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Specular microscopy of the different regions of the cornea in enucleated swine eyes - *ex vivo* evaluation

Microscopia especular das diferentes regiões da córnea de suínos - estudo *ex vivo*

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Abstract
The objective of this study was to determine the endothelial cell density (ECD) and hexagonality of the cornea in the different regions of healthy swine corneal endothelium using specular microscopy. Twenty-four eyeballs from 12 male, 6-month-old Large White pigs (*Sus scrofa domesticus*) were studied. Contact specular microscopy was performed in the central, superior, inferior, lateral and medial regions. The corneal parameters analysed in this study were ECD and hexagonality. The ECD in the central region was 1865 cells/mm²; in the upper region, it was 1877 cells/mm², in the lower region, it was 1854 cells/mm², in the lateral region, it was 1847 cells/mm², in the medial region, it was 1831 cells/mm². Hexagonality in the central region, was 53%; in the upper region, it was 54%, in the lower region, it was 54%, in the lateral region, it was 54%, in the medial region, it was 54%. There was no significant difference regarding to the evaluated parameters in all corneal regions evaluated. No statistically significantly differences were observed in ECD and hexagonality between the left and the right eyes. This study demonstrates that ECD and hexagonality of the central cornea area represent the entire endothelial mosaic.
Keywords: cornea; endothelium; morphology; cell count, swine.

Resumo
O objetivo do presente estudo foi avaliar a densidade endotelial e a hexagonalidade das células endoteliais nas diferentes regioes da córnea de suinos utilizando a microscopia especular de contato. Foram estudados 24 bulbos oculares de 12 suinos (*Sus scrofa domesticus*), machos, com seis meses de idade e da raça Large White. A microscopia especular de contato foi realizada nas regioes central, superior, inferior, lateral e medial. A densidade endotelial media na regiao central foi de 1865 celulas/mm², na regiao superior foi de 1877 celulas/mm², na regiao inferior foi de 1854 celulas/mm², na regiao lateral foi de 1847 celulas/mm2 e na regiao medial foi de 1831 celulas/mm². Na regiao central, a hexagonalidade foi de 53%, na regiao superior foi de 54%, na regiao inferior foi de 54%, na regiao lateral foi de 54%, na regiao medial foi de 54%. Nao foram observadas diferenças significativas na densidade celular e na hexagonalidade nas diferentes regioes da córnea analisadas. Este estudo demonstrou que a densidade endotelial e a hexagonalidade da área central da córnea representam todo o mosaico endotelial.
Palavras-chave: córnea; endotélio; morfologia; contagem celular; suíno.

1.Introduction

The endothelium is the innermost layer of the cornea and is formed by a monolayer of polygonal cells.[1] This layer can be examined using histological techniques[2,3], scanning electron microscopy[4], confocal microscopy,[5] and specular microscopy[6]. For *in vivo* evaluation, specular microscopy is the most widely used methods to analyse the corneal endothelium[6]. Knowledge of preoperative endothelial parameters of the corneal endothelium can help to minimise the incidence of postoperative complications, especially in the surgical procedures used for cataract removal[7]. Specular microscopy has also been used to study the parameters of the corneal endothelium in different animal species.

The corneal endothelium has been documented with specular microscopy in dogs[8-10], cats[11,12], sheep[13], horses[14], llamas and alpacas[15], chickens[16], chinchillas[17], rabbits[18,19] and goats,[20] among others animals. Pig eyes are employed as an experimental model in ophthalmology[3,21-26]. Due to the similarities between swine and human, the interest in the possibility of using swine corneas for transplantation in humans has increased[27,28]. However, studies evaluating the corneal endothelium of this species are scarce[28-31].

Because of the variations in corneal endothelial parameters among different species, knowledge of normal parameters of each species is fundamental. In addition, knowledge of endothelial parameters is a prerequisite for

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the recognition of pathological changes related to this layer of the cornea. Among the corneal endothelial parameters that can be quantified are endothelial cell density (ECD), cell size coefficient of variation (CV), cell area (CA) and hexagonality.[6] The ECD is the number of cells in the corneal endothelium per mm^2. Hexagonality is a percentage of hexagonal cells. The data collected in the present study will assist in future evaluations of the swine corneal endothelium. The purpose of this study was to determine the ECD and hexagonality in the different regions of healthy swine corneal endothelium using specular contact microscopy. It was also compared the values obtained in the central region with those obtained in peripheral regions of the cornea.

2. Material and methods

Overall, 24 healthy eyes from 12 swine (*Sus scrofa domesticus*), 6 months of age, males, Large White breed, were used in this study. The eyes were obtained from a local slaughterhouse (Avisui, Santa Maria, RS, Brazil) with Federal Inspection according to the technical and humanitarian precepts in force in the specific legislation. The animals were slaughtered for reasons unrelated to this project. This project was approved by the Research Committee of the College of Veterinary of the Federal University of Rio Grande do Sul. The animals had been desensitised by electronarcosis, followed by immediate bleeding with effective death. Before scaling, the right and left eyes of each animal were enucleated and were examined with slit lamp biomicroscopy (Slit Lamp SL 15, Kowa, Japan) and fluorescein staining (Fluorescein, Ophthalmos, SP, Brazil). Eyes that showed evidence of corneal alteration were not included in the experiment. After the enucleation, all eyes were stored in a moist chamber. The humid chamber was coated with gauze moistened with sterile saline solution and the eye was kept with the cornea up.

2.1 Specular microscopy

Eyes were evaluated within 4 hours after enucleation. After being removed from the humid chamber, the eyes were placed in a holder and examined with a specular contact microscope (Figure 1).

The objective lens of the microscope was positioned over the central, superior, inferior, lateral and medial regions for the digital photographic recording of the endothelium. Parameters studied included ECD and percentage of hexagonal cells. Two specular micrographs of each corneal region were taken. From each image, 50 cells were marked with a mouse by the examiner for analysis ECD by a built-in software programme (Celmax® software). The method used was labelling the centres of the cells. Endothelial morphology The morphology of 50 endothelial cells was obtained by means of an imaging software (Adobe photoshop). The number of sides of each cell was analysed, and the percentage of hexagonal cells was calculated. All analyses were carried out by the same researcher.

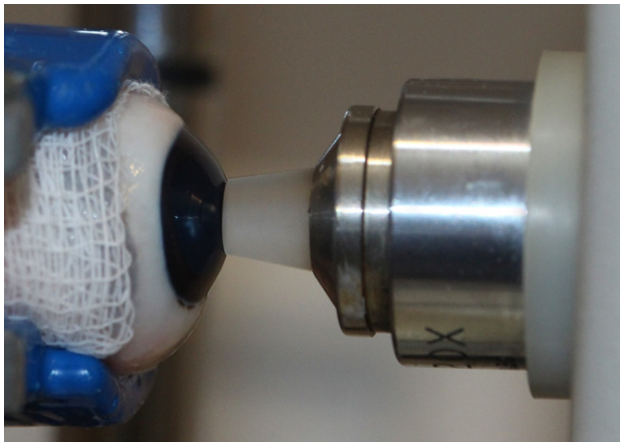

Figure 1. Positioning of the specular microscope objective with the porcine cornea.

2.2 Statistical analysis

Statistical analysis was performed using ANOVA and Tukey´s test. Values of $P<0.05$ were considered significant for all analyses. All data were analysed using a computer software (SPSS version 21).

3. Results

All enucleated eyes were included in the study. In all images captured, it was possible to analyse the endothelium of the cornea. With the specular contact microscope, it was possible to observe a regular pattern of polygonal cells with sharp, uniform and juxtaposed edges in all regions studied (Figure 2).

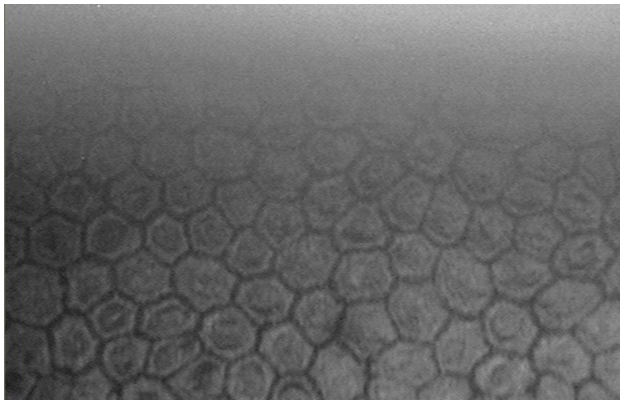

Figure 2. Image of the corneal endothelium of a 6-month-old swine obtained with specular microscopy.

In all eyes examined with specular microscopy, endothelial images were obtained easily. The ECD 1865

cells/mm² in the central region, 1877 cells/mm² in the upper region, 1854 cells/mm² in the lower region, 1847 cells/mm² in the lateral region and 1831 cells/mm² in the medial region. There was no significant difference regarding the ECD in all corneal regions evaluated (P = 0.307). In all corneal regions analysed, endothelial cells with four, five, six, seven and eight sides were found. Considering the morphology of all cells analysed, 54% of the cells had six sides, 21% of the cells had five sides, and 18% of the cells had seven sides. In addition, 5% of the cells had four sides and 2% of the cells had eight sides. In the central region, the average percentage of hexagonal cells was 53%. In the upper region, it was 54%, in the lower region, it was 54%, in the lateral region, it was 54%, in the medial region, it was 54%. There was no significant difference regarding the ECD in all corneal regions evaluated (P = 0.735). No differences were observed in ECD and hexagonality between the left and the right eyes.

4. Discussion

Specular microscopy is the primary technique for evaluating the endothelium *in vivo*[2]. Knowledge of the endothelial characteristics of the healthy cornea is critical for the interpretation of specular microscopy. Analysis of the morphometric and morphological parameters of healthy corneal endothelium has been performed using specular microscopy in some animal species in living animals[15,17,19] and in enucleated eyes[14,10,12,13,16,20].

The swine has widely been used as an experimental model in ophthalmology due to similarities of its cornea to the human cornea[27,32,33,28]. However, studies evaluating the healthy corneal endothelium of pigs are scarce[28,30,31,34], and interspecies variations in the characteristics of the corneal endothelium prevent the extrapolation of data obtained from one species to another. Due to this variation in endothelial parameters, the ideal is to establish reference values in healthy corneas for each species.

Various studies have been performed on animals with a specular microscope, but only the central region of the cornea was analysed[10,12,35]. This study reports endothelial cell data from different regions of healthy porcine corneas using a contact specular microscope. This is the first study performed to analyze these data in pigs. The use of eyes from swine intended for slaughter was a viable alternative, preventing animals from being sacrificed for reasons exclusively related to research. In the present study, post-mortem eye collection and individual storage in a humid chamber for up to 4 hours allowed the preservation of endothelial integrity for specular microscopy. This methodology has already been used by other authors and proved to be effective, allowing the analysis of the corneal endothelium up to 6 hours after death[10,12,16,17]. Specular microscopy can be used for both *in vivo* and *ex vivo* evaluation and is one of the most validated methods for evaluating corneal endothelium parameters[36-38]. Unlike other *ex vivo* techniques in specular microscopy, there is no deformation or distortion of endothelial cells during fixation or staining[39]. Eyes examined with a specular microscope within 6 h after enucleation still show a preserved endothelial structure[12,13,20]. Even so, all the data obtained can be used in future studies where live animals will be examined.

According to the contact or not of the objective with the cornea, there are two models of specular microscopes available for the evaluation of the endothelium[15,38,40]. In the current study, it was used contact specular microscopy. With the specular contact microscope, it was possible photograph the corneal endothelium cells of healthy porcine corneas. Furthermore, with a specular contact microscope, it was possible to examine the endothelium of different areas of the cornea. In the current study, enucleation was performed before scalding the animals to avoid corneal damage due to hot water. Thus, it was possible to select only eyes with intact epithelium. All eyes were examined with a slit lamp and fluorescein dye before the start of the experiment. In eyes with damaged epithelium, it is not possible to image the corneal endothelium with a specular microscope. Moreover, specular microscopy is not suitable for evaluating areas with endothelial trauma because of the edema it is difficult to obtain images in these regions[2]. In the present study, in all enucleated eyes, it was possible to analyse and photograph the corneal endothelium because only healthy eyes were examined.

The most used parameters for endothelial analysis are the hexagonality, the average cell area, the coefficient of variation of the cell area and the ECD[6,38]. In the present study, cell density and hexagonality from different regions of the cornea were analysed.

Different methodologies have been used to perform the analysis of the corneal endothelium[6,38]. Manual determination of the ECD by the centre method represents an accurate analysis by specular microscopy[40]. The accuracy of the assessment depends on the quality of the endothelial image obtained by specular microscopy. In the present study, only healthy eyes with a transparent cornea were selected to obtain images with good quality. With the software available in the specular microscope, it was possible to perform the endothelial count by the semi-automated method. The cell centre labelling method was used to determine the ECD. After labelling the selected cells, the software included in the device provided the cell density. There is no uniformity regarding the total of endothelial cells counted in each study performed to obtain a maximum accuracy. Andrew et al.[15] selected 15 endothelial cells in a study on specular microscopy to analyse the corneal endothelium of llamas and alpacas.

Some authors included 30 endothelial cells in the analysis[20] whereas others included 50 to 100 endothelial cells in each analysed cornea[12]. In the present study, 100 cells from each corneal region were analysed to assess cell morphology. Further studies must be performed to determine a minimum number of cells that should be counted in each sample to minimise possible sampling error.

Considering all analysed images, in the study reported here, the mean ECD was 1855 cells/mm^2. Mean cell density values of 2669 cells/mm^2 for llamas and 2275 cells/mm^2 for alpacas have been found using a non-contact specular microscope[15]. Brambatti and collaborators[19] found similar values in rabbits, with a mean cell density of around 1867/mm^2. In chinchillas, the average cell density calculated through specular microscopy in groups of different ages was between 2124 and 3423 cells/mm^2, with the highest average found in animals aged 2 to 4 months[17]. In owl, other authors obtained values between 2602 and 2864 cells/mm^2, also demonstrating higher cell density in younger animals[41]. The effect of age on endothelial density has already been studied by means of specular microscopy in humans[42], dogs[8], rabbits[18], horses[15], llamas/alpacas[15], cats[12], pigs[28], sheep[13], chinchillas[17] and other animal species. The ECD decreased with age[15]. In the present study, as all animals were of the same age, the effects of aging were not analysed. Both in humans and in animals, there is a decrease in ECD with aging[6,12,13,28,42].

In the present study, there was no significant difference in ECD among the five regions studied. There is no consensus in the consulted literature regarding the differences in ECD between the central and peripheral regions of a healthy cornea. According to Coyo et al.[13] this controversy may be due to the different methods used and to the different distances of the peripheral images obtained in relation to the limbus. In humans, some authors have already compared the ECD of the central and peripheral regions of healthy corneas and found no statistical difference[43,44].

There are different types of specular microscopes with built-in software that allow automatic or semi-automatic analysis, and many software packages automatically calculate endothelial parameters. In our study, the morphology of the corneal endothelium was studied using Adobe photoshop. A regular hexagonal pattern is the most geometrically and thermodynamically stable configuration. Lower percentages indicate a diminishing state of health of the endothelium. In the present study, it was observed that the swine endothelium consists of a monolayer of polygonal cells, with a predominance of hexagonal cells in all regions evaluated. Considering all analysed images, 54% of the endothelial cells were hexagonal in shape. In humans, in the normal healthy cornea, 70%-80% of corneal endothelial cells have a hexagonal shape[38]. In the different animal species in which endothelial morphology has already been studied in healthy corneas, most cells have six sides[10,12]. The percentage of hexagonality found depends on the species and the age of the animals evaluated.

Smeringova et al.[3] studied the healthy corneal endothelium of swines aged between 5 and 6 months and found a percentage of 51% of cells with six sides. Similar to our study, Lee et al.[28] using a confocal microscope, found a percentage of hexagonal cells of 54% in pigs aged between 5 and 10 months. In humans, the percentage of hexagonal cells is expected to decrease with age[6]. However, in swine, with regard to morphology, there is no correlation between age and the percentage of hexagonal cells[28]. In the current study, there was no significant difference in the percentage of hexagonal cells among the five regions evaluated. Similarly, Clerot et al.[31], using optical microscopy and vital dyes, also found no significant differences in hexagonality from different regions of a healthy swine cornea. The results obtained in the current study show that there were no differences in ECD and hexagonality when the right and left eyes were compared. In healthy corneas, there are no differences in the endothelial parameters when the left eye is compared to the right eye[10, 12,16,17,19,41].

In the present study, only eyes of male animals were analysed. Previous studies have shown that in healthy corneas, there is no difference in endothelial parameters related to the gender of the animals studied[16].

5. Conclusions

This study demonstrates that the endothelial cell density (ECD) and hexagonality of the central of swine cornea area represent the entire endothelial mosaic.

Conflict of interests

The authors declare no conflict of interest.

Author Contributions

Conceptualization: E.V.B. Vargas and J.A.T. Pigatto. *Methodology*: E.V.B. Vargas, A.M. Pigatto, R.S. Rocha, M.E.M. Franceschini and J.A.T. Pigatto. *Investigation*: E.V.B. Vargas, A.M. Pigatto, R.S. Rocha, M.E.M. Franceschini, J.A.T. Pigatto. *Project administration*: J.A.T. Pigatto. *Writing (original draft, review & editing):* E.V.B. Vargas, R.S. Rocha and J.A.T. Pigatto.

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