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Specular microscopy of the corneal endothelial cells of bovines: an *ex vivo* study

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Abstract

Background: The endothelium is the most posterior layer of the cornea and is essential for maintaining corneal transparency. Due to variations in corneal endothelial parameters among different species, knowledge of the normal parameters for each species is crucial.

Aim: To evaluate the corneal endothelium of bovines using contact specular microscopy.

Methods: Twenty eyeballs from 10 male Brangus (*Bos taurus*) aged 24 months were evaluated. Contact specular microscopy was performed on the central corneal area. The analyzed parameters were endothelial cell density (ECD) and endothelial cell morphology.

Results: The ECD in the central area was 1,277 cells/mm². Regarding the morphology, mainly cells with six (74.3%), five (14.7%) and seven sides (10%) were found. There were no significant differences in ECD and morphology between left and right eyes.

Conclusion: Contact specular microscopy facilitated the analysis and measurement of corneal endothelial parameters in bovines. The data obtained will serve as a reference for the analysis of bovine corneal endothelium.

Keywords: Cornea, Endothelium, Morphology, Cattle.

Introduction

The endothelium constitutes the posterior layer of the cornea (Miller, 2001). During embryogenesis, it derives from the neural crest and is formed by a single layer of polygonal cells arranged in an organized mosaic; in most species, the predominant endothelial morphological pattern is hexagonal (Bahn *et al*., 1986; Tuft and Coster, 1990; Joyce, 2003). The main function of the endothelium is the preservation of corneal transparency via the regulation of stromal hydration through the endothelial barrier function (Taylor and Hunt, 1981; Tuft and Coster, 1990; Wen *et al.*, 2001; Mimura *et al.*, 2013).

In most species, endothelial repair is limited. When endothelial cells are damaged, adjacent cells migrate toward the injury, resulting in hypertrophy and reestablishment of their function (MacCallum *et al.*, 1983; Nishida *et al.*, 2022). In humans, it is believed that the minimum cell density required for the functional maintenance of the corneal endothelium is 500 cells/mm² (Zavala *et al.,* 2013). Endothelial density is influenced by and declines due to factors related to aging, surgical or inflammatory trauma, and endothelial diseases. In cases of significant endothelial loss, where the remaining cells do not restore their function, endothelial decompensation occurs, causing corneal edema and visual deficits (Tuft and Coster, 1990; Abib and Barreto, 2001; Spinozzi *et al.,* 2021).

Endothelial evaluation through specular microscopy provides a non-invasive, accurate and reliable clinical analysis of quantitative and qualitative parameters of the corneal endothelium. Therefore, this technique is also employed for research purposes in the evaluation of intraocular drug toxicity and corneal surgical procedures (Abib, 2000; De Sanctis *et al*., 2006; Mccarey *et al*., 2008; Benetz and Lass, 2018; Coyo *et al.,* 2018a, 2018b; Chaurasia and Vanathi, 2021).

Endothelial parameters have been determined in several species, including humans, lagomorphs, alpacas, llamas, goats, horses, birds, pigs, dogs, and cats (Abib and Barreto, 2001; Pigatto *et al*., 2008; Tamayo-Arango *et al.,* 2009; Franzen *et al.,* 2010; Coyo *et al.,* 2018a, 2018b; Chaurasia and Vanathi, 2021).

Due to variations in corneal endothelial parameters among different species, knowledge of the normal parameters for each species is essential. Such knowledge is also a prerequisite for recognizing pathological alterations related to this layer of the cornea. However, studies related to endothelial parameters of bovine species are scarce (Bahn *et al.*, 1986). The aim of this study is to analyze and quantify the parameters of the

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corneal endothelium of a bovine species (*Bos taurus*) using contact specular microscopy.

Materials and Methods

The study was approved by the Research of Committee of the Federal University of Rio Grande do Sul. The eyes were donated by a local slaughterhouse. Twenty eyeballs from 10 Brangus breed male bovines aged 24 months were studied. Enucleation was made immediately after slaughtering, and the eyes were labeled and stored in a moist chamber until analysis under a specular microscope. All ocular bulbs underwent an ophthalmic examination which included biomicroscopy with a slit lamp (Kowa SL-15, Nagoya, Aichi, Japan) (Fig. 1) and a fluorescein test (1% sodium fluorescein, Allergan, Sao Paulo, Brazil) (Fig. 2). Only healthy eyes were selected. Only eyes with transparent corneas and eyes with a negative fluorescein test were included in the study. All analyses were carried out within 4 hours after slaughter. For endothelial evaluation, a contact specular microscope (Celmax, Medical Service, Sao Carlos, Brazil) was used. All evaluations were carried out by the same examiner. After being removed from the wet chamber, the eyes were placed on a support adapted to the contact specular microscope and lubricated with ophthalmic eye drops (Lacri, carboxymethylcellulose). The objective lens of the specular microscope was positioned in the central region of the cornea, at a 90° angle between the evaluated structure and the device (Figs. 3 and 4). From each cornea, we obtained one micrograph, and 30 endothelial cells were analyzed in each image. Cell density was calculated using software coupled to the specular microscope (Celmax® specular microscope). Endothelial morphology was obtained through manual assessment of the number of sides of each cell. The values obtained were the means of each identified parameter.

Statistical analysis

Quantitative variables were analyzed by entering the data into Excel and subsequently exporting them to IBM* SPSS v. 20.0 (Statistical Package for the Social Sciences) for statistical analysis. Endothelial cell density (ECD) and endothelial cell morphology were described by means and 95% confidence intervals. Comparison among eyes was performed using Student's *t*-test for paired samples. A significance level of 5% was considered for established comparisons.

Ethical approval

The study was approved by the Research of Committee of the Federal University of Rio Grande do Sul. The eyes were donated by a local slaughterhouse. The animals were slaughtered for the production of meat and meat products and not for reasons related to the study.

Results

All enucleated eyes were included in the study. All eyes had a transparent cornea. Using the contact specular microscope, it was possible to assess the corneal endothelium of the bovine eyes and obtain clear images of all analyzed eyes. A regular and continuous pattern of juxtaposed cells, with sharp borders, was observed in the central region of the bovine corneal endothelium (Fig. 5). The average values of cell density and endothelial morphology are different from the values found in other species already studied (Table 1).

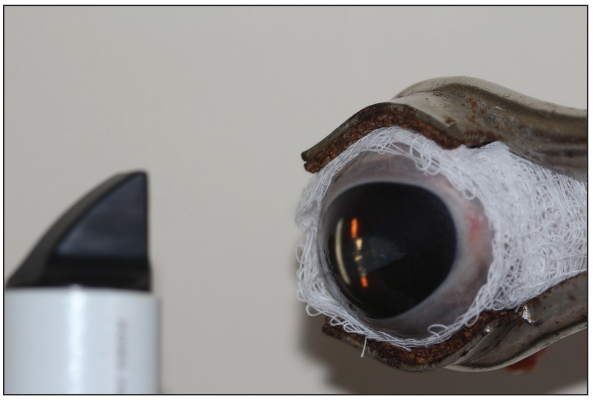

Fig. 1. Slit lamp examination of a bovine eye.

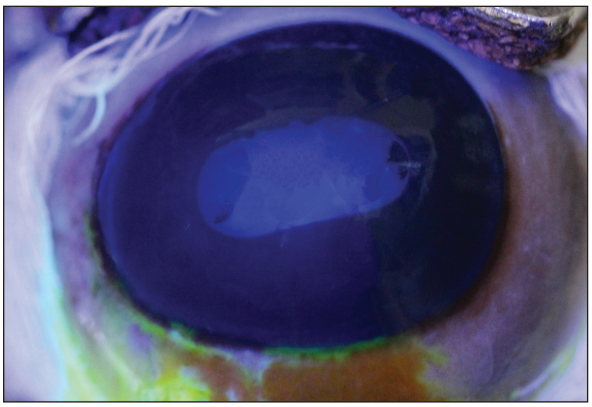

Fig. 2. Bovine eye after fluorescein staining. It is observed that the dye did not adhere to the cornea.

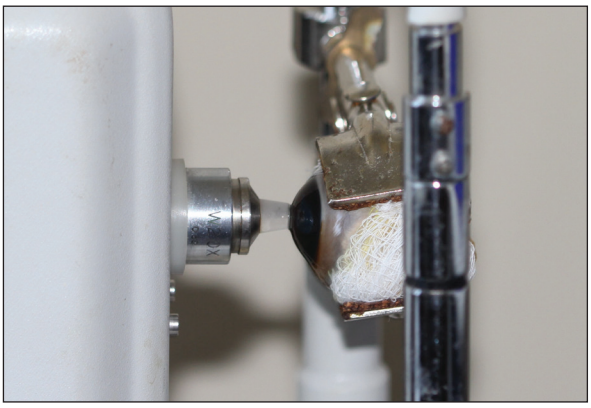

Fig. 3. Image during contact specular microscopy of a bovine eye.

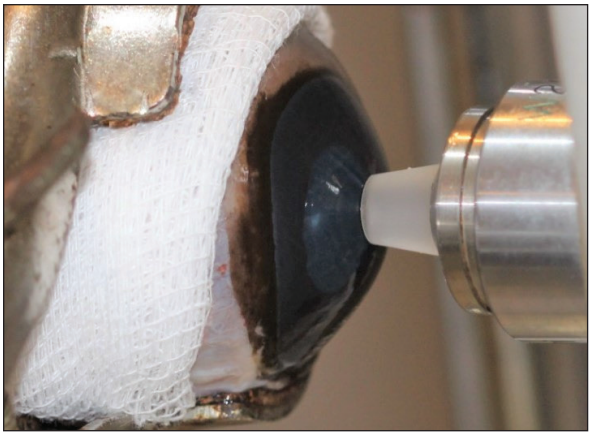

Fig. 4. Objective lens of the specular microscope in contact with the cornea of a bovine eye.

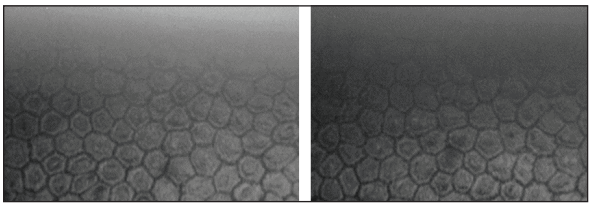

Fig. 5. Specular photomicrographs of the corneal endothelium of a bovine obtained using a contact specular microscope.

The mean ECD of the central area was 1,277 cells/mm². Regarding the morphology, mainly cells with six (74.3%), five (14.7%), and seven sides (10%) were found, along with four-sided and eight-sided cells (Table 2).

There was no significant difference in the morphological parameters of the endothelial cells when comparing the right eye to the left eye. We also found no statistically significant difference in ECD (p = 0.491) when comparing both eyes (Table 3).

Discussion

Corneal endothelial parameters have already been determined in some species, including humans (Abib and Barreto, 2001), alligators (Pigatto *et al.,* 2004), ostriches (Pigatto *et al.,* 2009), chinchillas (Bercht *et al*., 2015), chickens (Albuquerque *et al*., 2015), rabbits (Brambatti *et al*., 2017), dogs (Pigatto *et al.,* 2006; Kobashigawa *et al*., 2015; Hünning *et al*., 2018), sheep (Brandao *et al.,* 2006; Coyo *et al.,* 2016) goats (Coyo *et al.,* 2018a, 2018b), marmosets (Morita and Shimomura, 1996), horses (Andrew *et al.,* 2001; Faganello *et al*., 2016), owls (Coyo *et al.,* 2018a, 2018b), llamas and alpacas (Andrew *et al.,* 2001), Magellan penguins (Pigatto *et al.,* 2005), and pigs (Tamayo-Arango *et al.,* 2009). However, there are few ophthalmological studies related to endothelial parameters in bovine species (Tofflemire *et al*., 2015).

Several methods have been tested to evaluate the proliferative capacity of the corneal endothelium (Katz *et al*., 1994; Maurizi *et al*., 2020; Pei *et al*., 2021; Maurizi *et al*., 2022). Faye and collaborators reviewed different aspects related to the cultivation of endothelial cells in vitro (Faye *et al*., 2021). The proliferative capacity of corneal endothelial cells differs between species. While in larger mammals corneal endothelial cells are arrested in a quiescent state, rabbit corneal endothelial cells retain a high proliferative capacity and can repopulate the endothelium upon injury (Català *et al*., 2023).

Due to the differences in corneal endothelial parameters among species, it is important to know the parameters of each species. Only after obtaining these reference values, it is possible to recognize pathological changes related to this layer of the cornea and to perform surgical procedures with greater safety.

In the present study, eyes from slaughterhouse discards were evaluated. This methodology was feasible and allowed for scientific research without the need for euthanasia of experimental models for this purpose. Previous studies performed within 6 hours *post-mortem* have shown that endothelial integrity was maintained (Andrew *et al.,* 2001; Pigatto *et al.,* 2008, Bercht *et al*., 2015; Faganello *et al*., 2016). Although the present study was conducted on eyes obtained after enucleation, as no endothelial alterations occurred due to preparation for analysis, the results obtained can be used as a reference and compared with future data obtained from live animals. Unlike optical and scanning electron microscopy, no cellular retraction occurs during cornea preparation with the specular microscope. The specular microscope has been used to analyze both live animal corneas and enucleated eyes (Andrew *et al.,* 2001; Pigatto *et al.,* 2006; Pigatto *et al.,* 2008; Albuquerque *et al*., 2015; Bercht *et al*., 2015).

The cornea of bovines has characteristics that have been referenced in the literature, where the corneal thickness is 1,015 ± 104 µm, the horizontal corneal diameter is 29.8 ± 1.3 mm, and the vertical diameter is 23.9 ± 1.5 mm. The bovine corneal endothelium is an organized arrangement of polygonal cells of uniform size, of which 67.1% ± 2.7% demonstrated hexagonal morphology. Lwigale (1999), studying bovine corneas, found endothelial cell nuclei with a lobulated morphological pattern, arranged in a regular manner (Doughty *et al*., 1995; Lwigale, 1999).

Specular microscopy is an excellent method for the evaluation of corneal endothelium cells in humans and animals (Price and Chang, 1981; Klais *et al*., 2003; Pigatto *et al*., 2006; Coyo *et al*., 2016; Jirsova *et al.,* 2017). This non-invasive methodology can reliably be employed in the evaluation of corneal endothelium cells. The normal and pathological structure of the endothelium can be analyzed, and parameters can be quantified and established for clinical or experimental conditions (Abib, 2000; Benetz and Lass, 2018;

Table 1. Comparison of endothelial parameters found in different species.

Authors	Year	Specie	Parameters
Andrew, Willis, and Anderson	2002	Llamas	Mean density of corneal endothelial cells: 2,669 ± 56 cells/mm²
Andrew, Willis, and Anderson	2002	Alpacas	Mean density of corneal endothelial cells: 2,275 ± 90 cells/mm²
Coyo et al.	2018	Goats	Mean density of corneal endothelial cells: 2,966 cells/mm² Mean pleomorphism: 74.14%
Coyo et al.	2018	Tawny Owl	Mean density of corneal endothelial cells: 2,733 cells/mm² Mean pleomorphism: 75.79%
Tamayo-Arango et al.	2009	Swine	Mean density of corneal endothelial cells: 7,625.2 ± 998.2 cells/mm² Mean pleomorphism: 57.45%
Franzen et al.	2010	Cats	Mean density of corneal endothelial cells: 4,547.5 cells/mm² Mean pleomorphism: 55.6%
Pigatto et al.	2006	Dogs	Mean density of corneal endothelial cells: 2,535 cells/mm²
Faganello et al.	2016	Equine	Mean pleomorphism: 57.58% ± 5.32%
Brambatti et al.	2017	Rabbits	Mean density of corneal endothelial cells: 2,006.6 cells/mm² Mean pleomorphism: 70.06%
Abib and Barreto	2001	Human	Mean density of corneal endothelial cells: 2,530.3 cells/mm²

Table 2. Estimated mean ECD (cells/mm²) and polygonal cells in the central region of bovine eyes using contact specular microscopy (n = 20 eyes).

Data presented as the mean and 95% confidence interval.

Variables	Mean (CI 95%)
CED	1,237.3 (1,178.1–1,296.5)
% Pentagonal cells	14 (12.0–17.4)
% Hexagonal cells (pleomorphism)	74.3 (69.7–78.9)
% Heptagonal cells	10.0 (7.8–12.2)

Chaurasia and Vanathi, 2021). This allows the evaluation of endothelial diseases, healing processes, and aging as well as corneal transplants and the study of intracameral drug toxicity, aiding in the diagnosis and treatment of corneal disorders and improving surgical techniques in different species (Collin and Collin, 1998; Pigatto *et al.,* 2006; Albuquerque *et al*., 2015; Coyo *et al*., 2016; Benetz and Lass, 2018; Chaurasia and Vanathi, 2021).

In the present study, contact specular microscopy was employed to analyze and determine the parameters of the corneal endothelium in bovines. One limitation of specular microscopy is the difficulty in obtaining images of oedematous areas of the cornea (Doughty, 2006; Mccarey *et al*., 2008). In this experiment, using a specular microscope, the corneal endothelium could be analyzed and photographed in all enucleated eyes because only healthy eyes were included and examined. Various methods are employed to quantify endothelial parameters, among which the fixed and variable frame method, the center-to-center method, the corner method, and the comparison method are the most common ones. Regardless of the software used, the accuracy of the evaluation depends on the quality and sharpness of the obtained specular photo (Chaurasia and Vanathi, 2021). In this study, the central

Table 3. Comparative table of endothelial parameters in right and left eyes.

Variables	Right—Mean (CI 95%)	Left—Mean (CI 95%)	*P*
Density	1,202.6 (1,124.8–1,280.4)	1,271.9 (1,172.6–1,371.2)	0.043
% Pentagonal cells	14.3 (9.4–19.2)	15.0 (11.6–18.4)	0.809
% Hexagonal cells	75.7 (67.8–83.5)	73.0 (66.7–79.3)	0.574
% Heptagonal cells	9.0 (6.0–12.0)	11.0 (7.3–14.7)	0.405

Data presented as the mean and 95% confidence interval and compared using the paired Student's *t*-test.

endothelial density (CED) was determined using the center marking method in the equipment software. The number of delimited cells varies among studies, and the minimum recommended cell count for maximum accuracy of the analyzed area is 30 cells (Binder *et al*., 1979; Laing *et al*., 1979). In the current study, 30 endothelial cells were selected and analyzed from each evaluated cornea. Endothelial analysis mainly includes the analysis of parameters such as cell density (cells/mm²), cell area, polymegathism (cellular variation coefficient) and pleomorphism (hexagonality index) (Abib, 2000; Chaurasia and Vanathi, 2021). In this study, the ECD and endothelial cell morphology were determined, and only parameters of the central area were analyzed. Although the peripheral regions were not analyzed in this study, previous studies found no significant difference in endothelial parameters when comparing peripheral regions with the central region (Mccarey *et al*., 2008; Bercht *et al*., 2015; Faganello *et al*., 2016; Brambatti *et al*., 2017; Hünning *et al*., 2018). Based on this, the data obtained in the central region can typically be extrapolated to the peripheral regions.

Only one study was found where corneal endothelial analysis of bovines corneal was performed using specular microscopy (Bahn *et al*., 1986). In this study, the authors evaluated the central corneal density of adult and juvenile bovines and found an average endothelial density of 2,500 cells/mm². However, the number of endothelial cells per bovine cornea in this study was estimated by multiplying the area of the endothelial surface by the CED, determined by specular microscopy. This measurement of endothelial density is probably more accurate for spherical corneas (dogs, cats, and rabbits) and produces significant sampling errors (about 23%) when applied to the elliptical bovine cornea, overestimating the endothelial density of this species (Bahn *et al*., 1986).

Mean ECD of 2,669 cells/mm² for llamas and 2,275 cells/mm² for alpacas were obtained using a non-contact specular microscope (Andrew *et al*., 1999). In chinchillas, ECD calculated through specular microscopy in groups of different ages was between 2,124 and 3,423 cells/mm². These previous studies already carried out demonstrate that there is a difference in endothelial density according to the studied species. In the present study, the mean ECD of adult bovines was 1,277 cells/mm². In the current study, as all the animals were of the same age, the effects of aging were not analyzed. The effect of age on endothelial density has already been studied by means of specular microscopy in horses (Andrew *et al*., 2001), lhamas/alpacas (Andrew *et al*., 2001), dogs (Pigatto et al., 2006, 2008), cats (Franzen *et al*., 2010), sheep (Coyo *et al*., 2016), among others. ECD decreased with age (Coyo *et al*., 2018a, 2018b). There was no significant difference in the central ECD between the right and left eyes.

In most vertebrates the shape of the normal corneal endothelial cells was mainly hexagonal, pentagonal, and tetragonal cells (Yee *et al*., 1987; Collin and Collin, 1998). In this study, the predominant morphological pattern was hexagonal (74.3%). There was no significant difference between in the endothelial morphological findings for the right and left eyes, which is in agreement with previous findings (Albuquerque *et al*., 2015; Bercht *et al*., 2015; Brambatti *et al*., 2017; Coyo *et al.,* 2018a, 2018b).

In this study, only corneas from male bovines were analyzed, and sex was not a variable considered in this research. Previous studies have shown that in healthy corneas, there is no difference in endothelial parameters between male or female animals (Tuft and Coster, 1990; Tamayo-Arango *et al.,* 2009; Franzen *et al.,* 2010; Brambatti *et al*., 2017). Experimental studies have evaluated the properties of bovine endothelial cells, demonstrating their proliferative capacity *in vitro*. Researchers suggest that the culture model of endothelial cells preserves the ability to pump fluids, where the endothelial cell pumping rate was quantified for the first time, constituting an experimental model that could contribute to the development of corneal transplant methods in the future (Narula *et al.,* 1992; Huang *et al.,* 2010).

One study reported the *in vivo* heterologous transplantation in cat corneas, in which the endothelial layer was removed and replaced with endothelial cells from the cornea of bovines previously preserved in culture medium (Gospodarowicz *et al*., 1979). Cellular integrity was analyzed using the red alizarin dye, which demonstrated the high capacity for reorganization and formation of a monolayer of the bovine endothelial graft in the cat cornea within 8 days, maintaining corneal transparency for months after the experiment. The results showed that bovine corneal endothelial cells

maintained in culture remain functional, presenting an alternative to xenotransplantation (Gospodarowicz *et al*., 1979).

In another study, the corneal endothelium of cattle served as an experimental model and was successfully replicated *ex vivo* by supplementation with growth factors, temperature control, and human plasma culture medium. The supplemented culture medium ensured the maintenance of corneal transparency and hexagonal morphology. Therefore, considering the limited capacity of corneal endothelium regeneration, this study provides a basis for elucidating bovine corneal parameters in the future. Thus, the corneal endothelium of cattle, if kept in an adequate substrate, can serve as an alternative for xenotransplantation (Chou *et al*., 2014).

Using contact specular microscopy, it was possible to analyze and measure the corneal endothelial parameters of bovines, providing a reference for the analysis of bovine corneal endothelium.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

MGA: conceptualization, methodology, investigation, writing, review and editing; NPM: methodology, investigation, review and editing; LSC: methodology, investigation; RSR: methodology, investigation; MPS: methodology, investigation; AFS: methodology, investigation; JATP: conceptualization, supervision, writing, funding acquisition.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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