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Glaucoma

Measurement of Aqueous Humor Viscosity in an Experimental Rabbit Model With Corneal Neovascularization

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Purpose: This study investigated the relationship between aqueous humor (AH) viscosity and vascular endothelial growth factor-A (VEGF-A) in a corneal neovascularization (CNV) model.

Methods: Ten female New Zealand rabbits were divided into two groups (n = 5 each). CNV was induced by alkaline burns on the right corneas of group B, whereas group A underwent a sham procedure. After 14 days, at least 150 µL of AH was extracted from both eyes. VEGF-A concentration was measured using ELISA, and AH viscosity was determined using a viscometer. Correlations between VEGF-A concentration, total protein concentration, and AH viscosity were assessed.

Results: VEGF-A concentration was significantly elevated in CNV-induced eyes than in noninduced eyes (6029.06 ± 7116.50 pg/mL vs. 115.63 ± 33.19 pg/mL, $P < 0.01$). Total protein concentration was also elevated in CNV-induced eyes (11.66 ± 9.86 mg/mL) than in noninduced eyes (0.69 ± 0.06 mg/mL, $P < 0.01$), and correlated positively with VEGF-A ($r = 0.84$, $P < 0.01$). AH viscosity was significantly increased in CNV-induced eyes (1.82 ± 1.28 mPa-s) compared to noninduced eyes (1.05 ± 0.01 mPa-s, $P < 0.01$) and correlated strongly with VEGF-A concentration in CNV-induced eyes ($r = 1.00$, $p = 0.02$).

Conclusions: AH viscosity positively correlated with VEGF-A concentration, particularly in CNV-induced eyes with elevated VEGF-A levels.

Translational Relevance: The correlation between VEGF-A levels and AH viscosity in an experimental model of corneal neovascularization suggests that AH viscosity could serve as a biomarker for predicting IOP or surgical outcomes in conditions like neovascular glaucoma.

Introduction

Neovascular glaucoma (NVG) is a type of secondary glaucoma with a severe manifestation and poor prognosis, marked by the abnormal growth of new vessels within the anterior segment of the eye.[1] Specifically, the formation of new vessels in the iris or the anterior chamber angle leads to fibrovascular proliferation, aqueous outflow obstruction, increased intraocular pressure (IOP), and subsequent optic nerve damage.[2,3] The angiogenesis observed in NVG is closely linked to elevated levels of vascular endothelial growth factor-A (VEGF-A) in the eye.[4,5] The major causes of NVG include proliferative diabetic retinopathy, central retinal vein occlusion, and ocular ischemic syndrome,[2,6–8] all of which share the common feature of retinal ischemia; namely, the stimulation of VEGF-A secretion.[9,10]

Surgical outcomes of patients with NVG have significantly improved with the use of antimetabolites and anti-VEGF agents.[11,12] However, the surgical success rate still lags behind that of other types of glaucoma, owing to the aggressive nature of NVG and its associated complications.[13,14] Surgical strategies for controlling IOP in patients with NVG include

trabeculectomy, valved and nonvalved aqueous shunts, and microinvasive glaucoma surgery.[15] Although trabeculectomy is considered the gold standard, there is an increasing trend in the use of aqueous shunts, particularly in patients for whom traditional filtering surgery has a high risk of failure owing to neovascularization.[16] Among aqueous shunts, nonvalved devices are used based on the principle of drainage, which is governed by the Hagen-Poiseuille equation.[17–19] According to the Hagen-Poiseuille equation, pressure changes are determined by the radius and length of the tube, flow rate, and viscosity of the aqueous humor (AH) passing through the tube.[20]

Viscosity, an intrinsic property of AH, plays a critical role in determining resistance to outflow, which is essential for maintaining optimal IOP.[19,21] The viscosity of protein solutions such as AH is influenced by factors such as protein concentration and temperature.[22] In the NVG, elevated VEGF-A levels can lead to an increased protein concentration in the AH, potentially raising its viscosity.[23] This increase in viscosity can impede AH outflow, thereby exacerbating IOP elevation and complicating surgical intervention. Despite its potential impact, the importance of the AH viscosity has not been adequately emphasized in the literature.

We hypothesized that AH viscosity could play a significant role among the various factors associated with suboptimal surgical outcomes in NVG. Higher AH viscosity may increase the resistance to outflow, thus affecting IOP control and surgical success. Despite this potential impact, changes in AH viscosity in NVG and its relationship with VEGF-A concentration remain unclear. Therefore this study aimed to investigate the relationship between AH viscosity and VEGF-A concentration, a key factor in NVG pathophysiology, using an animal model of corneal neovascularization (CNV).

Methods

Animals

All procedures in this study that involved animal experiments were conducted following the review and approval of the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine, Samsung Medical Center (IACUC: 20230525001). The study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research in compliance with the principles of eye and vision research. Ten healthy female New Zealand White rabbits (Samtako, Osan, Korea) that weighed between 2.0 and 2.5 kg were used for the experiment. Before the experiment, the rabbits underwent a one-week acclimatization period in individual cages, during which they were provided with unrestricted access to water and standard rabbit chow.

Study Design

Ten rabbits were divided into two groups, A and B, with five rabbits assigned to each group. In group B, CNV was induced in the right eye, whereas in group A, the right eye underwent a sham procedure to serve as a control. The left eyes of both groups remained unchanged and were designated as naïve eyes. In this study, CNV-noninduced eyes refer to both sham-procedure eyes and naïve eyes.

After 14 days, AH was extracted from both eyes of all rabbits. The extraction was performed from the anterior chamber using a syringe equipped with a 26-gauge needle. Immediately after extraction, each sample was divided into two portions and stored in a freezer at −80°C until analysis. At least 70 µL was used for the measurement of VEGF-A and total protein concentration, and 80 µL was used for the AH viscosity measurement. Subsequently, the rabbits were euthanized, and the VEGF-A concentration and AH viscosity were compared between the right (sham-induced) and left (naïve) eyes of group A (Fig. 1A) and between the right (CNV-induced) and left (naïve) eyes of group B (Fig. 1B). Additionally, the correlation between VEGF-A concentration and AH viscosity was analyzed in eyes with (n = 5) and without (n = 15) induced CNV (Fig. 1C).

Corneal Neovascularization Model

All procedures were performed under anesthesia by injecting a mixture of Zoletil (15 g/kg) and Rompun (5 mg/kg) into the femoral muscles of the rabbits. After anesthesia, a 0.5% proparacaine solution was instilled onto the corneal surface. After instillation, the corneal surface was dried with a cotton applicator, and CNV was induced via alkaline burns in the right eye of five rabbits in group B (Fig. 2A). CNV induction was performed as previously described by Ahmed et al.[24] A Whatman paper disk (Advantec, Tokyo, Japan) with a diameter of 8 mm, absorbed with 50 µL of 4N NaOH, was placed on the central cornea for 90 seconds (Fig. 2B). Subsequently, the ocular surface was rinsed with 10 mL of 0.9% sodium chloride (NaCl) solution (0.9% saline solution; UFC Bio, Buffalo, NY, USA). For the sham-induced procedure, the right eyes of

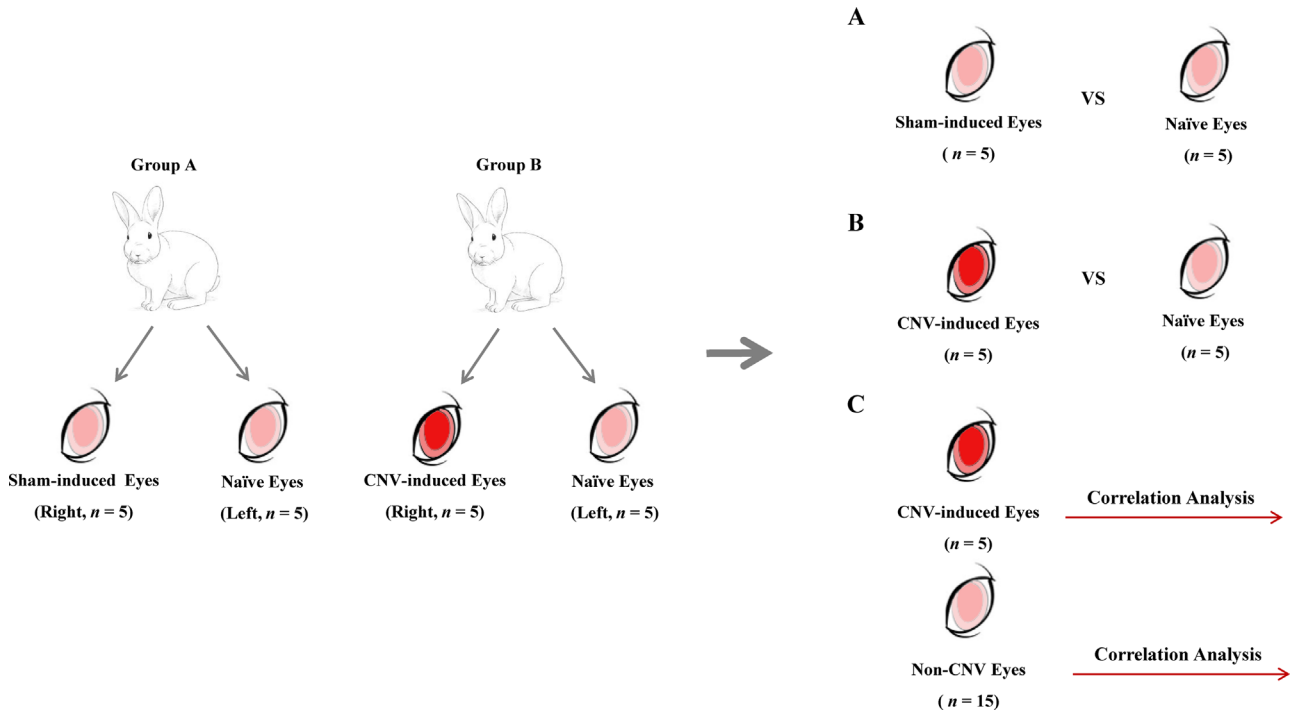

Figure 1. Schematic illustration of the study design. (**A**) Comparison between the right (sham-induced) and left (naïve) eyes of rabbits in group A. (**B**) Comparison between the right (CNV-induced) and left (naïve) eyes of rabbits in group B. (**C**) Correlation analysis between CNV-induced and noninduced eyes.

group A were treated by placing a paper disk soaked with 50 µL of 0.9% NaCl solution on the central cornea for 90 seconds, followed by rinsing the ocular surface with 10 mL of 0.9% NaCl solution (Fig. 2C). This procedure was performed under the same anesthetic conditions as the CNV-induced group to control for handling and procedural effects. The left eyes of both groups remained unchanged, with no procedures or interventions performed, and were designated as naïve eyes (Fig. 2D).

Measurement of VEGF-A Concentration in Aqueous Humor

The Human VEGF Quantikine ELISA Kit (DVE00; R&D Systems, Minneapolis, MN, USA), which has been previously used in other studies,[25,26] was used for this analysis. The Human VEGF ELISA Kit exhibited cross-reactivity with rabbit VEGF-A, enabling analysis.[27] All procedures were performed according to manufacturer's instructions. For eyes without induced CNV, the VEGF-A concentration was measured and then multiplied by a dilution factor of two, to obtain the final value. For eyes with induced CNV, VEGF-A concentration was measured after a 1:20 dilution and then multiplied by a dilution factor of 20.

Measurement of Total Protein Concentration in Aqueous Humor

Total protein concentration in the AH was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin (BSA) used as the standard reference. All AH samples were diluted in PBS at a ratio of 1:10–1:100 to ensure that they were within the detection range of the assay. Each sample was measured in triplicate by mixing 2 µL of diluted AH with 3 µL of distilled water and 500 µL of Bradford reagent. The absorbance was assessed at 595 nm using an X-Mark Microplate Spectrophotometer (Bio-Rad, Mississauga, ON, Canada). The protein concentrations of all the samples were calculated based on a linearized BSA absorbance curve.

Aqueous Humor Viscosity Measurement Protocol

The preprocessing steps for the viscosity measurements in this study involved filtering the samples using a centrifugal filter (Merck Millipore, Bedford, MA, USA) with a pore size of 5.0 µm, as described by Kingsbury et al.[28] The samples were measured using an automated viscometer (VROC Initium; Rheosense,

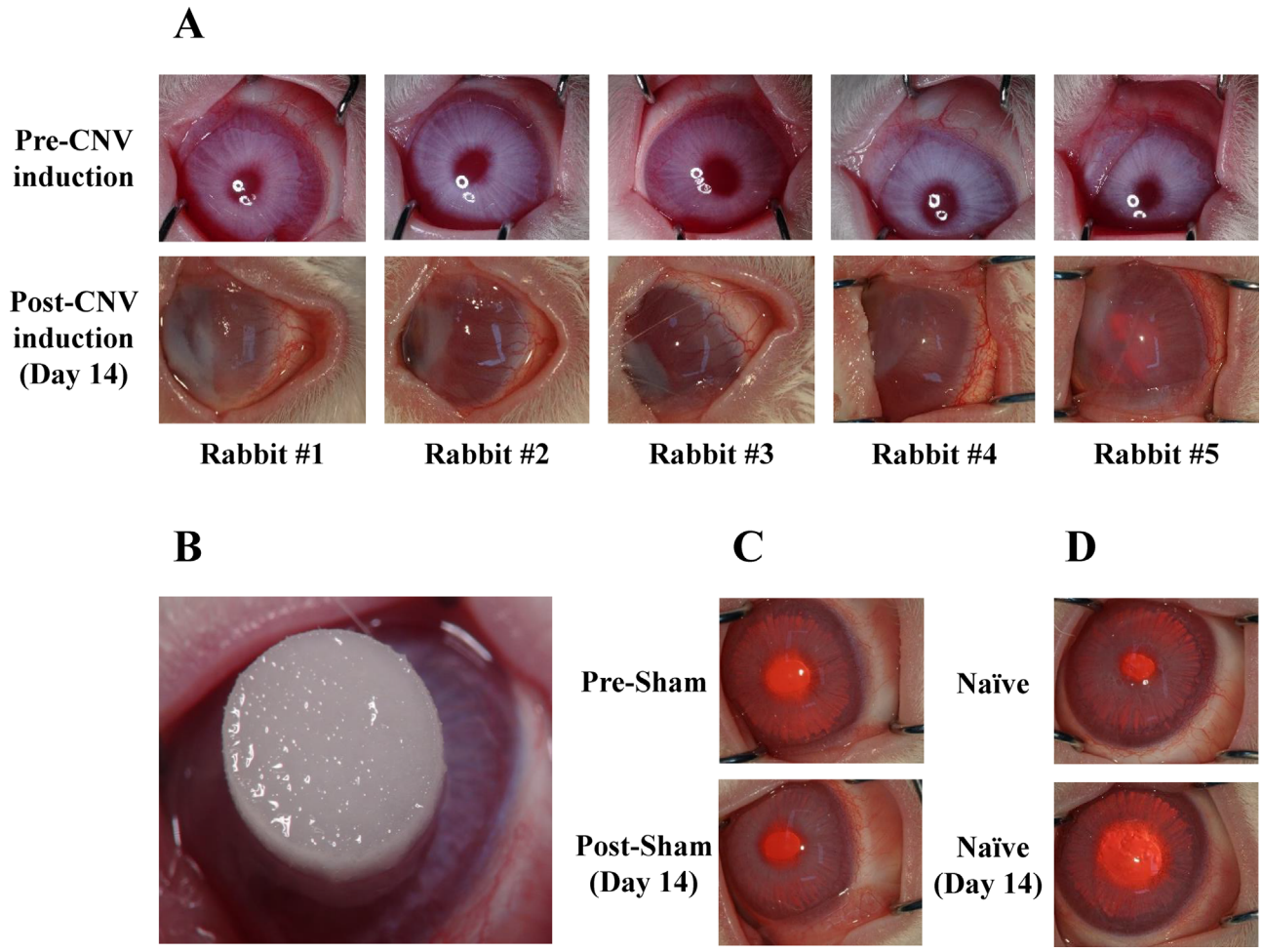

Figure 2. Representative images of CNV induction, sham-induced eyes, and naïve eyes. (**A**) Serial images of the right eyes of five rabbits before (Pre-CNV induction) and 14 days after (Post-CNV induction) alkaline burn–induced CNV, showing marked corneal neovascularization after CNV induction. (**B**) Image of the CNV induction procedure using an 8 mm Whatman paper disk soaked in 4N NaOH, applied to the central cornea for 90 seconds. (**C**) Representative images of the right eyes subjected to the sham procedure before (Pre-Sham) and 14 days after (Post-Sham), showing no evidence of neovascularization. (**D**) Images of contralateral naïve eyes at baseline (Naïve) and after 14 days (Naïve, Day 14), with no signs of neovascularization or corneal changes.

San Ramon, CA, USA), which allowed for multiple repeated measurements with 15 repetitions for each sample. The B05 chip, which can measure viscosity within the range of 0.2 to 3,000 mPa-s and a shear rate up to 100,000 s^{-1}, was used. Measurements were conducted at a shear rate of 20,000 s^{-1}, adjusted according to the viscosity of the sample, at 20°C.

Preparation of Protein Solutions and Viscosity Measurement

Protein solutions ranging from 50 to 300 mg/mL were prepared by diluting BSA (A8022; Sigma Aldrich, St. Louis, MO, USA), a protein standard, in distilled water. The preprocessing method, viscometer, and chip used for the viscosity measurements were conducted in the same manner as those used for the AH viscosity measurements. Each solution was measured 15 times at an adjusted shear rate within 22,000 s^{-1} at 20°C.

Statistical Analysis

All statistical analyses were performed using the open source statistical software Jamovi version 2.3 (Sydney, Australia). Each procedural mean comparison was performed using the Mann-Whitney U test, and correlation analysis was conducted using Spearman's rank correlation. Unless otherwise specified, the data are presented as mean standard deviation. Differences with $P < 0.05$ were considered statistically significant.

Results

Changes in Viscosity With Protein Concentration

To investigate the relationship between protein concentration and viscosity using the viscometer employed in this study, we examined changes in BSA solution viscosity according to variations in BSA concentration. These results confirmed that the viscosity of the BSA solution increased with increasing BSA concentration. At 20°C, the viscosity measurements for BSA concentrations of 0, 50, 100, 150, 200, 250, and 300 mg/mL were 1.03 ± 0.00, 1.29 ± 0.01, 1.76 ± 0.01, 2.43 ± 0.02, 3.44 ± 0.02, 5.07 ± 0.01, and 7.52 ± 0.06 mPa-s, respectively (Fig. 3).

Measurement of VEGF-A Concentration, Total Protein Concentration, and Viscosity in the Aqueous Humor

The concentrations of VEGF-A and total protein and the viscosity of the AH were measured in 20 eyes from 10 rabbits. This included five CNV-induced eyes, five sham-induced eyes, and 10 naïve eyes from groups A and B. Individual measurements for eyes with induced CNV and those without CNV induction are summarized in Table 1. Viscosity values represent the mean ± SD obtained from 15 measurements per sample following the manufacturer's instructions, with an R^2 value of 0.9980 or higher. In the control eyes without induced CNV, the mean VEGF-A concentration, total protein concentration, and viscosity values were 115.63 ± 33.19 pg/mL, 0.69 ± 0.06 mg/mL, and 1.05 ± 0.01 mPa-s, respectively. In contrast, CNV-induced eyes exhibited significantly elevated levels, with VEGF-A at 6029.06 ± 7116.50 pg/mL, total protein concentration at 11.66 ± 9.86 mg/mL, and viscosity at 1.82 ± 1.28 mPa-s (all $P < 0.01$, Table 2). Furthermore, VEGF-A concentration exhibited a significant positive correlation with total protein concentration ($r = 0.84$, $P < 0.01$).

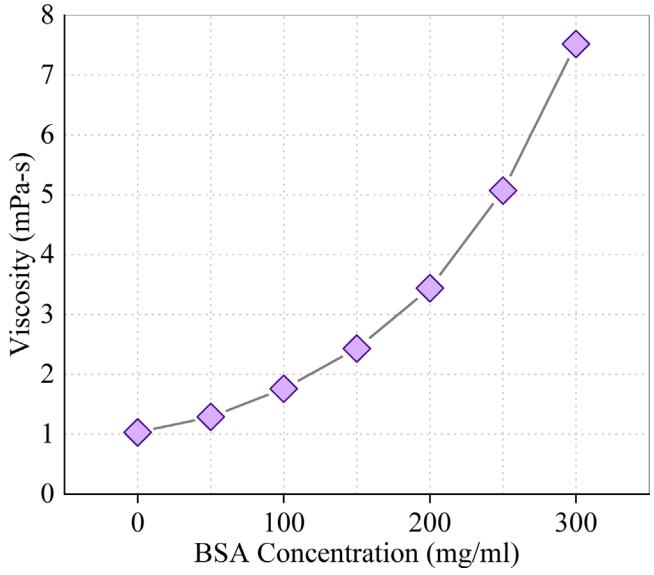

Figure 3. Relationship between BSA concentration and viscosity. The graph shows the viscosity (mPa-s) of BSA solutions at varying concentrations (0–300 mg/mL), measured at 20°C using an automated viscometer (VROC Initium; Rheosense, San Ramon, CA, USA). A concentration-dependent increase in viscosity is observed with increasing BSA concentrations.

Comparison of VEGF-A Concentration and Aqueous Humor Viscosity in CNV-induced and Control Eyes

In group A, which included sham-induced and naïve eyes, the VEGF-A concentrations in the right (sham-induced) and left (naïve) eyes were 144.45 ± 23.15 pg/mL and 119.41 ± 17.33 pg/mL, respectively, with no statistically significant difference ($P = 0.10$). The AH viscosity values were 1.05 ± 0.01 mPa-s and 1.04 ± 0.01 mPa-s, respectively, also with no statistically significant difference ($P = 0.08$) (Fig. 4A).

In group B, which comprised CNV-induced and naïve eyes, the VEGF-A concentration in the right (CNV-induced) eyes was 6029.06 ± 7116.50 pg/mL, which was significantly higher than in the value of 83.05 ± 25.22 pg/mL that was measured in the contralateral left (naïve) eyes ($P < 0.01$). The AH viscosity in the CNV-induced eyes was also significantly higher than the value in the contralateral naïve eyes (1.82 ± 1.28 mPa-s vs. 1.05 ± 0.01 mPa-s, $P < 0.01$) (Fig. 4B).

Comparing the sham-induced eyes (group A) to the CNV-induced eyes (group B), the VEGF-A concentrations were 144.45 ± 23.15 pg/mL and 6029.06 ± 7116.50 pg/mL, respectively, with a significantly higher VEGF-A concentration in the CNV-induced eyes ($P < 0.01$). The AH viscosity values were also significantly higher in the CNV-induced eyes than in the sham-induced eyes (1.82 ± 1.28 mPa-s vs.1.05 ± 0.01 mPa-s, $P < 0.01$) (Fig. 4C).

Correlation Between VEGF-A Concentration and Viscosity in Aqueous Humor

The correlation analysis between VEGF-A concentration in the AH and AH viscosity revealed that, in the control eyes (comprising the sham-induced eyes from

Table 1. Summary of VEGF-A Concentration, Total Protein Concentration, and Aqueous Humor Viscosity in CNV-Induced Eyes and Control Eyes

Group	Eye	Rabbit No.	VEGF-A Concentration (pg/mL)	Total Protein Concentration (mg/mL)	Aqueous Humor Viscosity (mPa-s)
A	Sham-induced	1	119.74	0.75 ± 0.02	1.06 ± 0.01
		2	177.20	0.78 ± 0.04	1.05 ± 0.00
		3	126.69	0.70 ± 0.02	1.06 ± 0.00
		4	156.27	0.78 ± 0.03	1.05 ± 0.00
		5	142.33	0.69 ± 0.05	1.05 ± 0.00
	Naïve	1	93.74	0.74 ± 0.03	1.05 ± 0.00
		2	118.01	0.63 ± 0.04	1.05 ± 0.01
		3	128.43	0.67 ± 0.06	1.03 ± 0.00
		4	140.59	0.76 ± 0.04	1.05 ± 0.00
		5	116.27	0.64 ± 0.04	1.04 ± 0.00
B	CNV-induced	1	18448.30	28.10 ± 0.36	4.09 ± 0.06
		2	3260.54	9.27 ± 0.19	1.20 ± 0.03
		3	5300.49	12.44 ± 0.29	1.49 ± 0.04
		4	1981.92	5.19 ± 0.19	1.17 ± 0.03
		5	1154.05	3.28 ± 0.12	1.16 ± 0.03
	Naïve	1	109.33	0.75 ± 0.05	1.05 ± 0.00
		2	111.07	0.64 ± 0.05	1.06 ± 0.01
		3	67.82	0.64 ± 0.03	1.05 ± 0.00
		4	69.55	0.58 ± 0.06	1.05 ± 0.00
		5	57.47	0.66 ± 0.02	1.04 ± 0.01

CNV, Corneal Neovascularization.

Table 2. Comparison of VEGF-A Concentration, Total Protein Concentration, and Aqueous Humor Viscosity Between Control and CNV-induced Eyes

	Control Eyes (n = 15)	CNV-Induced Eyes (n = 5)	*P* Value
VEGF-A Concentration (pg/mL)	115.63 ± 33.19	6029.06 ± 7116.50	<0.01
Total Protein Concentration (mg/mL)	0.69 ± 0.06	11.66 ± 9.86	<0.01
Aqueous Humor Viscosity (mPa-s)	1.05 ± 0.01	1.82 ± 1.28	<0.01

group A and the naïve eyes from both group A and group B, 15 eyes), there was no statistically significant correlation ($P = 0.68$, $r = 0.12$). In contrast, in CNV-induced eyes (group B, five eyes), a statistically significant correlation between VEGF-A concentration and AH viscosity was observed ($P = 0.02$, $r = 1.00$) (Fig. 5).

Discussion

In this study, we induced CNV in rabbits and measured VEGF-A levels and AH viscosity. We found that eyes with induced CNV had higher AH viscosity than control eyes, and viscosity strongly correlated with VEGF-A concentration in eyes with induced CNV. To the best of our knowledge, this is the first study to investigate the relationship between the AH viscosity and VEGF-A levels in eyes with neovascularization. Our findings provide significant insights into the role of AH viscosity in IOP elevation in ocular conditions with elevated VEGF-A levels, such as NVG, and its impact on surgical outcomes.

Research on the AH viscosity is sparse,[23,29–31] and most existing studies have focused on the composition and flow dynamics of AH rather than its viscosity. In the context of NVG, several studies have reported elevated levels of various growth factors, including VEGF-A, and inflammatory cytokines in the AH.[4,5,32] These factors are implicated in the promotion of

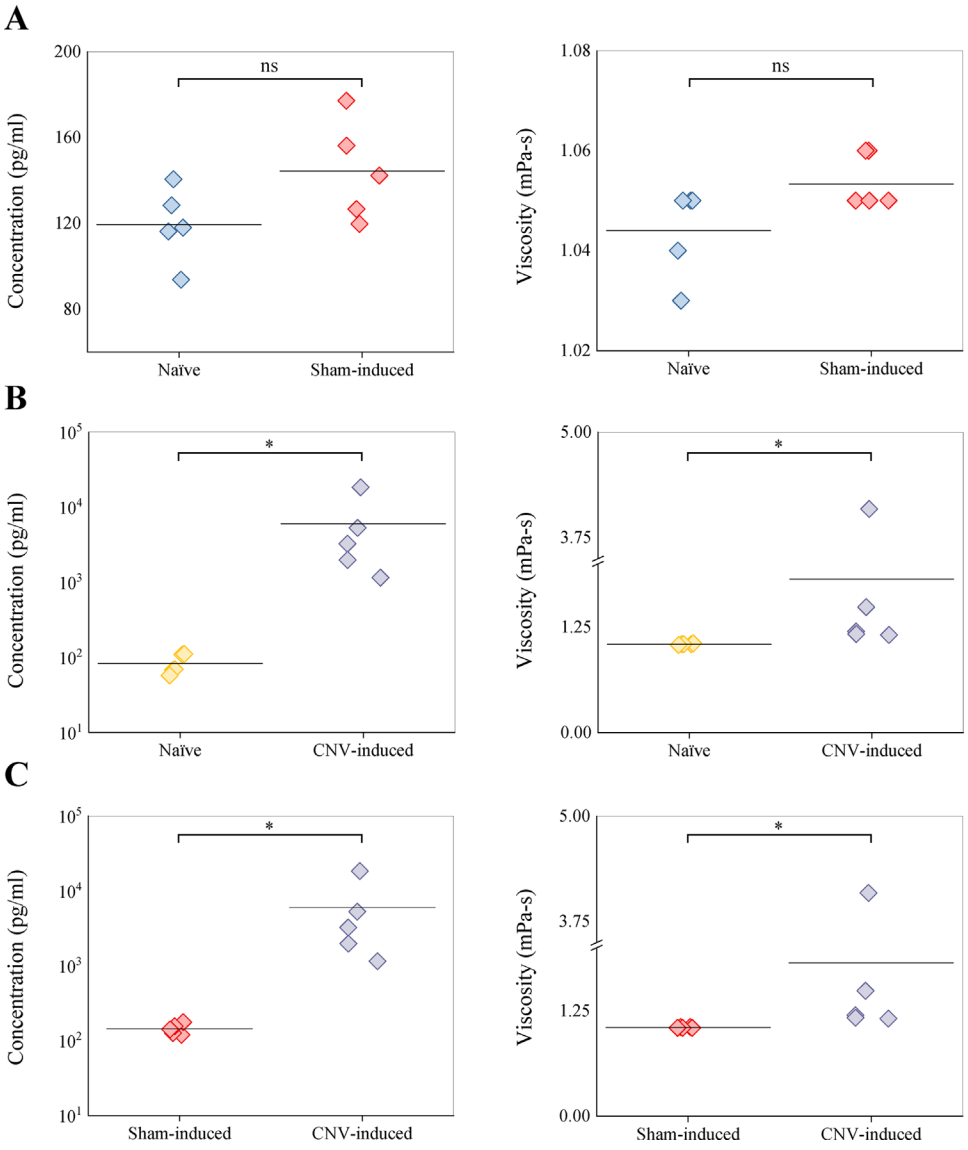

Figure 4. Mean comparison of VEGF-A concentration in aqueous humor and aqueous humor viscosity. (**A**) Comparison between the right (sham-induced) and left (naïve) eyes of group A. (**B**) Comparison between the right (CNV-induced) and left (naïve) eyes of group B. (**C**) Comparison between the sham-induced (group A) and CNV-induced (group B) eyes. ns, not significant; *$P < 0.05$.

angiogenesis and the exacerbation of inflammation, thereby contributing to NVG pathogenesis. However, few studies have explored how these components influence AH viscosity and consequently affect outflow resistance and IOP. The primary advantage of this study lies in its detailed examination of AH viscosity in the context of neovascularization.

To ensure the reliability of our viscosity measurements, we first confirmed the relationship between protein concentration and viscosity using BSA solutions. Our results show a clear concentration-dependent increase in viscosity,[33,34] which validated the precision and accuracy of our viscometer and ensured the reliability of the AH viscosity measurements in the rabbit CNV model. Our animal experiments further revealed that VEGF-A concentrations were significantly higher in CNV-treated eyes than in sham-induced or naïve eyes. This elevation in VEGF-A levels was accompanied by a marked increase in AH viscosity. These findings suggest that VEGF-A not only contributes to neovascularization, but also affects the physical properties of the AH, such as viscosity. The strong positive correlation ($P < 0.02$, $r = 1.00$) observed in our CNV-induced model underscores the potential of AH viscosity as a biomarker of NVG disease severity and prognosis.

Increased AH viscosity has significant clinical implications in glaucoma surgery. Surgical interventions

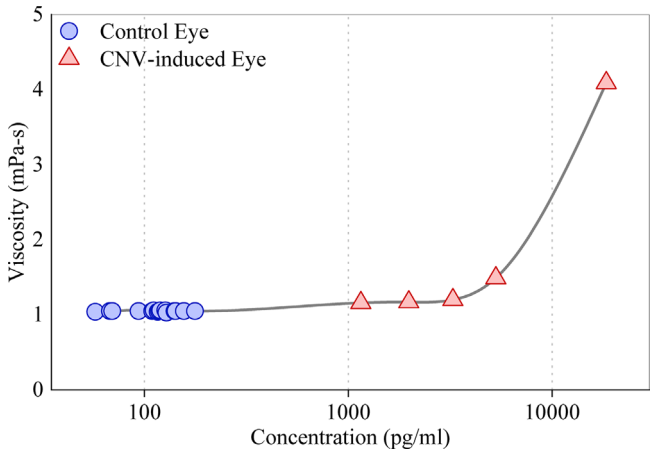

Figure 5. Scatter plot showing the relationship between VEGF-A concentration and aqueous humor viscosity for both control and CNV-induced eyes. The control eyes (comprising the sham-induced eyes from group A and the naïve eyes from both group A and group B, 15 eyes) and the CNV-induced eyes (group B, five eyes) are represented together in this plot.

for NVG, such as trabeculectomy and aqueous shunt implantation, rely on the efficient outflow of the AH through surgical channels or drainage devices to control IOP.[1,35] Although we did not directly measure changes in IOP according to AH viscosity, our findings suggest that increased AH viscosity in the NVG might influence the resistance to AH outflow, which is critical for the success of these surgical procedures. If future research confirms that AH viscosity affects outflow resistance, AH viscosity could be considered in the preoperative evaluation and surgical planning of patients with NVG. In addition, it may be necessary to modify surgical techniques or develop new devices that can better accommodate variations in the AH viscosity. For instance, adjusting the diameter or material of the shunt devices to optimize the flow dynamics in the context of altered viscosity should be considered in future studies.

In this study, we observed a significant correlation between AH viscosity and VEGF-A concentration, similar to that observed for total protein concentration. These findings suggest that elevated VEGF-A levels observed in the CNV-induced model may have contributed to increased vascular permeability, leading to a concurrent increase in total protein levels. This is consistent with the findings of a previous study that reported a positive correlation between VEGF-A and total protein concentrations in the aqueous humor of patients with NVG.[36] This raises the possibility that the increase in AH viscosity observed in our study could be influenced not solely by VEGF-A levels, but also by the associated elevation in total protein concentration. However, it is important to note that under neovascular conditions, not all proteins are equally elevated, nor do they contribute uniformly to viscosity changes. Thus VEGF-A, a pivotal mediator of NVG pathophysiology, may serve as a potential marker of viscosity-related alterations that affect AH outflow. Future studies analyzing the relationship between the total protein concentration and viscosity following anti-VEGF-A therapy in neovascular conditions could provide valuable insights into the direct impact of VEGF-A on AH viscosity.

This study had several limitations that should be noted when interpreting our findings. First, although the CNV model effectively induced elevated VEGF-A levels and allowed us to investigate its relationship with AH viscosity, it did not fully replicate the extensive neovascularization and pathophysiology observed in NVG. Unlike NVG that involves widespread neovascularization of the iris and anterior chamber angle and leads to pronounced IOP elevation, the CNV model provides a localized response. Additionally, we did not measure the corresponding changes in IOP, as the severe corneal alkaline burns used in the model made accurate IOP measurements through corneal contact challenging. Despite these limitations, the CNV model serves as a practical and reproducible system for isolating and studying VEGF-A-induced changes in AH viscosity. Future studies should address these gaps by using more advanced models that better replicate the multifactorial nature of NVG, including its effects on IOP. Second, the sample size was relatively small, with only 10 rabbits used in this study. Larger studies with other animal models and potentially human clinical studies are needed to confirm these findings and explore the variability in AH viscosity among a broader population. Third, although the correlation between VEGF-A and AH viscosity is evident, its potential impact on surgical outcomes, specifically in the context of glaucoma shunt surgery, requires further investigation. Future studies should evaluate the surgical outcomes in patients with varying AH viscosity levels, particularly in those undergoing shunt surgeries. Analyzing postoperative outcomes and complications in these patients could provide valuable insights into the role of AH viscosity in surgical prognosis.

In conclusion, this study highlights the significant correlation between the VEGF-A concentration and AH viscosity in a CNV-induced rabbit model. These findings suggest that NVG, which is characterized by uncontrolled IOP and poor surgical outcomes, may be associated not only with elevated VEGF-A levels in the AH, but also with increased AH viscosity. Further research is required to determine the effect of AH viscosity on aqueous outflow resistance and IOP elevation in all forms of glaucoma.

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* DEK and DYP contributed equally to this work.

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