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Sheep Corneal Endothelium Morphology - Evaluation with Trypan Blue and Alizarin Red

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ABSTRACT

***Background*:** The endothelium is a layer fundamental to maintaining corneal transparency. In ophthalmology, sheep eyes have been used as a model in research related to corneal transplantation. Different techniques have been used to evaluate the corneal endothelium. Concerning vital dyes, corneal endothelial cell analyses have not yet been studied in ovines. The purpose of the present study was to evaluate the morphology of endothelial cells from different regions of the cornea of sheep after staining with alizarin red and trypan blue using an optical microscope.

***Materials, Methods & Results*:** Twenty healthy eyes of 10 male sheep obtained from a licensed commercial slaughterhouse were studied. The study was approved by the Research Committee of the Faculty of Veterinary at UFRGS and followed the ethical standards of the Association for Research in Vision and Ophthalmology (ARVO). Immediately after the slaughter, the eyes were enucleated and underwent eye examination. The corneal endothelium was stained with trypan blue and alizarin red and examined and photographed using an optical microscope. The central, superior, inferior, nasal and temporal areas of the cornea were evaluated for cell morphology. Data were compared by *t*-tests. Differences were considered statistically significant at $P < 0.05$. Immediately after staining the corneal endothelium, it was possible to examine with an optical microscope, obtain images and analyse the shape of endothelial cells from all regions of the sheep cornea. Polygonal, uniform and continuous cells were observed in all samples studied. Considering all the corneas analysed, cells with 6 sides (75.11%), 5 sides (12.76%) and 4 sides (12.12%) were found. In the central region of the cornea 75.91% of cells with 6 sides, 12.6% of cells with 5 sides and 11.48% with 7 sides were found. In the superior region of the cornea 76.07% of cells with 6 sides, 13.25% with 5 sides and 10.68% with 7 sides were found. In the lower region were found 74.72% of cells with 6 sides, 13% with 5 sides and 12.27% with 7 sides. In the temporal region, 74.14% were 6-sided cells, 11.42% had 5 sides, and 14.43% had 7 sides. Furthermore, in the nasal region, 74.72% of the cells had 6 sides, 13.54% had 5 sides, and 11.73% had 7 sides. No significant differences were found between cell morphology in all corneal regions evaluated. In addition, no significant difference was found when comparing the right eye with the left eye.

***Discussion*:** Different methods are used for the analysis of corneal endothelium. For ex vivo research optical microscopy after endothelial staining is an alternative low-cost technique that allows the analysis of all regions of the cornea. Quantitative analyses must characterise the endothelial parameters of the different species. The analysis of the morphology of corneal endothelium with an optic microscope after staining with alizarin red has been described as an effective, rapid and cost-efficient method, since this dye blends with the borated cells, allowing identification. In the present study, using optical microscopy and coloration with alizarin red it was possible to explore and obtain images of the ovine endothelium of all regions of the cornea. In the current study, the endothelium had a predominance of cells will 6 sides in all regions studied. This study allowed us to obtain images of the endothelium as well as quantitative data on the morphology of the different regions of the sheep cornea. This study demonstrated that morphology did not differ between the central and peripheral regions. The findings of this study represent a further source of reproducible data that should be considered when using sheep cornea as ex vivo model for experimental research.

Keywords: ovine, endothelial cells, *ex vivo*, vital staining, hexagonality.

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INTRODUCTION

The corneal endothelium is a layer of polygonal cells [3]. Among the endothelial parameters that can be quantified are cell density and endothelial morphology [19,21,22,30].

The most used techniques for the evaluation of the corneal endothelium are specular microscopy, confocal microscopy, scanning electron microscopy and optical microscopy after staining endothelial cells with vital dyes [9,28,29]. Alizarin red stains the corneal endothelium cell wall allowing cell morphology analysis [27,28]. Vital dyes have been a fast, simple and inexpensive method that allows obtaining images for evaluation of the corneal endothelium, both in humans and animals [29].

Endothelial parameters have already been studied in humans [15] and in some animal species, including swine [32], rabbits [21], dogs [23], horses [3,14], llamas, alpacas [4], cats [16], birds [10], yacare [22], penguins [25], ostrich [24], chinchillas [6], chickens [2] and goats [11], among others.

Sheep have been used as a model in cornea-related research [1,7,13,26,34]. However, experimental evaluations of the main parameters of the corneal endothelium of sheep are scarce in the literature [12,17]. Concerning vital dyes, corneal endothelial cell analyses have not yet been performed in this species. The goal of this study was to assess the morphology of endothelial cells from different regions of the cornea of sheep after trypan blue and alizarin red staining using optical microscopy. The results obtained allowed an increase in the knowledge about the normal cornea of sheep allowing a better understanding and interpretation of changes that might occur in sheep and for future research using sheep as an experimental model.

MATERIALS AND METHODS

Samples

Twenty healthy eyes of 10 sheep (*Ovis aires*), aged 2 to 4 years that were males and crosses between the Ideal and Corriedale breeds, were studied. Eyes were obtained from a licensed slaughterhouse (Carneiros do Sul, Sapiranga, RS, Brazil) and transferred to the laboratory in a transport solution at 4°C. All eyes were submitted to examinations with fluorescein dye[1] and biomicroscopy with a portable slit lamp[2] to assess the cornea. This study was approved by the Research Committee of the Faculty of Veterinary at UFRGS and followed the ethical standards of the Association for Research in Vision and Ophthalmology (ARVO).

Morphological analysis

Corneal endothelium staining and analysis were performed within 1 h after enucleation. The cornea, with approximately 3 mm of the sclera, was collected with a scalpel and scissors. After irrigation with balanced saline solution (BSS)[3], the scleral corneal button was placed on a glass slide with the endothelial side up. To better accommodate the cornea on the blade and delimit each region that will be studied 4 radial incisions were made in each button with a scalpel (flat mounting technique). The endothelium was covered with drops of 0.25% solution of trypan blue[4] for 90 s and after that rinsing with BSS. The corneal endothelium was covered with alizarin red S[5] (0-2%; pH 4.2) for 1.5 min and again after rinsing twice in BSS.

Subsequently, with an optical microscope[6], the endothelium was examined. Five random photomicrographs of the different corneal regions (superior, temporal, central, nasal and temporal) were taken with 40x magnification with an image capture system[7]. The number of sides of 100 cells from each corneal region were counted using the Microsoft Windows Paint program. The data obtained were recorded in a spreadsheet for further analysis. All evaluations were performed by the same evaluator.

Statistical analysis

The data obtained were submitted to analysis of variance (ANOVA), the means of the regions were compared by an unpaired *t*-test, and the means of the regions between the right and left eyes were compared by a paired *t*-test with a significance level of 5%.

RESULTS

All enucleated eyes were included in the study. Previous examinations performed with fluorescein and slit-lamp biomicroscopy did not reveal any lesions, opacity or scarring on the corneas. After staining with the optical microscope, it was possible to examine, obtain images and analyse the shape of endothelial cells from all regions of the sheep cornea. Polygonal, uniform and continuous cells were observed in all regions studied (Figure 1).

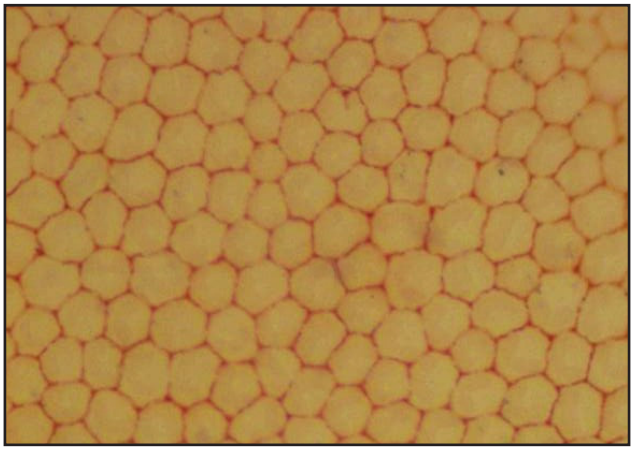

Figure 1. Optical photomicrograph of the ovine corneal endothelium after staining with tripan blue and alizarin red. Polygonal and uniform cells are observed [40x].

Of all the corneas studied, cells with 6 sides (75.11%), 5 sides (12.76%) and 4 sides (12.12%) were found. In the central region of the cornea, 75.91% of the cells had 6 sides, 12.6% of the cells had 5 sides, and 11.48% had 7 sides. In the superior region of the cornea, 76.07% of the cells had 6 sides, 13.25% had 5 sides, and 10.68% had 7 sides. In the inferior region of the cornea, 74.72% of the cells had 6 sides, 13% had 5 sides, and 12.27% had 7 sides. In the temporal region of the cornea, 74.14% of the cells had 6 sides, 11.42% had 5 sides and 14.43% had 7 sides. In the nasal region of the cornea, 74.72% of the cells had 6 sides, 13.54% had 5 sides, and 11.73% had 7 sides. No significant difference was found when comparing the right eye with the left eye, and there was no difference between the regions compared ($P > 0.05$).

DISCUSSION

Corneal endothelium analysis has already been performed in several animal species including horses, chinchillas, cats, dogs and swine, among others [6,14,16,23,32]. Due to the variation in endothelial parameters found in different species, it is necessary to know normal data on the endothelium in each species.

In ophthalmology, sheep have been used as experimental animals in research related to corneal transplantation [1,7,12,13,17,26,34]. However, experimental evaluations of the main parameters of the corneal endothelium of sheep are scarce in the literature [12,17]. Concerning vital dyes, corneal endothelial cell analyses have not yet been performed in this species. Different techniques have been used to evaluate the corneal endothelium including specular microscopy, scanning electron microscopy, confocal microscopy and light microscopy after staining the endothelium with vital dyes [28,29,32].

In the present study, the morphology of endothelial cells from different regions of the cornea was analysed using a microscope after staining the endothelium with trypan blue and alizarin red. In animals, both for clinical analysis and for experimental research, specular microscopy is the most used technique [2,4,13,16,23]. However, specular microscopy has limitations such as the cost of equipment and the difficulty of obtaining images in corneas with oedema and the difficulty of capturing images of peripheral regions of the cornea [29].

In this sense, for *ex vivo* studies, it would be interesting to use alternative low-cost techniques that allow the analysis of all regions of the cornea. For these studies to be designed, quantitative analyses must characterise the endothelial parameters of the different species used as experimental models in ophthalmology. In the present study, it was possible with the coloration employed and an optical microscope to analyse, photograph and study the shape of the endothelium in all regions of the cornea.

In this research, optical microscopy was chosen due to its low cost, ease of execution and because this technique has already been well used in previous studies with good results [9,14]. The association of trypan blue and alizarin red dyes is widely used to assess the health of the corneal endothelium [29,31,33].

Previous studies have shown that endothelial structure is preserved for up to 6 h after death [2,5,16,20,23]. In the current study, corneal endothelium staining and analysis were performed within 1 h after enucleation. In the current study, in all cells evaluated, it was possible to clearly identify the cell borders stained in red and evaluate the number of sides of each endothelial cell. For proper staining of corneal endothelium cells with alizarin red, adjusting the pH of alizarin red to 4.2 has been reported in previous studies that [33]. In the present study, this care was taken, and the pH was adjusted with hydrochloric acid to 4.2. In addition to the care with the preparation of the dye, adequate accommodation of the corneas was made on the glass slide to allow staining and analysis of endothelial cells in all regions of the cornea. To improve the accommodation

of the cornea on the blade and allow adequate contact of the dyes with the endothelial cells, four radial incisions were made with a scalpel. This technique, which is called flat mounting, has been previously used for the preparation of corneas, demonstrating excellent results in obtaining images of endothelial cells [8,18].

With 40x magnification selected for the present analysis, it was possible to identify the shape of a large number of cells in each captured image. In the present study, 100 endothelial cells were analysed from each corneal region, and with the acquisition of 5 microphotographs of each region, it was possible to analyse 100 cells from each region.

In addition to cell density, pleomorphism (% of hexagonal cells or hexagonality) has been used as an important parameter to assess the health of the corneal endothelium. In the animals studied, the normal corneal endothelium had a majority of normal hexagonal-shaped cells [14,23,32]. In the present study, most cells had 6 sides (75.11%). However, endothelial cells with 5 sides (12.76%) and 4 sides (12.12%) were also found. Coyo *et al*. [11], using specular microscopy to evaluate corneal endothelial cells in enucleated sheep eyes, determined that approximately 75.64% of cells were hexagonal in lambs (3-6 months) and 69.9% were in adults (2-5 years); the study showed that age and breed influence the corneal endothelial parameters in sheep, with an expected decrease in the percentage of 6-sided cells with aging.

The results obtained in the present study showed that there were no differences for endothelial morphology when the right and left eyes were compared. Normally, in healthy corneas, there is no difference in terms of endothelial parameters when the left eye is compared to the right eye [2,16]. In the present study, sex was not considered as a variable. Previous studies have shown that there is no difference in endothelial parameters between males and females [2,6,16].

CONCLUSION

This study has demonstrated that the morphology of the healthy endothelial cells of sheep did not differ between the central and peripheral regions. The findings of this study represent a further source of reproducible data that should be considered when using sheep corneas as *ex vivo* models for experimental research.

MANUFACTURERS

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