

Gas Stress Test for Assessment of Corneal Endothelial Function

Nobuyuki Ohguro,* Mamoru Matsuda,*
Masakatsu Fukuda,* Shigeru Kinoshita[†] and Yasuo Tano*

**Department of Ophthalmology, Osaka University Medical School, Osaka, Japan;*

[†]*Department of Ophthalmology, Kyoto Prefectural College of Medicine, Kyoto, Japan*

Purpose: This study was undertaken to evaluate the endothelial pump function by monitoring both corneal swelling response under hypoxia and dehydration response following hypoxia in vivo.

Methods: Humidified nitrogen gas was used to obtain corneal swelling, and humidified gas mixed with oxygen and nitrogen was used for corneal dehydration. First, in 6 young volunteers, we investigated the most suitable oxygen level for evaluating pump function by changing oxygen levels. Then, with the optimal oxygen level, we attempted to evaluate pump function in 53 normal subjects, 5 Fuchs' dystrophy patients, and 3 iridocorneal endothelial syndrome (ICE) patients.

Results: Swelling rate showed similar values regardless of age, but both dehydration rate and swelling rate plus dehydration rate decreased with aging. The swelling rate of 5 guttata corneas was significantly higher than that of age-matched control corneas. In contrast, dehydration rate markedly decreased in guttata corneas, while the swelling rate plus dehydration rate of guttata corneas was comparable to that of age-matched corneas. In the 3 ICE corneas, however, swelling rate, dehydration rate, and swelling rate plus dehydration rate were markedly lower than those of both the fellow corneas and the age-matched control corneas.

Conclusion: These observations lead us to conclude that in order to evaluate pump function, it is necessary to monitor not only dehydration response following hypoxia but also swelling response under hypoxia. **Jpn J Ophthalmol 2000;44:325-333** © 2000 Japanese Ophthalmological Society

Key Words: Corneal endothelium, gas stress test, pump function.

Introduction

The integrity of the corneal endothelium is necessary for the maintenance of normal corneal transparency. The main function of the endothelium is to control corneal hydration for optimum clarity. This is accomplished by means of an active metabolic pump and a barrier to fluid flow between endothelial cells. The mechanism of corneal hydration control can be explained by the "pump-leak" theory of Mau-

rice, which states that hydraulic pressure pulls water into the stroma through intercellular spaces (endothelial barrier) and the fluids are pumped back out by the active endothelial pump function.¹ The leak rate through the endothelial barrier normally equals the metabolic pump rate; however, if corneal oxygen supply is sufficiently reduced, corneal metabolic activity is adversely affected, thereby increasing stromal lactate, reducing active pump function, and increasing corneal hydration.²⁻⁶ These responses appear to be reversible when normal oxygen levels are restored.

Several investigators have monitored corneal dehydration following hypoxic stress to evaluate endo-

Received: March 8, 1999

Correspondence and reprint requests to: Nobuyuki OHGURO, MD, Department of Ophthalmology, Osaka University Medical School E7, 2-2 Yamadagaoka, Suita, Osaka 565-0871, Japan

thelial pump function in vivo (stress test).^{7–14} Corneal dehydration results from the difference between the restored endothelial pump rate and the fluid leak rate through the endothelial barrier, while corneal swelling under hypoxia is induced by the difference between the decreased endothelial pump rate and the fluid leak rate. For this reason, we believed that monitoring both corneal swelling response under hypoxia and dehydration response following hypoxia might provide a more reliable indicator of endothelial pump function in vivo.

In previous stress test studies, hypoxia was obtained using thick hydrogel contact lenses with eyes closed, and dehydration response was then observed with naked eyes closed (contact lens stress test).^{7–14} This method of contact lens stress test, however, has two problems. One is that a corneal swelling response using thick hydrogel contact lens with eyes closed might be affected by hypoxia as well as a variety of other factors, including decreased corneal endothelial pH,¹⁵ increased corneal metabolic activity accompanying contact lens wear,¹⁶ and higher corneal temperature when contact lenses are worn with eyes closed.¹⁶ In fact, Holden et al¹⁷ have already demonstrated that a swelling response under hypoxia obtained with contact lens was significantly greater than that obtained with humidified nitrogen gas. The second problem is that although corneal dehydration with closed eyes is considered to be induced by the endothelial pump function under humidified 7% oxygen concentration ($PO_2 = 55$ mm Hg),¹⁸ there has been no evidence to date that corneal dehydration following hypoxia is affected by various oxygen concentrations.

In light of these problems, we chose to use humidified nitrogen gas to evaluate the corneal swelling response induced by hypoxia alone, and humidified gas mixed with oxygen and nitrogen to monitor corneal dehydration in order to control oxygen concentration (gas stress test). First, we tried to find the optimal oxygen level for evaluating endothelial pump function in the gas stress test. Then, with that oxygen level, we attempted to evaluate endothelial pump function by monitoring both swelling response under hypoxia and dehydration response following hypoxia.

Materials and Methods

Subjects

Fifty-three volunteers (mean age, 35.8 ± 18.0 ; range, 19–72) were enrolled in this study. Each volunteer was free of ocular disease and had no prior contact lens experience. Three patients with iri-

docorneal endothelial syndrome (ICE) corneas and 5 with Fuchs' dystrophy (5 corneas) were also enrolled in this study. All patients with ICE syndrome had typical abnormal corneal endothelium, peripheral anterior synechiae, and distortion of the iris (pupillary irregularity or anterior stromal traction tears) in 1 eye only.^{19–22} In the 5 guttata corneas, the guttata were present across the width of the cornea without corneal edema. These were designated as "moderate" guttata, as reported by Krachmer et al²³ and Wilson and Bourne.²⁴ Corneas with stromal edema or abnormal intraocular pressure were eliminated from the study in the selection of ICE syndrome and guttata corneas. The purpose and methods of this study were explained fully to all subjects, and an informed consent was obtained from each subject.

Gas Stress Test

In order to obtain a hypoxic stimulus, a gas-tight eye-cup swimmer's goggle was fitted to each subject, thus forming a separate atmospheric chamber in front of the eyes. The goggle was equipped with inlet and outlet tubes through which humidified gas was circulated. Humidified 100% nitrogen gas was sent to each eye after being bubbled through water at 25°C through the inlet port of the goggle (N_2 phase). After monitoring corneal hydration during the N_2 phase (swelling response), humidified gas mixed with oxygen and nitrogen (O_2 phase) was directed into each chamber to monitor corneal dehydration (dehydration response). During gas stress test, the room temperature was kept constant at 25°C and the flow rate of the gases was also kept constant at 1 L/h. The goggles were taken off only for a few minutes when corneal thickness was being measured.

Corneal Thickness

Central corneal thickness was measured by lightly touching the gel-filled probe of an ultrasonic pachymeter (UP2000; Nidek, Gamagori, ultrasonic speed 1640 m/s) to the anterior surface of the cornea. The probe was fixed with slit-lamp microscope in the same way that Goldmann's applanation tonometer is, and the position of the central cornea was controlled by fixation and alignment light. Baseline corneal thickness was measured at least 3 hours after subjects awoke in order to eliminate any influence that sleep may have had on corneal thickness. Changes in corneal thickness during N_2 phase and O_2 phase were monitored every 30 minutes. Eight readings were obtained, and then both the highest

and the lowest values were omitted. Finally, the mean of the remaining six readings was used as the recorded measurement. Before measurement, a topical anesthetic was used on each cornea, and each corneal surface was examined by slit-lamp microscope with fluorescein before and after gas stress test.

Endothelial Morphology

Ten to 15 photographs of the central 3–4 mm of each volunteer's cornea were taken with a wide-field specular microscope. The photograph with the most clearly visible cell boundaries was chosen for each eye. One hundred adjacent cells were analyzed with a computerized digitizer.^{25,26} Endothelial morphology was quantified by measuring a variety of factors, including cell density, coefficient of variation in cell size, and percentage of hexagonal cells.

Experiment

In the first part of the experiment, 6 normal corneas (right eye) from 6 young volunteers (2 men and 4 women: mean age, 20.5 ± 2.1 ; range, 19–24 years) were used to investigate whether corneal dehydration following hypoxia depends on oxygen concentrations. We selected corneas with similar baseline corneal thickness because corneal thickness may affect swelling pressure.¹ After baseline corneal thickness was measured, the gas stress test was performed four times with four different O₂ phases on the same 6 volunteers on separate days; that is, each subject was exposed four different times to a combination of 3 hours of 100% humidified nitrogen gas and 3 hours of the following four oxygen environments, each at a different time: (1) humidified 7%, (2) humidified 20%, (3) humidified 50%, and (4) humidified 95% oxygen gas. Since both swelling during N₂ phase and dehydration during the O₂ phase of all corneas happened in a linear manner (Figure 1), both swelling and dehydration rates ($\mu\text{m}/\text{h}$) were calculated by linear regression analysis. In the end, humidified 50% oxygen gas was selected for the O₂ phase.

In the second part of the experiment, the same 6 volunteers were used to study whether both swelling and dehydration rate could be evaluated in a shorter duration gas stress test for clinical application. After baseline corneal thickness was measured, each subject was exposed to humidified 100% nitrogen gas for 1 hour and then to humidified 50% oxygen gas for 1 hour (1-hour gas stress test). Since all corneas again swelled during the N₂ phase and dehydrated during the O₂ phase in a linear manner, both swelling and dehydration rates ($\mu\text{m}/\text{h}$) were again

calculated by linear regression analysis. In the third part of the experiment, 53 subjects, ages 19 to 72 years, with normal corneas were enrolled to investigate the effect of aging on the swelling response under hypoxia and dehydration response following hypoxia with the 1-hour gas stress test. In addition, this 1-hour gas stress test was applied to the 3 patients with ICE syndrome and the 5 patients with guttata corneas (right eye) to study whether the test could be used to successfully evaluate abnormal endothelial functions in these corneas.^{27–30}

Analysis

Corneal swelling under hypoxia is induced by the difference between the decreased endothelial pump rate and the fluid leak rate, while corneal dehydration following hypoxia is caused by the difference between the restored endothelial pump rate under any given oxygen level and the fluid leak rate.^{1–6,31–33} Therefore, dehydration rate plus swelling rate might provide a more adequate indicator of the endothelial pump function than the dehydration rate.

In our analysis, statistical significance was evaluated by one-way analysis of variance (ANOVA), one-way repeated measures ANOVA, unpaired *t*-test, and all the relationships between age and swelling rate, dehydration rate, swelling rate plus dehydration rate, baseline corneal thickness, and endothelial morphology, respectively. Any differences detected by these statistical tests were interpreted as significant when the null hypothesis could be rejected with reasonable confidence ($P < .05$).

Results

Swelling rate in the first part of the experiment showed similar values in four trials on each subject, and baseline corneal thickness was comparable in each test (Table 1). Both dehydration rate and swelling rate plus dehydration rate significantly increased with oxygen concentration levels up to 50%, and achieved a plateau beyond 50% (Figure 2).

Swelling rate, dehydration rate, and swelling rate plus dehydration rate in the 3-hour gas stress test in which humidified 50% oxygen gas was used as the O₂ phase for 3 hours were almost equal to those in the 1-hour gas stress test (Table 2). There was no correlation between baseline corneal thickness and age, swelling rate and age, but both dehydration rate and swelling rate plus dehydration rate decreased with aging (Figure 3). As for the relationships between age and endothelial morphology, while the coefficient of variation in cell size increased with aging

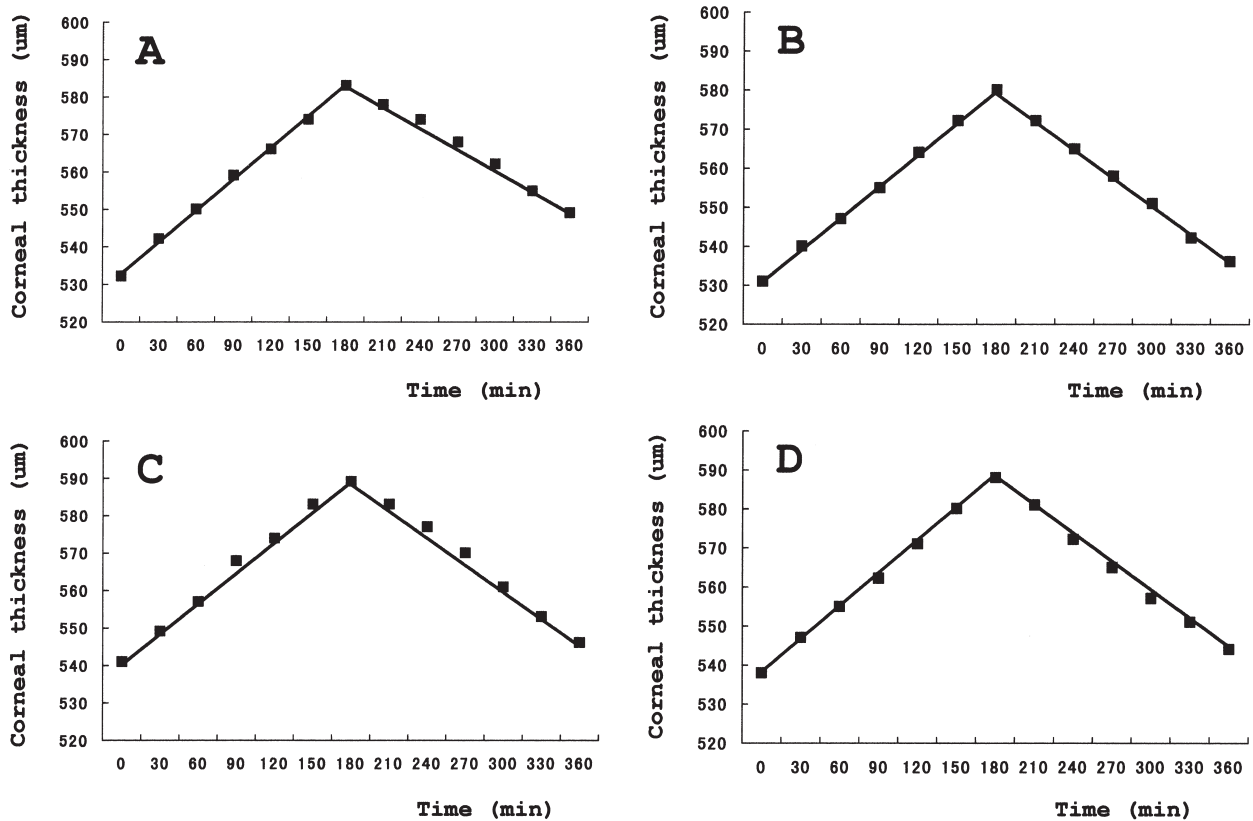


Figure 1. Typical swelling and dehydration using four different oxygen concentrations. Cornea swelled in N₂ phase and dehydrated in O₂ phase in almost linear manner. (A) N₂ phase: $Y = 533.036 + 16.643t, R^2 = 0.998, P < .0001, t = 55.857$. O₂ phase (7% oxygen): $Y = 584.143 - 11.429t, R^2 = 0.994, P < .0001, t = -28.284$. (B) N₂ phase; $Y = 531.143 + 16.286t, R^2 = 0.999, P < .0001, t = 90.125$. O₂ phase (20% oxygen): $Y = 579.786 - 14.714t, R^2 = 0.999, P < .0001, t = -63.878$. (C) N₂ phase: $Y = 541.143 + 16.571t, R^2 = 0.997, P < .0001, t = 41.012$. O₂ phase (50% oxygen): $Y = 590.857 - 14.857t, R^2 = 0.997, P < .0001, t = -41.110$. (D) N₂ phase: $Y = 538.143 + 16.571t, R^2 = 0.999, P < .0001, t = 74.878$. O₂ phase (95% oxygen): $Y = 587.607 - 14.786t, R^2 = 0.998, P < .0001, t = -47.489$. Each value represents mean of six readings remaining after omitting both highest and lowest values. Standard deviations were below 2% of each value.

($r = 1.537, P < .0001$), and percent of hexagonal cells decreased with aging ($r = -.248, P < .0001$), endothelial cell density had no correlation with aging ($r = -.995, P = .5761$).

While swelling rate of the 5 guttata corneas were significantly higher than that of 5 age-matched corneas and dehydration rate markedly decreased in the guttata corneas, swelling rate plus dehydration rate

Table 1. Dehydration Response at Several Oxygen Concentrations

Status of O ₂ Phase	Swelling Rate (μm/h) (Barrier Function)	Dehydration Rate (μm/h) (Apparent Pump Function)	Swelling Rate Plus Dehydration Rate (μm/h) (True Pump Function)	Baseline Corneal Thickness (μm)
7% oxygen (n = 6)	16.2 ± 0.4	9.1 ± 1.2	25.3 ± 1.4	538 ± 8
20% oxygen (n = 6)	16.1 ± 0.8	13.6 ± 0.7	29.7 ± 0.8	535 ± 5
50% oxygen (n = 6)	16.6 ± 0.6	14.6 ± 0.8	31.2 ± 1.3	537 ± 12
95% oxygen (n = 6)	16.3 ± 0.9	15.0 ± 0.4	31.3 ± 1.2	535 ± 7

There is statistically significant difference ($P < .001$) by one-way repeated measures analysis of variance. To isolate group or groups that differ from the others, all pairwise multiple comparison procedures (Tukey test) were performed. We found statistical significance ($P < .05$) in the following comparisons: 7% oxygen vs. 20% oxygen, 7% oxygen vs. 50% oxygen, 7% oxygen vs. 95% oxygen, and 20% oxygen vs. 95% oxygen in both dehydration rate and swelling rate plus dehydration rate.

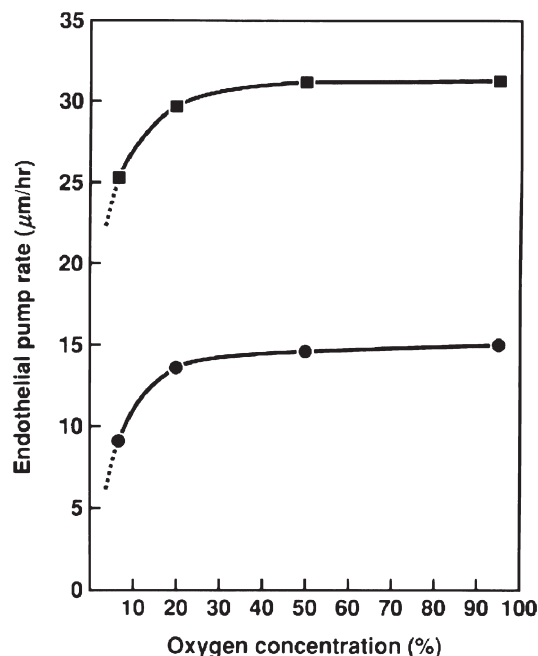


Figure 2. Relationship between oxygen concentration and swelling rate plus dehydration rate (■: true pump function) and between oxygen concentration and dehydration rate (●: apparent pump function). Both swelling rate plus dehydration rate and dehydration rate increased with oxygen concentrations to the 50% oxygen level, and reached plateau beyond the 50% oxygen level.

of the guttata corneas was comparable to that of age-matched corneas (Table 3). On the other hand, swelling rate, dehydration rate, and swelling rate plus dehydration rate of the 3 ICE corneas were markedly lower than those of both the fellow corneas and the age-matched control corneas (Table 4), although there was no significant difference in each parameter between the fellow corneas and age-matched control corneas. Baseline corneal thickness of the 5 guttata corneas and the 3 ICE corneas was comparable with that of age-matched corneas.

During and after the gas stress test, none of the subjects experienced any complications, and pooling of fluorescein was not observed by slit-lamp exami-

nation in any of the tested corneas after the gas stress test.

Discussion

In the first part of this study, we have three important findings: first, both swelling rate and dehydration rate can be calculated by linear regression analysis; second, dehydration rate depends on oxygen concentration levels on the corneal surface; and third, a 50% oxygen level is the most suitable concentration in the gas stress test.

Although previous contact lens stress test studies demonstrated that dehydration response following hypoxia was not linear,^{7,8} our findings showed that dehydration rate could be calculated by linear regression analysis. While corneal edema did decrease in an exponential fashion following hypoxia in those studies, the data also suggested that dehydration response seemed to follow a linear pattern up to 3 hours.^{7,8} Moreover, previous results showing that swelling response under 100% humidified nitrogen gas was linear for up to 3 hours agreed with our results.⁴

Therefore, we believe that it was reasonable to calculate both swelling and dehydration response with linear regression analysis in the gas stress test.

As to the issue of whether dehydration rate depends on oxygen concentration levels, our findings conflict with O'Neal and Polse's⁷ previous contact lens stress test study. In that study, dehydration response following hypoxia with eyes open in humidified air (20% humidified oxygen gas) was similar to that with eyes closed (7% humidified oxygen gas¹⁸), while our study indicates that dehydration rate depends on oxygen concentration levels. This difference may be due to the higher corneal temperature with eyes closed compared to that with eyes open in humidified air.¹⁶ It is generally known that enzyme activity depends on temperature, and previous temperature reversal experiments^{34,35} have indicated that the activity of Na₂K-ATPase, which is a key enzyme in active endothelial pump function,³⁶ also de-

Table 2. Comparison of Both Swelling rate and Dehydration Rate Between 3-Hour and 1-Hour Gas Stress Tests

	Swelling Rate (µm/h) (Barrier Function)	Dehydration Rate (µm/h) (Apparent Pump Function)	Swelling Rate Plus Dehydration Rate (µm/h)
3-hour gas stress test (n = 6)	16.6 ± 0.6	14.6 ± 0.8	31.2 ± 1.3
1-hour gas stress test (n = 6)	15.3 ± 1.4	14.2 ± 1.1	29.5 ± 2.4
P-value (unpaired t-test)	P = 0.0732	P = 0.4253	P = 0.1617

50% humidified oxygen gas was used as O₂ phase in both 3-hour and 1-hour gas stress test.

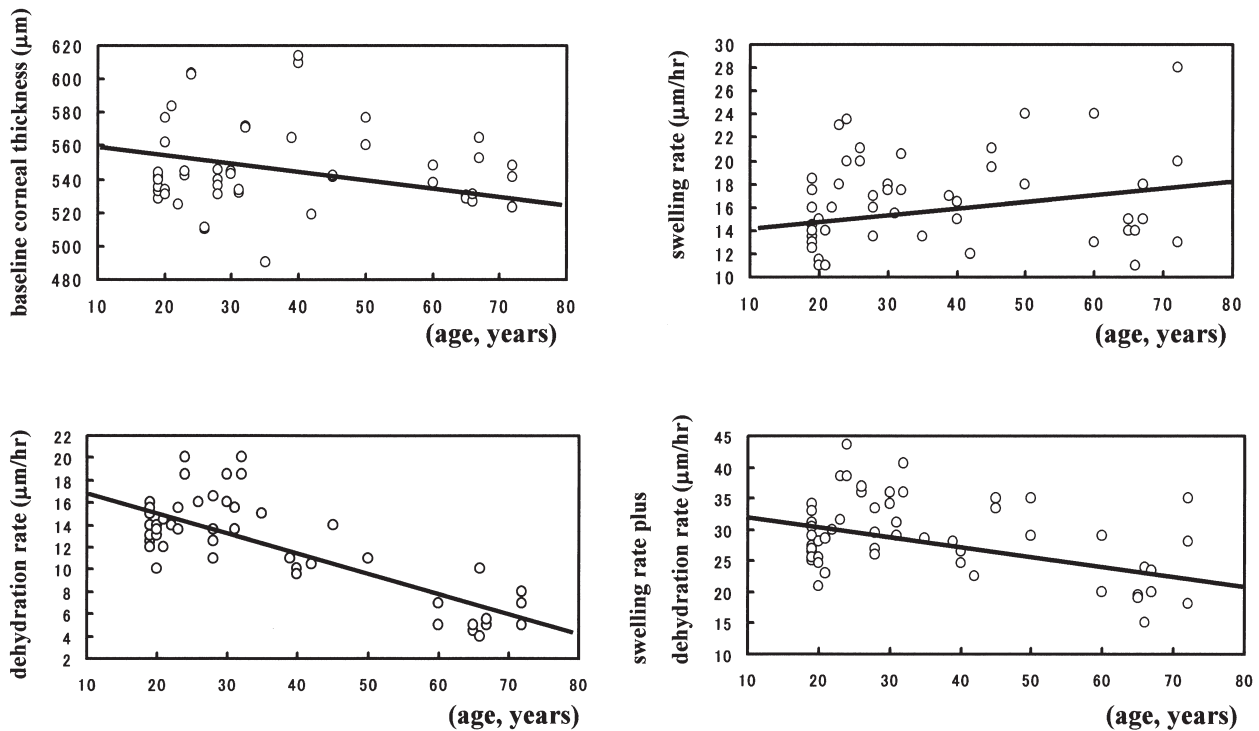


Figure 3. Relationship between age and baseline corneal thickness, swelling rate, dehydration rate, and swelling rate plus dehydration rate. Neither baseline corneal thickness nor swelling rate had any correlation with age ($r = -0.093, P = .6365$; $r = 0.043, P = .1417$). On the other hand, both dehydration rate and swelling rate plus dehydration rate decreased with age ($r = -0.179, P < .0001$; $r = -0.142, P = .0018$).

depends on corneal temperature. This suggests that, when evaluating dehydration response following hypoxia, it is very important to keep all other factors besides hypoxia constant. By using humidified gas, we can keep these factors constant, and at the same time control oxygen concentration.

Our third finding, that swelling rate plus dehydration rate is affected by oxygen concentration levels on the corneal surface, is perhaps the most interesting finding in the first part of this study. Since pump function under hypoxia can be considered as the constant factor in each cornea, we can see from Figure 2 that endothelial pump function increases with oxygen concentration levels up to 50% and plateaus

beyond 50%. Since the increase of oxygen concentration on the corneal surface results in the increase of available oxygen tension in the corneal endothelium,^{37,38} it is clear that endothelial pump function depends on oxygen concentration levels, and it follows that since endothelial pump function was found to be working to its maximum at 50%, that level is best for evaluating total pump function.

In the second part of this study, we found that there was virtually no difference in the results from the 3-hour gas stress test and 1-hour gas stress test. This clearly shows that the 1-hour gas stress test is sufficient to evaluate the swelling response under hypoxia and dehydration response following hypoxia.

Table 3. Swelling and Dehydration Response in Guttata Corneas

	Swelling rate (µm/h) (Barrier Function)	Dehydration Rate (µm/h) (Apparent Pump Function)	Swelling Rate Plus Dehydration Rate (µm/h) (True Pump Function)	Baseline Corneal Thickness (µm)	Age (Years)
Guttata corneas (n = 5)	26.2 ± 6.1	5.2 ± 1.8	31.4 ± 6.1	555 ± 38	52.8 ± 15.0
Age-matched corneas (n = 5)	16.4 ± 2.7	9.8 ± 3.7	26.2 ± 1.8	539 ± 52	52.4 ± 15.9
P-value (unpaired t-test)	P = .0112*	P = .0368*	P = .1535	P = .6082	P = .9684

*p < .05.

Table 4. Swelling and Dehydration Response in Iridocorneal Endothelial (ICE) Syndrome Patients

	Swelling Rate ($\mu\text{m}/\text{h}$) (Barrier Function)	Dehydration rate ($\mu\text{m}/\text{h}$) (Apparent Pump Function)	Swelling Rate Plus Dehydration Rate ($\mu\text{m}/\text{h}$) (True Pump Function)	Baseline Corneal Thickness (μm)	Age (Years)
ICE corneas (n = 3)	5.7 \pm 1.5	3.7 \pm 1.2	9.3 \pm 2.5	547 \pm 60	40.7 \pm 6.0
Fellow corneas (n = 3)	13.7 \pm 0.6	9.7 \pm 0.6	23.3 \pm 0.6	522 \pm 49	40.7 \pm 6.0
Age-matched corneas (n = 3)	14.5 \pm 3.1	12.2 \pm 2.5	26.7 \pm 3.6	529 \pm 44	40.0 \pm 5.0

There is a statistically significant difference ($P = .003$) by one-way analysis of variance. To isolate group or groups that differ from the others, all pairwise multiple comparison procedures (Tukey test) were performed. We found statistical significance ($P < .05$) in following comparisons. ICE corneas vs. fellow corneas, ICE corneas vs. age-matched corneas in swelling rate, dehydration rate, and swelling rate plus dehydration rate.

In the third part of this study, we concluded, based on our findings, that 1-hour gas stress test is a reliable method to evaluate endothelial pump function in vivo. This conclusion, however, requires some explanation, as the findings on which it is based at times appear to contradict the results of several previous studies.

Our 1-hour gas stress test showed that both swelling rate plus dehydration rate and dehydration rate decreased with aging. With pump function under 50% oxygen considered as the total pump function and pump function under hypoxia considered as the constant factor in each cornea, our results indicate that the total pump function decreases with aging, contrary to previous results which indicate that Na₂K-ATPase pump site density remains constant with aging.³⁹ This present finding suggests that pump activity decreases with aging, as pump capacity is a function of both the number of pumps and their activity. The swelling rate, on the other hand, was found not to correlate with aging, indicating the barrier function would have to be constant with aging. This agrees with previous studies showing that there is no correlation between age and the permeability to fluorescein.⁴⁰ Thus, in normal aging, the total pump function decreases while the barrier function keeps constant, and apparent pump function (dehydration rate) has a good correlation with the true pump function (swelling rate plus dehydration rate).

Our finding that the total pump function of moderate guttata corneas is comparable to that of age-matched control corneas, indicated by the normal swelling rate plus dehydration rate of the guttata corneas in our study, concurs with recent findings that the Na₂K-ATPase pump site density of moderate guttata corneas increases significantly more than that of corneas with normal endothelium.²⁹ The markedly higher swelling rate of the guttata corneas in our study, which agrees with clinical evidence that

some patients with guttata corneas experience morning blur, indicates that the barrier function markedly decreases in guttata corneas. This agrees with previous studies showing that endothelial permeability of guttata corneas significantly increases compared to that of normal corneas.^{27,28} Thus, it would appear that in moderate guttata corneas, normal pump function is maintained in order to keep corneal transparency, despite their markedly decreased barrier function.

It is interesting to note that while at least one previous contact lens stress test study concluded that lower dehydration response indicates decreased endothelial pump function in guttata corneas,¹⁰ our results indicated that lower dehydration rate of guttata corneas results from decreased endothelial barrier function. It is our belief that this lower dehydration rate suggests only that the recovery from corneal edema caused by some stress will be slower in guttata corneas. This agrees with previous clinical findings that, after cataract extraction, guttata corneas tend toward longer periods of postoperative corneal edema, which occasionally results in pseudophakic bullous keratopathy.⁴¹ Monitoring swelling response, therefore, seems to be important in evaluating endothelial pump function.

Finally, regarding the ICE corneas in our study, we reached two conclusions. In these ICE corneas, the total pump function could not be estimated because of markedly lower swelling response. On the other hand, the markedly decreased swelling rate must have been induced by increased barrier function. This is supported by previous studies indicating that in ICE patients, endothelial permeability to fluorescein was within normal limits in the fellow corneas, yet the permeability in the fellow corneas was six times that in the abnormal corneas.³⁰ Epithelial-like cells on the posterior corneal surface in ICE corneas have also been reported, and tight junctions were observed between these cells.⁴² As for ICE syn-

drome, although it has been viewed primarily as a unilateral disease, endothelial morphological changes observed in the contralateral eyes have been reported.⁴³ Although we cannot be sure, our findings in our patients with ICE syndrome suggest that they might have functionally abnormal corneal endothelium in 1 eye only. Further investigations must be done about ICE syndrome.

The findings reported here and the analyses presented above lead us to conclude that corneal hydration control is not yet fully understood. Analysis of the dehydration response following hypoxia represents only the apparent endothelial pump function, which has a good correlation with the true pump function only in normal endothelium, not in abnormal endothelium such as guttata corneas. Endothelial function, including pump function and barrier function, can best be evaluated by analyzing both swelling and dehydration responses in the gas stress test.

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