MORPHOMETRIC ANALYSIS OF THE CORNEAL ENDOTHELIUM WITH 3 DIFFERENT SPECULAR MICROSCOPES

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Morphometric analysis of the corneal endothelium with three different specular microscopes

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Abstract. The morphometry of the central corneal endothelium of 10 eyes in 10 subjects was analyzed with three different specular microscopes. Computer-assisted analysis was performed with only two microscopes (Zeiss and Keeler Konan sp 3300) because the third microscope (Topcon sp 1000) could not be adapted to our computerized system. With this Topcon microscope a grid with standard densities was used to compare the images with, in addition, we also performed manual cell counting on the same Topcon images. The coefficient of variation of the cell analysis of three different images per cornea with the four methods varied between 3.4 and 4.7 percent. One-way analysis of variance showed a significant difference between the Zeiss and the other microscopes. So only the Keeler Konan and the Topcon microscopes could be used interchangeably. The computerized image analysis permitted also an evaluation of the hexagonality. The results of polygonality were not significantly different between the Zeiss and the Keeler Konan. For clinical purposes the Topcon specular microscope is more advantageous than the other two methods, since it is the most rapid way to record and analyze specular images. But for more precise measurements an image processing system is indispensable.

Introduction

Morphometric analysis of the corneal endothelium has become an accepted practice both clinically and in research [1, 2]. Since the introduction of several contact and non-contact specular microscopes different methods have been developed to process such specular images [3–7]. The processing methods have become more and more automated to analyze the endothelium efficiently [8–15]. Sophisticated computer technology has been developed to determine cell boundaries directly from the original photograph or video image. This computerized cell analysis not only provides in cell density but also quantifies the frequency distribution of cell areas and analyzes the polygonality.

* None of the authors have proprietary interest in the equipment described.

Commercially available specular microscopes have become more user's friendly, recording images with an autofocus device. More and more specular microscopes are provided with an incorporated image analyzing program.

The aim of our study was to compare three specular microscopes, of which two are adapted to our own computerized image processor, and discuss the agreements and differences.

Subjects and methods

Subjects

We recorded the central corneal endothelium in 10 right eyes of 10 individuals (5 females, and 5 males) with healthy corneas, which appeared normal by biomicroscopy. None of these eyes had a history of trauma, ocular disease, or surgery. Three images were recorded of each eye with each specular microscope and were analyzed with the technique described. The subjects' age ranged from 24 to 48 years (mean: 37 years). The results were analyzed with Systat (Systat, Inc., Evanston, IL, USA).

Recording methods

Zeiss. A clinical non-contact specular microscope was used in combination with a photo slit lamp (Zeiss SL75) to visualize the corneal endothelium. The images were recorded with a black-and-white video camera (HTH MO High Technology Holland BV, Eindhoven, The Netherlands), fixed on the slit lamp. A frame grabber (PC Vision) digitized the images, which were displayed on the monitor (Sony, PVM 1442QM). The clearest images were selected with the use of its snap function, put in an image analyzer (TIM, Difa Measuring System BV, Breda, The Netherlands), and processed with a microcomputer (PC AT 486, 33 MHz) where the images were stored on the hard disk.

Keeler Konan. The Keeler Konan sp 3300 is a contact widefield specular microscope which was also used with the above described video recording technique. We used the 40x magnification cone which applanates the central part of the cornea.

Topcon. The Topcon sp 1000 is a non-contact specular microscope which is provided with an auto-focus device. The images are displayed on an incorporated screen, which can be printed with a video printer. Only three images per eye can be stored at the same time. When new recordings are made the old images are overwritten.

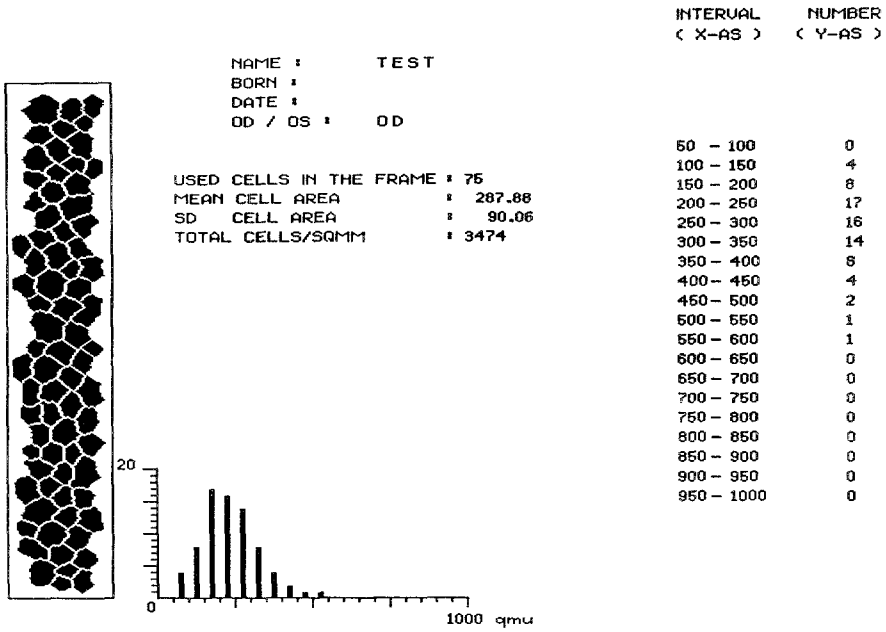


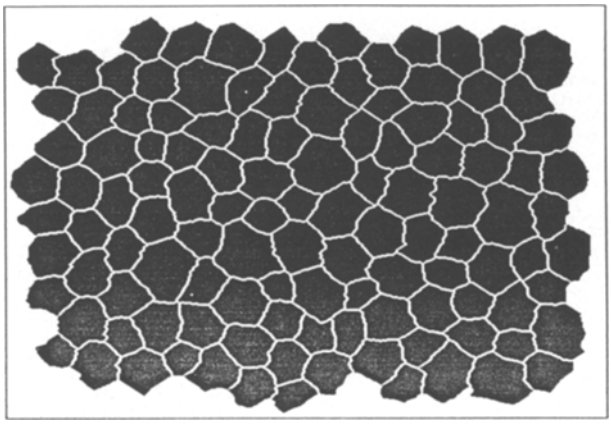
Fig. 1. Output after image analysis with the Zeiss specular microscope, displaying the mean cell area, cell density, and histogram.

Image analysis

We used the same semi-automated image analysis for both the Zeiss and the Keeler Konan specular microscopes. Both microscopes were calibrated with a haematocytometer prior to adapting the program. More details on the image processing software are described elsewhere [16]. This software is capable of determining cell boundaries of specular images. During the processing, missed or wrongly determined boundaries can be corrected and restored manually. The individual cell areas are calculated, resulting in the mean cell area, standard deviation, and mean cell density. The frequency distribution of cell areas are represented in a histogram (Figs. 1 and 2).

The polygonality of the cells is determined by framing each cell with a rectangle. With this algorithm the pixels of the analyzed cell boundary create rectangles with those belonging to the neighboring cells. The number of triangles calculated determines the polygonality of the analyzed cell. As a consequence the cells that are not completely surrounded by other cells, such as on the margin of the image, are incorrectly calculated. Therefore a manual correction is needed.

The images made by the Topcon specular microscope can be analyzed in two different ways;



name : test
born :
date :
OD / OS : od

USED CELLS IN THE FRAME : 119
MEAN CELL AREA : 374.53
SD CELL AREA : 104.42
TOTAL CELLS/SQMM : 2670

interval (X-as)	number (Y-as)
50 - 100	0
100 - 150	0
150 - 200	3
200 - 250	7
250 - 300	19
300 - 350	26
350 - 400	20
400 - 450	22
450 - 500	8
500 - 550	6
550 - 600	3
600 - 650	2
650 - 700	3
700 - 750	0
750 - 800	0
800 - 850	0
850 - 900	0
900 - 950	0
950 - 1000	0

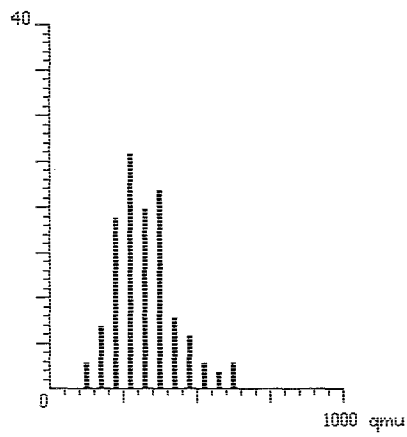


Fig. 2. Output after image analysis with the Keeler Konan specular microscope, displaying the mean cell area, cell density, and histogram.

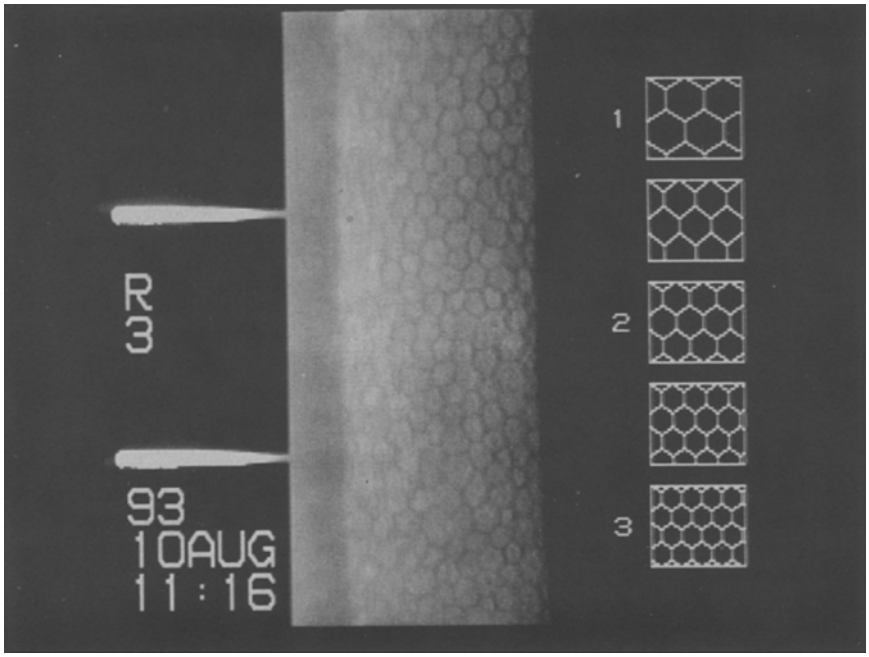


Fig. 3. Video out print of the Topcon specular microscope, where the image can be compared to a grid.

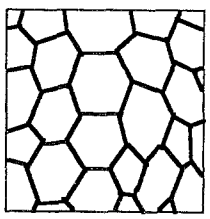


Fig. 4. Schematic drawing of an enlarged square of an image recorded with the Topcon microscope in which individual cells can be counted, including those touching two adjacent sides of the square.

Topcon-grid: Grids varying from 1000 to 3000 cells/mm^2, with steps of 500 cells/mm^2, are projected next to the image so one can compare the image with the grids (Fig. 3). This gives an estimate of the cell density.

Topcon-count: There is also a possibility to enlarge an area of 0.01 mm^2 on three fixed positions for each image, in which the individual cells can be

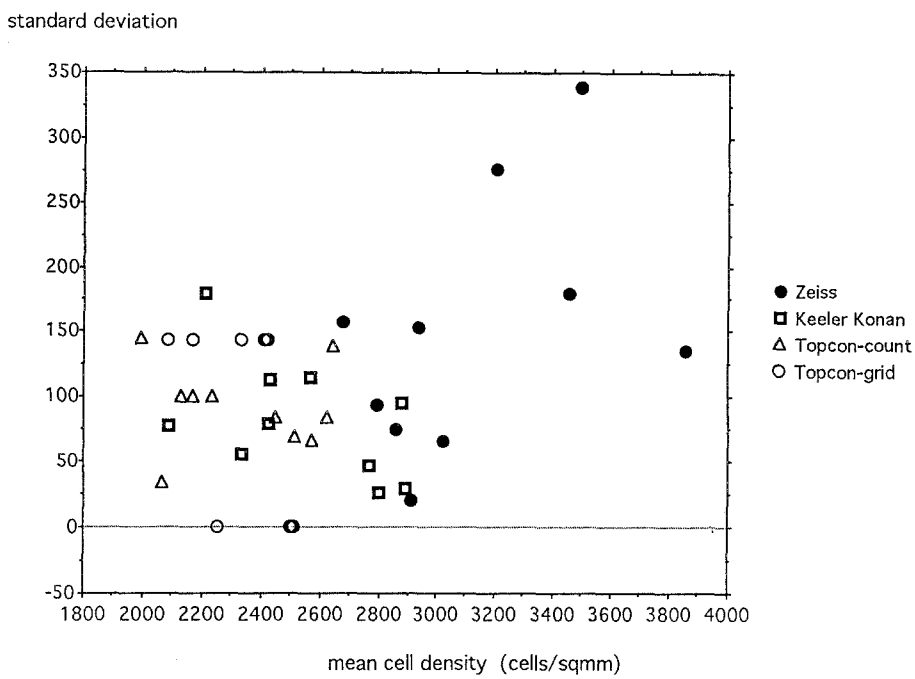


Fig. 5. Scattergram of the standard deviation of three measurements per eye and per method against the mean.

counted (Fig. 4). The total number of cells counted is than divided by the number of squares. This number has to be multiplied a 100-fold to obtain the mean cell density. Since there is no modality to calculate the polygonality, except by hand, we did not analyze the polygonlity with this microscope.

Results

Mean cell density

We compared the results of the mean cell density for all four methods (Table 1). We plotted the standard deviation of the three measurements, for each eye and for each method, against the mean (Fig. 5). This graph displays a lack of agreement between the Zeiss and the three other methods. We see the same difference occurring in Figure 6, representing the mean cell density of the ten corneas for all four analyzing methods, showing a significantly higher mean cell density measured with the Zeiss. The mean difference between the Zeiss and the other three methods varied between 18% and 25%. Whereas the mean difference between the Keeler Konan and the Topcon-count, and Topcon-grid measurements were 6.9% and 7.85%, respectively. The one-way analysis of

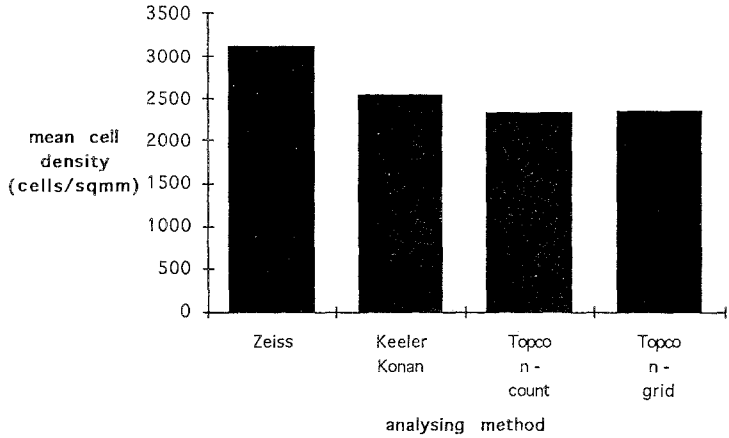


Fig. 6. Mean cell density with for each method. One-way analysis of variance (ANOVA) showed a significant difference between the four methods (F=20.7, p=0.0001).

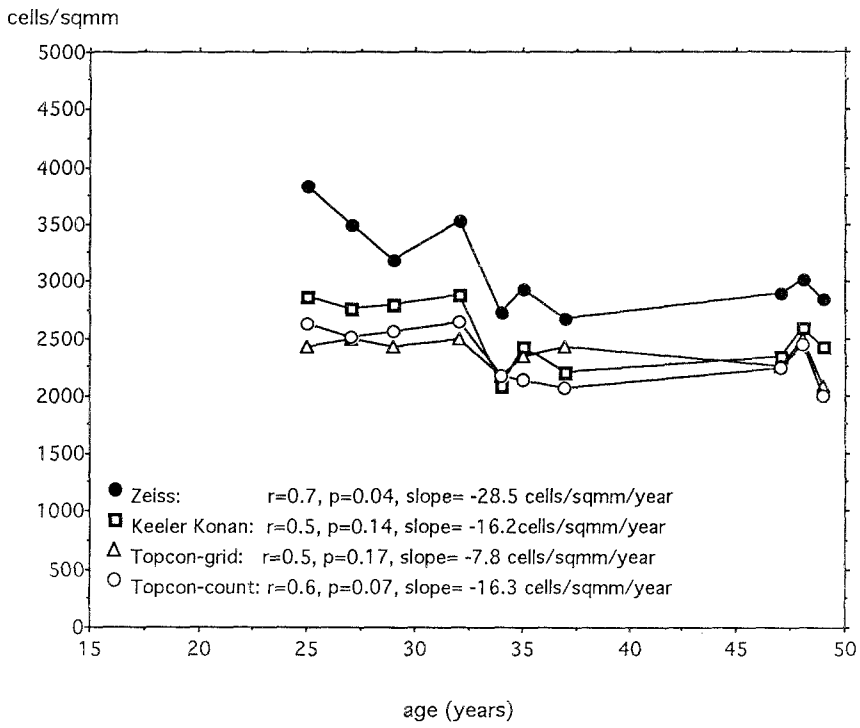


Fig. 7. Mean cell density of each cornea plotted against the age. Regression analysis was performed for all four methods.

Table 1. Cell density of each measurement per cornea and per method

subject	Zeiss		Keeler Konan		Topcon count		Topcon estimate	
	density	cv	density	cv	density	cv	density	cv
A1	3737		2719		2533		2700	
2	3806	3.5	2561	3.3	2700	3.2	2250	6.0
3	4000		2494		2633		2500	
B1	2828		2349		2250		2133	
2	2856	5.2	2560	4.7	2033	4.7	2250	6.2
3	3106		2382		2233		2500	
C1	3005		2719		2433		2500	
2	2961	2.2	2561	4.5	2533	3.4	2500	0
3	3092		2494		2366		2500	
D1	3506		2709		2533		2500	
2	3147	9.7	2783	1.8	2566	2.8	2500	0
3	2824		2800		2433		2500	
E1	2938		2469		2033		2000	
2	2802	2.6	2325	3.3	2250	7.4	2067	6.9
3	2816		2459		1800		2000	
F1	2839		2045		2067		2250	
2	2674	3.5	2035	3.6	2267	4.9	2000	6.7
3	2676		2171		2167		2250	
G1	2881		2267		2333		2250	
2	2914	0.7	2354	2.4	2233	3.2	2250	0
3	2919		2373		2133		2250	
H1	3036		2267		2633		2500	
2	3044	8.6	2354	0.9	2500	2.6	2500	6.0
3	3518		2373		2576		2250	
I1	2494		2004		2033		2500	
2	2781	5.6	2344	8.2	2067	1.6	2250	5.6
3	2754		2279		2100		2500	
J1	3444		2920		2800		2500	
2	3634	5.2	2890	1.0	2600	5.3	2500	0
3	3274		2860		2533		2500	

density expressed in cells/sqmm.
cv= coefficient of variation (%).

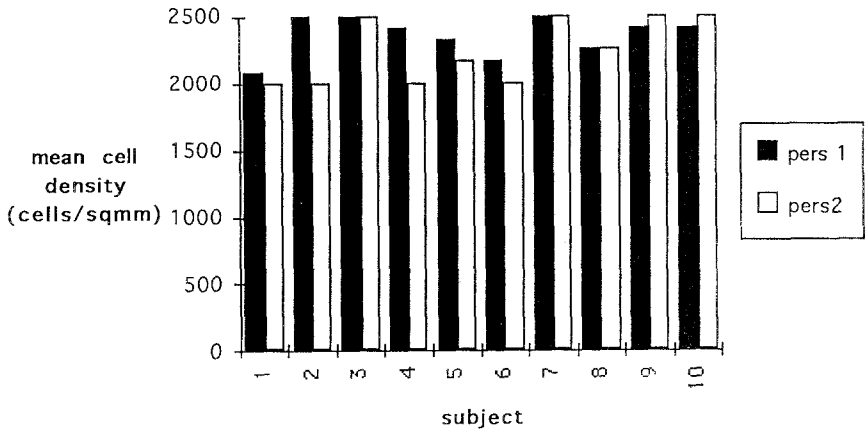


Fig. 8. Topcon-grid measurements of the mean cell density of the 10 corneas performed by 2 different observers. One-way analysis of variance showed no significant difference (F=3.4, p=0.1).

Table 2. Repeatability

	Zeiss	Keeler Konan	Topcon-count	Topcon-grid
Mean c.v.	4.7%	3.4%	3.9%	3.8%
ANOVA $p > 0.05^*$	10/10	9/10	n.p.	n.p.

c.v. = coefficient of variation.
* Number of corneas out of ten whith no significant difference between three measurements.
n.p. = not performed.

variance between these three counting methods showed a non-significant difference (F=1.9, p=0.2). Plotting the individual mean cell density against the age also shows a higher mean cell density for the Zeiss microscope (Fig. 7). Nevertheless, all four methods show a negative correlation with age.

Repeatability

We calculated the coefficient of variation for all measurements (Table 1). This was also performed for the Topcon-grid values, eventhough it was not appropriate, because three similar estimations do not give any variation. Table 2 shows that the repeatability for all methods is practically the same. For analyzing the significant difference between three measurements we needed the

individual cell sizes per image, so we could only perform a one-way analysis of variance on measurements made with the Zeiss and Keeler Konan.

For the Topcon-grid method a second observer processed the same images (Fig. 8). The mean difference with the first observer was 4.9%, which is not significant.

Hexagonality

Although we analyzed the total polygonality, we only represent the percentage of hexagonal cells in Table 3. The chi-square test was performed on the total polygonality for the two methods and separately for the three measurements per cornea. We did not find a significant difference between the Zeiss and the Keeler Konan measurements ($p=0.23$). There was a significant difference in two cases out of ten both for the Zeiss and Keeler Konan.

Discussion

Our results show that the accuracy of measuring the mean cell density of the corneal endothelium with the Zeiss, Keeler Konan and Topcon specular microscopes are practically the same. For clinical purposes however, the Topcon specular microscope is more advantageous than the other two methods, since it is the most rapid way to record and analyze specular images. But for more precise measurements an image processing system is indispensable. We could not adapt our computer analyzing program to the Topcon microscope, because the auto-focus device could not focus on the heamatocytometer, which is needed for calibration. One of the advantages of using the computerized analysis system for the Zeiss an Keeler Konan microscopes, is the possibility of storing images. With the Topcon only three images per eye can be stored till the next recording session, during which the new images overwrite the old ones.

Alteration in morphology such as abnormalities in cell size and shape may be more reliable indices of endothelial distress than the mean cell density alone [2]. The other advantage of using a computerized system is the possibility to calculate the individual cell area, with which the coefficient of variation can be deduced to measure the degree of polymegatism. In addition, the modality of analyzing the polygonality (i.e. the percentage of hexagonality), measures the change in cell shape.

We analyzed 39 to 78 cells per image with the Zeiss and 85 to 128 cells per image with the Keeler Konan (Table 4). With the Topcon-count method we analyzed approximately 54 to 76 cells. Recommendations have been made to use a sample of at least 75 to 100 cells [2, 7], which could be performed with the Keeler Konan. The relatively small sample sizes of

Table 3. Percentage of hexagonal cells

Subject	Zeiss		Keeler Konan	
	hexagonal cells (%)	Chi-square test (p)	hexagonal cells (%)	Chi-square test (p)
A1	68		52	
2	60	0.04	60	0.72
3	51		56	
B1	61		64	
2	62	0.5	57	0.83
3	61		65	
C1	48		70	
2	61	0.15	67	0.48
3	64		57	
D1	64		49	
2	60	0.15	67	0.02
3	61		71	
E1	68		67	
2	53	0.09	60	0.4
3	64		51	
F1	65		60	
2	57	0.18	60	0.95
3	57		65	
G1	58		60	
2	53	0.1	59	0.93
3	68		61	
H1	60		64	
2	63	0.35	56	0.64
3	70		53	
I1	65		61	
2	82	0.03	72	0.52
3	75		63	
J1	55		67	
2	70	0.1	47	0.04
3	68		61	

Table 4. Number of cells counted

Subject	Zeiss	Keeler Konan
A1	74	120
2	78	105
3	64	115
B1	63	89
2	56	107
3	56	90
C1	62	105
2	54	101
3	60	110
D1	65	115
2	64	103
3	68	117
E1	63	93
2	61	103
3	60	111
F1	43	98
2	54	89
3	55	85
G1	66	100
2	62	95
3	68	92
H1	67	114
2	56	109
3	55	126
I1	51	97
2	41	100
3	39	88
J1	56	124
2	50	128
3	60	123

the Zeiss can be explained by the fact that our system consists of a variable frame analysis enclosed in a fixed frame, as a consequence the sample size is inversely proportional to the cell size. More importantly the non-contact specular microscope only visualizes a small central field of the endothelium [17]. With the wide-field specular microscope the field is larger.

The automated determination of the polygonality showed about the same percentage of hexagonality as Nishi and Hanasaki [15] reported in their study (between 48% and 70%, and between 49% and 70% for the Zeiss and Keeler Konan, respectively, vs 43% and 63.6%).

In accordance with other studies we found a negative correlation between the cell density and increase in age [17]. The decrease of cell density in our study varied between 0.3% and 0.7% per year.

Mean cell densities have been described between 1500 and 3500 cells/mm^2 for an age between 40 and 90 years [1], 2200 and 4000 cells/mm^2 for an age between 20 and 60 years [8], and 2300 and 3215 cells/mm^2 for an age between 21 and 64 years [14]. The Zeiss measured mean cell densities between 2676 and 3848 cells/mm^2, the Keeler Konan between 2084 and 2890 cells/mm^2, the Topcon, counting the cells in squares, between 1997 and 2644 cells/mm^2 and the Topcon, using the grids, between 2093 and 2510 cells/mm^2. The results of the Zeiss are more in accordance with the other studies described than the other results.

Although we found a statistically significant difference between the Zeiss measurements and the measurements of the other three methods, we do not think this is clinically meaningful. But the three microscopes can not be used interchangeably. This could only be possible with the Keeler Konan and Topcon specular microscopes. However, for more precise measurements as in carefully designed research the difference between the three specular microscopes may be important. In that case only one specular microscope should be used consistently. Also if more morphometric precision is required a computer analyzing system is preferable.

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