

Freeze-controlled Crystallisation of the Aqueous Humour in Glaucomatous and Non-glaucomatous Patients

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Abstract

The purpose of this study was to investigate the aqueous humour of glaucomatous and non-glaucomatous patients by freeze-controlled crystallisation qualitatively to detect a possible difference. Aqueous humour was taken from 20 patients with different kinds of glaucoma (11 primary open-angle glaucoma, eight pseudo-exfoliation glaucoma, one pigmentary glaucoma) and compared with samples from 40 patients with cataracts and to samples from seven patients with cataract and glaucoma; 1µl of each sample was applied to a microscope slide and put in a deep-freezer at a constant temperature of -20°C for 24 hours. During this time, the water evaporated and distinct crystal patterns developed. These patterns were examined stereo-microscopically, stored digitally and compared with each other. Many distinct crystals of different sizes and formations were obtained. No obvious differences in the crystal patterns could be found between the three groups (patients with glaucoma, cataract or cataract and glaucoma). As a multifactorial disease, open-angle glaucoma is characterised by specific changes to the trabecular meshwork cells with an accumulation of deposits of extracellular matrix. Since the trabeculum is rinsed consistently by aqueous humour, changes of the compounds might influence these cells. Alterations concerning this were published. In contrast to the ferning test, one of several methods of examining fluids with patterns that are highly dependent on temperature and humidity, our examinations with freeze-controlled crystallisation at a constant and controlled temperature of -20°C gave more valid results. In our study we could not find obvious differences in the crystal patterns of patients with cataract, glaucoma or cataract and glaucoma.

Keywords

Crystallisation, aqueous humour, glaucoma

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In eyes with open-angle glaucoma, increased deposits of extracellular matrix have been found in the trabecular meshwork.¹ As the trabecular meshwork is rinsed by aqueous humour, it seems possible and plausible that alterations of the aqueous humour might influence the behaviour of the trabecular meshwork cells.²

Fluids and their chemical compounds can be analysed quantitatively and qualitatively. The ferning phenomenon, known since 1946, represents a qualitative method for analysing different kinds of fluid. Papanicolaou et al. found that drying of vaginal fluid on a glass plate led to the formation of a homogenous dendritic crystallisation pattern.³ In 1955 Sole et al. discovered that dried tear fluid showed similar crystallisation patterns.⁴ There is a broad spectrum of possible substrates for performing the ferning procedure. It has been used to examine the quality of body fluids such as saliva,⁵ cervical mucous,⁶ tears,⁷ sub-retinal fluid⁸ and aqueous humour.⁹ Since the results of the ferning test are strongly dependent on humidity and temperature, it is mandatory to provide stable conditions in terms of temperature and humidity to obtain reproducible results when performing this test, otherwise the comparison of ferning patterns and subsequent grading by established systems might not be accurate.¹⁰

The so-called 'deep-freezing crystallisation' procedure limits the influence of changes in temperature on the crystallisation process. Using this method it is possible to obtain a pattern of single crystals that can be compared with other samples. The method and the analysis of freeze-crystallisation patterns of human tear fluid and aqueous humour have been described before.^{11–13} In order to find a difference in the crystallisation behaviour of the aqueous humour we investigated glaucomatous and non-glaucomatous eyes under a constant temperature of -20°C.

Patients and Methods

We investigated samples of aqueous humour from 20 patients (11 female, nine male) with different kinds of open-angle glaucoma (11 primary open-angle glaucoma, eight pseudo-exfoliation glaucoma, one pigmentary glaucoma), who were operated on by trabeculectomy due to uncontrolled levels of intraocular pressure and/or progression of disease. The mean age was 70.1±10.9 years (range 41–86).

The control group consisted of 40 patients (18 female, 22 male) with cataract but without signs of glaucoma or pseudo-exfoliation syndrome, who were operated on by phaco-emulsification. The

Figure 1: Patient with Primary Open-angle Glaucoma Treated with Trabeculectomy



Patient, 68 years of age. There are different crystal formations with small and large diameters and of varying shapes.

Figure 2: Patient with Pseudo-exfoliation Glaucoma Treated with Trabeculectomy



Patient, 75 years of age. There is a similar pattern to that seen in primary open-angle glaucoma.

Figure 3: Patient with Cataract Treated with Phaco-emulsification



Patient, 86 years of age. There is a similar crystal pattern to that seen in patients with glaucoma.

Figure 4: Patient with Cataract and Glaucoma Treated with Phaco-emulsification



Patient, 82 years of age. There is a similar crystal pattern to that seen in patients with either glaucoma or cataract alone.

mean age in the control group was 75.6 ± 9.5 years (range 49–87). Furthermore, we investigated the aqueous humour of seven patients (six female, one male) with cataract and well-controlled primary open-angle glaucoma, who underwent phaco-emulsification only. The mean age in this group was 79.7 ± 9.7 years (range 59–90).

The aqueous humour was aspirated using an insulin syringe via a paracentesis at the beginning of the cataract or glaucoma surgery. Since the freeze-controlled crystallisation pattern develops best on fat-, dust- and fluff-free microscope slides, we achieved satisfactory results by washing brand new slides with alcohol and then drying them with an aseptic towel. Application of 1µl aqueous humour onto the slide was performed using an Eppendorf reference pipette. To limit the influence of wind, air-flow and other environmental factors on the crystallisation process, we decided to site the workplace in the laboratory very close to the deep-freezer (Liebherr, Austria).

The microscope slide with the drop of aqueous humour on it was frozen at a constant temperature of -20°C for at least 24 hours. During this time a distinct pattern of crystals of the freeze-dried aqueous humour developed, which was then evaluated stereo-microscopically. All of the samples were stored digitally and printed, and the patterns were compared with each other independently by two of the authors (FC, RDF).

Results

The samples of the freeze-controlled crystallisation showed crystals of different shapes and patterns. In the periphery of the frozen drops the crystals were of a smaller diameter but of higher density. In the centre of the frozen drop we found the largest crystals with the most developed structures. One part of these structures resembled four-edged crystals exhibiting regular shapes such as quadrates, rhombs, trapezoids or deltoids, while others showed an irregular shape. Rarely, five- or six-edged crystals were

detected. Many of these crystals had a central dent with two lines perpendicular to each other. The second structures found were needle-like formations, sometimes with a second perpendicular line or three lines crossing at 60° to each other. Some of these lines were rather short, forming flower-like crystals. Presumably, these formations indicate an intermediate step in the development of a four- or six-edged structure to a line formation.

With regard to form and density, the crystallisation products within the three groups did not differ. Furthermore, no obvious differences have been found between the types of glaucoma (see *Figures 1–4*).

Discussion

Glaucoma is a multifactorial neurodegenerative disease of the optic nerve with the main risk factor being increased intraocular pressure. In addition to other risk factors (i.e. age, race, genetic or vascular status), a different composition of the aqueous humour may be relevant in the development of glaucoma. Deposits of the extracellular matrix (so-called Rohen bodies¹) have been found in the trabecular meshwork of glaucomatous eyes. Since trabecular cells are rinsed by aqueous humour, it has been suggested that an altered composition of the aqueous humour might influence the function of these trabecular cells.¹⁴

Chronic oxidative stress might participate in the pathogenesis of open-angle glaucoma.^{15,16} In an animal model of glaucoma, the reductive power of aqueous humour was found to be decreased as a consequence of chronic oxidative stress, which resulted in reduced concentrations of uric and ascorbic acid.¹⁷

The quality of aqueous humour can also be examined, similarly to other fluids, by using the ferning test. In dry-eye conditions, in addition to the simple comparison of ferning patterns, a grading of the dendritic ferns with the aid of the Rolando system provides information about the composition of the tear fluid and helps to identify adequate therapy.⁷ According to the appearance of the ferning patterns, four grades are introduced in which patients suffering from ocular surface disorders such as keratoconjunctivitis sicca tend to have grade three or four patterns.¹⁸

The formation of the ferns depends on the equilibrium of lipids, water, mucin, electrolytes, pH value and osmolarity.¹⁹ Scanning-electron microscope findings suggest that ferns of tears are composed of sodium and potassium chloride, with proteinaceous material controlling the crystallisation.²⁰

A different composition of the aqueous humour has been reported in glaucoma patients. Specific proteins in the range of 130,000–135,000 daltons²¹ and 140,000–160,000 daltons²² have been detected in patients with glaucoma and not in cataract patients. Increased levels of malondialdehyde, a reactive oxidative product, have been found in the aqueous humour of glaucomatous eyes.¹⁶ By contrast, by means of the thiobarbituric acid-reacting substances method it was not possible to find statistically significant differences of malondialdehyde between eyes with glaucoma and cataract.²³ Moreover, Recupero et al., who performed the ferning test with the aqueous humour of six patients with

pseudo-exfoliation syndrome, found tetragonal shaped masses, most of them probably corresponding to exfoliation material.⁹ However, the fern patterns themselves did not differ significantly between patients with pseudo-exfoliation syndrome and subjects with uncomplicated cataracts.

Since the result of the ferning test is strongly dependent on examination settings such as temperature and humidity,¹⁰ we performed freeze-controlled crystallisation under a constant temperature of -20°C independent of environmental conditions as far as attainable. Immediately after acquiring the aqueous humour the samples were put into a deep-freezer, where the water evaporated slowly. By this well-known physical principle called lyophilisation, it is possible to almost completely remove water from a sample. After completion of this process a stable crystal pattern of the sample's components can be analysed.

The results obtained by the current authors using this method revealed distinct patterns of crystals with different shapes and formations. No obvious differences were observed between the crystal pattern formations of the aqueous humour of patients with cataract or glaucoma. Likewise, there were no differences between the different types of open-angle glaucoma.

The patterns of the aqueous humour differed from those of freeze-dried tear fluid. The freeze-controlled crystallisation of human tears shows a 3D crystallisation pattern in concentric rings. In the centre cubic crystals can be observed, in the intermediate zone arborescent structures and at the margin delicate star-like crystals are present.^{11,13}

The weakness of our study is that we did not investigate the influence of humidity in the freezer, but it might be assumed that the values should be relatively constant during the 24-hour freezing process. Furthermore, the aqueous humour of the control group was obtained from patients with cataracts, a disease caused by a certain amount of reactive oxygen species at least.²⁴ Physico-chemical analysis of the solitary freeze-dried crystals of aqueous humour should bring further insight into the role of its altered composition in ocular pathologies like glaucoma and cataract.

Experiments showed that further analysis of frozen crystals by different physico-chemical methods is promising. Crystals of freeze-dried human tear fluid have been studied with the help of electron microscopy, X-ray powder diffractometry and Raman spectroscopy.¹³ ■



Christoph Faschinger works in the Department of Ophthalmology at the Medical University of Graz. His special interests include electro-physiology, perimetry, glaucoma, lacrimal and oculoplastic surgery and ocular traumatology, and he is the author of many published articles. Dr Faschinger has also worked in numerous surgical eye camps in Namibia, Zambia, Zimbabwe and Nepal. He completed his internship and residency in the Department of Ophthalmology at the University of Graz, Austria in 1978.

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