SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

CONTACT SPECULAR MICROSCOPY ANALYSIS OF THE HUMAN CORNEA AND ITS CLINICAL RELEVANCE

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DOCTORAL SCHOOL OF CLINICAL MEDICINE

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The Examination takes place at the Department of Gynecology, Faculty of Medicine, University of Debrecen; 11 a.m., November 27, 2015

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen; at 13:30 p.m., November 27, 2015

BACKGROUND

The cornea is the transparent front part of the eye. It has a diameter of about 11.5 mm and a thickness of 0.5–0.6 mm in the center and 0.6–0.8 mm at the periphery. The radius of the cornea is 7.6-7.8 mm. In humans, the refractive power of the cornea is approximately 42-44 diopters. Because transparency is of prime importance, the cornea does not have blood vessels; it receives nutrients via diffusion from the tear fluid through the outside surface and the aqueous humor through the inside surface, and also from neurotrophins supplied by nerve fibers that innervate it. Transparency, avascularity, the presence of immature resident immune cells, and immunologic privilege make the cornea a very special tissue. The cornea has no blood supply; it gets oxygen directly through the air. Oxygen first dissolves in the tears and then diffuses throughout the cornea to keep it healthy. The human cornea has five layers. The outermost layer of the human cornea is the corneal epithelium: an exceedingly thin multicellular epithelial tissue layer. It is continuous with the conjunctival epithelium, and is composed of about 6 layers of cells, which are shed constantly on the exposed layer and are regenerated by multiplication in the basal layer. The second layer is Bowman's Layer, a tough layer composed of collagen, laminin, nidogen, perlecan and other HSPGs that protect the corneal stroma. Bowman's Layer can be described as an acellular, condensed region of the apical stroma, composed primarily of randomly organized yet tightly woven collagen fibrils. These fibrils interact with and attach onto each other. The third layer is the corneal stroma a thick, transparent middle layer, consisting of regularly arranged collagen fibers along with sparsely distributed interconnected keratocytes, which are the cells for general repair and maintenance. Up to 90% of the corneal thickness is composed of stroma. The fourth layer is the Descemet's membrane a thin acellular layer that serves as the modified basement membrane of the corneal endothelium, from which the cells are derived. This layer is composed mainly of collagen type IV fibrils, less rigid than collagen type I fibrils, and is around 5-20 μm thick, depending on the subject's age. The innermost layer is the corneal endothelium: a simple squamous or low cubical monolayer, approx 5 μm thick, of mitochondria-rich cells. These cells are responsible for regulating fluid and solute transport between the aqueous and corneal stromal compartments. The corneal endothelium cells do not regenerate. Instead, they stretch to compensate for dead cells, which reduce the overall cell density of the endothelium, which in turn has an impact on fluid regulation. If the endothelium can no longer maintain a proper fluid balance, stromal swelling due to excess fluids and subsequent loss of transparency will occur and this may cause

corneal edema and interference with the transparency of the cornea, thus impairing the image formed. Our corneal endothelial cells decrease as we age. A normal cell count for the population at large would be expressed as 2500. The range for normal cell count is much larger, 2000-3200. Central endothelial cell density decreases throughout life at an average rate of about 0.6%/year. Endothelial cell density decreases more after intraocular surgery, in glaucoma, contact lens wear, in Fuchs dystrophy and in diabetes mellitus. Corneal decompensation occurs when cell density is under 1000 per square millimeter. Endothelial cell density is an important marker of corneal health; endothelial cell loss can be linked to aging, several disorders, trauma or chemical agents.

Assessment of the cornea is an important part of the routine ophthalmological examination, which has traditionally been performed with slit lamp. However, this method cannot provide an objective and quantifiable description of the anterior segment structures. Modern imaging techniques have been developing rapidly and novel anterior segment diagnostic instruments promise to overcome these limitations.

Specular microscopy allows for structural and functional in vivo examination of the corneal endothelium.

PURPOSE

The purpose of my work was to ensure a safe and useful examination technique of the new instrument as well as to examine corneal parameters in healthy and pathologic corneas. After statistical analysis the results were compared with the data of the literature.

1. To expand a quick, safe, trusty examination method for donor corneas.

2. To follow the quantitative and morphological changes of implanted corneas after penetrating keratoplasty.

3. To examine the cornea of in vivo diabetic patients. To find correlation among endothelial morphology and patient age, glucose level and duration of diabetes.

4. To examine the influence of modern contact lenses on the cornea and endothelial cells.

PATIENTS AND METHODS

Specular microscopy

Specular microscopy is a noninvasive photographic technique that allows visualization and analysis of the corneal endothelium. Using computer-assisted morphometry, modern specular microscopes analyze the size, shape and population of the endothelial cells. The instrument projects light onto the cornea and captures the image reflected from the optical interface between the corneal endothelium and the aqueous humor. The reflected image is analyzed by the instrument and displayed as a specular photomicrograph. In clinical practice, specular microscopy is the most accurate way to examine the corneal endothelium.

The result obtained via specular microscopy is based on reflection. Thus, it must be considered that magnification depends on the light path length. Consequently, in thick corneas, cell density will be under-estimated and vice versa. Hence we corrected the determined cell density. The correction was then calculated using the equation below (given by the Tomey for use):

$Z\ (corr) = Z \times (F / 10{,}566)^2$, where:

Z (corr): corrected cell density;

Z: actual cell density;

F: focus, namely, the thickness of the cornea;

10.566: calibration data from the manufacturer

Before taking the photo, the cornea was anaesthetized with a drop of local anesthetic (0.4% oxybuprocain hydrochloride or 1% tetracain hydrochloride FoNo VII.).

PATIENTS AND METHODS

Our study was in full compliance with Good Clinical Practices, the Declaration of Helsinki (1996), and the guidelines of the University of Debrecen. All patients underwent a consenting procedure and were informed of the treatment, the use of local anesthesia, as well as possible risks and benefits of the examinations. All patients signed a written informed consent for the publication of the study results regarding the investigational process, expected results, and possible complications.

1. **Specular microscopy examination of donor corneas**

100 eyeballs of 62 donors were examined. The enucleation was performed within 12 hours after death. The corneal endothelium was examined with Tomey EM 1000 contact specular microscope (Tomey, Erlangen, Germany) after administration of a drop of antibiotics (Neomycin, FoNo VII.) In cases of hypotonia, the eye ball was filled with gas (air) or with fluid (NaCl) at pars plana or in the optic nerve. The eyeball was put in an eyeball holder, to prevent movement.

We examined the central part of the cornea. We took at least 3 pictures, than the best was used for further investigation. The pictures were analyzed by the EM 1200, V 1.5.1, Tomey software. We compared the examination methods.

We recorded donor age, corneal thickness, and endothelial cell density. The Region of Interest was fixed, 0.040 ± 0.001 mm², which contains 100 cells. Afterwards three cell analyses were compared for counting endothelial cell density:

1. Normal program: the basic software without any corrections.

2. Manual correction: the unrecognized cell borders were signed manually.

3. Contrast effect: exaltation of the cell boarders by software

2. **Corneal thickness and cell density measurements followed by corneal transplantation**

68 postkeratoplastic patients were involved in our investigations at least one year after penetrating keratoplasty. Causative diagnoses were keratoconus (n=36), bullous keratopathy (n=14), corneal leucoma (n=9), herpetic keratitis (n=7), Acanthamoeba keratitis (n=1), or corneal dystrophy (n=1).

29 of the donor corneas were preserved in Optisol corneal storage medium (Chiron Ophthalmics, Irvine, California), 39 were stored in moist chamber. The donor corneal grafts were between 6.5 and 7.5 mm in diameter. The recipients' corneas were between 6.0 and 7.0 mm in diameter. The grafts were sutured using a 16 bit 10-0 nylon running suture.

The parameters of donor endothelial cells were detected at an average of 67.8 months postoperatively (ranging from 12 months to 23 years). Patients with a history of contact lens wear were excluded from this study. At follow-up visits, after using tetracaine hydrochloride as topical anesthesia, central corneal endothelial photographs were taken of 3 different central areas of the recommended sample size of 50-100 cells with contact specular microscope (EM 1200, Tomey, Tennenlohe, Germany) [2-4]. Mean central endothelial cell density (ECD) and the coefficient of variation of endothelial cell size describing polymegethism were calculated with the built-in image analysis software (v. 1.5.1) of the device. Regarding corneal thickness, the normalized magnification conversion table provided by the manufacturer was used to ensure accurate cell density. The annual endothelial cell loss was calculated from the pre- and postoperative cell density. All data were assessed in relation to the preoperative diagnosis and the type of preservation.

3. Corneal endothelial layer in diabetes mellitus

Forty-one eyes of 21 patients with insulin dependent type I diabetes mellitus and 59 eyes of 30 patients with non-insulin dependent type II diabetes mellitus were recruited. For all patients in the type I group insulin therapy was initiated immediately after diagnosis. Patients with previous ophthalmic disorder, contact lens use, glaucoma and intraocular surgery were excluded from the study.

Both diabetic groups were compared with age-matched normal subjects. Control group I (served as a control for diabetes type I) included 40 eyes of 22 subjects. Control group II (served as a control for diabetes type II) consisted of 60 eyes of 30 subjects. Normal subjects were defined as having no previous or present ocular disease and a negative history of contact lens use and intraocular surgery.

Patients underwent slit-lamp examination, IOP measurement and were submitted to specular microscopy investigation. To avoid diurnal fluctuation of corneal thickness, measurements were carried out after 2:00 pm as suggested earlier.

Ophthalmoscopic investigation was performed in dilated pupil in all patients. After fundus photography, if necessary, patients were submitted to fluorescein angiography. The International Clinical Diabetic Retinopathy Disease Severity Scale was used to classify the stage of diabetic retinopathy: 0 = No apparent retinopathy, 1 = Mild non-proliferative diabetic retinopathy, 2 = Moderate non-proliferative diabetic retinopathy, 3 = Severe non-proliferative diabetic retinopathy, 4 = Proliferative diabetic retinopathy.

4. Contact lens-induced corneal changes

In this study 55 people (110 eyes) were enrolled. The subjects were divided into two groups. To Group 1, we assigned 56 eyes of 28 subjects. These were habitual, non-silicone hydrogel soft contact lens-wearers (one male and 27 females with a mean age 25±7.1 years), with a mean contact lens wear time of 5.93±6.02 years (minimum: two years, maximum: 31 years). The reason for refitting these subjects with more modern contact lenses was to preserve the physiological status of the eye. In Group 2, 27 neophytes (three male and 24 females with a mean age 20±2.15 years) had never worn contact lenses before they were fitted with lotrafilcon B lenses. The subjects were examined before being fitted with the silicone hydrogel lenses. They were then examined at two weeks, four weeks, three and six months, and 1 and 2 and 3 years thereafter. At every visit, we recorded visual acuity by Snellen chart. This was defined by clinical measurements and biomicroscopical examination results (lens centration and movement, morphological alterations of the contact lenses, anterior segment of the eye and corneal staining). Only the subjects who appeared at all the examinations and had appraisable data were included in our study analysis.

With the help of self-administrated questionnaires (yes or no choices), we collected the subjective experiences of the subjects. We created the response format applying several similar configurations. Inquiries included: uncomfortable sensation at the end of the day, itchy or irritating sensation, dryness, redness, inability to wear contact lenses for an entire day, blurred vision and fluctuating visual acuity with the subjects' old lenses and with their new lotrafilcon B lenses. The questionnaires were filled in before patients were fitted with the silicone hydrogel lenses and then four weeks later. Endothelium cell density was measured using an EM 1100 (Tomey, Tennenlohe, Germany) contact specular microscope. The examinations were performed at scheduled intervals, with photos taken before the lotrafilcon B lenses were worn and then again at one month, six months, one, two and three years.

Apart from cell density, the following parameters were determined by specular microscopy: corneal thickness, average endothelium cell size, coefficient of variation of endothelial cells and the percentage of endothelial hexagonal cells.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, Illinois, USA) and Medcalc 10.2.0 (MedCalc Software, Mariakerke, Belgium). Descriptive statistical results were described with mean, standard deviation (SD), as well as minimum and maximum values. Differences between groups were recorded using the paired test of Wilcoxon. Associations between groups were established with Spearman's correlation of rho (r). Comparisons were made using the Student's t-tests. Repeated measures analysis of variances was used to compare each parameter at different time points. Mann-Whitney U unpaired sample test was applied for comparison between groups. The p value of 0.05 was considered as the level of significance. For linear correlation Pearson correlation coefficient (r) was used.

RESULTS

1. Specular microscopy examination of donor corneas

Mean age of donors was 65 ± 11 years. The youngest donor was 43, the eldest was 87 years old.

Corneal thickness measurement was 0.64 ± 0.07 mm on average. We got the lowest corneal cell density with the normal software (without any changes) 1823 ± 253 cell/mm². For corneal thickness corrected cell density - as mentioned in the specular microscopy chapter - was 1853 ± 257 cell/mm² ($p<0.0001$). With manual correction cell density was 2103 ± 194 cell/mm², after correction it was 2138 ± 207 cell/mm² ($p<0.0001$). By using contrast effect cell density was 2126 ± 180 cell/mm², after correction it was 2160 ± 185 cell/mm² ($p < 0.0001$).

Thereafter we used only the corrected data for further examinations. It was a significant lower cell density with the normal cell count program, then in the other two (manual and contrast effect corrected) software ($p<0.0001$). There was no significant difference between manual correction and contrast effect usage ($p= 0.25$). We found no significant correlation between age and cell density.

2. Corneal thickness and cell density measurements followed by corneal transplantation

At all follow-up visits, each transplanted cornea was clear at slit-lamp examination. At examination time the recipient patients' mean age was 42.4±17.1 years. Mean age of donors was 66.2±14.3 years. The average postoperative follow-up time was 67.8±74.1 months (ranging from 12 to 276 months).

Endothelial parameters were counted in 3 different central donor corneal areas of 80.3±34 cell/mm^2 each. At examination time, the overall ECD was 1501±249 cell/mm^2 (ranging from 1100 to 2225 cell/mm^2) (Fig. 1, 2). In the keratoconus group, it was 1483±244 cell/mm^2, in the bullous keratopathy group 1528±279 cell/mm^2, in the herpetic keratitis group 1573±263 cell/mm^2, and in the leucoma group it was 1509±334 cell/mm^2. No difference in ECD was detected between the keratoconus and the bullous keratopathy ($p=0.58$), herpetic keratitis ($p=0.42$) or the leucoma ($p=0.82$) groups. 5.4 years after PKP average ECD was 1545±237 cell/mm^2 in the preserved group, and 1467±256 cell/mm^2 in the moist chamber group

(p=0.2). Average cell size was 665.4±118.8 μm^2 (ranging from 447.5 to 915.5 μm^2), the coefficient of variation of cell size was 0.61±0.11 (ranging from 0.37 to 0.85). Corneal thickness was 0.56±0.06 mm (ranging from 0.45 to 0.73 mm). A statistically significant positive correlation can be observed between postoperative time and corneal thickness (r=0.36, p=0.002).

Correlation between ECD and postoperative time was r=0.02 (p=0.85). At examination time, there was no significant correlation between patient's age and ECD (r=0.11, p=0.35). The rate of endothelial cell loss was 15.8 %/years in the first 2 years in the preserved group.

The coefficient of variation of cell size was 0.61±0.11 (ranging from 0.37 to 0.85). Mean endothelial cell size was 673.6±98.3 μm^2 (ranging from 447.5 to 915.5 μm^2). Corneal thickness was 0.56±0.06 mm (ranging from 0.45 to 0.73 mm) and was measured by contact specular microscopy. No significant correlation was found between ECD recipient (r=0.11; p=0.35) or donor age (r=0.04; p=0.83). There was no correlation between coefficient of variation of cell size and postoperative time (r=-0.01, p=0.92) on the one hand, and between ECD and corneal thickness (r=0.01, p=0.92) on the other.

3. Corneal endothelial layer in diabetes mellitus

Forty-one eyes of 21 patients (9 females, 12 males mean age 40,97 ± 15,46) with insulin dependent type I diabetes mellitus and 59 eyes of 30 patients with non-insulin dependent type II diabetes mellitus (20 females, 10 males 64.36 ± 10.47) were recruited.

Control group I (served as a control for diabetes type I) included 40 eyes of 22 subjects (9 females, 13 males) with the mean age of 40.45±15.16 years. Control group II (served as a control for diabetes type II) consisted of 60 eyes of 30 subjects (15 females, 15 males) with the mean age of 62.69±13.38 years.

Type I diabetes mellitus:

There was a statistically significant decreased cell density (p = 0.024), increased mean endothelial cell area (p = 0.001), coefficient of variation of cell area (p = 0.002), and corneal thickness (p = 0.001) in diabetic corneas as compared to the normal subjects. No difference was present in the IOP values (p = 0.25). The HbA1c level was inversely and significantly correlated with endothelial cell density (r = -0.60; p < 0.0001) and significantly correlated with mean endothelial cell area (r = 0.60, p < 0.0001). A statistically significant correlation was observed

between the glucose level and the morphologic parameters ($r = -0.35$, $p = 0.023$, endothelial cell density; $r = 0.36$, $p = 0.022$, endothelial cell area), and pachymetry values ($r = 0.33$, $p = 0.037$). Statistical analysis revealed a significant negative correlation between cell density and duration of the disease/insulin therapy ($r = -0.38$, $p = 0.014$). Stage of diabetic retinopathy reflected by fundus appearance correlated significantly with endothelial cell density ($r = -0.40$, $p = 0.01$) and mean cell area ($r = 0.38$, $p = 0.015$).

In the type I diabetes group, endothelial cell density ($r = -0.38$, $p = 0.013$) and mean cell area ($r = 0.41$, $p = 0.008$) correlated significantly with patient age.

Type II diabetes mellitus:

No statistically significant difference was found in the endothelial morphology, corneal thickness, and IOP between diseased and normal eyes. In contrast to type I disease, no correlation was detected either between HbA1c or blood glucose level and endothelial parameters. The duration of the disease did not correlate significantly with the corneal morphologic results ($r = 0.02$, $p = 0.891$, endothelial cell density; $r = -0.01$, $p = 0.932$, corneal thickness). Spearman's test did not disclose a significant correlation between the severity of diabetic retinopathy and endothelial cell parameters ($r = -0.01$, $p = 0.967$, endothelial cell density; $r = 0.01$, $p = 0.921$, endothelial cell area).

For type II patients, none of the endothelial parameters was found to correlate with age ($r = 0.05$, $p = 0.718$, endothelial cell density; $r = -0.05$, $p = 0.713$, endothelial cell area); however, central corneal thickness correlated negatively with patient age ($r = -0.44$, $p < 0.0001$).

4. Contact lens-induced corneal changes

One male and 27 females with a mean age 25±7.1 years were in Group 1., with a mean contact lens wear time of 5.93±6.02 years (minimum: two years, maximum: 31 years). The reason for refitting these subjects with more modern contact lenses was to preserve the physiological status of their eyes. Group 2 included 27 neophytes: three males and 24 females with a mean age of 20±2.15 years.

In all cases, distance- corrected visual acuity was 20/20. All lenses were well centered with a 1-2 mm lens movement. The anterior segment findings were normal and staining was

not more than grade I (Efron Grading Scale). Limbal hyperemia was reduced in Group 1 but had not developed in Group 2.

Approximately 60% (16/28) of the subjects in Group 1 found their current habitual lenses to be uncomfortable. However, after the subjects were refitted with lotrafilcon B lenses, this percentage decreased to 6% (1/28). Similar results were obtained concerning self-assessed redness of the eye. Approximately 53% (14/28) of the investigated subjects reported lens awareness and irritation with their previous, habitual lenses. This decreased to 0% after the subjects were refitted with lotrafilcon B lenses. Dryness was a problem for 60% (16/28) of the subjects but, after wearing lotrafilcon B lenses, this complaint completely disappeared (0%). Approximately 53% (14/28) of the investigated subjects said that they could wear lotrafilcon B lenses longer than their habitual lenses. With their previous lenses, approximately 60% (16/28) of the investigated subjects complained of blurred vision. However, none reported this symptom when wearing lotrafilcon B lenses.

Approximately 44% (12/27) of the subjects in Group 2 reported lens awareness and mild irritation in the first two to four weeks but not thereafter. This feeling of discomfort is incredibly common among new lens wearers. It develops at the beginning stages of wearing lenses of all types and decreases after a period of adaptation.

Hypoxia-related complications (microcysts, Descemet's striae, corneal staining) were not discovered by slit lamp examination. However, a decrease in limbal vascularization was observed in Group 1

Repeated measures analysis of variances disclosed no statistically significant difference in the measured parameters in either group during the follow up period ($p=0.06 – 0.96$).

The change in corneal thickness was not statistically significant in either group during the three-year period. The same was true for endothelial cell density. Nevertheless, we noted an interesting trend in Group 2: cell density slightly decreased in the first month, which was not observed after six months of lens use. Cell density decreased in Group 1 by 1.62% after one year, by 0.85% after two years, and by 6.43% after three years. ($p=0.25; 0.26; 0.59$) This is in contrast with the cell density found in Group 2, which increased slightly in the first two years (0.78% after 1 year and 0.46% after two years) but decreased by 3.7% after three years. ($p= 0.28; 0.06; 0.93$) There was a significant difference in the ages of the two groups (the average

age was 25.3 years in Group 1 and 19.89 years in Group 2; p=0.024). However, cell densities were not significantly different at baseline (2554.76 cells/mm2 and 2629.27 cells/mm2, respectively; p=0.17). Cell size and cell density are interdependent variables. In a given field, cell density is higher when the cells are smaller and vice versa.

In this study, there was a correlation between the coefficient of variation and the time of lens wear. In Group 1, the coefficient of variation decreased significantly after six months compared to baseline values (the baseline coefficient of variation was 0.47, whereas, after six months, it was 0.44; p=0.049). In Group 2, there was no significant change.

The percentage of hexagonal cells increased significantly after 1 month in Group 2 (before the lenses were worn, it was 27.78%, whereas, after 1 month, it was 28.25%; p=0.025). In Group 1, there was no significant change.

In Group 1, we observed and documented the regression of limbal hyperemia and neovascularization in the subjects during lotrafilcon B lens wear.

Age is known to be inversely proportional to cell density. This outcome was also observed in this study because cell density decreased with the progression of age (but without statistical significant level) (r=-0.43; p=0.094; Fig. 5.).

This study also found that lens wearing time was directly proportional to the coefficient of variation (r=0.28; p=0.045)

The first correlation was examined in Group 2 using parameters that were obtained prior to the lenses being worn. This was to eliminate the influence of any previous contact lens usage on cell density. The second correlation was investigated in Group 1 using data that were obtained before the subjects were fitted with lotrafilcon B lenses.

DISCUSSION

For the anatomical and functional characterization of human corneas corneal thickness and endothelial cell density are oblique. In 1968, Maurice'6 introduced a microscope for the examination of the cornea1 endothelium in intact eyes at high (400X) magnification. For clinical practice Ronald A. Laing, William M. Bourne and Herbert E. Kaufman used it in 1975/1976. Specular microscopy can provide a non-invasive morphological analysis of the corneal endothelial cell layer. The analysis provides a measure of the endothelial cell physiological reserve from aging, ocular surgical procedures, pharmaceutical exposure, and the general health of the corneal endothelium such as bullosus keratopathia, hereditery dystrophias, corneal ectasia, contact lens wear, diabetic keratopathia, and glaucoma, posttraumatic or postoperative corneas.

1. Specular microscopic examination of donor corneas

It is necessary to determie endothelial cell density in donor corneas before implantation. The European Eye Bank Association (EEBA) prescribes a minimum of 2000 cell/mm² density for donor corneas before conservation. In our study the eye balls were enucleated within 12 hours after death. In spite of this, because of hypotony and the beginning of autolysis, the corneas were swollen. After the eyeballs were filled the specular microscopic images were of good quality. That is why we suggest filling the eyeballs before examination.

Corneal thickness was 0.64 mm, like in other studies with contact specular microscopy. There is a study with Orbscan topography, in which the corneal thickness of enucleated eyes was 0.766 mm. But we know that Orbscan topography measures the cornea thicker than the widely accepted ultrasound measurement.

It has been suggested that the number of cells counted to obtain a maximum accuracy per image is at least 75 cells per image. The average of the 3 images is used to define cell density. Not enough cells or not visible cell boarders can lead to wrong results, hence the importance of using cell analysis programs, such as manual cell count or using the contrast effect. In our study we compared the normal, the manual correction and the use of the contrast effect. We stated that the normal software could not recognize cell boarders quite sufficiently. If we sign up the cell boarders with manual correction or by using the contrast effect, the cell boarders become visible. We stated that there is no significant difference between the last two options. In the past 20 years cell count variability has been high. Our results can be compared to Seitz and coworkers, who used the same microscope. No correlation was observed between

age and cell density. This could be because all of the donors were older. It is known that at birth, the human endothelium comprises a monolayer of up to 500,000 cells, with a density as high as 7,500 cells/mm². During life, cell density is progressively reduced. An initial rapid decline occurs in the first year, reflecting hypertrophy of a fixed population of endothelial cells in response to continued corneal growth. Cell density continues to fall at a reduced rate until the mid-twenties due to endothelial cell loss, and there follows a more gradual decline into old age. It has been estimated that between the ages of 20 and 80 years the reduction in cell density averages 0.52% per year.

2. Corneal thickness and cell density measurements followed by corneal transplantation

Penetrating keratoplasty (PKP) was the most frequently employed allograft transplantation surgery. Pseudophakic bullous keratopathy, Fuch's dystrophy, keratoconus, corneal scarring, and aphakic bullous keratopathy are considered to be main indications for PKP.

Different studies on PKP have reported that the one-year survival rate of donor cornea is up to 90 %, at 5 years it is 88 %, at 10 years it is 80 %, and in a 2007 Australian graft report it is 60 %. Graft clarity rate can reach the extent of 97% after 4 years in a low-risk group. In transplanted corneas, the endothelium can keep its function in some cases even for 30 years, with an initial cell density above 2500 cells/mm2. Minimal cell density at the time of penetrating keratoplasty is reported to range between 2000 and 2500 cells/mm2 and the minimal, critical cell density limit for corneal decompensation is 250-500 cells/mm2. Donor corneas with initial cell densities under 2000 cells/mm2 could reach this critical cell density in less than 20 years. In normal eyes the half- time for the slow component of ECD loss due to ageing is 224 years, which decreases to 21-26 years after intraocular surgeries.

In our study, corneal endothelium of 68 eyes with an optically clear donor cornea at slit lamp examinations were evaluated after an average of 5.4 postoperative years with a minimum cell density of 1100 cell/mm^2. No higher endothelial cell loss associated with higher initial endothelial cell number was observed.

Human corneal endothelial cells were first examined and photographed in vivo with a specular microscope in 1968. Age-related and postoperative changes, such as increasing pleomorphism, polymegethism, decreasing cell density in corneal endothelial layer and increasing corneal thickness have already been observed and well described in several studies;

Bourne [27] reported that the markedly enlarged endothelial cells in long-term corneal transplants have a reduced ability to keep the cornea clear. Møller-Pedersen described higher rates of cell loss in younger groups. His data about an annual loss of 2.9% up to 14 years and 0.3% after 14 years postoperatively is lower than those in our study.

Several studies report a rate of 0.3-0.6 % endothelial cell loss per year in normal corneas. After intraocular surgery, the rate of endothelial cell loss is increased. After cataract surgery, the rate of ECD loss increases up to 2.5%/year during the first 10 years. ECD decrease is also known after posterior lamellar keratoplasty. Endothelial cell loss was more rapid after penetrating keratoplasty than that of postcataract patients in 5 to10 postoperative years, although donor corneas obviously lose endothelial cells during preservation and transplantation procedure. Decrease is generally observed in studies, but there is one reported case finding with no endothelial cell decrease after PKP. Relatively few attempts have been made to study at least 5 years' graft survival with at least 500 eyes.

Endothelial cell loss after surgeries like penetrating keratoplasty is mostly described with the biexponential model with two periods: a rapid period in the first postoperative year and a slow one that persists for years. In the first two postoperative years after penetrating keratoplasty, an overall endothelial cell loss of 33% has been reported, similarly to our data of 15.8%/year in that period. After these two initial years, ECD continues to decrease with a 3-7 times higher rate than normally for up to 20 years after surgery. 10 years after keratoplasty, density reduction is at an average of 50-65%. After penetrating keratoplasty, the rapid period of endothelial cell loss is longer than after cataract surgery, but after 4 years this difference becomes negligible [16]. Another model for describing cell loss called the monoexponential model underestimates early cell loss and overestimates long-term cell loss when applied for long-term data interpretation.

ECD decreases with age so donor age can be a significant risk factor for late endothelial donor failure. Only donor corneas with an ECD of at least 2000 cells/mm2 were suitable for penetrating keratoplasty in our study. One researcher has observed higher endothelial cell loss in grafts for keratoconus, others have reported higher ECD loss after penetrating keratoplasty for Fuchs' dystrophy or corneal edema. Opposing studies, similarly to our data, found no significant correlation between endothelial cell loss and recipient or donor age or preoperative diagnosis, even over a longer period.

In one of his publications, Kus reported an ECD of 808±194 cells/mm2 with a mean thickness of 608±75 μm correlated with neither thickness nor follow-up interval. Coefficient of variation of the cell area was approximately 0.29 in the first 5 years in Ing's study, which was smaller than the corresponding data in our study. Corneal thickness was between 0.54 and 0.57 mm in the first 5 postoperative years, results similar to our data.

In conclusion, the process of endothelial cell loss is highly accelerated after penetrating keratoplasty. There are relatively few studies with more than 5 years postoperative donor endothelial data like in our study. The rate of loss was similar in different preoperative causative lesions, and did not differ in consideration of preoperative donor endothelial cell number, donor or recipient's age.

3. Corneal endothelial layer in diabetes mellitus

The most conspicuous finding of the present study is the altered morphology of the corneal endothelium in patients with type I diabetes mellitus. The reduction of mean cell density was associated with increased mean endothelial cell area and coefficient of variation in comparison with age-matched normal controls. These morphologic changes were accompanied by increased corneal thickness and normal intraocular pressure. However, these alterations could not be detected in type II diabetes mellitus.

The present observations are similar to those reported in the pathologic alterations of the corneal endothelium in diabetes mellitus. Previous studies also proved significantly increased mean endothelial cell areas, coefficient of variation and decreased hexagonality in patients with diabetes mellitus types I and II. However, most of the earlier papers found no significant difference in mean endothelial cell density between the diseased and normal group. In contrast, the recent studies have described significantly lower endothelial cell density in diabetic corneas compared with the normal population. Our findings are similar to these recent reports.

These different results on endothelial cell density may derive from the different specular and image analysis techniques, diabetic control, duration of the disease, and statistical methods used in the different studies. It was described earlier that it is essential to record a minimum of 3 good quality images, preferably by the same investigator. More importantly, that for the evaluation of the accurate cell density, the correction of the cell count is essential to normalize magnification after proper calibration of the instrument. Specular image

magnification is influenced by corneal thickness. There is a linear correlation between such values, and an increase in corneal thickness results in an increase in cell count. An additional important factor for proper image analysis is the number of analyzed cells. As mentioned above, at least 75 cells are necessary for precise analysis. The present investigation considered these suggestions during study design and implementation. Before the study the microscope was re-calibrated, the same investigator counted approximately 90 cells and the cell density was recorded after correction of thickness because, in our opinion these factors are essential for proper image analysis technique.

Opposite to type I disease, endothelial cell density and morphology was normal in type II diabetic patients in comparison with healthy age-matched subjects. It is known that endothelial cell density and hexagonality gradually decreases with age. These patients and their controls were from an older population; therefore these changes are similar and mimic age related alterations as described earlier.

A further striking feature of this report was the inverse correlation between HbA1c and endothelial cell density in diabetes mellitus type I. This result suggests that endothelial morphology may be related to hyperglycemia, especially insulin deficiency. This serves as further evidence that type I diabetic corneas with poor diabetic control are more susceptible to intra-, and extraocular alterations, such as iatrogenic trauma (intraocular surgery) in the microenvironment. Our finding contradicted previous investigations- which demonstrated no such relationship between endothelial morphology and glycosylated hemoglobin levels. However, some studies disclosed a correlation between cell density and duration or severity of the disease, and even with the grade of retinopathy. In cases of type I diabetic patients the present study proved a significant correlation between the severity of diabetic retinopathy and keratopathy.

With the reduction of cell density we also detected significantly thicker corneas in the type I diabetic group. This was consistent with most previous studies evaluating corneal endothelial morphology and corneal thickness. The presumed mechanism is that reduced endothelial cell density causes reduced function, resulting in the swelling of the corneal tissue. However, these changes in corneal structure and function are not clearly understood. Aldose reductase as the first enzyme of the polyol pathway is detected both in the epithelium and endothelium of the cornea by immunohistological studies. This enzyme is responsible for the intracellular accumulation of polyols to extremely high levels, creating an osmotic imbalance

leading to the swelling and rupturing of cells, and may be responsible for the endothelial alterations in diabetic corneas.

Hyperglycemia can also help the formation of advanced glycation end-products (AGEs), which alter protein structure and function, and participate in diabetic long-term complications. These heterogeneous molecules interact with their receptors (receptors for AGEs, RAGEs) found on many cell types, especially on those which play a role in diabetes. This interaction leads to the production of free radicals, inflammatory molecules and has a considerable role in diabetic complications, such as retinopathy, cataract, atherosclerosis, neuropathy, and delayed wound healing.

In summary, the present study disclosed the alteration of the corneal endothelial morphology in type I diabetes mellitus compared to normal subjects after using proper endothelial image analysis technique. The mean level of HbA1c demonstrated a linear and significant correlation with the mean cell area, and an inverse correlation with cell density. Moreover, in type I patients a significant correlation was present between the severity of diabetic retinopathy and keratopathy. Therefore, glycaemic control is essential not only for the control of diabetic retinopathy, but also in the management of corneal complications. These changes indicate that type I diabetic corneas are more susceptible to environmental changes as compared to type II corneas.

4. Contact lens-induced corneal changes

Oxygen passes through a contact lens by diffusion. The International Organization for Standardization (ISO) standard measure of the oxygen permeability of a lens material (at a uniform, standardized thickness) is called Dk. Dk has the unit of 10-11 (cm2/s) x [ml O2 / (ml x hPa) or (cm/sec) x (ml O2 /ml x mmHg)]. The actual oxygen transmissibility of the lens is called Dk/t. This measurement takes into account the central thickness (t) of a -3.00 D lens (ISO standard), or the t at any other place of the lens. Dk/t has the unit of 10-9 (cm/s) x [ml O2) / (ml x hPa)] or (cm/sec) x (ml O2 /ml x mmHg).

A transmissibility graph shows the Dk/t values across the entirety of any given lens and describes the distribution of maximum and minimum oxygen transmissibility (power, base curve).

In silicone hydrogel (SiH) contact lenses, silicone rubber is combined with conventional hydrogel monomers. The silicone component of these lens materials provides extremely high oxygen permeability. The hydrogel component facilitates flexibility, wettability and fluid transport. This aids lens movement. Their oxygen transmissibility (Dk/t) is high because silicone is a better oxygen transmitter than water. These properties may improve the comfort of wearing contact lenses. However, a disadvantage of these lenses is the higher rigidity moduli, due to their high silicone content. First generation SiH contact lenses (lotrafilcon A, balafilcon A) have a lower water content and higher rigidity moduli, when compared with second generation SiH contact lenses (lotrafilcon B, senofilcon A, galyfilcon A, lotrafilcon B). Second generation SiH contact lenses are more comfortable, even though their oxygen permeability is lower than that of first generation SiH contact lenses, because they have increased water contents and reduced moduli.

Our investigation found that wearing lenses with low oxygen permeability (called conventional hydrogel lenses) generated irreversible damage to the corneal endothelium (a comparison of the baseline values of the non-lens-wearing vs. lens-wearing groups). Furthermore, it showed that not even high Dk/t lotrafilcon B contact lenses were able to stop the consequential increase in cell destruction. The decrease observed in cell density in Group 1 exceeded 0.56%, which is the mean annual decrease that has been reported. The fact that neither of the examined parameters significantly decreased in Group 2 suggests that lotrafilcon B provides the cornea with enough oxygen over the three- year period.

By comparing changes in cell density over different age segments and examining the correlation between the coefficient of variation and lens-wearing time, we came to the same conclusions as Sheng et al. Their research found that age is inversely proportional to cell density and that years of contact lens wear is directly proportional to the coefficient of variation. The two groups in our study are not age-matched, it is matching at the beginning age of lens wearing.

The coefficient of variation of the endothelial cells is a measure of the diversity in cell size. A lower coefficient of variation is considered to be better because it indicates that the cells are more similar to one another. Healthy eyes are known to possess more similar, regular shapes. The coefficient of variation is lower when, for the same or a slightly smaller mean, the standard deviation becomes smaller (which is likely to be the case for the corneal endothelium). Additionally, it is lower when, for the same or a slightly increased standard deviation, the mean

becomes larger or when they both change for the better (for example, a relatively lower standard deviation and a corresponding smaller change in the mean).

The present study investigated the central region of the cornea. This is important because, according to Amann et al., the cornea has a larger endothelial cell density in the paracentral and peripheral regions, in comparison to that in the central region. However, this difference cannot be seen in contact lens wearers. This suggests that contact lens wear may cause a mild redistribution of the endothelial cells from the center to the periphery of the cornea. Only a few published studies have examined lotrafilcon B contact lenses. Our results support the outcomes of these papers. The daily wear of lotrafilcon B lenses improved corneal signs and health (conjunctival and limbal redness, corneal neovascularization, corneal edema, corneal and conjunctival staining, etc.,) and subjects' symptoms (uncomfortable lens wear, redness, dryness, irritation, blurred vision, etc.). Half the subjects in Group 2 had mild irritation in the first two to four weeks but not thereafter. This feeling of discomfort is exceptionally common among new lens wearers. It develops at the beginning stages of wearing lenses of all types and decreases after a period of adaptation.

Additionally, it provides excellent vision and comfort. Subjects preferred these new lenses over their habitual lenses. Silicone hydrogel contact lenses provide enough oxygen for the cornea. Thus, they protect the cornea from changes caused by hypoxia. Furthermore, Santos et al. have proven that silicone hydrogel contact lenses are generally less susceptible to microbial adhesion in comparison to conventional hydrogels. This feature facilitates better lens resistance to bacteria. According to a study by Lira et al. silicone hydrogel contact lenses are less susceptible to damage over time, resulting in sustained biocompatibility for longer periods of time. This contributes to the clinical success of this type of lens.

The Gothenburg study has demonstrated that prolonged wearing of low Dk/t contact lenses disturbs the metabolism of the epithelium, decreases the oxygen absorption of the eye and thin the epithelium. Jalbert et al. have recently shown that this effect can be significantly reduced via use of silicone hydrogel contact lenses.

Dumbleton et al. refitted successful soft lens wearers with other high Dk/t silicone hydrogel contact lenses. They then evaluated the objective and subjective responses of subjects. Their results demonstrate that bulbar and limbal hyperemia significantly decreased in all quadrants. This was also observed in the subjects of our study. In addition, dryness

diminished and end-of-day comfort improved. Doughty et al. experienced improvement in the mean bulbar and limbal redness after six months of silicone hydrogel lens usage. Consequently, high oxygen availability ensured better comfort for the wearer and in this investigation lotrafilcon B supported the physiological metabolism and functions of the cornea by improving oxygen provision. Thus, it can be argued that contact lens wear does not provoke corneal damage.

A state of hypoxia is caused by the prolonged wearing of older hydrogel low Dk/t contact lenses. Nearly all contact lens wearers report instances when they do not remove their contact lenses before sleeping ("closed-eye contact lens wear"). At such times, tear flow stops between the contact lens and the anterior surface of the cornea, which, in just a few minutes, induces metabolic changes in the micro-environment of the corneal epithelium. After some minutes, both the stroma and the endothelial cells automatically undergo anaerobic glycolysis. This leads to corneal swelling. A short-term disorder of the metabolism does not lead to irreversible deviations in the structure of the cornea. However, prolonged, frequent hypoxia results in secondary morphological changes in the epithelium, stroma, and endothelium, which are only minimally reversible. Oxygen deprivation has been associated with the appearance of micro cysts in the epithelium, epithelial thinning, slowed mitosis, the loss of hemidesmosomes, reduced epithelial oxygen consumption and an increased superficial cell size in the epithelium. Stromal changes include a chronic loss of glycosaminoglycans and thinning, and the endothelium shows signs of increased polymegethism. Furthermore, conjunctival hyperemia, corneal neovascularization, corneal edema, corneal staining, myopic shift and a decreased resistance against microbial keratitis develop due to oxygen deprivation. These effects lead to subjective symptoms, including a decreased or fluctuant visual acuity, blurred vision, seeing a rainbow circle around lights, dryness and lens awareness. Schafer et al. examined the stability of dryness symptoms after refitting subjects with high-Dk/t silicone hydrogel contact lenses. According to their results, the during-the-day and end-of-day dryness symptoms significantly improved during the first week after refitting with lotrafilcon B lenses and remained stable for three years. The presence of dryness symptoms after 1 week was associated with the discontinuation of contact lens wear.

Hypoxia that affects the periphery of the cornea is an even more important problem than hypoxia of the central cornea. This is because the limbus is the only source of epithelial stem cells that ensure unlimited new epithelial cells and fast regeneration after surface damage. Any

stem cell deprivation or damage consequently results in recurrent erosion, chronic keratitis or vascularization.

NEW RESULTS AND THEIR CLINICAL SIGNIFICANCE

1. Specular microscopy examination of donor corneas

Specular microscopy is useful in testing donor corneas. The hypotensive eyeballs should be filled with liquid or gas in order to reduce the Descemet's folds. To analyze the morphology of endothelial cells an analysis of a minimum of 75 cells is recommended, and during cell analysis the use of manual technique or the contrast effect method offered by the software should be recommended. The latter technique is faster and is not determined by individuals. The correction of corneal endothelial cell count depending on corneal thickness by using the appropriate function of the equation is also required.

2. Corneal thickness and cell density measurements followed by corneal transplantation

We can conclude that endothelial cell loss after corneal transplantation is not negligible. Our results correlate with the literature data. Long-term cell loss is not influenced by any diagnosis that indicates keratoplasty surgery, or the age of the donor, or the initial cell density above the minimum limit number of cells.

3. Corneal endothelial layer in diabetes mellitus

In our survey, decreased corneal endothelial cell count, consequentially enlarged average cell area, and corneal thickening were detected in patients with diabetes mellitus compared to healthy controls. In patients with diabetes mellitus the cornea is constantly in a state of stress, therefore, it has more sensible reactions to external and internal traumas (e.g. injuries, intraocular surgeries). It was determined that these characteristics can be seen in particular in type I of the diabetes mellitus disease, mostly in those patients whose HbA1c and serum glucose levels are not properly set. We drew attention to the fact that besides the known ophthalmic complications of diabetes mellitus, diabetic keratopathy should also be taken into consideration.

4. Contact lens-induced corneal changes

Second-generation lotrafilcon B lenses can be worn safely and without complaints. Both subjective and objective parameters proved to be of higher quality in comparison with conventional hydrogel lenses.

KEYWORDS

cornea, corneal endothelial cells, contact lens, diabetes mellitus, silicone hydrogel, specular microscopy, keratoplasty

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List of publications related to the dissertation

1. **Kettesy, B.**, Várdai, J., Berta, A., Módis, L., Kemény-Beke, Á.: A survey of corneal changes caused by daily wear silicone hydrogel contact lenses.
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