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## Morphology of endothelial cells from different regions of the equine cornea

### Morfologia das células do endotélio de diferentes regiões da córnea de equinos

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#### ABSTRACT

The objective of this study was to evaluate the morphology of different regions of the equine cornea using optical microscopy. Both healthy eyes of eight horses, male or female, of different ages were evaluated. Corneas were stained with alizarin red vital dye and subsequently examined and photographed using optical microscopy. Corneal endothelial morphology of central, superior, inferior, temporal and nasal areas was assessed. One hundred endothelial cells from each corneal area were analyzed. The shape of the corneal endothelial cells of each corneal region was analyzed. Statistical data analysis was conducted using the Student's *t* test. Values of  $P < 0.01$  were considered significant. Regarding morphological analysis, no statistically significant differences were reported between the equine corneal regions analyzed. The present research suggested that there are no morphological differences between regions of the equine cornea. The values obtained in any region of the equine cornea can be extrapolated to other regions of the cornea and are representative of the cell morphology present in all regions of the cornea.

**Key words:** horses, corneal endothelium, pleomorphism, alizarin red.

#### RESUMO

O objetivo deste estudo foi o de avaliar a morfologia das diferentes regiões da córnea equina usando microscopia óptica. Foram avaliados ambos os olhos saudáveis de oito equinos, machos ou fêmeas, de diferentes idades. As córneas foram coradas com corante vital vermelho de alizarina, examinadas com microscópio óptico e fotografadas. A morfologia endotélio corneano de áreas centrais, superior, inferior, temporal e nasal foi avaliada. Foram analisadas células endoteliais da córnea de cada área. A forma das células endoteliais da córnea de cada região da córnea foi analisada. Análise estatística dos dados foi realizada por meio do teste *t*. Valores de  $P < 0.01$  foram considerados significativos.

*Em relação à análise morfológica estatisticamente significativa, não foram encontradas diferenças entre as regiões da córnea equina analisadas. O presente trabalho sugere que não houve diferença entre a morfologia das regiões da córnea de equino. Os valores obtidos em qualquer região da córnea equina podem ser extrapolados para outras regiões da córnea e são representativos da morfologia celular em todas as regiões da córnea.*

**Palavras-chave:** equinos, endotélio da córnea, pleomorfismo, vermelho de alizarina.

#### INTRODUCTION

The corneal endothelium maintains stromal deturgescence, which is a prerequisite for corneal transparency (SRINIVAS, 2010). Endothelial parameters that can be quantified principally included mean cell area, cell density, polymegathism and pleomorphism (PIGATTO et al., 2004; PIGATTO et al., 2005b; SELIG et al., 2015). Pleomorphism is the variation in cell shape represented by the percentage of hexagonal cells (LAING et al., 1979). Principal among the techniques used to study the corneal endothelium are specular microscopy and scanning electron microscopy (SEM) (PIGATTO et al., 2005a, PIGATTO et al., 2005b; McCAREY et al., 2008). Aside from these techniques, the analysis of the corneal endothelium using optical microscopy after cell staining with alizarin red has also been used (RODRIGUES et al., 2009). Basic

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morphometric and morphological endothelial cell studies provided reference information for the investigation of clinical diseases, such as uveitis, glaucoma, and the effects of aging on different regions of the cornea. Furthermore, the only way to treat horses with cataract is to surgically remove the opacified lens (MILLICHAMP & DZIEZC, 2000; HARDMAN et al., 2001; FIFE et al., 2006; HARRINGTON et al., 2013). Regarding equine corneal endothelium, there are only two published studies (ANDREW et al., 2001; LEDBETTER & SCARLETT, 2009). However, none of these previous studies analyzed the endothelial morphology with respect to different regions of the cornea of healthy horses. The objective of this study was to assess the morphology of endothelial cells from different areas of the cornea in normal eyes of horses.

## MATERIALS AND METHODS

Sixteen healthy eyes from eight horses, male or female, of different ages, obtained from a licensed Brazilian abattoir (Foresta abattoir, São Gabriel, RS) were studied. All experiments were carried out in accordance with the ARVO statement for the use of animals in ophthalmic and visual research. Eyes were enucleated immediately after slaughter and were kept at 4°C in moist chambers. An ophthalmic examination was performed before enucleation and consisted of evaluation by slit lamp biomicroscopy (Portable Slit Lamp SL 15, Kowa, Japan) and proof of fluorescein (Fluorescein Strips, Ophthalmos, SP, Brazil). Immediately following enucleation, corneas were dissected along with 2mm of surrounding scleral tissue from the eyeball and placed endothelial side up on a glass slide, covered with 0.2% red alizarin (Alizarin Red S, Sigma-Aldrich, St. Louis, USA) for two minutes, and again rinsed twice with balanced salt solution. Corneal endothelium was evaluated and photographed using an optical microscope (Nikon Eclipse E200, Japan) at 40X magnification. Three random images of the central, superior, inferior, temporal, and nasal cornea were taken. One hundred endothelial cells from each corneal area were analyzed. All assessments were performed by the same evaluator. Statistical analysis was performed using analysis of variance (ANOVA) and the measures were described as mean  $\pm$  SD. Values of eyes were compared by Student's t test for paired samples (t-paired) and the values of the quadrants to each other by repeated-measures ANOVA. Values of  $P < 0.01$  were considered significant.

## RESULTS

The median age for horses was 12 years (range, 4-20 years). In all samples, it was possible to analyze, capture images of and study the shape of cells in all regions of the cornea. Normal endothelium was seen to comprise mainly hexagonal ( $57.58 \pm 5.32\%$ ), pentagonal ( $20.66 \pm 2.95\%$ ), heptagonal ( $19.48 \pm 3.28\%$ ), and octagonal ( $2.25 \pm 1.18\%$ ) cells with a minimal number of cells of other shapes present (Figure 1). Average percentage of hexagonal cells in the central area was  $55.43 \pm 4.5\%$ ; in the superior area was  $57.78 \pm 3.1\%$ , in the inferior area was  $58.62 \pm 6.4\%$ ; in the temporal area was  $56.14 \pm 6.7\%$ ; and in the nasal area was  $56.88 \pm 6.29\%$  ( $P = 0.34$ ). The average percentage of cells with less than six sides in the central area was  $22.72 \pm 3.0\%$ ; in the superior area was  $20.81 \pm 3.5\%$ ; in the inferior area was  $20.14 \pm 3.82\%$ ; in the temporal area was  $21.66 \pm 4.04\%$ ; and in the nasal area was  $21.60 \pm 3.04\%$  ( $P = 0.82$ ). The average percentage of cells with more than six sides in the central area was  $21.85 \pm 3.99\%$ ; in the superior area was  $21.31 \pm 3.81\%$ ; in the inferior area was  $21.24 \pm 4.08\%$ ; in the temporal area was  $22.2 \pm 4.88\%$ ; and in the nasal area was  $21.52 \pm 4.71\%$  ( $P = 0.82$ ).

## DISCUSSION

Among the techniques used to record and later analyze images of the corneal endothelium there are specular microscopy, scanning electron microscopy and optical microscopy after staining with vital dyes (SAAD et al., 2008; RUGGERI, 2010). Using images obtained by specular microscopy, it is possible to examine the corneal endothelium and obtain data related to endothelial cell density and morphology. This technique has been used to quantify endothelial parameters in humans and other animal species including pigs (TAMAYO-ARANGO et al., 2009), rabbits (SAILSTAD & PEIFFER, 1981; OJEDA et al., 2001), dogs (GWIN et al., 1982; PIGATTO et al., 2006; RODRIGUES et al., 2006), horses (ANDREW et al., 2001; LEDBETTER & SCARLETT, 2009), llamas and alpacas (ANDREW et al., 2002), chinchillas (BERCHT et al., 2015), and cats (FRANZEN et al., 2010), among others. The high cost of specular microscopes and the difficulty in obtaining good images in injured endothelial areas are common challenges or barriers to the use of this technique (ANDREW et al., 2001; PIGATTO et al., 2005a; SAAD et al., 2008). SEM has been widely used to compare the endothelial ultrastructure of vertebrates

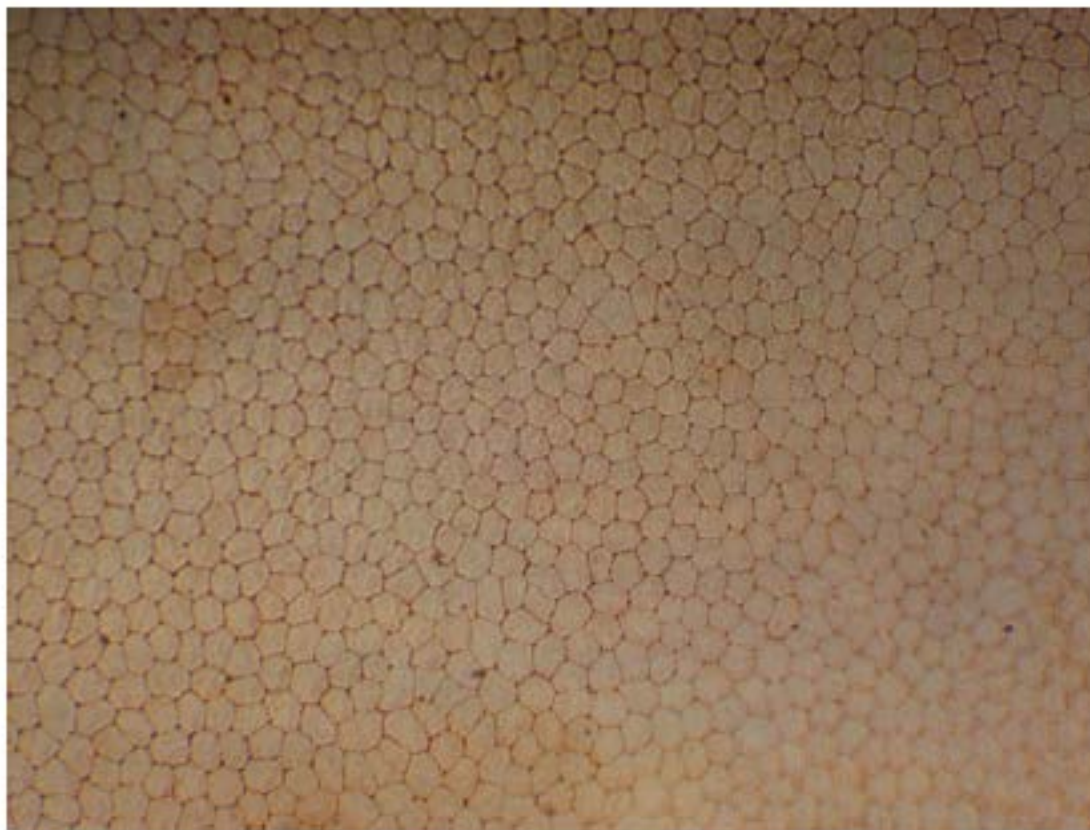


Figure 1 - Image of endothelium of healthy equine cornea stained with alizarin red and examined with an optical microscope (40X magnification).

and to evaluate the effects of medications, chemicals, or surgical procedures on the endothelium (COLLIN & COLLIN, 1998; PIGATTO et al., 2004; PIGATTO et al., 2005a; PIGATTO et al., 2005b; PIGATTO et al., 2009). Changes in endothelial cell dimensions can occur as a result of processing the cornea for SEM and the data obtained with regard to endothelial cell density cannot be compared with those obtained using specular microscopy (SCHUTTEN & VAN HORN, 1980). Dual staining of the corneal endothelium with vital dyes using 0.25% trypan blue and 0.2% alizarin red is the most common method for determining viable and nonviable cells in areas where cells cannot be identified using specular microscopy (SPERLING, 1977; TAYLOR & HUNT, 1981). Vital staining with alizarin red and optical microscopy is a rapid, simple, and inexpensive method for obtaining images for morphological and morphometric evaluation

of the endothelium in humans and animals (SAAD et al., 2008). In the present study, using an optical microscope was possible to obtain clear images of the endothelial mosaic in all regions of the cornea. Trypan blue was not used because the objective was to evaluate the shape of endothelial cells and staining of cell walls was obtained with alizarin red. In order to increase the visibility of the cell boundary and thus allow a more reliable estimate of morphological parameters, alizarin red dye was used in this study. Poor recognition of cell borders can result in errors of omitting cells or double entering cells during the analysis (McCAREY et al., 2008). In the present study, the photographic magnification was large enough to allow accurate tracing of individual cells.

In this study, immediately after slaughter, eyes were kept in a humid chamber with 0.9% saline solution. Previous studies performed with

enucleated eye balls have proven that it is possible to evaluate the corneal endothelium within six hours post-mortem without structural changes occurring (ANDREW et al., 2001; PIGATTO et al., 2006; RODRIGUES et al., 2006; PIGATTO et al., 2008; FRANZEN, et al., 2010).

The number of cells counted per image to obtain maximum accuracy varies according to the analyzed study. Some authors recommend counting at least 30 cells, others recommend 50, 75 and 100 cells per image. In the present study, 100 cells were evaluated in each region of the cornea. Studies reported in the literature concerning the comparison of endothelial density in the central and peripheral regions of the cornea by specular microscopy showed controversial results. In humans, rabbits and dogs, there was no significant difference in endothelial parameters between central and peripheral regions of healthy corneas (TAYLOR & HUNT, 1981; GWIN et al., 1982; McCAREY et al., 2008). However, AMANN et al. (2003) concluded using specular microscopy and histological methods that human corneal endothelium has a higher density in peripheral regions compared with the central region. Normally, corneal endothelium is a monolayer consisting mostly of hexagonal cells, but may have four, five, seven, or even eight sides (TUFT & COSTER, 1990; DOUGHTY, 1998). Results of previous studies reported no significant difference in polymorphism according to age (SPERLING 1977; GWIN et al., 1982) and the lack of differences in relation to endothelial parameters obtained from the bulbs of the right and left eyes in dogs, pigs, horses, and cats (GWIN et al., 1982; ANDREW et al., 2001; AMANN et al., 2003; TAMAYO-ARANGO et al., 2009; FRANZEN et al., 2010). Results obtained in this study showed that the normal equine endothelium consisted of a monolayer of hexagonal cells; most, however, have also been observed with cells having four, five, seven, eight and nine sides. The polymorphism among the studied quadrants was not significantly different, with similar values between 52.23% and 64.54%. The regular pattern of polygonal cells with a predominantly hexagonal shape assessed by optical microscopy was similar to that described in other species (SAILSTAD & PEIFFER, 1981; PEIFFER et al., 1981; MORITA, 1985; PIGATTO et al., 2008; FRANZEN et al., 2010), which corroborates the results in the present study.

In the current study, no correlation was made between gender and endothelial parameters. Previous studies in humans, dogs, pigs, and rabbits observed that the corneal endothelium was not

influenced by gender, with respect to the endothelial parameters analyzed (LAULE et al., 1978; GWIN et al., 1982; MORITA, 1995; TAMAYO-ARANGO et al., 2009). In the current study, there was no significant difference between the right and left eyes. There was no difference in relation to endothelial parameters obtained between fellow eyes of the same individual (MORITA, 1985; ANDREW et al., 2001; PIGATTO et al., 2004; PIGATTO et al., 2005b; PIGATTO et al., 2008; PIGATTO et al., 2009; FRANZEN et al., 2010).

## CONCLUSION

The cell shape values obtained in any region of the normal equine cornea can be extrapolated to other regions of the cornea and are representative of cell morphology in all regions of the cornea. This information will assist in tests using the specular microscope for the evaluation of endothelial morphology in any region of the cornea.

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