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# Morphology of bovine corneal endothelial cells obtained with alizarin red and optical microscopy

## Morfologia das células endoteliais corneanas bovinas obtidas com alizarina vermelha e microscopia óptica

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**Abstract:** The aim of this study was to determine the endothelial cell morphology in the different regions of healthy bovine corneal endothelium using optical microscopy. Twenty eyeballs from 10 male Brangus cattle, aged 24 months, were studied. The corneal endothelium was stained with the vital dye alizarin red and then examined with an optical microscope and photographed. Thirty endothelial cells from each corneal region were included in the analysis. Endothelial cell morphology was analysed in the central, superior, inferior, lateral and medial regions of the cornea. Comparisons between regions were performed using repeated measures analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$. Normal endothelial cells were mainly hexagonal (83.7%), pentagonal (7.45%) and heptagonal (8.8%), with a minimal number of cells of other shapes present. No statistical differences were observed in the endothelial cell morphology when comparing different regions of the cornea. Regarding endothelial cell morphology, there were no differences between the corneal regions.

**Keywords:** cornea; endothelium; morphology; vital dye, cow

**Resumo:** O objetivo deste estudo foi determinar a morfologia das células endoteliais nas diferentes regiões do endotélio da córnea bovina saudável por meio de microscopia óptica. Foram estudados 20 globos oculares de 10 bovinos machos da raça Brangus, com idade de 24 meses. O endotélio da córnea foi corado com o corante vital vermelho de alizarina e, em seguida, examinado no microscópio óptico e fotografado. Trinta células endoteliais de cada região da córnea foram incluídas na análise. A morfologia das células endoteliais foi analisada nas regiões central, superior, inferior, lateral e medial da córnea. As comparações entre as regiões foram realizadas usando medidas repetidas de análise de variância (ANOVA). As diferenças foram consideradas estatisticamente significativas em $P < 0,05$. As células endoteliais normais eram principalmente hexagonais (83,7%), pentagonais (7,45%) e heptagonais (8,8%), com um número mínimo de células de outras formas presentes. Não foram observadas diferenças estatísticas na morfologia das células endoteliais quando comparadas as

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diferentes regiões da córnea. Em relação à morfologia das células endoteliais não houve diferenças entre as regiões da córnea estudadas.

**Palavras-chave:** córnea; endotélio; morfologia; corante vital; bovinos.

## 1. Introduction

The corneal endothelium is composed of polygonal cells arranged in a single layer in the posterior portion of the cornea. In most vertebrates, the endothelial mosaic is formed by cells with six sides[1]. The density and morphology of endothelial cells change throughout life[2]. Furthermore, the number of these cells decreases with advancing age, traumatic processes, inflammation and other dystrophies. This cellular decrease results in a displacement and remodelling of cells in order to fill the remaining spaces without leaving gaps in the mosaic[3]. The objective of corneal evaluation is to determine the overall condition of the tissue, particularly that of endothelial cells[4]. Knowledge of the shape and size of corneal endothelial cells is important, as these are the best indicators of cell function and integrity. These morphological parameters, which estimate corneal health, have been referenced in other studies with different species, including chickens[5], rabbits[6], chinchillas[7], sheep[8,9], goats[10], dogs[11,12,13,14], marmosets[15], horses[16,17], owls[18], Magellanic penguins[19], ostriches[20], Pantanal alligators[21]and pigs[22], among others.

Alizarin red staining and observation under light microscopy is an *ex vivo* technique that is easy, rapid and inexpensive[23,24]. Alizarin red has been used in various studies and has been shown to be effective as a specific indicator of intercellular borders of endothelial cells[12,16,24,25]. As there are variations in corneal endothelial parameters among species, knowledge of the normal parameters for each species is fundamental. Studies regarding the bovine cornea are scarce[26]. There are few studies in which the morphology of bovine corneal endothelial cells has been analysed[27,28]. The aim of this study was to determine the endothelial cell morphology in the different regions of healthy bovine corneal endothelium.

## 2. Materials and methods

Twenty eyeballs from 10 male Brangus cattle, aged 24 months were studied. The eyeballs were obtained from an abattoir inspected by the Division of Inspection of Animal Origin Products (CISPOA). According to Concea Normative Resolution n°. 55, dated October 5, 2022, which updates the text of the Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities, validation by the CEUA (Ethics Committee on Animal Use) for this study was not required as it involves a substitutive method of teaching and the ethical use of ethically obtained cadavers [29]. Enucleation was performed immediately after slaughter, and the eyeballs were stored individually in humidified chambers. All eyes underwent ophthalmic examination, including biomicroscopy with a slit lamp (Kowa SL-15, Japan) and fluorescein staining (Fludiag; 1% sodium fluorescein, Brazil) in the Veterinary Ophthalmology Section of the Federal University of Rio Grande do Sul. Eyes that presented any opacity observed on

slit lamp examination or positive staining of the cornea with fluorescein dye were discarded and not included in the study. The corneoscleral button was removed and placed in a Petri dish with the endothelium facing upwards. Staining was performed with 0.2% diluted alizarin red (Alizarin Red S, Sigma-Aldrich, St. Louis, USA). The preparation of the dye involved homogenizing 0.2 g of alizarin red powder in 100 ml of 0.9% saline solution using a magnetic stirrer for two hours. The pH of the mixture was adjusted to 4.2 using diluted ammonium hydroxide (0.1% of the substance in isotonic saline solution), with the pH controlled using a portable digital pH meter (PH-100B, Phtek).

For the staining technique, four drops of the dye were instilled, covering the entire posterior surface of the cornea. After 2 minutes, the corneal endothelium was washed with 0.9% saline solution. The corneoscleral button was placed on top of a glass slide, with the epithelial side in contact with the glass slide and the endothelial side facing upward. Photomicrographs of the central, superior, inferior, lateral and medial regions of the corneas were randomly captured (Figure 1). An optical microscope (Nikon Eclipse E200) with an attached image capture system (EO-0813C Edmund Optics) was used. With a magnification of 40×, one image was obtained for each studied region. With the paint software, the morphology of 30 endothelial cells from each region of the cornea was analysed. The information was recorded in a data spreadsheet, and all analyses were performed by the same evaluator. The data were entered into Microsoft Excel and subsequently exported to SPSS v. 20.0 for statistical analysis. Cell percentages were described using mean and standard deviation[12].

### Statistical analysis

Comparisons between regions were performed using repeated measures analysis of variance (ANOVA). Student's t-test for paired samples was used for comparisons between eyes. A significance level of 5% was considered for the established comparisons.

## 3. Results

With optical microscopy and after staining with alizarin red, it was possible to obtain images of all samples and regions examined. Furthermore, it was possible to document and study the morphology of corneal endothelial cells. Out of the 20 corneas studied, normal endothelial cells were mainly hexagonal (74.1%), pentagonal (14.17%) and heptagonal (10.95%), with a minimal number of cells of other shapes present (four-sided, eight–sided, nine-sided). The average percentage of hexagonal cells was 72.3±9.1% in the central region, 73.5±8.4% in the inferior region, 72.8±9.3% in the lateral region, 75.0±7.0% in the medial region and 76.8±9.7% in the superior region. The average percentage of pentagonal cells was 15.6±5.7% in the central region, 15.0±4.8% in the inferior region, 15.3±5.4% in the lateral region, 12.7±5.2 in the medial region and 12.1±5.0% in the superior region. The average percentage of heptagonal cells was 11.2±3.5% in the central region, 11.1±4.2% in the inferior region, 10.8±5.5% in the lateral region; 11.0±4.2% in the medial region and 10.6±4.1% in the superior region. No significant difference was observed in the cellular shape of the endothelium when

comparing the right and left eyes. No statistical differences were observed in the endothelial cell morphology when comparing different regions of the cornea.

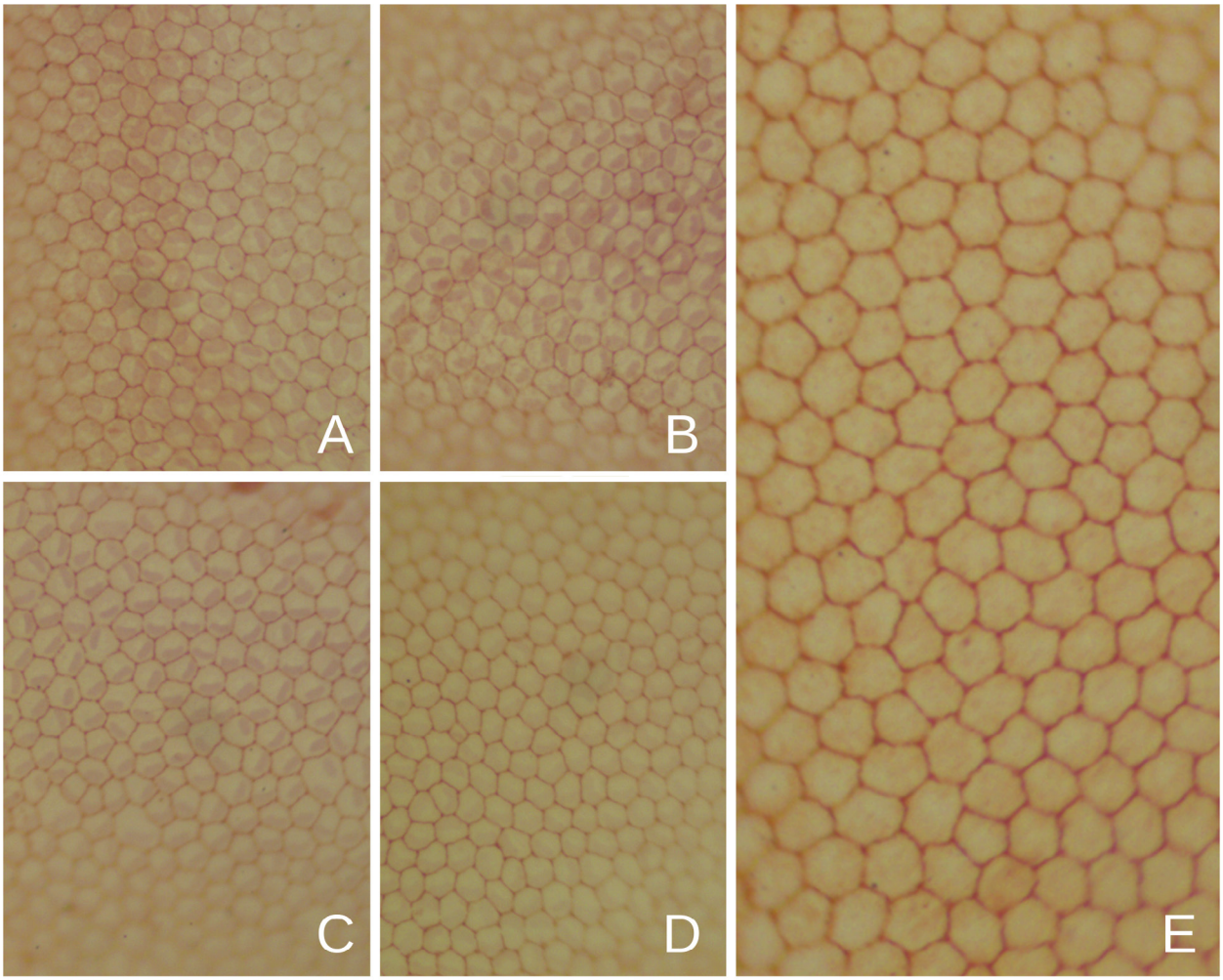


**Figure 1** Optical photomicrograph of healthy bovine corneal endothelium stained with alizarin red. 40× magnification. A: Inferior region of corneal endothelium; B: Lateral region of corneal endothelium; C: Superior region of corneal endothelium; D: Medial region of corneal endothelium; E: Central region of corneal endothelium.

## 4. Discussion

The corneal endothelial layer is critical for maintaining a clear cornea. As endothelial parameters of the cornea vary between species, it is important to know the normal parameters for each species For *in vivo* studies, specular microscopy and confocal microscopy stand out among the most applied techniques for endothelial analysis[4]. For *ex vivo* analysis, the most used techniques are scanning electron microscopy and optical microscopy using vital stains[1,7,12-14,16,25,30-33].

Specular microscopy is the standard method for the evaluation of corneal endothelium cells[2,4,22]. However, specular microscopy has a limitation, which is the difficulty in obtaining images in non-transparent corneas. Therefore, under these conditions, *ex vivo* studies are often necessary to analyse the corneal endothelial cells.

In the current study, eyes obtained from slaughterhouse and abattoir discards were evaluated, which represents a practical alternative for the development of scientific research without the need to sacrifice animals solely for this purpose. In the present study, maintaining the eyeball in a humid chamber proved to be effective, as all corneas remained transparent until the moment of optical microscopy analysis. Previous studies mentioned that the technique of subconjunctival enucleation, combined with storage of samples in a wet chamber and analysis of the eyeballs within six hours post-mortem, preserved corneal integrity[12,14,16,19]. Although this study was conducted in enucleated eyes, the data obtained in the present study can be compared with those found in future analyses with specular and confocal microscopy. Endothelial morphology does not change as a result of alizarin red staining.

Knowledge of the shape and size of corneal endothelial cells is important, as these are the best indicators of cell function and integrity. These morphological parameters, which estimate corneal health, have been referenced. Studies regarding the parameters of the corneal endothelium of bovines are rare[26]. In the present study, analysis of the endothelium of healthy bovine corneas was performed using 0.2% alizarin red, which allowed for sharp identification of cell borders. The staining technique described by Taylor & Hunt[25] was used, with only a modification in the immersion time of the sample, along with optical microscopy for image capture. Evaluation of the corneal endothelium through vital staining is a simple, low-cost, fast and practical method[12,16,25]. The number of cells that should be counted to obtain reproducible and reliable data has not been established, but previous studies recommended analysing at least 30 cells from each cornea[12]. In this study, 30 endothelial cells were analysed in each region of the cornea.

In the current research, clear images of the corneal endothelium of bovines from the central, inferior, lateral, medial, and superior regions were successfully captured using optical microscopy in all evaluated corneas. Only corneas from male bovines were examined in this study. However, gender was not considered as a variable, as there are reports in research on other species that found no significant differences in the corneal endothelium between male and female animals[6,34-36].

The most used parameters for endothelial evaluation are cell density and endothelial morphology[37]. In the present study, the morphology of endothelial cells from different regions of the cornea was analysed. As cell retraction and changes in the endothelial cell count occur after staining with alizarin red, the density was not analysed in the present study. The percentage of hexagonal cells is one of the most used parameters to indicate the health of the corneal endothelium.

In the study reported here, considering all analysed images, the variation in the percentage of hexagonal cells was between 71% and 79%. In healthy corneas of other species in which endothelial morphology has been studied, with different methods of evaluation, most cells are hexagonal, according to Table 1[6,12,14,16,19,28,36,38].

**Table 1 - Comparison of the percentage of hexagonality of corneal endothelial cells in different species and evaluation methods.**

| Authors | Age | Specie | Evaluation Method | % Hexagonal Cells |
|---|---|---|---|---|
| Hünning et al | Mean: 3 years | Dogs | Optical microscopy with alizarin red staining | Mean pleomorphism: 78,4% |
| Bercht et al | Group 1: 2-4 months<br>Group 2: 48 months<br>Group 3: 10 years | Chinchillas | Specular microscopy | Mean pleomorphism group 1: 70,05%<br>Mean pleomorphism group 2: 65,18%<br>Mean pleomorphism group 3: 62,28% |
| Pigatto et al | 6 years | Dogs | Specular microscopy | Mean pleomorphism: 68% |
| Franzen et al | Group 1: 1-3 months<br>Group 2: 5-12 months<br>Group 3: 24-40 months | Cats | Specular microscopy | Mean pleomorphism: 39% - 74% (increases with age) |
| Pigatto et al | Juveniles | Penguin | Scanning electron microscopy | Mean pleomorphism: 80% |
| Faganello et al | Mean: 12 years | Equine | Optical microscopy with alizarin red staining | Mean pleomorphism: 57.5% |
| Gallicchio et al | Mean: 12-24 months | Bovine | Specular microscopy | Mean pleomorphism: 74,3% |
| Bu et al | 5 months | Mice | Confocal microscopy | Mean pleomorphism: 66% |

*Source: the author himself.*

The percentage of hexagonality found depends on the species and the age of the animals evaluated. In a study conducted on equine corneas, Faganello and collaborators[16] found a percentage of hexagonal cells between 55% and 57%. Hünning and collaborators[12], in their study on corneal endothelial cells in dogs, found an average percentage of hexagonal cells between 77% and 80%. A limitation of the present study is that all animals were the same age. This occurred because eyes donated after slaughter were used. All slaughtered animals were males of the same age. Despite this limitation, the study was able to quantify the morphology of the corneal endothelium cells in two-year-old cattle. In addition, it was possible to compare the data obtained from different regions of healthy corneas. Bu and collaborators[38] found

66% of hexagonal cells in mice. In the human species it was possible to observe that the percentage of hexagonal cells of the corneal endothelium decreases with aging[37]. This study is essential to enable similar research in the corneas of cattle affected by diseases that lead to diffuse corneal opacity, along with other ophthalmological signs contributing to the loss of corneal transparency in cattle.

## 5. Conclusion

In the present study, optical microscopy and alizarin red staining allowed for the analysis and documentation of the bovine corneal endothelium. Regarding endothelial cell morphology, there are no differences between regions of the healthy bovine cornea. This information on endothelial morphology will be key to future clinical and experimental studies where bovine corneas will be analysed.

### Conflict of interests

The authors declare no conflict of interest.

### Authors' contributions

**Conceptualization**: N. P. Méndez and J. A. T. Pigatto. **Data curation**: N. P. Méndez, and J. A. T. Pigatto. **Formal analysis**: N. P. Méndez, M. G. Azevedo and J. A. T. Pigatto. **Investigation**: N. P. Méndez, M. G. Azevedo, L. S. Cargnin, M. P. Seibel, A. F. Silva, M. E. M. Franceschini, R. S. Rocha and J. A. T. Pigatto. **Writing (original draft):** N. P. Méndez, M. G. Azevedo and J. A. T. Pigatto. **Writing (proof-reading & editing):** N. P. Méndez and J. A. T. Pigatto.

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