

**Growth Factors in the Aqueous Humor of Diabetic and Non-Diabetic Eyes Undergoing
Cataract Extraction**

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Introduction:

The aqueous humor plays an important function in the health of the anterior segment of the eye, serving to both nourish the eye and maintain the structural integrity of the anterior chamber. Although aqueous humor production and outflow has been extensively studied, little has been done to investigate its nourishing effects. Prior studies have only quantified electrolytes and basic molecules in the aqueous humor of normal humans. Although the protein concentration in the aqueous humor is only 0.2 mg/mL, proteins play an important role in the health of the anterior segment. More specifically, the interactions of different growth factors regulate most living cells in the eye along with the rest of the human body¹. For example, imbalances in transforming growth factor-beta (TGF-beta) have been implicated in diseases such as keratoconus and pseudoexfoliation syndrome^{2,3}, and exuberant vascular endothelial growth factor (VEGF) has been shown to cause neovascularization in the eye⁴.

Previously, the small volume of aqueous humor that could be obtained presented a barrier to a large scale analysis of growth factors. Traditionally, enzyme-linked immunosorbant assays (ELISA) or Western blots were used for protein level testing. These testing methods require large volumes to test multiple proteins and the low levels of many factors of interest in the aqueous humor made dilution of the sample problematic due to detection limits of these testing modalities. With the advent of multiplex bead testing, such as the Luminex, it is now possible to simultaneously test multiple factors on a small volume sample^{5,6}.

Although growth factors have been individually investigated with regards to specific diseases, no study has been done to determine an aggregate of normal values for these growth factors. Diabetes mellitus is known to cause degradation of the blood-ocular barrier but the effect of this on growth factor and cytokine levels in the aqueous humor has also not been previously investigated. Quantification of the normal levels of growth factors is important when trying to determine growth factor imbalances. With this knowledge, the aqueous humor can be tested to determine its role in anterior segment diseases in the future.

Methods:

Patients undergoing cataract surgery were selected for an aqueous tap prior to surgery. Patients in this study were treated with concordance to Health Insurance Portability and Accountability Act standards. The study was approved by the Texas Tech University Health Sciences Center institutional review board.

Sample collection:

100-200 μ L of aqueous humor were collected from 25 healthy and 25 diabetic patients undergoing cataract extraction. Exclusion criteria included eye drops that may alter intraocular protein levels, such as cyclosporine or prostaglandins, inflammatory eye conditions, such as uveitis, or age less than 40 years. These samples were collected through a paracentesis port prior to surgery as previously described⁷. No complications were encountered during sample collection. The samples were centrifuged at 10,000 revolutions per minute for 1 minute and the

supernatant was extracted. These samples were then stored in a minus 80 freezer until all samples were ready for processing.

Sample Analysis:

The samples were then processed using Luminex multiplex bead technology (Luminex Corporation, Austin, TX) and tested for platelet derived growth factor (PDGF), brain derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), transforming growth factor beta (TGF β), epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), tumor necrosis factor α (TNF α), interferon alpha (IFN α), interferon gamma (IFN γ), and interleukin (IL) 1, 2, 4, 5, 6, 8, and 10. The samples were diluted if needed to insure adequate aqueous humor for analysis. The samples were then mixed with the proper Luminex beads (Invitrogen Corporation, Carlsbad, CA) and placed in the assay plate. The Luminex machine was calibrated, and the 96-well plate was then inserted into the Luminex S100 for sample analysis.

Statistical Analysis:

For the PDGF, EGF, FGF, and VEGF assays, the concentrations were converted into 0's (effect absent) and 1's (effect present) to reflect the finding that the growth factors were either strongly detected in a subject or not detected. The control-diabetic difference for these assays was then evaluated in terms of the proportion of subjects with detectable effects using a Cochran-Armitage statistic for two samples. For all other assays, the difference was investigated using a two-tailed Student's t-test of the mean concentration difference. *Multiplicity-adjusted* probability values were derived to take into consideration the many assays simultaneously

acquired from each patient (Westfall & Young, 1993). The p-values were determined using a computer-intensive method based on 10,000 (bootstrap) samples drawn randomly and independently from the residual errors pooled from the control and test groups.

Results:

The control group showed an average age of 69.2 years with male:female ratio of 14:11. The diabetic group had an average age of 67.9 years with male:female ratio of 11:14.

Aqueous levels:

When tested for inflammatory markers, most of the samples showed undetectable levels of inflammation in both the control and the diabetic group. Growth factor testing did not show any statistically significant difference between the control group and the diabetic group. The results taken collectively did not reveal any obvious difference between the control and diabetic groups ($p = 0.47$). None of the test for differences in individual growth factor was significant (Table 1).

Conclusions:

The aqueous humor has long been known to help maintain the health of the eye. The intraocular pressure (IOP) is dependent on aqueous production and outflow. The IOP is important in both maintaining the shape of the globe and health of the optic nerve. The aqueous also provides nutrients to the anterior segment. Due to the avascular nature of the cornea and the lens, these structures require nourishment, including glucose and oxygen, from the aqueous

humor. Additionally, growth factors and other cell signaling factors direct the function of the anterior segment¹. For example, it has been well documented that production and outflow can be controlled by altering prostaglandin levels. However, the small quantity of aqueous fluid has limited protein studies in the past.

The advent of multiplex bead technology provides a new tool in protein analysis. Recently, a few studies have used this technology to investigate intraocular disease states. First, Funding et.al. had investigated inflammatory factors in failed penetrating grafts. They noted that 17 inflammatory factors were elevated in the aqueous of failed corneal grafts compared to patients with Fuchs endothelial dystrophy. Next, Sato et.al. had measured 27 different factors in the vitreous of retinopathy of prematurity (ROP) patients. They collected vitreous samples from the eyes with stage 4 ROP. With the multiplex bead technology, they were able to measure 27 different factors simultaneous. In addition to discovering that VEGF was elevated in vascularly active eyes, there were significant differences found in the levels of IL-6, IL-7, IL10, IL-15, Eotaxin, FGF, granulocyte-colony stimulating factor, granulocyte macrophage colony stimulating factor, interferon- γ -inducible protein 10, and RANTES (regulated on activation, normal T cells expressed and secreted). Although VEGF was likely the key protein in ROP, they highlighted several other factors that could be studied.

Similarly, we had applied the same technology to investigate growth factors in diabetic eyes however there are some inherent shortcomings to this study due to its design. First, the small sample size may not have the power to achieve significance in the testing. In addition to the sample size, only patients undergoing cataract surgery were sampled. As a result,

noncataractous eyes, or truly non-diseased eyes, could not be used. Next, the dilutions of the samples also skew the results. The dilutions may render a few of the growth factors undetectable. Lastly, by testing a large number of factors, the chance of randomly finding a difference is increased. Statistically adjusting for this type 1 error creates a higher threshold for achieving significance in this study.

Despite these limitations, this study does provide a methodology for future testing. The strength of multiplex bead technology is its ability to potentially identify numerous proteins of interest in various disease states. As expected, inflammatory markers are not present in most eyes undergoing routine cataract surgery; therefore, future studies can solely focus on growth factors and eliminate the issues with dilution. Further testing would also add more data to the existing study possibly achieving significance. Trends in growth factor levels in this study may be verified with a larger sampling size.

This study provides the first quantification of a broad spectrum of growth factors and cytokines in the aqueous humor in patients undergoing cataract surgery in both diabetic and non-diabetic eyes. This information will likely be useful for other future studies analyzing these aqueous components in various ocular disease states. Future studies utilizing multiplex bead technology will likely further elucidate the pathogenesis of ocular disease and allow more targeted therapies.

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Figures:

Table 1

Growth factor/cytokine	Control group	SD	Diabetic group	SD	P value
PDGF	6.04 pg/mL	30.18	13.52 pg/mL	35.53	0.78
BDNF	26.81 pg/mL	36.16	36.63 pg/mL	42.52	1.00
GDNF	114.42 pg/mL	109.26	146.75 pg/mL	127.47	0.83
HGF	12.22 ng/mL	9.42	17.98 ng/mL	31.43	1.00
TGF-beta	0.08 ng/mL	0.10	0.09 ng/mL	0.11	1.00
EGF	3.28 pg/mL	10.43	1.04 pg/mL	2.67	0.90
FGF basic	0.46 pg/mL	1.79	0.09 pg/mL	0.47	0.47
VEGF	undetectable	0	0.13 pg/mL	0.36	1.00