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Evaluation of equine corneal endothelium after exposure to 0.5% indocyanine green - *in vitro* study

Avaliação do endotélio corneano de equinos após exposição à indocianina verde 0,5% - estudo *in vitro*

Vanessa Ruiz Moura da Silva[1]; Maria Cristina Caldart de Andrade[1]; Claudia Skilhan Faganello[1]; Marcela Torikachvili[2]; Andre Silva Carissimi[3]; João Antonio Tadeu Pigatto[3*]

Abstract

The purpose of the study was to investigate whether indocyanine green (ICG) dye damages the corneal endothelium of horses. Twenty-four corneas of 12 healthy equines, males or females, of different ages were used in this study. Only eyes with no ocular findings were used. Randomly, one eye was included in the treatment group and one in the control group. The eyes of the treatment group were exposed for 1 minute to dye ICG 0.5%. After that the endothelium of all eyes was stained with trypan blue and alizarin red S and analyzed and photographed under an optical microscope. Areas with damaged endothelial cells were manually measured and quantified using software for morphometric analysis and expressed as a percentage of cell damage. In all eyes examined areas of cell damage were observed in both corneas of the control group and the treatment group. The mean endothelial damage was 0.8 ± 0.37% in the treatment group and 0.97 ± 0.39% in the control. The Qui-square test stated that treatment and control group were not different. The ICG 0.5% did not cause acute damage to equine corneal endothelium.
Key words: Cataract. Indocyanine green. Corneal endothelium. Alizarin red.

Resumo

O objetivo do estudo foi investigar se indocianina verde (ICG) induz dano nas células do endotélio da córnea de equinos. Vinte e quatro córneas de 12 equinos saudáveis, machos ou fêmeas, de diferentes idades foram estudadas. Somente olhos hígidos foram utilizados. Aleatoriamente, um olho foi incluído no grupo controle e outro no grupo tratamento. Os olhos do grupo tratamento foram expostos durante um minuto à indocianina verde a 0,5%. Posteriormente, o endotélio da córnea foi corado com azul de tripano e vermelho de alizarina, analisado e fotografado usando microscópio óptico. As áreas com células endoteliais danificadas foram aferidas e quantificadas utilizando um *software* para análise morfométrica. Os valores encontrados foram expressos como percentual de perda celular. Em todos os olhos examinados foram observadas áreas de dano celular, tanto no grupo controle quanto no grupo tratamento. A perda celular endotelial média foi de 0,8±0,37% no grupo tratamento e 0,97 ± 0,39% no grupo controle. O teste Qui-quadrado confirmou que os grupos tratamento e controle não diferiram. Foi possível concluir que a ICG 0,5% não causou dano agudo nas células do endotélio da córnea de equinos.
Palavras-chave: Catarata. Indocianina verde. Endotélio corneano. Vermelho de alizarina.

[1] Discentes, Programa de Pós-Graduação em Medicina Animal, Equinos, Faculdade de Veterinária, UFRGS, Porto Alegre, RS, Brasil. E-mail: vanessaruizms@hotmail.com; mccaldart@hotmail.com; kkfaganello@yahoo.com.br
[2] Discente de Graduação, UFRGS, Porto Alegre, RS, Brasil. E-mail: marcelatorika@gmail.com
[3] Profs., Departamento de Medicina Animal, UFRGS, Porto Alegre, RS, Brasil. E-mail: pigatto@ufrgs.br; asc@ufrgs.br
[*] Author for correspondence

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Introduction

A healthy corneal endothelium is essential for corneal transparency. Insult to this monolayer, such as surgical trauma or intracameral injection, poses a risk for permanent harmful effects on the corneal endothelium (PIGATTO et al., 2008; SAAD et al., 2008). In cases of blindness due to cataract, surgical removal of the lens is the standard management. Phacoemulsification is the most useful technique in horses for the treatment of cataract (FIFE et al., 2006; HARDMAN et al., 2001; MILLICHAMP; DZIEZYC, 2000). Continuous curvilinear capsulorhexis (CCC) is considered the most important step of cataract removal, and the visualization of the lens capsule may impaired by the presence of white cataract, dense, with no reflection of the eye fundus (CHUNG et al., 2005; MELLES et al., 1999). Staining of the anterior capsule has become a popular method of increasing visibility when performing CCC. Several kinds of dye-enhanced techniques for CCC have been developed (HOLLEY et al., 2002; PANDEY et al., 2000).

Currently, specular microscopy (ANDREW et al., 2001; BERCHT et al., 2015; FRANZEN et al., 2010; PIGATTO et al., 2006, 2008), scanning electron microscopy (SEM) (PIGATTO et al., 2004, 2005a,b, 2009; RODRIGUES et al., 2006; TAMAYO-ARANGO et al., 2009) and confocal microscopy (LEDBETTER; SCARLETT, 2009) are accepted methods for endothelial analysis. The most common method of determining cell death or injury, in the areas not seen by specular microscopy, is through the use of vital dye such as trypan blue and alizarin red (PARK et al., 2012; RUÍZ et al., 1991; SAAD et al., 2008; TERRY et al., 2009). The toxicity of vital dyes to the corneal endothelium has been evaluated in humans and in rabbits (CHANG et al., 2005; CHUNG et al., 2005; RODRIGUES et al., 2009). However, the effects of vital dyes on the endothelium horses have not been studied. The aim of this study was to investigate whether ICG dye damages the endothelium of the horse cornea.

Materials and Methods

Twenty-four corneas from 12 healthy equines, male or female, of different ages, were used in this study. These eyes were obtained from a licensed Brazilian commercial slaughterhouse (Foresta, São Gabriel, RS). All procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement on the use of animals in ophthalmic and vision research. Eyes were enucleated immediately after death and were kept at 4ºC in moist chambers. Ophthalmic examination was performed before the experiment and included slit-lamp biomicroscopy, (Portable Slit Lamp SL 15, Kowa, Japan) and proof of fluorescein (Fluorescein Strips, Ophthalmos, SP, Brazil). Eyes that showed evidence of ocular disease were excluded. Immediately after the enucleation, the corneas were carefully dissected and washed with cold saline so as to remove any adhering pigments. Afterwards, a six mm corneal trephine was used to remove a central corneal button. Twenty-five mg of ICG (Ophthalmos, SP, Brazil) was dissolved in 0.5 mL aqueous solvent in 4.5 mL balanced salt solution (BSS). ICG was applied to one randomly selected eye from each horse and the contralateral eye served as control. Group 1: Corneal endothelium was perfused with 0.5 mL of ICG 0.5% for one minute (Figure 1A), followed by washout with BSS. Group 2: Corneas were used as controls. Corneal endothelium of this group was perfused with 0.5 mL BSS for one minute. Corneas were placed endothelial side up, on a glass slide, and 0.25% trypan blue (Sigma-Aldrich, St. Louis, USA) was added dropwise to cover the endothelium. After 1.5 minutes, the stain was poured off and the cornea briefly rinsed twice in BSS. The endothelial layer was then covered with 0.2% alizarin red S (Sigma-Aldrich, St. Louis, USA) for 1.5 minutes (Figure 1A), and again rinsed twice in saline after pouring away the staining reagent. Corneal endothelium was evaluated and photographed using an optical microscope (Nikon Eclipse E200, Japan) at 40X magnification. Three

images of corneal endothelium of each cornea were chosen for analysis. For each eye, the mean of measurements made on three micrographs was calculated. Areas with damaged endothelial cells were manually measured, quantified using a software for morphometric analysis (UTHSCSA Image tool 3.0, Texas, USA), and described as mean ± SD. In the quantitative analysis, the percentage of cellular damage of each image (three images from each horse) was measured and was attributed a binary variable for representation. This way, a binomial model was used to verify the influence of the treatment in the experiment. Statistical analysis was performed using Qui-square test. A P value < 0.05 was considered significant.

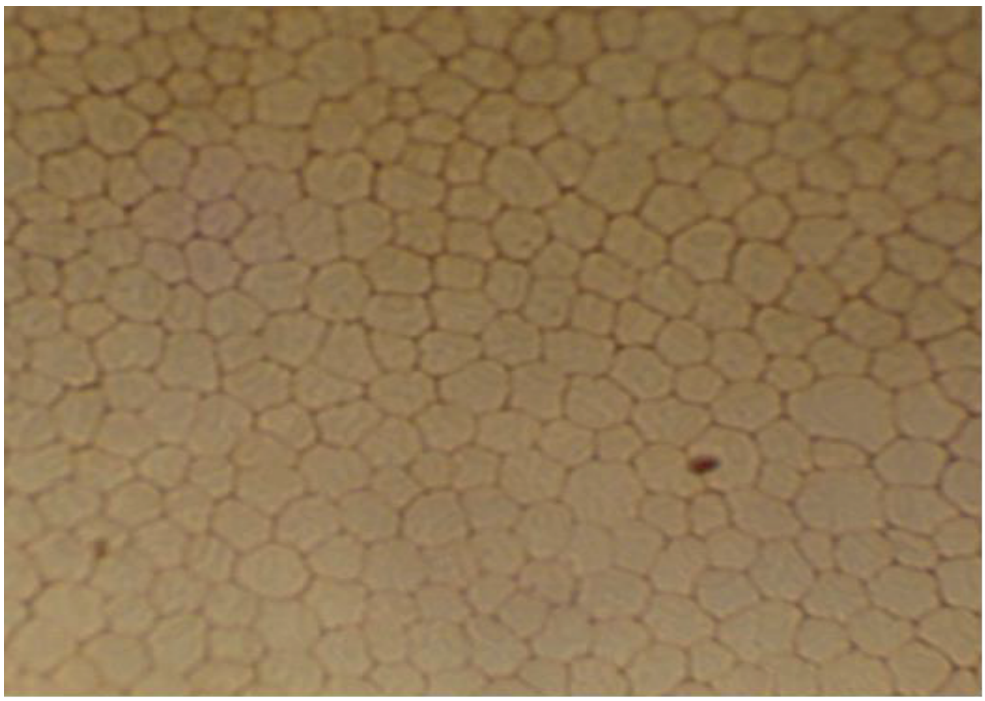
Figure 1. A.Excised corneoscleral button of horse eye with the endothelial side face up covered with ICG. B. Excised corneoscleral button of horse eye with the endothelial side face up after alizarin red staining.

A **B**

Results

With optical microscopy after the use of the dyes, it was possible to observe sharp cellular borders in all analysed images (Figure 2). Light microscopy demonstrated that the corneal endothelium was continuous, and with minimal areas of endothelial cell damage, in a linear format. Furthermore, it was possible to identify and delineate the areas with cellular damage in all analyzed images. These changes were observed in endothelial cells in both groups. The mean endothelial damage was 0.8 ± 0.37% in the ICG group and 0.97 ± 0.39% in the control group. The percentage of endothelial damage was not statistically different between the two groups (P=0.31). The treatment and control groups differences were not significant by the Chi-square test at the 5 % level of significance. This indicates that there is no influence of treatment on cell damage percentage.

Figure 2. Optical photomicrograph of equine corneal endothelium after perfusion with ICG 0.5% for 1 minute and stained with alizarin red and trypan blue. The endothelium has a regular polygonal appearance. X40 magnification.



Discussion

Since endothelial cells have limited capacity mitosis, it is important that the intraocular substances are non-toxic to corneal endothelium. Previously, the toxicity of vital dyes, such as trypan blue, ICG, and brilliant blue, to corneal endothelium has been evaluated in humans, swine, and rabbits (CHANG et al., 2005; HORIGUCHI et al., 1998; TERZARIOL et al., 2016).

Specular microscopy has become a standard technique to determine endothelial cell counting and cell morphology in humans and animals (ABIB; BARRETO 2001; ANDREW et al., 2001; BERCHT et al., 2015; FRANZEN et al., 2010; PIGATTO et al., 2006, 2008). Furthermore, this technique has been used to study the toxicity studies of different substances on the corneal endothelium, including vital stains, antiseptic and anesthetic intraocular (CHUNG et al., 2005; HORIGUCHI et al., 1998; KIM et al., 1998; NAOR et al., 2001). The high cost of specular microscope and the difficulty in obtaining good images in injured endothelial areas, make the use of this technique difficult or impossible (ANDREW et al., 2001; PIGATTO et al., 2005a). In the present study, this method has not been employed because the specular microscope was not available.

SEM has been widely used to compare the endothelial ultrastructure of vertebrates, and to evaluate the effects of medications, chemicals or surgical procedures on the endothelium (PIGATTO et al., 2004, 2005a,b, 2009; RODRIGUES et al., 2006; TAMAYO-ARANGO et al., 2009). However, it requires processing of samples and sophisticated equipment for utilisation.

In the current study, acquiring the eyes were from slaughterhouse avoided the use of live animals for research. In the present study, the utilization

of eyes within one hour after enucleation allowed the maintenance of endothelial structure. Corneal endothelial evaluations using eyes immediately after the death of the animals have been used in studies with specular microscopy, SEM, and optical microscopy with excellent results (ALBUQUERQUE et al., 2015; FAGANELLO et al., 2016; PIGATTO et al., 2004, 2005a,b, 2009; RODRIGUES et al., 2006).

In the present study, corneal endothelium was exposed to the dye for one minute. This time was chosen because it is normally the maximum time that the dye remains within the eye during surgery for cataract removal (CHANG et al., 2008; XIAO et al., 2004). Among the methods used for assessing cell death or damaged areas, is a vital staining technique using trypan blue and alizarin red (PARK et al., 2012; RUÍZ et al., 1991; SAAD et al., 2008; SPERLING, 1977; TAYLOR; HUNT, 1981; TERRY et al., 2009). Many studies on endothelial evaluation for corneal transplantation, and studies on the effects of substances to the endothelium, intraocular administration of anesthetics and other vital staining using this technique to analyze, by means of photographic images, to quantitatively and qualitatively analyze cell loss (CHANG et al., 2005; EGGELING et al., 2000; LIOU et al., 2004; SAAD et al., 2008; TERRY et al., 2009; WERNER et al., 1998). This *in vitro* technique has been used in the laboratory to determine acute cell trauma and death from surgical manipulations. Trypan blue stains the nuclei of dead cells, in which the plasma membranes are no longer intact. In normal eyes, the endothelial cell is impervious to trypan blue and does not stain (PARK et al., 2012; TAYLOR; HUNT, 1981). Alizarin red S stains intercellular spaces and the Descemet membrane in areas with detached or necrosed endothelial cells. Combined staining with trypan blue and alizarin red, allows the analysis of endothelial cell morphology and integrity, with determination of areas presenting cell damage or loss (PARK et al., 2012; SAAD et al., 2008; TAYLOR; HUNT, 1981). In the present study, the staining protocol described by Taylor and Hunt (1981) was used with excellent results. This made it possible to examine the whole endothelial surface selected. Moreover, the technique proved easy to perform, simple, practical, and inexpensive for the analysis of equine cornea endothelial cells.

The most important advance observed in performing the capsulotomy was the use of dyes to stain the anterior capsule, which facilitated the realization of this procedure (HISATOMI et al., 2006; LINEBARGER et al., 1999). As the endothelium presents minimal mitotic activity, it is important that applied intraocular substance are not toxic to the corneal endothelium. ICG staining for capsule visualization has been reported since end of the 1990s (RODRIGUES et al., 2009). ICG concentration usually used for staining of the anterior lens capsule ranges from 0.125% to 0.5% (HORIGUCHI et al., 1998; RODRIGUES et al., 2009). In a study by Horiguchi et al. (1998), 0.5% ICG was used to stain the anterior capsule for CCC in phacoemulsification in humans and concluded that the ICG was not toxic to the corneal endothelium. Holley et al. (2002), studied the effects of ICG on the human and rabbit corneal endothelium. They showed that corneas exposed to ICG had no corneal endothelial damage. On the other hand, Chang et al. (2005), in their rabbit corneal endothelial cells culture observed, that a one-minute exposure to 0.5% ICG resulted in significant increase in the percentage of damage cells. In the current study was used the ICG with the highest concentration. A solution of ICG 0.5% was left in the eye for up to one minute to determine whether exposure to the dye was toxic to corneal endothelium equine. ICG was prepared as in the study by Chang et al. (2005), 25 mg of ICG was dissolved in 0.5 mL aqueous solvent in 4.5 mL BSS to obtain a 0.5% solution.

In the present study, only the central region of the corneal endothelium was subjected to analysis. Previous reports have demonstrated that humans, and other mammals, have no significant differences in endothelial parameters between the central and

peripheral regions of healthy corneas (BERCHT et al., 2015; GWIN et al., 1982; McCAREY et al., 2008; PIGATTO et al., 2004; TAYLOR; HUNT, 1981). In the current study, areas with minimal cell loss were occasionally detected in both groups and, possibly, occurred due to manipulation of the corneas. Based on the present data, it is suggested that ICG can be used as a dye for the anterior lens capsule during cataract surgery in horses.

Conclusion

In accordance with the presented results, it was concluded that ICG not damaged the cells of the equine corneal endothelium.

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References

ABIB, F. C.; BARRETO, J. J. Behavior of corneal endothelial density over a lifetime. *Journal of Cataract and Refractive Surgery*, New York, v. 27, n. 10, p. 1574-1578, 2001.

ALBUQUERQUE, L.; FREITAS, L. V. R.; PIGATTO, J. A. T. Analysis of the corneal endothelium in eyes of chickens using contact specular microscopy. *Semina: Ciências Agrárias*, Londrina, v. 36, n. 6, p. 4199-4206, 2015.

ANDREW, S. E.; RAMSEY, D. T.; HAUPTMAN, J. G.; BROOKS, D. E. Density of corneal endothelial cells and corneal thickness in eyes of euthanatized horses. *American Journal of Veterinary Research*, Chicago, v. 62, n. 4, p. 479-482, 2001.

BERCHT, B. S.; ALBUQUERQUE, L.; ARAUJO, A. C.; PIGATTO, J. A. T. Specular microscopy to determine corneal endothelial cell morphology and morphometry in chinchilas (*Chinchilla lanigera*) in vivo. *Veterinary Ophthalmology*, Oxford, v. 18, n. 1, p. 137-142, 2015.

CHANG, Y. S.; HSIAO, J. H.; TSENG, S. H.; KUO, P. H.; CHEN, F. K. Indocyanine green-assisted phacoemulsification in cases of complicated or simple advanced cataracts. *Journal of the Formosan Medical Association*, Singapore, v. 107, n. 9, p. 710-719, 2008.

CHANG, Y. S.; TSENG, S. Y.; TSENG, S. H.; CHEN, Y. T.; HSIAO, J. H. Comparison of dyes for cataract surgery part 1: cytotoxicity to corneal endothelial cells in a rabbit model. *Journal of Cataract and Refractive Surgery*, New York, v. 31, n. 4, p. 792-798, 2005.

CHUNG, C. F.; LIANG, C. C.; LAI, J. S.; LO, E. S.; LAM, D. S. Safety of trypan blue 1% and indocyanine green 0. 5% in assisting visualization of anterior capsule during phacoemulsification in mature cataract. *Journal of Cataract and Refractive Surgery,* New York, v. 31, n. 5, p. 938-942, 2005.

EGGELING, P.; PLEYER, U.; HARTMANN, C.; RIECK, P. W. Corneal endothelial toxicity of different lidocaine concentrations. *Journal of Cataract and Refractive Surgery*, New York, v. 26, n. 9, p. 1403-1408, 2000.

FAGANELLO, C. S.; SILVA, V. R. M.; ANDRADE, M. C. C.; CARISSIMI, A. S.; PIGATTO, J. A. T. Morphology of endothelial cells from different regions of the equine cornea. *Ciência Rural,* Santa Maria, v. 46, n. 12, p. 2223-2228, 2016.

FIFE, T. M.; GEMENSKY-METZLER, A. J.; WILKIE, D. A.; COLITZ, C. M.; BRAS, I. D.; KLAGES, D. C. Clinical features and outcomes of phacoemulsification in 39 horses: a retrospective study (1993-2003). *Veterinary Ophthalmology*, Oxford, v. 9, n. 5, p. 362-368, 2006.

FRANZEN, A. A.; PIGATTO, J. A. T.; ABIB, F. C.; ALBUQUERQUE, L.; LAUS, J. L. Use of specular microscopy to determine corneal endothelial cell morphology and morphometry in enucleated cat eyes. *Veterinary Ophthalmology,* Oxford, v. 13, n. 4, p. 222-226, 2010.

GWIN, R. M.; LERNER, I.; WARREN, J. K.; GUM, G. Decrease in canine corneal endothelial cell density and increase in corneal thickness as functions of age. *Investigative Ophthalmology and Visual Science*, St. Louis, v. 22, n. 2, p. 267-271, 1982.

HARDMAN, C.; McILNAY, T. R.; DUGAN, S. J. Phacofragmentation for morganian cataract in a horse. *Veterinary Ophthalmology,* Oxford, v. 4, n. 3, p. 221-225, 2001.

HISATOMI, T.; ENAIDA, H.; MATSUMOTO, H.; KAGIMOTO, T.; UENO, A.; HATA, Y.; KUBOTA, T.; GOTO, Y.; ISHIBASHI, T. Staining ability and biocompatibility of brilliant blue G. *Archives of Ophthalmology*, Chicago, v. 124, n. 4, p. 514-519, 2006.

HOLLEY, G. P.; ALAM, A.; KIRI, A.; EDELHAUSER, H. F. Effect of indocyanine green intraocular stain on human and rabbit corneal endothelial structure and viability: an in vitro study. *Journal of Cataract and Refractive Surgery*, New York, v. 28, n. 6, p. 1027-1033, 2002.

HORIGUCHI, M.; MIYAKE, K.; OHTA, I.; ITO, Y. Staining of the lens capsule for circular continuous capsulorrhexis in eyes with white cataract. *Archives of Ophthalmology*, Chicago, v. 116, n. 4, p. 535-537, 1998.

KIM, T.; HOLLEY, G. P.; LEE, J. H.; BROOCKER, G.; EDELHAUSER, H. F. The effects of intraocular lidocaine on the corneal endothelium. *Ophthalmology*, Rochester, v. 105, n. 1, p. 125-130, 1998.

LEDBETTER, E. C.; SCARLETT, J. M. In vivo confocal microscopy of the normal equine cornea and limbus. *Veterinary Ophthalmology*, Oxford, v. 12, n. 1, p. 57-64, 2009.

LINEBARGER, E. J.; HARDTEN, D. R.; SHAH, G. K.; LINDSTROM, R. L. Phacoemulsification and modern cataract surgery. *Survey of Ophthalmology*, New York, v. 44, n. 2, p. 123-147, 1999.

LIOU, S. W.; CHIU, C. J.; WANG, I. J. Effect of intracameral injection of lidocaine and carbachol on the rabbit corneal endothelium. *Journal of Cataract and Refractive Surgery*, New York, v. 30, n. 6, p. 1351-1355, 2004.

McCAREY, B. E.; EDELHAUSER, H. F.; LYNN, M. J. Review of corneal endothelial specular microscopy for FDA clinical trials of refractive procedures, surgical devices and new intraocular drugs and solutions. *Cornea*, Hagerstown, v. 27, n. 1, p. 1-16, 2008.

MELLES, G. R. J.; WAARD, P. W.; PAMEYER, J. H.; BEEKHUIS, W. H. Trypan blue capsule staining to visualize the capsulorhexis in cataract surgery. *Journal of Cataract and Refractive Surgery*, New York, v. 25, n. 1, p. 7-9, 1999.

MILLICHAMP, N. J.; DZIEZYC, J. Cataract phacofragmentation in horses. *Veterinary Ophthalmology*, Oxford, v. 3, n. 2-3, p. 157-164, 2000.

NAOR, J.; SAVION, N.; BLUMENTHAL, M.; ASSIA, E. I. Corneal endothelial cytotoxicity of diluted povidone-iodine. *Journal of Cataract and Refractive Surgery*, New York, v. 27, n. 6, p. 941-947, 2001.

PANDEY, S. K.; WERNER, L.; ESCOBAR-GOMEZ, M.; ROIG-MELO, E. A.; APPLE, D. J. Dye-enhanced cataract surgery part 1: anterior capsule staining for capsulorhexis in advanced/white cataract. *Journal of Cataract and Refractive Surgery*, New York, v. 26, n. 7, p. 1052-1050, 2000.

PARK, S.; FONG, A. C.; CHO, H.; ZHANG, C.; GRITZ, D. C.; MIAN, G.; HERZLICH, A. A.; GORE, P.; MORGANTI, A.; CHUCK, R. S. Protocol for vital dye staining of corneal endothelial cells. *Cornea*, Hagerstown, v. 31, n. 12, p. 1476-1479, 2012.

PIGATTO, J. A. T.; ABIB, F. C.; PEREIRA, G. T.; BARROS, P. S. M.; FREIRE, C. D.; LAUS, J. L. Density of corneal endothelial cells in eyes of dogs using specular microscopy. *Brazilian Journal of Veterinary Research and Animal Science*, São Paulo, v. 43, n. 4, p. 476-480, 2006.

PIGATTO, J. A. T.; ABIB, F. C.; PIZZETI, J. C.; LAUS, J. L.; SANTOS, J. M.; BARROS, P. S. M. Análise morfométrica do endotélio corneano de coelhos à microscopia eletrônica de varredura. *Acta Scientiae Veterinariae*, Porto Alegre, v. 33, n. 1, p. 41-45, 2005a.

PIGATTO, J. A. T.; ANDRADE, M. C.; LAUS, J. L.; SANTOS, J. M.; BROOKS, D. E.; GUEDES, P. M.; BARROS, P. S. Morphometric analysis of the corneal endothelium of yacare caiman (*Caiman yacare*) using scanning electron microscopy. *Veterinary Ophthalmology*, Oxford, v. 7, n. 3, p. 205-208, 2004.

PIGATTO, J. A. T.; CERVA, C.; FREIRE, C. D.; ABIB, F. C.; BELLINI, L. P.; BARROS, P. S. M.; LAUS, J. L. Morphological analysis of the corneal endothelium in eyes of dogs using specular microscopy. *Pesquisa Veterinária Brasileira*, Rio de Janeiro, v. 28, n. 9, p. 427-430, 2008.

PIGATTO, J. A. T.; FRANZEN, A. A.; PEREIRA, F. Q.; ALMEIDA, A. C. V. R.; LAUS, J. L.; SANTOS, J. M.; GUEDES, P. M.; BARROS, P. S. M. Scanning electron microscopy of the corneal endothelium of ostrich. *Ciência Rural*, Santa Maria, v. 39, n. 3, p. 926-929, 2009.

PIGATTO, J. A. T.; LAUS, J. L.; SANTOS, J. M.; CERVA, C.; CUNHA, L. S.; RUOPPOLO, V.; BARROS, P. S. Corneal endothelium of the magellanic penguin (*Spheniscus magellanicus*) by scanning electron microscopy. *Journal of Zoo and Wildlife Medicine*, Lawrence, v. 36, n. 4, p. 702-705, 2005b.

RODRIGUES, E. B.; COSTA, E. F.; PENHA, F. M.; MELO, G. B.; BOTTÓS, J.; DIB, E.; FURLANI, B.; LIMA, V. C.; MAIA, M.; MEYER, C. H.; HÖFLING-LIMA, A. L.; FARAH, M. E. The use of vital dyes in ocular surgery. *Survey of Ophthalmology*, New York, v. 54, n. 5, p. 576-617, 2009.

RODRIGUES, G. N.; LAUS, J. L.; SANTOS, J. M.; RIQUEIRO, M. P.; SMITH, R. L. Corneal endothelial cell morphology of normal dogs in different ages. *Veterinary Ophthalmology*, Oxford, v. 9, n. 2, p. 101-107, 2006.

RUÍZ, J. M.; MEDRANO, M.; ALIÓ, J. L. An improved method of vital staining of the corneal endothelium. *Ophthalmic Research*, Basel, v. 23, n. 1, p. 27-30, 1991.

SAAD, H. A.; TERRY, M. A.; SHAMIE, N.; CHEN, E. S.; FRIEND, D. F.; HOLIMAN, J. D.; STOEGER, C. An easy and inexpensive method for quantitative analysis of endothelial damage by using vital dye staining and adobe photoshop software. *Cornea*, Hagerstown, v. 27, n. 7, p. 818-824, 2008.

SPERLING, S. Combined staining of corneal endothelium by alizarin red and trypan blue. *Acta Ophthalmologica*, Medford, v. 55, n. 4, p. 573-580, 1977.

TAMAYO-ARANGO, L. J.; BARALDI-ARTONI, S. M.; LAUS, J. L.; MENDES-VICENTI, F. A.; PIGATTO, J. A. T.; ABIB, F. C. Ultrastructural morphology and morphometry of the normal corneal endothelium of adult crossbred pig. *Ciência Rural*, Santa Maria, v. 39, n. 1, p. 117-122, 2009.

TAYLOR, M. L.; HUNT, C. J. Dual staining of corneal endothelium with trypan blue and alizarin red S: importance of pH for the dye-lake reaction. *British Journal of Ophthalmology*, London, v. 65, n. 12, p. 815-819, 1981.

TERRY, M. A.; SAAD, H. A.; SHAMIE, N.; CHEN, E. S.; PHILLIPS, P. M.; FRIEND, D. J.; HOLIMAN, J. D.; STOEGER, C. Endothelial keratoplasty: the influence of insertion techniques and incision size on donor endothelial survival. *Cornea*, Hagerstown, v. 28, n. 1, p. 24-31, 2009.

TERZARIOL, M.; HÜNNING, P. S.; BRAMBATTI, G.; ALBUQUERQUE, L.; NEUMANN, C.; PIGATTO, J. A. T. Effects of intracameral brilliant blue on the corneal endothelium of swine: in vitro study. *Pesquisa Veterinária Brasileira*, Rio de Janeiro, v. 36, n. 8, p. 775-780, 2016.

WERNER, L. P.; LEGEAIS, J. M.; OBSLER, C.; DURAND, J.; RENARD, G. Toxicity of xylocaine to rabbit corneal endothelium. *Journal of Cataract and Refractive Surgery*, New York, v. 24, n. 10, p. 1371-1376, 1998.

XIAO, Y.; WANG, Y. H.; FU, Z. Y.; HONG, H. Staining the anterior capsule with indocyanine green or trypan blue for capsulorhexis in eyes with white cataract. *International Ophthalmology*, Dordrecht, v. 25, n. 5, p. 273-276, 2004.