given that the viscoelastic properties of the vitreous are mostly unknown, a more direct measurement method is required to quantify these properties. It has been shown previously that rheological experiments can reliably measure the mechanical characteristics of fluid, in particular the vitreous [2, 3, 5, 24]. Rheology is the study of flow behaviors, and rheological experiments provide specific information on the characteristics of fluid [25]. Previous rheological studies have used creep flow [5–7, 21–24] and oscillatory flow [1, 7] to quantify vitreous humor properties. Studies recommend that many vitreoretinal diseases such as Posterior Vitreous Detachment (PVD) occur as a result of changes in the rheology of the vitreous gel [5, 7, 17]. However, the role of the vitreous in a variety of vitreoretinal diseases is still not well understood [26]. Better understanding of the rheological properties and behavior of the vitreous gel may lead to improved or new therapeutic approaches.

The novel technique developed by our group enabled us to measure rheological properties of porcine vitreous *in situ* [24]. In this method, no dissection is required and the vitreous network is preserved. A cylindrical probe enters the eye globe through a small incision. Previously, the porcine and bovine eyes were tested using this method [24].

The objective of this study is to determine the effect of the collagenase type II on the structure of the vitreous humor. The change of structure is monitored through *in situ* measurement of the viscoelastic properties of the vitreous fluid at different times. The measured properties are then compared to that of a control experiment where the eyes are injected with PBS.

2. Material and Methods

2.1. Preparation

Thirty-eight porcine eyes were assigned to either the study group (*n*=27, group 1) or the control group (*n*=11, group 2). During the entire procedure, the eyes were kept hydrated in a container and were surrounded by diluted Phosphate Buffered Saline solution (PBS) with calcium and magnesium chloride (D1283 Sigma-Aldrich), to prevent evaporation of the water content of the vitreous during the experiments. The specimens were transported on ice to the lab less than 6 hours after enucleation and were prepared for the experiment immediately at room temperature. Experiments were done using a stress-controlled shear rheometer (TA instruments, AR-2000, New Castle, DE, USA) using our patented probe [24]. Using surgical tools, a triangle shaped incision with a base of 3 mm and height of 2mm, was made through the sclera on the pars plana to allow the rheometer's probe (0.86 mm in radius) to enter into the vitreous cavity.

2.2. Rheological experiment

Each eye was placed on a 3-D printed cube (Fig. 1.b) to hold the eye in a secure position (radius of the cut out hole is 13mm) to avoid any movement during the experiment. The probe was directed towards the center of the vitreous to avoid undesired contact with the surrounding tissues that can drastically affect the rheological data acquisition. The probe was fully inserted in the vitreous so that the marked point of 10 mm reached the sclera. This helped us to make sure the full diamond-covered part of the probe was in contact with the center of vitreous network. The probe location inside the vitreous is shown in the schematic (Fig. 2) and in the real experiment setting (Fig. 1.c). Lyophilized powder of Collagenase type II (C1764 SIGMA, Type II-S, 0.5-5.0 FALGPA, Sigma-Aldrich) was diluted to a concentration of 0.7 mg/mL in PBS with Calcium and Magnesium Chloride. The eyes were injected (through the incision on the vitreous cavity) with 50 µL of collagenase in the first group and 50 µL PBS (X1) in the second group. The

eyes were tested by a 6 minute long rotational creep flow test with a constant torque of *M*=0.1 μNm before the injection (time 0), 1 hour and 24 hours after the injection. The probe was inserted close to the injection site to carry out the measurements. Collagenase and PBS solutions, both water-based solutions, are injected slowly in small volumes using a micropipette. This ensures that the injected fluid mixes with the vitreous main solvent which is water (more than 99%). There are no large size molecules in the injected fluid; therefore, the rheology of the vitreous is not affected. Eyes were stored at 4°C after the 1 hour tests and removed about 2 hours before the 24 hour creep test to equilibrate to the room temperature of 20°C.

2.3. Creep

In a creep test, a constant torque is applied (Fig. 3.a) and the angular displacement of the flow is recorded [27]. In a theoretical creep curve, viscosity is derived from the slope of the fitted line on the curve at the steady state region (i.e. liquid state), shown in Fig. 3.b. Equation (1):

$$\mu = \frac{\tau}{\dot{\gamma}} \tag{1}$$

where $\dot{\gamma}$ shows the rate of strain caused by the shear stress, τ, applied on the fluid.

Creep compliance is an indicator of the instantaneous elasticity of the fluid and is defined as:

$$J = \frac{\gamma}{\tau} \tag{2}$$

where γ is the strain and τ is the shear stress applied on the sample. The intercept of the fitted line on the creep curve with the y-axis is referred to as the creep compliance intercept, $J_s(0)$ [1/Pa], that is used as an elasticity parameter and in this study is one of the important features to describe the viscoelastic behavior of vitreous. More details can be found in the previous rheological studies using the creep test [5, 24].

2.4. Data analysis

Matlab is used to find the fitted line in the steady state region of the fluid from which viscosity and creep compliance values are extracted and reported in the result section. An unpaired t-test is performed on the viscosity and creep compliance intercept values when comparing the two groups. By contrast, a paired t-test is carried out when comparing the results of different time points from one group. *P*-values are reported and $P<0.05$ indicates the significant statistical differences between any of the two groups in the comparison. R-studio software is used to calculate the *P*-values and plot the results.

3. Results

Creep curves are the reported output of each creep test in this study, similar to the previous *in situ* study of the vitreous [24]. Comparison between a typical creep curve for one eye from each group is shown before and 24 hours after the intravitreal injection (Fig. 4). The linear segment of the creep curve indicates the viscoelastic region of the flow. The change in the slope of the linear segment of the creep curve for group 1 from $t=0$ to $t=24$ is significant. Group 2 creep curves do not change over the same period. The mean and variance values are reported in Table 1 for each group at each time point.

3.1. Creep compliance intercept

The creep compliance intercept values (J_s) of both groups are compared at every time point (group 1 vs group 2) as well as within each group (Fig. 5). There is no statistically significant difference between the values of creep compliance from group 1 versus group 2 before the injection (with mean of 0.196 vs 0.16 [Pa^{-1}] respectively). Data comparison within each group from $t=0$ to $t=1$ indicates that the change in the J_s values of group 1 is statistically significant (Table 2.b), average increased from 0.196 to 0.326 [Pa^{-1}], compared to group 2 (the average

decreased from 0.16 to 0.15 [Pa^{-1}]) over the same time period (Table 2.c). One hour after the injections, creep compliance values are significantly different between group 1 and 2 ($P< 2.4\times10^{-6}$) as reported in Table 2.a. The same trend is observed 24 hours after the injection and the result of the unpaired t-test is $P< 1.0\times10^{-7}$ with the average creep compliance intercept values of 0.715 [Pa^{-1}] and 0.207 [Pa^{-1}] for group 1 and group 2 respectively.

3.2. Viscosity

At t=0 viscosity values are not significantly different between the two groups (with mean values of 14.5×10^{3} vs 16.8×10^{3} [Pa.s]). However, the statistical results reported in Table 2.a shows that the viscosity values of the two groups are significantly different from one another 1 hour and 24 hours after the injection. Table 2.b indicates the statistically significant decreases in group 1 between each two time points (e.g. t=0 vs t=24) individually. The same comparison on group 2 data showed no statistically significant changes for the data over time, except for t=0 vs t=1 which had a P value just under 0.05 (Table 2.c). Figure 6 indicates the changes in the viscosity values for each group at every time point, where the overall trend within each group is shown as well as in comparison with the other group.

3.3 P-values

Exact P values for the comparisons between the groups are reported in Table 2. For each eye the steady state values of the viscosity and creep compliance intercept are used. Therefore, each eye has three viscosity and three creep compliance intercept values. The unpaired t-test compares the values from all the eyes from group 1 versus all the eyes in group 2 at each time point, Table 2.a. The paired t-test is used to compare the changes of the values over time within each group. In addition, values from different time points within each group are compared over time and reported here, (Table 2.b and c).

4. Discussion

Previous studies have investigated the vitreous gel in many aspects including but not limited to appearance [6], molecular structure [21], and biochemistry [28, 29]. In addition, recent studies have focused on the potential effects of changes in the vitreous characteristics on the vitreoretinal diseases [11, 13, 15, 30, 31]. However, the limited number of studies on the viscoelastic behavior [5, 24, 32–36] of the vitreous minimizes our ability to understand its relationship to the pathology of many vitreoretinal diseases.

As previously mentioned, rheology is a suitable method to quantify the fluid properties of the vitreous gel. To be more precise, rheological studies can measure bulk or molecular level of flow behaviors. Generally, rheology refers to measurements of the bulk fluid behavior instead of the molecular level which is known as micro-rheology. Previous rheological experiments studied the mechanical features [5] of the vitreous and the effects of enzymes on vitreous properties by parallel plate setup [7] on a low number of samples. In a parallel setup, the vitreous is extracted from the vitreous cavity and the vitreoretinal connection is broken. After this connection is broken, the vitreous starts to dehydrate, and major alteration to the structure happens quickly that might alter the captured rheological data. In addition, the volume of each sample varies due to the dissection which may impact the accuracy and reliability of the results [7, 24]. Micro-rheology is a method that provides data on the microscopic properties of material by tracing the thermal motion of the tracer particles in the substance [23]. Micro-rheology measures the local properties and has many advantages [23, 37] due to its setup but lacks the certainty provided by bulk rheology methods [38]. There is ongoing research in this field to improve this method and to increase its accuracy [23]. Micro-rheology is used to measure the mechanical properties of the dissected vitreous humor [39] but this method provides less precise information. Similar to the parallel plate setup, micro-rheology requires the vitreous to be extracted from the eye.

The results reported in this *in situ* study, provide a better understanding of the mechanical changes of the vitreous structure due to the injection of collagenase type II in comparison with PBS injection over time. Alteration to the network structure of the vitreous is minimized as there is no need for a dissection. It is noteworthy that, the vitreous structure is primarily comprised of collagen type II [1]. Evidence suggests that the presence of collagen type II bundles may be a sign of the vitreous liquefaction [40]. Therefore, it is crucial to quantify the mechanical effects of the collagen type II degradation in the vitreous, which can be achieved by collagenase type II injection.

The data evaluations show that the slope of the typical creep curve of group 1 is steeper and that the intercept of the line with the *J* axis is greater 24 hours after the injection compared to the curve prior to the injection (Fig. 4). This result suggests that the viscosity and elasticity of the vitreous in this group has decreased. The usual creep curve of group 2 is reported before the injection and 24 hours afterwards (Fig. 4). There are no significant changes in the slope of the steady state region of the creep curve or the *J* axis intercept. Hence, the viscosity and elasticity of the vitreous are not significantly changing after the PBS intravitreal injection. The reported changes in group 2 might be due to the degradation of the vitreous over time. In addition, PBS diffuses over time which might lead to a decrease in the viscosity. These changes are not significant compared to group 1.

It is hypothesized that vitreous mainly owes its elastic behavior to the collagen in its network [3]. The increase in the creep compliance intercept for group 1 indicates the decrease in the elasticity of the vitreous (Fig. 5). Collagenase breaks the peptide bonds that exist in the collagen content of the vitreous. The reduced number of bonds in the chemical structure provides a network that is easier to deform which leads to a decrease in the elasticity of the flow. The results in this study are aligned with the expectations of the biochemistry.

The viscosity values decreased significantly in group 1 compared to group 2 at each time point (Fig. 6). This result suggests that collagen fibrils have an important role in establishing the viscous behavior of the vitreous. In addition to the comparison between the groups, the experiments for each group are also evaluated individually over time. *P*-values of the statistical analysis indicates no significant variation in the viscosity and creep compliance intercept values of group 2 over time (Table 2). By contrast, in group 1 the changes are statistically significant after the injection in comparison with the pre-injection results. The difference in values between one hour and 24 hours after the injection is also statistically significant. The exact trend of variations is not the focus of this study.

In this study, it is assumed that the evaporation of the vitreous content was kept to a minimum using constant hydration therefore its effect did not significantly change the results. The results verify the hypothesis of the expected changes on the vitreous content due to the injection of collagenase type II compared to PBS.

Conclusions

Consequently, new information on this extracellular matrix and mechanical properties of the vitreous humor after different enzyme injections can lead to improved diagnostic and treatment tools. In addition, it could expand the new era of intravitreal drug delivery [41] and might potentially prevent the need for surgical treatments in some cases.

We studied the changes caused by collagenase type II on the vitreous network structure and its effects on the mechanical behavior of the vitreous network over time in comparison with a control group (PBS injected). The analysis showed a significant decrease in the elasticity of the vitreous as a result of the active enzyme effect on the collagen content in the network. Future studies can use the same method to understand the effectiveness of other active enzymes on the vitreous network that can be helpful in further understanding the macromolecular structure

of the vitreous and its potential role in some vitreoretinal conditions. In addition, modeling the trend of the effectiveness of active enzymes could help to predict the result and develop new potential therapeutic options. We believe this method can be useful in the investigation of the effectiveness of current pharmacological injections used to treat vitreoretinal conditions such as liquefaction or aging.

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Table captions

Table 1. Variance and mean values for the viscosity and creep compliance intercept are presented for both groups

Table 2. Each group of injection was tested at three time points, prior to the injection, one hour and 24 hours after the injection. a) The unpaired t-test results of the comparison between the viscosity and compliance intercept values of the two groups at each time point; b) The results of the paired t-test of the viscosity and creep compliance intercept values for group 1 between two different time points; c) The results of the paired t-test of the viscosity and creep compliance intercept values for group 2 between two different time points

Table 1. Variance and mean values for the viscosity and creep compliance intercept are presented for both groups

Injection	t (hour)	Viscosity $[Pa.s]$		Creep compliance $[Pa^{-1}]$	
		Mean× 10^3	Variance× 10^6	Mean	Variance× 10^{-3}
Collagenase	0	14.5	24.5	0.196	4.7
Collagenase	1	10.1	25.7	0.326	11.5
Collagenase	24	6.96	19.9	0.715	130.4
PBS	0	16.8	36.2	0.16	6.6
PBS	1	21.6	109.3	0.15	5.1
PBS	24	1.4	16.6	0.207	8.3

Table 2. Each group of injection was tested at three time points, prior to the injection, one and 24 hours after the injection

a. The unpaired t-test results of the comparison between the viscosity and compliance intercept values of the two groups at each time point

p-value for Group 1 vs Group 2	$t = 0$	$t = 1$	$t = 24$
Creep compliance intercept (J_s)	0.21	2.4×10^{-6}	1.0×10^{-7}
Viscosity (μ)	0.29	4.5×10^{-3}	8.3×10^{-5}

b. The results of the paired t-test of the viscosity and creep compliance intercept values for group 1 between two different time points

p-value Group 1	$t = 0$ vs $t = 1$	$t = 0$ vs $t = 24$	$t = 1$ vs $t = 24$
Creep compliance intercept (J_s)	6.33×10^{-9}	4.70×10^{-8}	4.87×10^{-6}
Viscosity (μ)	3.577×10^{-3}	4.87×10^{-8}	7.105×10^{-3}

c. The results of the paired t-test of the viscosity and creep compliance intercept values for group 2 between two different time points

p-value Group 2	$t = 0$ vs $t = 1$	$t = 0$ vs $t = 24$	$t = 1$ vs $t = 24$
Creep compliance intercept (J_s)	0.5799	0.3388	0.2518
Viscosity (μ)	4.825×10^{-2}	0.8112	7.069×10^{-2}

Figure captions

Figure 1. a) The mounting block with a half sphere cut out (13mm radius) which was 3D printed to keep the eye in place and prevent unnecessary movements, b) The location of the incision is consistent for all of the specimens, the eye is secured in the 3D printed mounting block and the block is taped to the surface of the rheometer, c) The rough surface of the probe is fully inserted in a porcine eye with minimal interaction with retina or sclera

Figure 2. Schematic of the eye during the *in situ* rheological test probe towards center. The roughened end of the probe has a 0.86 mm radius. We insert 10 mm of the probe into the eye. This ensures that the roughened part is fully in the vitreous and the angle is towards the center of the vitreous

Figure 3. a) Graphical representation of a creep test where a constant shear stress (τ) is applied to the sample for a constant period of time (t), during the experiment, b) Representation of a typical theoretical creep curve, which shows the creep compliance over time. J_s at t=0s shows the intercept of the steady state region of the sample with the y axis indicating the elasticity of the sample. In addition, the slope of the steady state part of the graph is inverse of the viscosity (μ) of the sample in the steady state

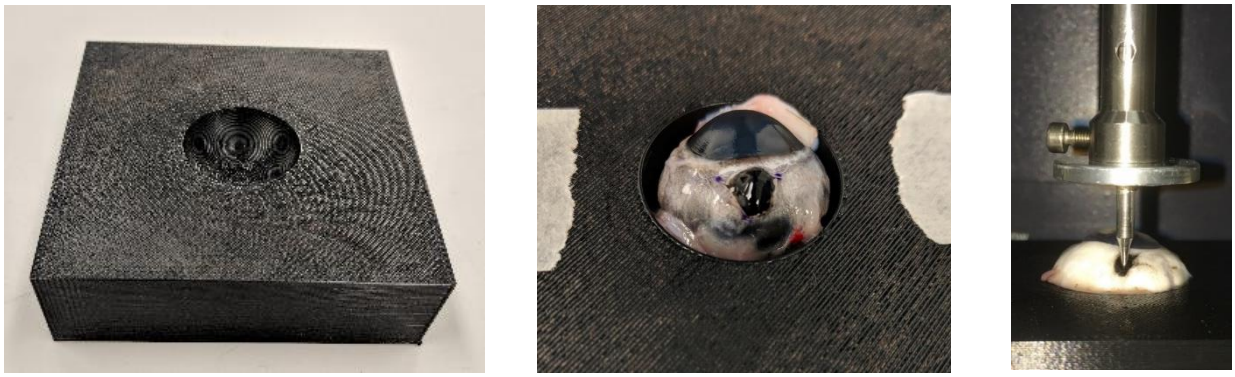
Figure 4. Examples of the creep compliance curve for one eye from each group (indicated with Collagenase or Control) before the injection (t=0), and 24 hours after the injection (t=24)

Figure 5. Comparison of compliance intercept values for the porcine eyes injected with PBS, Collagenase and no injection over time, Standard deviation of the values are shown with the bars. The differences between the collagenase injected eyes at 1 hour compared to the values of the same time from the PBS injected eyes is statistically significant (with the mean values of 0.326 compared to 0.161). The same holds for the difference between the groups at 24 hours (with the

mean values of 0.715 for group 1 and 0.207 for group 2). The eyes in group 1 show significant increases from t=0 to t=1, and t=1 to t=24. This does not hold for the eyes in group 2

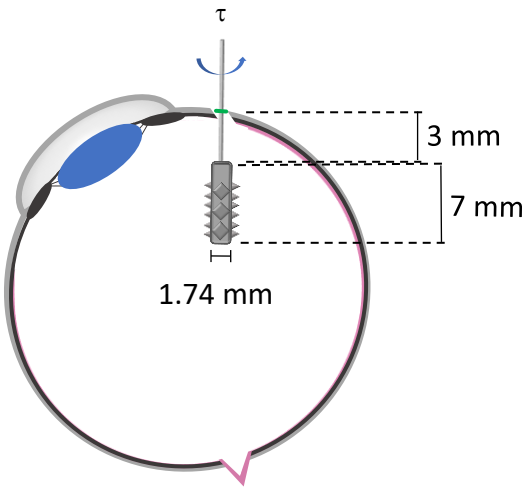
Figure 6. Comparison of viscosity values for the porcine eyes injected with PBS versus Collagenase over time, Standard deviation of the values are shown with the bars. The differences between the collagenase injected eyes at 1 hour compared to the values of the same time from the PBS injected eyes is statistically significant (with the mean values of 10.1×10^3 compared to 18.8×10^3). The same holds for the difference between the groups at 24 hours (with the mean values of 6.96×10^3 for group 1 and 14.2×10^3 for group 2). The eyes in group 1 show significant increases from t=0 to t=1, and t=1 to t=24. This does not hold for the eyes in group 2

Figures



(a) (b) (c)

Fig. 1. a) The mounting block with a half sphere cut out (13mm radius) which was 3D printed to keep the eye in place and prevent unnecessary movements, b) The location of the incision is consistent for all of the specimens, each eye is secured on the 3D printed mounting block and the block is taped to the surface of the rheometer, c) The rough surface of the probe is fully inserted in a porcine eye with minimal interaction with retina or sclera



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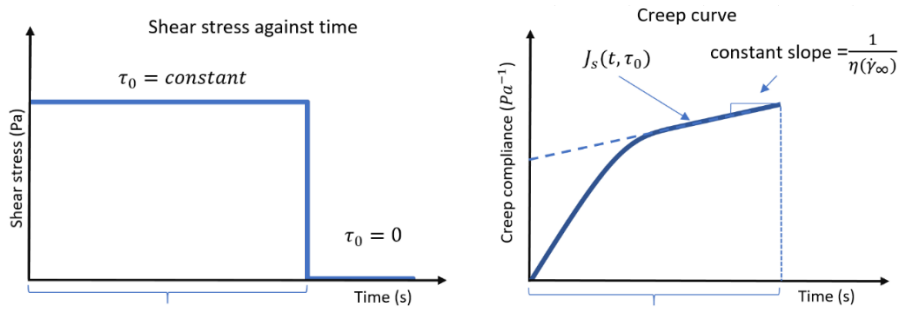

(a) (b)

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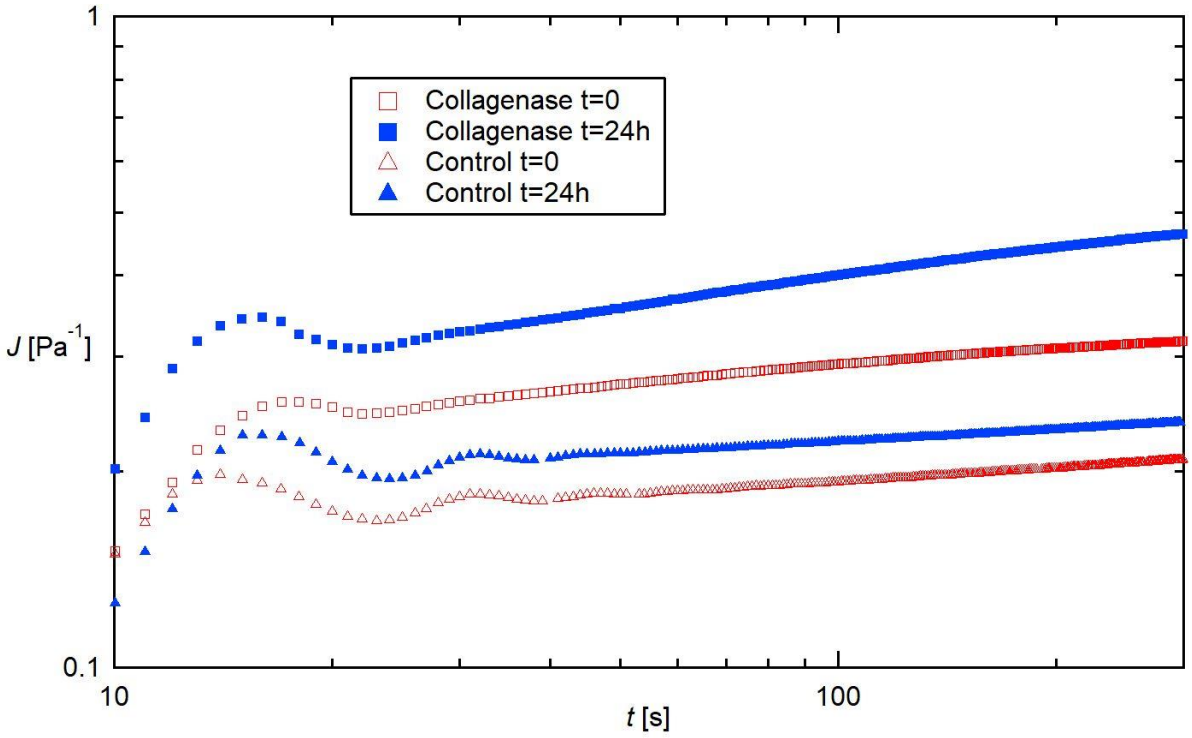

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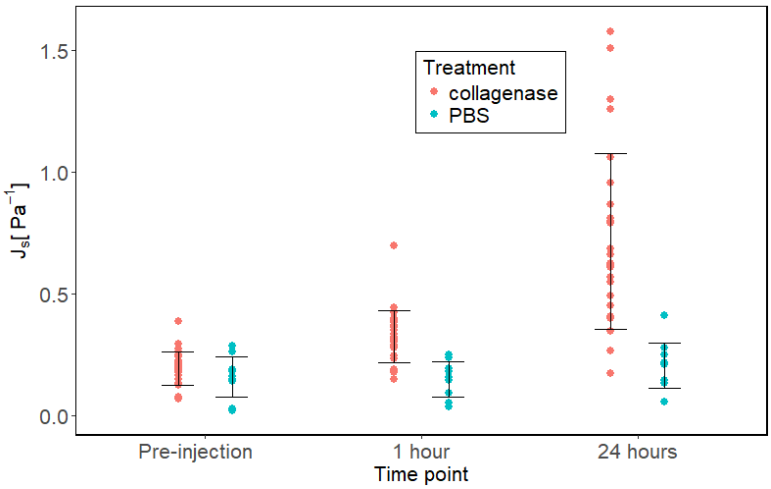

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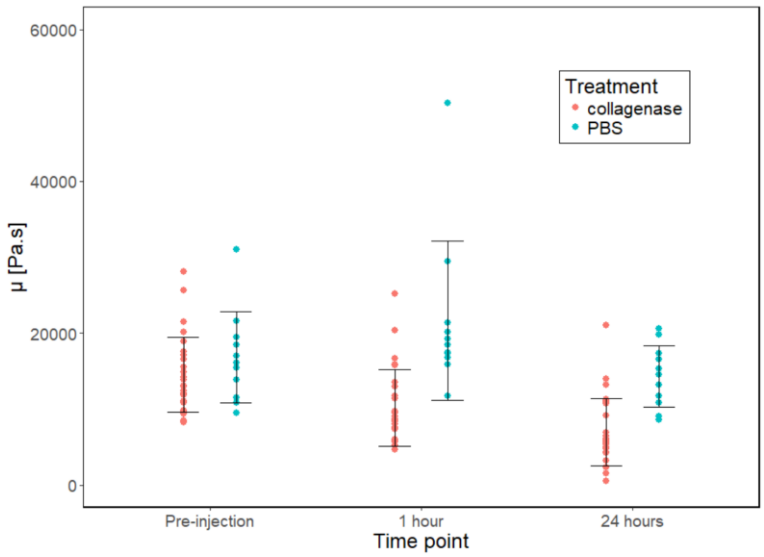

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