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Cornea & External Disease

Normal Corneal Thickness and Endothelial Cell Density in Rhesus Macaques (*Macaca mulatta*)

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Purpose: To define the normal range of central corneal thickness (CCT) and corneal endothelial cell density (ECD) in rhesus macaques *(Macaca mulatta*) and the effects of age, body weight, sex, and intraocular pressure (IOP) on these parameters.

Methods: Ophthalmic examinations were performed on 144 rhesus macaques without anterior segment pathology. The CCT was measured via ultrasound pachymetry (USP) and specular microscopy, and the ECD was semiautomatically and manually counted using specular microscopy. Rebound tonometry was used to measure IOP. Linear regression and mixed-effects linear regression models were used to evaluate the effects of age, body weight, sex, and IOP on CCT and ECD.

Results: We included 98 females and 46 males with an age range of 0.2 to 29.4 years. The mean CCT by USP and specular microscopy were 483 ± 39 and 463 ± 33 µm, respectively, and were statistically different ($P < 0.001$). The ECDs were 2717 ± 423 and 2747 ± 438 cells/mm^2 by semiautomated and manual analysis, respectively. Corneal endothelial degeneration was identified in one aged rhesus macaque.

Conclusions: The mean USP and specular microscopy CCT values differed significantly, whereas the semiautomatic and manual ECD did not. The CCT was associated with the IOP and sex, whereas the ECD was associated with body weight and age ($P < 0.05$). As in humans, corneal disease in rhesus macaques is uncommon.

Translational Relevance: Establishing reference values is fundamental to use rhesus macaques as a model for corneal disease or to identify toxicity in studies of ocular drugs or devices.

Introduction

In humans, corneal diseases are a common cause of blindness.[1] Nonhuman primates (NHP) are valuable animal models to study ocular diseases owing to their similar ocular development, anatomy and physiology with humans.[2–5] In particular, rhesus macaques (*Macaca mulatta*) have been useful in studying the development of therapies for diseases that affect the corneal stroma and endothelium.[2,6–10] The corneal endothelium is the innermost layer of the cornea and is composed of a single layer of highly metabolic cells responsible for maintaining corneal deturgescence, transparency, and refractive power.[11] Disturbance of the stromal architecture or corneal endothelial cell dysfunction can lead to changes in the corneal thickness with concomitant decreases in visual acuity.[11]

Normative data are a valuable reference for research and can aid in understanding the limitations and translatability of animal models to human biology. Normative data on central corneal thickness (CCT) and endothelial cell density (ECD) are useful to understand corneal physiology. A previous study reporting ECD in rhesus macaques[12] did not include cell area data or provide correlations with other ocular and biometric parameters. Thus, the purpose of this work was to provide normative data for CCT and ECD from 144 rhesus macaques and determine their relationship to body weight, age, sex, intraocular pressure (IOP). The axial length (AXL) of the eye and refractive error were also measured. Finally, we also compared agreement between manual and semiautomated analysis for determining ECD as well as use of ultrasound pachymetry (USP) versus specular microscopy for measuring CCT.

Methods

Animal Care

All rhesus macaques in the present study were cared for and examined at the California National Primate Research Center, an accredited Association for Assessment and Accreditation of Laboratory Animal Care International institution. All procedures were performed following the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocol approved by the Institutional Animal Care and Use Committee at UC Davis.

Ophthalmic Examination

A total of 144 rhesus macaques underwent a single, complete ophthalmic examination. All data were derived from normal animals either before use in a study, or from a screening program to identify animals with age-related or potentially inherited ocular abnormalities. Primates with corneal or anterior chamber abnormalities were excluded from this study. Ketamine (5–30 mg/kg intramuscularly [IM]), dexmedetomidine (0.015–0.075 mg/kg IM, and midazolam (0.10 mg/kg IM) were administered before the examination and imaging. In some of cases, an additional smaller dose of these medications was given to extend time under anesthesia when necessary. Anesthetics were administered by California National Primate Research Center staff under the direction of a veterinarian. Animals were always monitored by a trained technician and veterinarian. Ocular examinations were performed with the primate in supine position. The IOP measurements took place with the animal held upright. Ocular imaging tests were done prone with the chin on a chin rest. The eyelids were kept open using a speculum, and regular corneal lubrication (GenTeal tears, Alcon, Geneva, Switzerland) was applied regularly during the exam. Studies included external color photography (Rebel T3 EOS, Canon, New York, NY, USA) and rebound tonometry (TonoVet, Icare Oy, Vantaa, Finland) for both eyes. Noncontact specular microscopy (Topcon SP-2000P; Topcon Corp., Tokyo, Japan) was performed to evaluate the corneal endothelium in one eye per animal with laterality chosen at random. After hand-held slit lamp examination (SL-17, Kowa Optics, Los Angeles, CA), streak retinoscopy (Welch-Allyn, Inc., Skaneateles, NY) was performed following cycloplegia with tropicamide 1% (Akorn Inc, Lake Forest, IL), phenylephrine 2.5% (Paragon BioTeck, Inc., Portland, OR), and cyclopentolate 2% (Alcon Laboratories Inc, Fort Worth, TX) to estimate the refractive error in both eyes. Corneal thickness was measured using USP (Pachette 4, DGH Technology Inc., Exton, PA) in both eyes. Finally, A-scan ocular biometry (Sonomed Pacscan Plus, Escalon, Wayne, PA) was performed to determine the AXL of both globes. To reverse the anesthesia, atipamezole at a comparable dose to dexmedetomidine was used after examinations were completed.

Data for CCT was collected using specular microscopy and USP. The ECD was semiautomatically calculated with the same specular microscope with the Endothelial Cell Analysis Module in the IMAGEnet 2000 software package (Topcon Corp.). The ECD measurements from the central cornea were used for this study; the central ECD is considered to be representative of the full cornea.[12] A simplified cell analysis method was used in the IMAGEnet 2000 endothelial cell analysis software to determine the ECD and cell area using the center method, in which the analyst manually selects the center of the endothelial cell.[13,14] For this study, at least 30 contiguous corneal endothelial cells were selected. To ensure accuracy of the ECD values, one of the authors (M.I.C) estimated the corneal ECD by planimetry, which involved selecting images of good quality ($n = 114$) and manually calculating the area of five representative corneal endothelial cells within an area of 0.036 mm^2 using the standardized grid displayed by the endothelial cell analysis module as a reference.

Statistical Analyses

To calculate agreement between USP and specular microscope for CCT values and between manual and semiautomated ECD counts, a concordance correlation coefficient (CCC) and coefficient interval were calculated using values obtained from the same eye. For the CCC, the results were interpreted as previously described, with values of greater than 0.75 indicating

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good agreement, values between 0.40 and 0.75 indicating moderate agreement, and values of less than 0.4 indicating poor agreement.[15–17] Normality was determined by the Anderson–Darling test for normality, and the *t*-test with Welsh correction was used to compare statistical differences between USP and specular microscope for CCT values and between manual and semiautomated ECD counts.

A mixed-effects linear regression model was used to evaluate the correlation of an individual's body weight, age, and sex to the CCT and ECD. Each NHP was treated as a random effect and all other variables were considered fixed effects. Reference ranges were calculated as a range of ±2 standard deviations from the mean.[18] The statistical analysis was carried out in R using R packages epiR, *lme4*, and lmerTest and GraphPad Prism version 9.3.1.[19]

Results

A total of 144 rhesus macaques were examined in this study, of which 98 were female and 46 were male, with ages ranging from 0.2 to 29.4 years (Fig. 1). Of the 144 rhesus macaques examined, one presented an abnormal corneal ECD and cell morphology and was excluded from further analysis.

The mean IOP for the remaining 143 primates was 16 ± 4 mm Hg, with a range of 7 to 29 mm Hg (Table 1).

The mean CCT was 483 ± 39 µm using USP and 463 ± 33 µm using specular microscopy, a significant difference between the two techniques ($P < 0.001$) (Table 1). The CCC was 0.47 (95% confidence interval, 0.36–0.57), indicating moderate consistency between CCT generated by USP and specular microscopy (Fig. 2A1, 2A2). For the 114 NHPs that had ECD estimated by semiautomatic and manual analyses, mean ECD was 2719 ± 439 and 2747 ± 438 cells/mm^2, respectively (Table 1), which was not significantly different ($P = 0.24$). The CCC was 0.88 (95% confidence interval, 0.83–0.91), indicating a strong consistency between the ECD calculated by the two analysis techniques (Fig. 2B1, 2B2). The mean ECD and corneal endothelial cell area for the 143 primates undergoing specular microscopy were 2717 ± 423 cells/mm^2 and 377 ± 59 µm^2, respectively (Table 1). Regarding refractive error and AXL, one highly myopic NHP was found (−17 diopters [D] in both eyes) with a markedly high AXL (26.24 and 25.20 mm for the right eye [OD] and the left eye [OS], respectively). This primate was deemed to be an outlier and thus excluded from statistical analysis for these parameters. The median refractive error for the remaining 142 primates was 0.75 D (interquartile range, 0.25–1.25 D) and ranged from −3.75 to +11.5 D (Table 1). The median AXL calculated for 142 primates was 19.95 mm (interquartile range, 19.51–20.49 mm), and values ranged from 17.48 to 22.36 mm (Table 1). A linear model analysis found a direct relationship between AXL and age, with an increase of 1 mm in 24 years ($P < 0.0001$).

A mixed-effects linear regression model including sex, age, weight, and differences between left and right to study CCT by USP revealed that females had significantly thicker corneas than males, at 487 ± 41 and 477 ± 35 µm, respectively ($P = 0.024$). A significant correlation was found between IOP and CCT values, with an increase of 1.26 mm Hg for each 100-µm increase in CCT ($P = 0.015$) (Fig. 3). No significant correlations were observed between CCT and age ($P = 0.833$), CCT and weight ($P = 0.123$), CCT and refractive error ($P = 0.574$), or CCT and AXL ($P = 0.470$).

Using a mixed-effects linear regression model for semiautomated ECD measurements using the same parameters, body weight and age were significantly

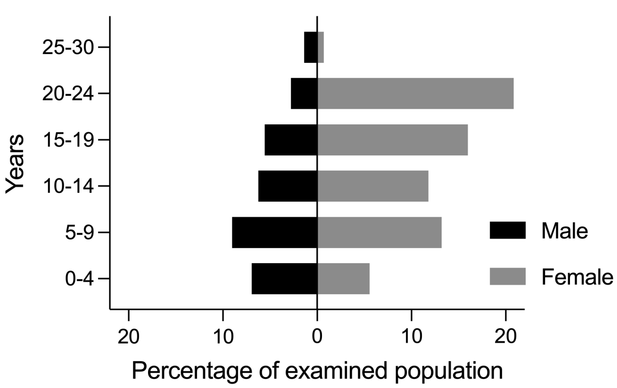

Figure 1. Age and sex of the 144 rhesus macaques examined in this study. The NHPs were divided into 6 age groups: 0 to 4 years (8 females, 10 males), 5 to 9 years (19 females, 13 males), 10 to 14 years (17 females, 9 males), 15 to 20 years, (23 females, 8 males), 20 to 24 years (30 females, 4 males), and 25 to 30 years (1 female, 2 males). Females were overrepresented ($n = 98$ [68%]).

Table 1. Age, Weight, and Ocular Biometric Findings, and Reference Ranges From 143 Rhesus Macaques With Healthy Corneas Included in the Study

	Mean ± Standard Deviation	Range	Reference Range
Age (years)	13.8 ± 7.1	0.2–29.4	
Weight (kg)	10.1 ± 3.8	1.0–23.9	
CCT USP (µm)	484 ± 39	367–593	406–562
CCT Specular (µm)	463 ± 33	388–568	403–529
ECD (cells/mm^2)	2717 ± 423	1853–3864	1871–3563
EC area (µm^2)	377 ± 59	258–539	259–495
IOP (mm Hg)	16 ± 4	7–29	8–24

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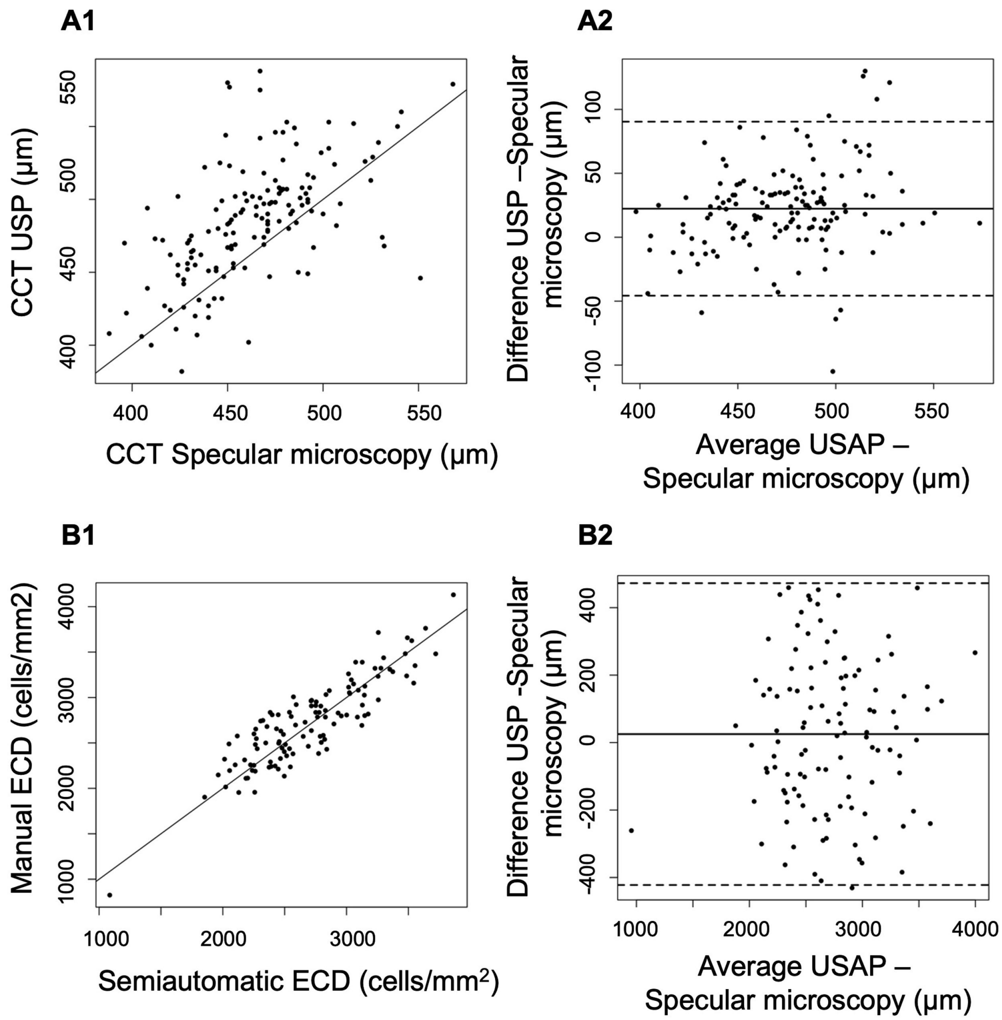

Figure 2. Scatterplots and Bland–Altman plots showing difference between USP and specular measurements for CCT (A1, A2), and difference between ECD semiautomatic and manual measurements (B1, B2) in 143 primates without corneal disease. For the Bland–Altman plots (A2, B2), the vertical axis shows the difference among the two types of measurements and the horizontal axis is the mean value of the two types of measurements. The *dashed lines* represent the 95% limits of agreement and the *black line* the mean of the differences between the two types of measurements. The CCC was 0.47 (0.36–0.57) for CCT measurements, and 0.88 (0.83–0.91) for ECD, indicating moderate and strong agreement between techniques, respectively.

negatively correlated with ECD (P = 0.006 and P < 0.0001, respectively). The ECD decreased 29 cells/mm^2 for every 1-kg increase in weight (Fig. 4A) and decreased 23 cells/mm^2 for each 1 year of increase in age (Fig. 4B and Fig. 5). As expected, the ECD and corneal endothelial cell area were strongly and significantly correlated (R^2 = 0.9985; P < 0.0001). A significant correlation was also observed between semiautomatic ECD and AXL (P = 0.032), with an increase of 80 cells/mm^2 per each millimeter of increase in the AXL; however, this correlation was not significant when using the manual ECD data (P = 0.860). No significant differences were observed between ECD and eye laterality (P = 0.380) or ECD and sex (P = 0.453). No significant correlations were observed between ECD and refractive error (P = 0.166), semiautomatic ECD and USP CCT (P = 0.894), or semiautomatic ECD and specular CCT (P = 0.891).

As for the excluded primate, when examining the ECD data for outliers (Fig. 6A), a geriatric male presented with transparent corneas (Figs. 6B, 6C) but low ECD in both OD and OS. The OD was examined when he was 19.6 years old and the ECD by semiautomatic specular microscopy was 1507 cells/mm^2 (average cell area, 663 ± 121 µm^2) (Fig. 6D). Three years later, the OS was examined and was also confirmed to have low ECD values, at 1086 cells/mm^2 (cell area, 920 ± 244 µm^2) (Fig. 6E). On ophthalmic examination of

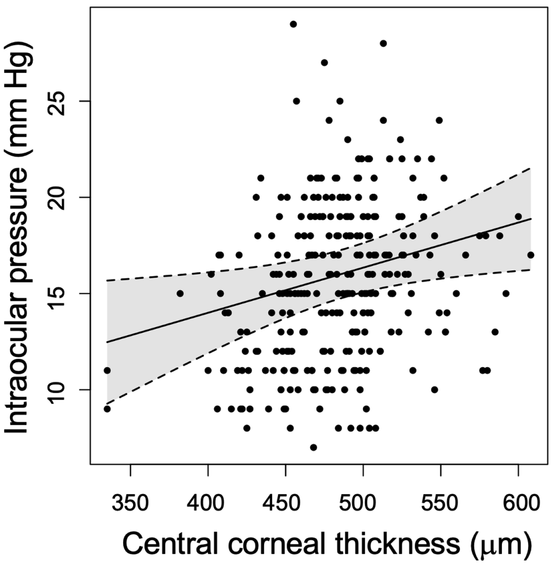

Figure 3. Scatterplot demonstrates a direct relationship between CCT measured with USP and IOP measured by rebound tonometry 286 eyes of 143 primates with healthy corneas. The area in gray corresponds with the 95% confidence interval. For every 1.26-mm Hg increase in IOP the CCT increases by 100 μm ($P = 0.015$; $R^2 = 0.07$).

this animal, nuclear sclerosis and anterior and posterior incipient cataracts were noted in both eyes, as well as vitreous degeneration. The IOPs were normal (19 mm Hg OD, 20 mm Hg OS), and no signs of active or chronic anterior uveitis were present. Although both corneas were transparent, the CCT was slightly thicker than the average CCT of the cohort examined at 541 and 538 μm for OD and OS, respectively. Thus, this NHP's corneas were considered abnormal, and his data were excluded from statistical analysis.

Discussion

The use of appropriate in vivo models that mimic the structure of the human cornea is key in the study of the pathophysiology of corneal diseases, as well as in the development of therapeutic strategies. Although other laboratory animal models have their own advantages, NHPs have similar corneal thicknesses, diameters, ECDs, and corneal endothelial cell regenerative capacities, all of which are properties that make them an excellent model for the study of corneal disease.[20,21]

The preservation of physiologic IOP values is essential for the maintenance of correct homeostasis and corneal characteristics. In this study, the mean IOP was similar to previous studies in captive[5] and free-ranging rhesus macaques[22] and humans.[23] As in humans and other studies in rhesus macaques, this study found that corneal thickness has a direct relationship with IOP.[5,24,25] Thus, CCT values should be taken into consideration when interpreting IOP measurements.

The determination of the CCT is essential in the diagnosis and monitoring of a wide range of corneal diseases and before ocular surgical procedures.[26,27]

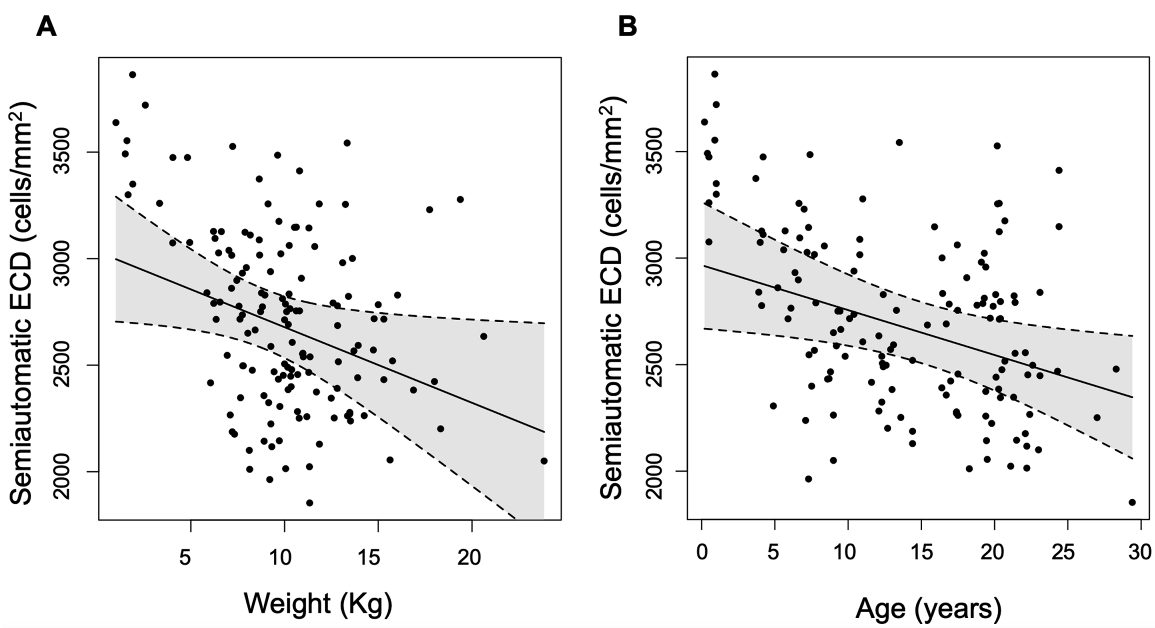

Figure 4. Scatterplot showing an indirect relationship between (A) ECD and body weight, and (B) ECD and age in 143 eyes of 143 primates with healthy corneas. For each 1-kg increase in body weight, the ECD decreases by 29 cells/mm² ($P = 0.006$; $R^2 = 0.17$). For each additional year of age, ECD decreases by 23 cells/mm² ($P < 0.0001$; $R^2 = 0.22$). The area in gray corresponds with the 95% confidence interval.

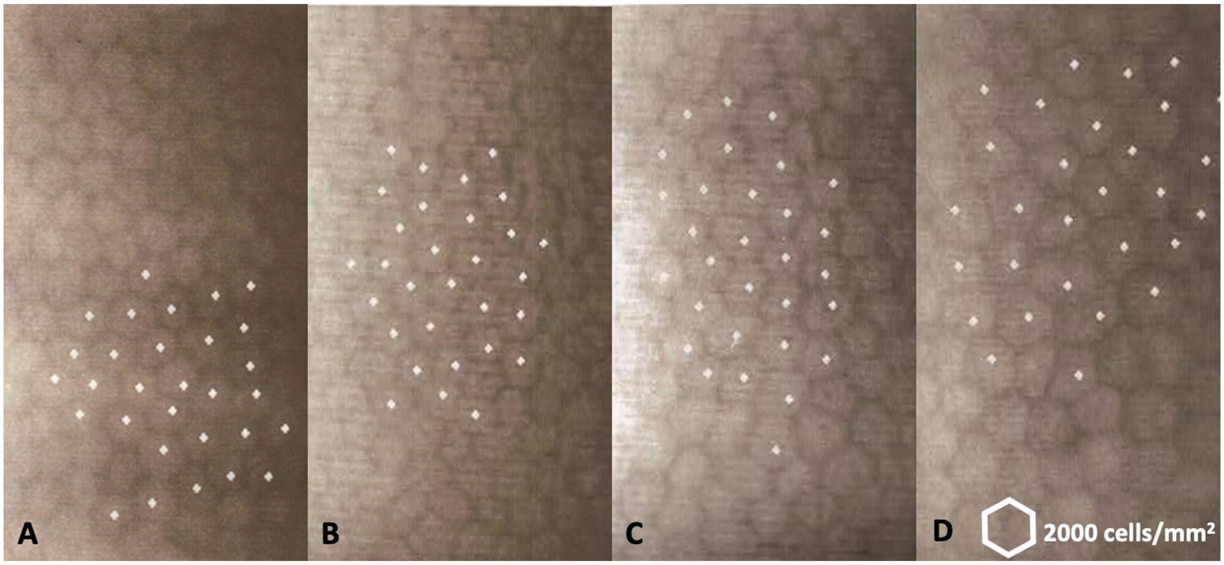

Figure 5. Corneal endothelial cell appearance using specular microscope in healthy rhesus macaques at different ages. Although the cell morphology remains regular and mostly hexagonal, a lower ECD and increased cell area were observed in older individuals. Rhesus macaques of 0.4 years (A) (3061 cells/mm^2; mean ± standard deviation cell area, 366 ± 69 µm^2), 10.8 years (B) (3088 cells/mm^2; mean cell area, 323 ± 51), 22.6 years (C) (2497 cells/mm^2; mean cell area, 400 ± 24 µm^2), and 29.4 years (D) (1853 cells/mm^2; mean cell area, 539 ± 17) are shown. *White dots* in the center of some cells were placed manually for analytical processing. Scale for reference in (D) applies to all images.

Although there are several techniques available to measure corneal thickness, USP remains the standard technique in humans.[26] Our study compared USP and specular microscopy for CCT measurements in rhesus macaques. Similar to human reports,[28,29] we reported the mean CCT by USP to be significantly higher than specular microscopy with moderate concordance between the two types of measurements, suggesting

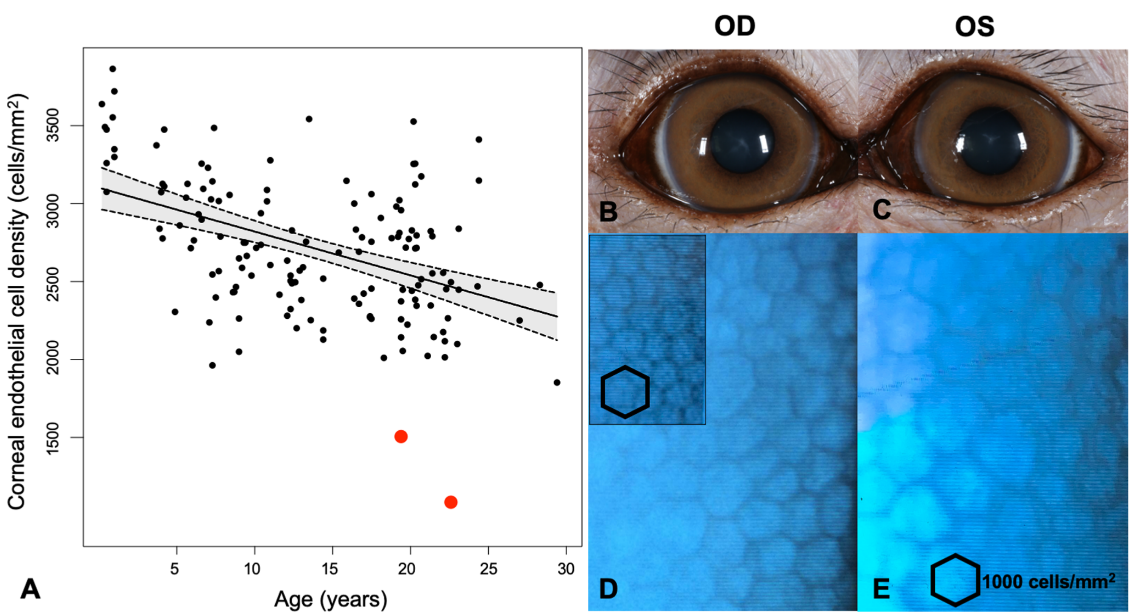

Figure 6. A geriatric male rhesus macaque with low corneal ECD, mild pleomorphism, and polymegathism in both eyes. In a scatterplot for analyzing outliers (A), the value for the right eye (OD) and the left eye (OS) of the rhesus macaque is outside of the cluster. The area in gray corresponds with the 95% confidence interval. Anterior segment appearance was normal OD (B) and OS (C). Specular microscopy revealed low ECD at 19.3 years old (D) (1507 cells/mm^2, OD) and at 22.6 years old (E) (1086 cells/mm^2, OS). Both eyes also had larger cells when compared with other primates (663 ± 121 µm^2 and 920 ± 244 µm^2 for OD and OS, respectively). Subjective loss of hexagonality of the endothelial cells was also apparent. The inset in (D) includes detail of the specular microscopy at same magnifications of a 21.4-year-old female with normal corneal endothelial morphology (ECD 2457 cells/mm^2).

that these two devices should not be used interchangeably. The USP CCT in this study was similar to previous studies in rhesus macaques using the same instrument (486 ± 38 µm),[5] and slightly thinner than human CCT measured by USP (535 ± 34 µm vs. 547 ± 35 µm).[30,31] In our study population, females had slightly thicker corneas than males by a mean of 10 µm and that difference was considered statistically significant using a mixed-effect linear regression analysis. Although most studies in humans do not find statistically significant differences between male and female CCT,[30,32] some studies have reported that variations in the CCT in females associate with the menstrual cycle.[33,34] Further studies would be required to deduce whether hormonal variation plays a role in corneal thickness in female rhesus macaques. In concordance with some previous studies done in humans, there were no significant age-dependent CCT differences found in our study.[32,35] However, there is an interesting debate regarding age-related changes in corneal thickness in humans, with some studies reporting increased CCT with age[36,37] and others finding the opposite relationship.[27,38] The reasoning for these differences may include inadequate sample size, genetic differences among sample populations, or sampling bias owing to the inclusion of patients with ocular, noncorneal alternations.[27]

Manual and semiautomatic ECD values were comparable in this study, supporting the reliability of semiautomatic determination of ECD with specular microscopy in rhesus macaques with healthy eyes. The ECD values obtained in this study are similar to the ones reported in humans (2800 cells/mm^2 and 2737 cells/mm^2 in 30-year-old adults).[39,40] Similar to humans, we have observed an age-related decline in ECD.[41,42] Our mixed-effects linear regression found a positive correlation between semiautomatic ECD and AXL that was not significant when the analysis was performed with manual ECD values. Although a correlation between the ECD and AXL has been described previously in humans,[43] the inconsistency between the results obtained using both datasets suggests that the effect of AXL in ECD is questionable. In accordance with Lin et al.,[5] we also found a correlation between AXL and age.

Fuchs endothelial corneal dystrophy (FECD) is the most common endothelial dystrophy in humans and is characterized by guttae formation on Descemet's membrane and premature degeneration and progressive loss of corneal endothelial cells that leads to corneal edema, bullous keratopathy, and vision loss.[11,44] With specular microscopy, patients diagnosed with FECD typically present with a low ECD, enlarged endothelial cells with loss of hexagonality, and small multifocal hyporeflective round excrescences of extracellular matrix known as guttae.[11,45] Of the 144 primates examined, one 22.6-year-old male had low corneal ECD with mild pleomorphism and polymegathism, which are characteristics described in humans with corneal endothelial disease.[46] Both eyes had an ECD below our lower limit of our reference range (1871–3563 cells/ mm^2), but were greater than what has been reported for corneal decompensation in humans (≤500 cells/mm ^{2}).[47,48] Both corneas were clear with a thickness approaching the upper limit of our reference range (406–562 µm). In aggregate, the findings in this primate are suggestive of a bilateral endothelial degeneration in a compensated stage. A primary cause for endothelial degeneration was not evident. Although guttae-like lesions were not observed in this NHP, it is possible that this rhesus macaque has a heritable corneal endothelial degeneration, similar to FECD in humans. In humans, peripheral corneal ECD has been shown to correlate with disease severity for FECD.[49]

Limitations of our study are the unbalanced number of females versus males included, as well as the lack of peripheral corneal endothelial cell analysis. Surveys of larger cohorts with specular microscopy that include the peripheral corneal endothelium may lead to the identification of additional NHPs with corneal endothelial disease and a better characterization of this condition.

In summary, in this study we report normative data and reference values of corneal ECD and corneal thickness and their relationships with age, body weight, sex, AXL, and IOP. As in humans, rhesus macaques with low ECD are rare, but can be found within the population. The measurements obtained expectedly align with human parameters, highlighting the similarity in anatomy as well as the value of NHP research models for the study of corneal diseases.

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