

Evaluation of the endothelium of the healthy equine cornea using a specular contact microscope

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[Avaliação do endotélio da córnea equina utilizando-se microscópio especular de contato]

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ABSTRACT

The corneal endothelium is important for maintaining corneal transparency. This study aimed to obtain data on endothelial cell density and the percentage of hexagonal cells in different regions of the equine corneal endothelium using contact specular microscopy. A total of 24 healthy eyes from 12 horses were included in this study. Images of the endothelium were obtained from five different regions of the cornea (central, superior, inferior, lateral, and medial regions); from each image, endothelial cell density and endothelial morphology values were obtained. The average endothelial density was 1705 cells/mm² in the central region, 1717 cells/mm² in the upper region, 1694 cells/mm² in the lower region, 1715 cells/mm² in the lateral region, and 1724 cells/mm² in the medial region. The percentage of hexagonal cells was 56.5% in the central region, 57.1% in the upper region, 56.5% in the lower region, 56.5% in the lateral region, 56.5% in the medial region. No significant differences in either cell density or hexagonality were observed between the different regions of the cornea. In a healthy cornea, the values of endothelial density and hexagonality are similar in all corneal regions of the cornea.

Keywords: corneal transplant, endothelial morphology, cell density, horse

RESUMO

O endotélio é importante para manter a transparência da córnea. Objetivou-se com este estudo obter dados sobre a densidade de células endoteliais e a porcentagem de células hexagonais em diferentes regiões do endotélio da córnea equina, por meio de microscopia especular de contato. Vinte e quatro olhos saudáveis de 12 cavalos foram analisados. Imagens do endotélio foram obtidas de cinco regiões diferentes da córnea (regiões central, superior, inferior, lateral e medial). De cada imagem foram obtidos os valores da densidade celular endotelial e da morfologia endotelial. A densidade endotelial média foi de 1705 células/mm² na região central, 1717 células/mm² na região superior, 1694 células/mm² na região inferior, 1715 células/mm² na região lateral e 1724 células/mm² na região mediana. A porcentagem média de células hexagonais foi de 56,5% na região central, 57,1% na região superior, 56,5% na região inferior, 56,5% na região lateral e 56,5% na região medial. Não foram observadas diferenças significativas na densidade celular ou na hexagonalidade entre as diferentes regiões da córnea analisadas. Na córnea saudável de equinos, os valores de densidade endotelial e da hexagonalidade são semelhantes em todas as regiões da córnea.

Palavras-chave: transplante de córnea, morfologia endotelial, densidade celular, cavalo

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INTRODUCTION

The endothelium is the innermost layer of the cornea. It is a monolayer of predominantly hexagonal cells with little variation in cell size (Joyce, 2003). The corneal endothelium is a layer of cells with minimal regenerative capacity (Joyce, 2003); endothelial losses can occur due to age or due to inflammation, increased intraocular pressure, trauma, or intraocular surgical procedures (Franzen *et al.*, 2010).

Specular microscopy is widely used in the analysis of endothelial parameters in humans (Abib and Barreto Jr, 2001). In animals, this technique has already been used to evaluate the corneal endothelium of some species, including dogs (Gwin *et al.*, 1982; Pigatto *et al.*, 2006, 2008), cats (Franzen *et al.*, 2010), chinchillas (Bercht *et al.*, 2015), chickens (Albuquerque *et al.*, 2015), pigs (Vargas *et al.*, 2023), and cows (Azevedo *et al.*, 2023), among other species. In addition to being useful for anatomical assessment, it is possible to use specular microscopy to study the safety of drugs and of corneal and intraocular surgical procedures (Eggeling *et al.*, 2000; Nagatsuyu *et al.*, 2014; Andrade *et al.*, 2019; Terzariol *et al.*, 2016). Using the microscope, specular parameters of the corneal endothelium can be quantified. Specular microscopy is also crucial in evaluating the endothelium before cataract surgery and in evaluating the donor cornea before transplantation.

The corneal endothelium of the equine species has been previously evaluated by non-contact specular microscopy (Andrew *et al.*, 2001), confocal microscopy (Ledbetter and Scarlett, 2009), and optical microscopy (Faganello *et al.*, 2016). Despite these published articles, no studies related to the analysis of endothelial parameters in different regions of the cornea using contact specular microscopy have yet been carried out. The aim of this study was to evaluate the equine corneal endothelium with a contact specular microscope and to quantify the endothelial cell density (ECD) and cell morphology in different regions of the healthy cornea.

MATERIAL AND METHODS

This study was carried out in accordance with ARVO (Association for Research in Vision and Ophthalmology) standards and was approved by the Research Committee of the College of Veterinary of the Federal University of Rio Grande do Sul. A total of 24 healthy eye bulbs from 12 horses were studied; the horses were male or female with ages ranging between 18 and 21 years. The eyes were provided by a local slaughterhouse (xxx). Immediately after slaughter both eyes from each animal were enucleated and kept in individual identified humid chambers until examination with specular microscope. Before endothelial analysis, all eyes underwent an ophthalmic examination that included fluorescein testing (1% Sodium Fluorescein, Allergan, Brazil) and biomicroscopy with a portable slit lamp (Portable Slit lamp SL15, Kowa, Japan). Only eyes with transparent corneas and eyes with a negative fluorescein test were included in the research.

Endothelial microscopy was performed within two hours after enucleation using a specular contact microscope (Celmax, Medical Service®, Brazil). After being removed from the humid chamber, the eyes were fixed on a support adapted to the contact specular microscope. The cornea was lubricated with saline solution and the objective lens of the microscope was positioned over the central, superior, inferior, lateral, and medial regions of the cornea for digital photographic recording of the endothelium. Two specular micrographs were captured from each region of the cornea; from each image, 30 cells were analyzed and both ECD and cellular morphology (hexagonality) values were obtained. All assessments were carried out by the same evaluator, using a process in which the center of each cell was marked by the examiner and the ECD was automatically obtained.

Quantitative variables with a normal distribution were described by mean and standard deviation and analyzed using the Analysis of Variance (ANOVA) test for repeated measures. A significance level of 5% was utilized, and all data were analyzed using the statistical program IBM® SPSS Statistics 21 (Statistical Package for the Social Sciences).

RESULTS

All enucleated eyes were included in this study. In images obtained using the contact specular

microscope, it was possible to observe a regular pattern of polygonal cells with clear, uniform, and juxtaposed edges in all of regions that were studied (Fig. 1).

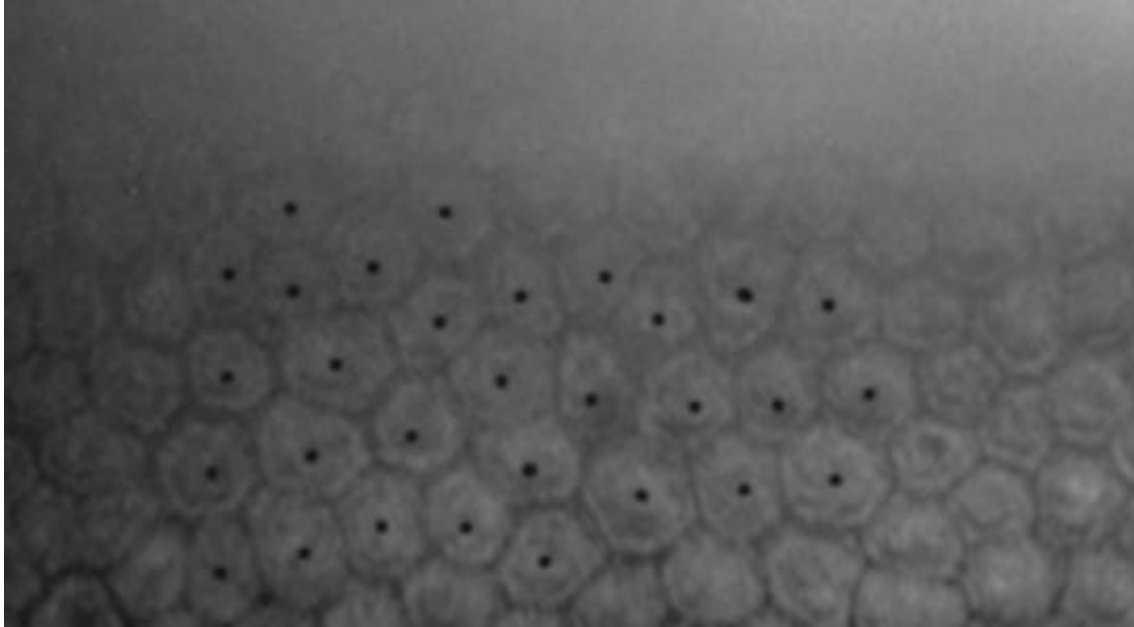


Figure 1. Specular microscopy of equine corneal endothelium. The center of selected endothelial cells was marked.

Average corneal ECD values in each region, in the left and right eyes, were as follows: central region, 1707 ± 195 cells/mm² in the right eye and 1704 ± 201 cells/mm² in the left eye; upper region, 1713 ± 194 cells/mm² in the right eye and 1722 ± 188 cells/mm² in the left eye; lower region, 1700 ± 237 cells/mm² in the right eye and 1689 ± 224 in the left eye; lateral region, 1722 ± 196 cells/mm² in the right eye and 1709 ± 200 cells/mm² in the left eye; and medial region, 1732 ± 206 cells/mm² in the right eye and 1717 ± 220 in the left eye. There were no significant differences in average corneal ECD between the five regions or between the right and left eyes.

Regarding the morphology of all of the cells that were analyzed, 56.6% of all cells had six sides, 21% had five sides, and 20.6% had seven sides; the proportion of cells with four or eight sides totaled 1.8%. The average percentage of hexagonal cells was 56.50% in the central region, 57.13% in the upper region, 56.58% in the lower region, 56.5% in the lateral region, and 56.7% in the medial region; there were no

significant differences in the percentages of hexagonal cells between the right and left eyes. There was also no significant difference in the hexagonality of corneal endothelial cells between the regions studied ($p > 0.05$ for all comparisons).

DISCUSSION

Specular microscopy has been the main technique used in humans to analyse the parameters of the corneal endothelium (Abib and Barreto Jr, 2001). In veterinary medicine, the specular microscope has been employed for evaluation of the healthy corneal endothelium of some animal species (Pigatto *et al.*, 2008; Franzen *et al.*, 2010; Albuquerque *et al.*, 2015; Bercht *et al.*, 2015; Brambatti *et al.*, 2017; Coyo *et al.*, 2018; Vargas *et al.*, 2023). Furthermore, it has been used to analyze the corneal endothelium in dogs with cataracts and in dogs after cataract removal (Nagatsuyu *et al.*, 2014).

Specular microscopy has a major limitation, which is the difficulty of obtaining images in non-transparent corneas. Therefore, *ex vivo*

studies are often necessary under these conditions to enable the analysis of corneal endothelial cells (Faganelo *et al.*, 2016). In the present study, it was possible to obtain images of all eyes that had been analyzed because all corneas were healthy and transparent at the time of the specular microscopy examination; keeping the eyes in a humid chamber helped to keep the corneas transparent. Previous studies had concluded that keeping the eyes stored in a humid chamber until six hours after death preserves the integrity of the cornea (Franzen *et al.*, 2010; Pigatto *et al.*, 2008).

In horses, few studies have been carried out to analyze the corneal endothelium (Andrew *et al.*, 2001; Ledbetter and Scarlett, 2009; Faganello *et al.*, 2016). Specular microscopy is a diagnostic modality used to obtain images and analyze the parameters of the corneal endothelium. There is no consensus regarding the regional distribution of corneal endothelial parameters (Coyo *et al.*, 2018; Faganelo *et al.*, 2016). To the best of the authors' knowledge, this is the first study that used the specular contact microscope to analyze the different regions of the cornea of healthy equine eyes. Knowledge of the parameters of endothelial density and morphology in different regions of the cornea is important to better understand endothelial behavior. Furthermore, the region with the greatest reserve of endothelial cells may be chosen for corneas that will be used as donors for transplantation.

In the present study, evaluation of the endothelium of the healthy equine cornea was performed using a specular contact microscope. The great advantage of contact specular microscopy is that it is possible to measure and analyze parameters from different regions of the cornea. In addition to specular microscopy, confocal microscopy has also been used to analyze animal corneas *in vivo* (Ledbetter and Scarlett, 2009). For *ex vivo* analyses, optical microscopy and scanning electron microscopy are alternative methods that have already been used to evaluate the corneal endothelium (Pigatto *et al.*, 2005; Faganelo *et al.*, 2016). Previous studies report that the preparation of corneas for studies using optical microscopy and scanning electron microscopy (SEM) results in variable degrees of cellular retraction, thus reducing the original cellular area and increasing endothelial density (Virtanen *et al.*, 1984; Tamayo-Arango

et al., 2009). Unlike other endothelial analysis methods such as SEM and optical microscopy, specular microscopy does not cause distortions in the corneal endothelium. Cell retraction occurs during the preparation of corneas for SEM analysis, which causes changes in the density of endothelial cells (Virtanen *et al.*, 1984; Tamayo-Arango *et al.*, 2009). These methods are applicable methodologies for analyzing the endothelial morphology and ultrastructure of corneal endothelial cells and for evaluating drug toxicity in the cornea (Pigatto *et al.*, 2005; Andrade *et al.*, 2019; Clerot *et al.*, 2019).

In the current study, despite the eyes having undergone enucleation, the data obtained can be compared with parameters obtained in live animals. Contact specular microscope used in the present study allows the analysis of the endothelium in enucleated eyes and in living patients.

Horses are animals that are highly dependent on vision, with a high propensity to develop eye diseases and experience trauma that often require surgical procedures. In the current study, the eyeballs that were collected and analyzed belonged to animals destined for slaughter. As a result, no animals were sacrificed for reasons exclusively related to research. The eyes were stored in a humid chamber within four hours after death, allowing the preservation of endothelial parameters for obtaining images with specular microscopy. The eyes collected after slaughter were promptly analyzed and the corneas did not suffer any changes resulting from pre-analytical preparation; all eyes were analyzed within 6 hours after the animals were slaughtered. Previous research using the same methodology has shown that the corneal endothelium was intact within six hours after slaughter (Pigatto *et al.*, 2008; Tamayo-Arango *et al.*, 2009; Franzen *et al.*, 2010; Albuquerque *et al.*, 2015; Brambatti *et al.*, 2017).

To estimate endothelial parameters in each image obtained using the specular microscope, a group of endothelial cells are analyzed (Villalba *et al.*, 2014). There is no consensus regarding the number of cells that should be analyzed, and the number of endothelial cells analyzed in each image varies depending on the study. Some authors have recommended counting 30 cells, whereas others recommended the inclusion of 50

to 100 cells per image (Melo *et al.*, 2008; Faganelo *et al.*, 2016). In the present study, two images of each region of the cornea were analyzed and 30 cells were included in each image.

When evaluating the corneal endothelium, the main parameters used are ECD and cell morphology (Abib and Barreto Jr, 2001; McCarey *et al.*, 2008). These parameters are also used to determine the quality of the cornea that will be used for transplantation.

In the present study, ECD and the percentage of hexagonal cells in different regions of the cornea were analyzed. The most commonly used methods for quantifying corneal endothelium parameters include the frame method (fixed or variable), center-to-center method, flexible center method, corner method, and comparison method (Villalba *et al.*, 2014). Regardless of the method used, the accuracy of the assessment depends on the quality of the endothelial image obtained by specular microscopy (Abib and Barreto Jr, 2001). In the present study, the cell-center-marking method, which was available in the equipment being utilized, was used to determine ECD; after the selected cells were marked, a cell density measure was provided by the software included in the device.

In horses, a pioneering study on the corneal endothelium was carried out using non-contact specular microscopy in 2001 (Andrew *et al.*, 2001). In the study by Andrew and colleagues, the average cell density was 3155 ± 765 cells/mm², and there was no statistical difference between the right and left eyes for ECD. The authors also observed that cell density decreased significantly with advancing age. In the present study, the average cell density among regions varied between 1694 and 1794 cells/mm². The animals in the present study were adults, ranging between 18 and 21 years old. In chinchillas, the reported average cell density obtained through specular microscopy in groups of different ages varied between 2124 and 3423 cells/mm², with animals between two and four months of age showing the highest average. (Bercht *et al.*, 2015). In rabbits, researchers found similar values, with average cell density varying between 1868 and 2307 cells/mm² (Brambatti *et al.*, 2017). In owls, cell density values between 2602 and 2864 cells/mm² were reported by a study that also demonstrated

higher average cell density in younger animals (Coyo *et al.*, 2018).

In the current study, it was possible to observe that the equine endothelium is made up of a monolayer of polygonal cells, with a predominance of hexagonal cells observed in all the regions that were evaluated. The percentage of hexagonal cells ranged between 71% and 79%. The hexagonality found in the central region was 56.5%, in the upper region it was 57.1%, in the lower region it was 56.6%, in the lateral region it was 56.5%, and in the medial region it was 56, 7%. On average, 21% of cells had five sides and 20.6% of cells had seven sides totaled. In a morphological study of equine endothelial cells that was performed using optical microscopy with vital dyes, Faganello and collaborators reported that the endothelial mosaic was composed mostly of hexagonal cells (57.6%), followed by pentagonal cells (20.7%), heptagonal cells (19.5%). and octagonal cells (2.3%). In the present research, there was no significant difference in the distribution of hexagonal cells between the five regions evaluated. Based on the results obtained, it can therefore be suggested that, when selecting equine corneas for transplantation, donor buttons can be chosen from any region of the cornea as there are no differences in endothelial parameters related to the region of the cornea.

In the present study, no differences in the parameters evaluated were observed between the right and left eyes. This corroborates what has already been observed in other species such as humans, dogs, cats, chickens, chinchillas, rabbits, and owls (Pigatto *et al.*, 2008 Franzen *et al.*, 2010; Albuquerque *et al.*, 2015; Bercht *et al.*, 2015; Brambatti *et al.*, 2017; Coyo *et al.*, 2018). To carry out the analysis, both male and female animals were used, as it has previously been reported that there are no significant differences in endothelial parameters between males and females (Tamayo-Arango *et al.*, 2009; Brambatti *et al.*, 2017).

CONCLUSIONS

Using contact specular microscopy, it was possible to systematically analyze the endothelium of five different regions of the equine cornea; the results showed that the values of ECD and hexagonality of the central region of

the endothelium can be extrapolated to the other regions of the cornea. The parameters obtained in the present study will help in the evaluation of the equine corneal endothelium as well as in the selection of corneas for transplantation.

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