



Evaluation of equine corneal endothelium after exposure to 0.05% brilliant blue – an *in vitro* study

[Avaliação do endotélio corneano de equinos após contato com o corante azul brilhante 0,05% - estudo *in vitro*]

M.C.C. Andrade¹, T.M. Moreno¹, M.S. Muccillo¹, J.A.T. Pigatto², E.V. Camilo³

¹ Aluno de pós-graduação – Faculdade de Veterinária – Universidade Federal do Rio Grande do Sul - Porto Alegre, RS

² Faculdade de Veterinária – Universidade do Rio Grande do Sul – Porto Alegre, RS

³ Aluno de pós-graduação – ESALQ – Universidade de São Paulo - Piracicaba, SP

ABSTRACT

The aim of this study was to evaluate the immediate effects of 0.05% brilliant blue on corneal endothelium of horses. Thirty-eight corneas of 19 horses, male or female, of different ages were studied. Corneas were randomly divided into two groups. Group 1: Corneal endothelium was covered with 0.3mL of brilliant blue 0.05% for 60 seconds followed by rinsing with a balanced salt solution. Group 2: Corneal endothelium was covered with BSS for 60 seconds. The corneas were excised with an 8mm trephine and prepared to analyze posterior endothelial surface using a light microscope (24 corneas) and a scanning electron microscope (14 corneas). The equine posterior corneal endothelium surface observed by optical microscopy and scanning electron microscopy revealed a continuous layer of polygonal cells of uniform size and shape in both the control and treatment groups. Due to non-normal residuals at ANOVA mean comparison, a generalized linear model was utilized at 5% level of significance. The chi-square test stated that treatment and control group were not different statistically. The 0.05% brilliant blue did not cause damage to equine corneal endothelium.

Keywords: equine, brilliant blue, vital dye, corneal endothelium

RESUMO

Objetivou-se avaliar os efeitos imediatos de uma solução de 0,05% de azul brilhante sobre o endotélio da córnea de equinos. Trinta e oito córneas de 19 cavalos, machos ou fêmeas, de diferentes idades foram estudadas. As córneas foram divididas aleatoriamente em dois grupos. Grupo 1: O endotélio corneano foi perfundido com 0,3mL de azul brilhante 0,05% durante 60 segundos seguido por irrigação com uma solução salina balanceada. Grupo 2: O endotélio corneano foi perfundido com BSS durante 60 segundos. As córneas foram posteriormente excisadas com trépano de 8mm e preparadas para análise endotelial utilizando um microscópio óptico (24 córneas) e um microscópio eletrônico de varredura (14 córneas). A análise da superfície posterior do endotélio da córnea equina observada por microscopia óptica e microscopia eletrônica de varredura revelou uma camada contínua de células poligonais de tamanho e forma uniformes tanto no grupo controle quanto no grupo tratamento. Devido aos resíduos não normais na comparação da média de ANOVA, utilizou-se um modelo linear generalizado com nível de significância de 5%. Evidenciou-se com o teste qui-quadrado que não houve diferença estatística entre o grupo controle e o grupo tratamento. O azul brilhante de 0,05% não causou dano ao endotélio corneano de equinos.

Palavras-chave: equino, azul brilhante, corante vital, endotélio da córnea

INTRODUCTION

Lens opacities are present in horses and lead to visual impairment. The most widely used technique for cataract surgery is the phacoemulsification, which consists in opening the anterior lens capsule for opacified lens extraction (Brooks, 2005). Capsulorhexis is considered by many to be one of the most important steps in cataract surgery (Hisatomi *et al.*, 2006). Poor visualization of the capsule results in an inadequate capsulorhexis with a high risk of radial tears toward or beyond the lens equator and associated complications, such as zonular and posterior capsular rupture, vitreous loss, nucleus drop, and intraocular lens (IOL) displacement (Pandey *et al.*, 2000). Staining the anterior capsule with dyes is a useful alternative for performing capsulotomy in cases of advanced cataracts (Pandey *et al.*, 2000). The corneal endothelium consists of a single cell layer between the corneal stroma and anterior chamber. The barrier and ‘‘pump’’ functions of the endothelium are responsible for maintaining corneal transparency by regulating stromal hydration. Lack of a vigorous proliferative response to cell loss in most species makes the endothelium a fragile tissue. Age and intraocular surgery, mainly in the anterior segment, can decrease the number of endothelial cells leading to decompensation and corneal edema, which is a frequent complication of phacoemulsification in horses (Brooks, 2005; McMullen & Utter, 2010). Thus, every substance that could possibly cause toxicity to the corneal endothelium should be carefully evaluated. Anterior lens capsule staining efficacy of trypan blue, indocyanine green, gentian violet, and brilliant blue were tested in eyes from different species and the best concentration of each substance was determined (Dada *et al.*, 2004; Chang *et al.*, 2005; Hisatomi *et al.*, 2006). The toxicity of vital dyes was tested in cultured rabbit endothelial cells (Chang *et al.*, 2005), rat eyes (Hisatomi *et al.*, 2006), and rabbit and human eyes (Holley *et al.*, 2002; Van Dooren *et al.*, 2004).

Brilliant blue was tested for cromovitrectomy and phacoemulsification, as a facilitator of capsulorhexis for improving anterior lens capsule visualization (Hisatomi *et al.*, 2006). Currently, specular microscopy, vital staining and scanning electron microscopy (SEM) are used to evaluate the corneal endothelium (Chang *et al.*, 2005;

Pigatto *et al.*, 2005a; Pigatto *et al.*, 2005b; Pigatto *et al.*, 2006; Pigatto *et al.*, 2009; Landry *et al.*, 2011; Bercht *et al.*, 2015; Faganello *et al.*, 2016). Light microscopy has been used to evaluate corneal endothelial toxicity due to different medications (Trivedi *et al.*, 2003; Saad *et al.*, 2008). The best visualization of endothelial cells is achieved with the aid of alizarin red that stains cellular borders and trypan blue that stains the nucleus of disrupted cells (Taylor & Hunt, 1981). Scanning electron microscopy has been widely used to evaluate and compare the ultrastructure of endothelial cells of vertebrates and to evaluate the effect of drugs and surgery on corneal endothelium (Doughty *et al.*, 1997; Pigatto *et al.*, 2005a; Pigatto *et al.*, 2005b; Pigatto *et al.*, 2009). The use of brilliant blue dye to facilitate capsulotomy has been studied (Hisatomi *et al.*, 2006; Udaondo *et al.*, 2007; Terzariol *et al.*, 2016)). However, the effects of brilliant blue dye on the corneal endothelium of horses have not been investigated. This study aimed to assess the immediate effects of 0.05% brilliant blue on the corneal endothelium of horses by light microscopy and SEM.

MATERIALS AND METHODS

Thirty-eight healthy corneas from 19 equines, including males and females of different ages, were studied. The eyes were obtained from a licensed Brazilian commercial slaughterhouse (Foresta, São Gabriel, RS) and examined only *post mortem*. In these cases, UFRGS’s ethical committee does not require analysis protocol. Besides that, all procedures were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and visual research. The eyes were enucleated and kept in a moist chamber containing saline solution and gauze until the time of examination. Ophthalmic examination was performed before the start of the experiment. The examination consisted of evaluation with slit-lamp biomicroscopy (Portable Slit Lamp SL 15, Kowa, Japan) and fluorescein stain (Fluorescein Strips, Ophthalmos, SP, Brazil). Eyes that showed evidence of ocular disease were excluded. Corneas were randomly divided into two groups. In Group 1, the corneal endothelium was covered with 0.3mL of brilliant blue 0.05% (Opht-Blue, Ophthalmos, São Paulo, Brazil) for 60 seconds

followed by rinsing with a balanced salt solution (BSS, Ophthamos, São Paulo, Brazil). In Group 2, the corneal endothelium was covered with BSS for 60 seconds. The corneas were subsequently punched with an 8mm trephine and the posterior endothelial surface was analyzed using a light microscope (24 corneas) and a scanning electron microscope (14 corneas).

For light microscopy corneal buttons were placed endothelial side up on a glass slide and stained with 0.1% trypan blue (Opht-Blue, Ophthamos, São Paulo, Brazil) followed by rinsing in BSS and staining with alizarin red (Sigma-Aldrich, St. Louis, USA) for 90 seconds. After a final rinse with BSS, the corneal disc was mounted endothelial side up on a microscope slide and examined and photographed using an optical microscope (Nikon Eclipse E200, Japan) at 40X magnification. Three random images of the corneal endothelium of each cornea were chosen for analysis. This part of the experiment was done at the slaughterhouse laboratory facilities. Posteriorly, images were analyzed with software (UTHSCSA Image tool 3.0) and areas with cellular damage were measured.

For scanning electron microscopy corneal buttons were fixed in 2.5% glutaraldehyde in a 0.1 cacodylate buffer (pH 7.4) for 24 hours and transported to the university for SEM. At the University, samples were washed in a cacodylate buffer and dehydrated through an increasing series of ethanol solutions. The specimens were submitted to critical point drying using liquid carbon dioxide. The corneas were placed on aluminum stubs with adhesive tape and sputter coated with gold-palladium. The posterior endothelial surfaces were examined and photographed using a scanning electron microscope (JSM 6060, JEOL, Tokyo, Japan) operating at 10 kV. Five random electron micrographs (x750 to x3500) of each sample were taken. Ultrastructural characteristics were observed and compared between groups using electron micrographs.

In the analysis of variance, the mean cell damage of the treatment and control groups did not differ statistically. However, the excessive number of "zeros" (images without cell damage) resulted in non-normal residuals and the analysis could not be validated. Therefore, "Generalized Linear Models" as proposed by Nelder and Wedderburn (1972) was used in this paper. In the quantitative analysis, corneas were classified as follows. First, the percentage of cellular damage of each image (three images from each horse) was measured. If only one image presented a valid percentage of cell damage (different from zero), it was attributed a binary variable for representation. This way, a binomial model was used with a canonical link to verify the influence of the treatment in the experiment.

RESULTS

With alizarin red and trypan blue staining and the use of light microscopy it was possible to obtain images from all analyzed samples. Minimal areas of endothelial cell damage in a linear format were observed in endothelial cells in both groups (Figure 1). The boxplot (Figure 2) represents the behavior of the data in relation to variability and statistical summaries. The mean values are represented by red dots, median values by black dots and the box extension represents variability (Figure 2). The treatment and control group differences were not significant by the Chi-square test at the 5 % level of significance. The equine posterior corneal endothelium surface observed by SEM revealed a continuous layer of polygonal cells of uniform size and shape. The electron micrographs showed that corneas in both the control and treatment groups presented regular and normal endothelium upon SEM (Figure 3). A polygonal pattern was seen with clear cell borders. Interdigitations, pinocytotic vesicles and microvilli were observed in all images. Punctual areas of cell damage was seen in both groups. The endothelial damage was not different between the two groups.

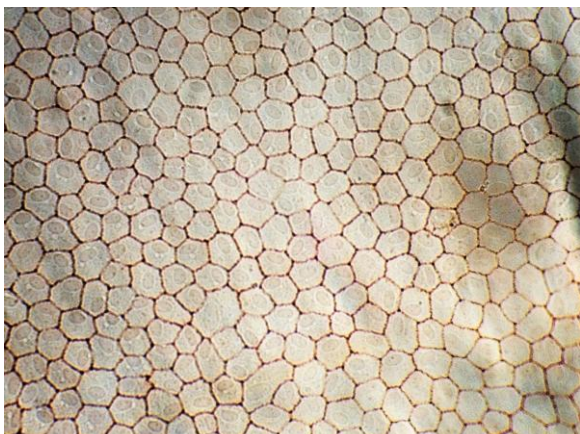


Figure 1. Image of the endothelium of equine cornea stained with alizarin red and examined with an optical microscope subjected to treatment with brilliant blue. The visible cell walls and the endothelial cells without changes are observed. (40X magnification).

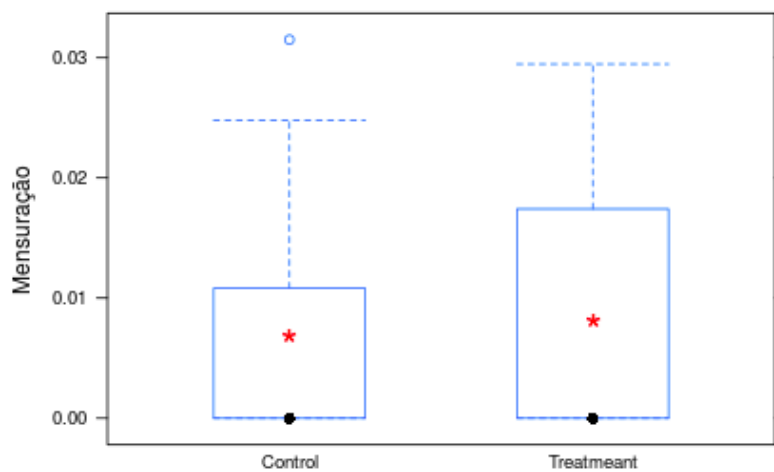


Figure 2. Box plot of mean percentage of damaged cells for treatment and control groups (red dots), median (black dots) and variability is represented.

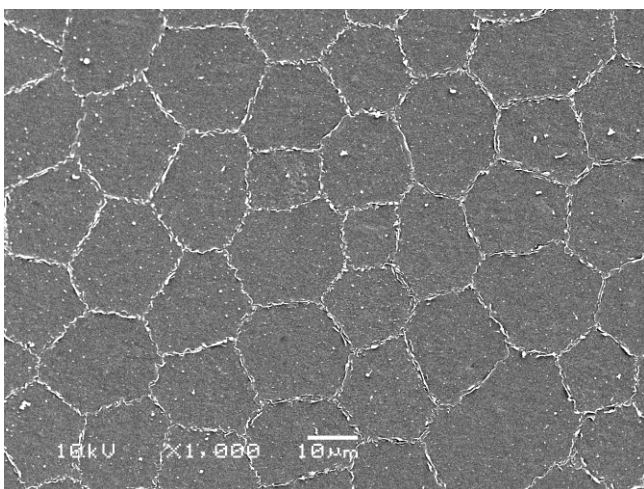


Figure 3. Electron micrograph of equine corneal endothelium subjected to treatment with brilliant blue. Endothelial cells without alterations.

DISCUSSION

The importance of dye-enhanced capsulotomy is widely known for white cataracts, and in cases of a pigmented fundus or vitreous disease. Good visualization of the lens capsule is important to maintain control of tears, to facilitate IOL implantation and, consequently, to prevent severe radial tears and IOL displacement (Pandey *et al.*, 2000; Jacobs *et al.*, 2006). The safety of different dyes used for capsulotomy has been assessed in humans and other species (Holley *et al.*, 2002; Dada *et al.*, 2004; Van Dooren *et al.*, 2004; Hisatomi *et al.*, 2006; Terzariol *et al.*, 2016). However, there is no information known about their effects on the equine corneal endothelium. For modern cataract surgery in horses, IOL implantation is considered mandatory for vision restoration (McMullen *et al.*, 2010). Additionally, the fact that posterior capsule opacification is diminished in the presence of an IOL (Pandey *et al.*, 2000) makes us commit to always perform the best capsulotomy. However, horses are not prone to glaucoma, due to their ocular anatomy and aqueous. The use of air, diathermy, an endoilluminator, vitrectomy and scissors, as well as staining the anterior capsule with dyes have also been suggested (Pandey *et al.*, 2000). In horses, a continuous curvilinear capsulorhexis is performed manually or with the assistance of a high-frequency diathermy tip (McMullen *et al.*, 2010; McMullen & Utter, 2010). There is a report on the intraocular use of trypan blue for phacoemulsification in horses, but there was no mention of its possible toxic effects (Brooks, 2005). Our choice for testing brilliant blue in the equine eye was based on availability of a commercial product, novelty of this dye for veterinary use and its safety regarding the corneal endothelium as reported by Hisatomi *et al.* (2006). According to this study, the osmolality of the solution is an important factor in its toxicity, and therefore in cell survival. The brilliant blue solution has an osmolality similar to that of the physiologic aqueous humor. Thus, the authors concluded that it is a good alternative dye for capsular staining, with superior staining ability and biocompatibility (Hisatomi *et al.*, 2006). Previous studies have shown that the normal equine corneal endothelium was characterized by a monolayer of polygonal cells (Andrew *et al.*, 2001; Ledbetter and Scarlett, 2009; Faganello, *et al.*, 2016).

The dual staining reported by Taylor and Hunt (1981) is an inexpensive, easy and very satisfactory method for assessing the equine corneal endothelium and evaluating toxicity of vital dyes. Alizarin red S stains intercellular borders and bare Descemet's membrane, while trypan blue penetrates the cell membrane staining the nuclei and cytoplasm of damaged cells. The combination of these dyes permits visualization of a wide pattern of regular endothelium, damaged cell areas and cell loss areas (Taylor & Hunt, 1981; Trivedi *et al.*, 2003; Saad *et al.*, 2008; Faganello, *et al.*, 2016). The efficacy and importance of this technique has already been demonstrated since it is used for assessments of endothelial graft quality during storage in eye banks (Saad *et al.*, 2008; Terry *et al.*, 2009). In light microscopy images, brilliant blue also did not have any toxic effects on the equine corneal endothelium compared to the control group. The small percentage of cell damage in both groups was believed to be related to sample preparation, as reported by Trivedi *et al.* (2003). Even in the control group, these linear areas are stained with trypan blue, representing corneal folds, and were due to manipulation during staining and cornea excision (Trivedi *et al.*, 2003). Terry *et al.* (2009) reported some faint striae damages, probably caused by the stretching "trampoline effect" from trephination. Although the majority of our samples were free of damage, one of each group presented these linear bands stained by trypan blue and with poor visualization of intercellular spaces. The manipulation during excision, performing trephination and staining procedures, along with the difficulties in flattening the cornea over the glass laminae, might have caused these slight damages.

SEM has been widely utilized for reporting ultrastructural characteristics of the endothelium and to analyze possible toxic effects of different substances (Eggeling *et al.*, 2000; Pigatto *et al.*, 2005a; Pigatto *et al.*, 2005b; Hisatomi *et al.*, 2006; Pigatto *et al.*, 2009; Terry *et al.*, 2009; Landry *et al.*, 2011). However, the morphometric evaluation was compromised by retraction of the specimens, as there is an expected shrinkage of 11 to 20% using glutaraldehyde 2% fixative solution (Doughty *et al.*, 1997).

In this study, SEM of the equine corneal endothelium revealed characteristics comparable to other species (Pigatto *et al.*, 2005b; Pigatto *et al.*, 2009; Bercht *et al.*, 2015). A monolayer with a polygonal pattern, microvilli, pinocytotic vesicles, interdigitated cell borders are in accordance with previous description of normal endothelium (Pigatto *et al.*, 2005a; Pigatto *et al.*, 2008; Pigatto *et al.*, 2009; Terzariol *et al.*, 2016). Cilia could not be well differentiated in our samples of equine endothelium.

Analysis of electron micrographs, regarding possible damage of brilliant blue, revealed no alterations in the endothelium ultrastructure compared to the BSS group. According to Hisatomi *et al.* (2006), cellular edema or shrinkage and some degenerated cells may be seen when toxic solutions contact the corneal endothelium. Eggeling *et al.* (2000) reported cellular necrosis, denuded Descemet's membrane, swollen cytoplasm and indistinguishable borders, as a result of lidocaine in high concentration exposure. Landry *et al.* (2011) found cell membrane disruption and missing cells at endothelium SEM microphotographs after using SF6 in the anterior chamber of cats (Eggeling *et al.*, 2000; Hisatomi *et al.*, 2006; Landry *et al.*, 2011).

Exposure to brilliant blue did not result in any severe damage to the equine corneal endothelium. Rare damaged cells were detected upon SEM electron micrographs in both groups and were possibly due to manipulation of samples. The few alterations were described as punctual cell membrane disruptions. It was not possible to perform statistical analysis, as the high number of samples with 0% damage decreased the power of statistical tests.

CONCLUSION

The results of this study showed that exposure to 0.05% brilliant blue for 60 seconds did not damage equine corneal endothelial cells.

ACKNOWLEDGMENTS

We thank Foresta abattoir (São Gabriel, RS, Brasil) for supplying the eyes used in this research and the Electron Microscopy Center (CME/UFRGS) for support for the realization of this research.

REFERENCES

- ANDREW, S.E.; RAMSEY, D.T.; HAUPTMAN, J.G. *et al.* Density of corneal endothelial cells and corneal thickness in eyes of euthanatized horses. *Am. J. Vet. Res.*, v.62, p.479-482, 2001.
- BERCHT, B.S.; ALBUQUERQUE, L.; ARAUJO, A.C.P. *et al.* Specular microscopy to determine corneal endothelial cell morphology and morphometry in chinchillas (*Chinchilla lanigera*) in vivo. *Vet. Ophthalmol.*, v.18, p.137-142, 2015.
- BROOKS, D.E. Phacoemulsification cataract surgery in the horse. *Clin. Tech. Equine Pract.*, v.4, p.11-20, 2005.
- CHANG, Y.S.; TSENG, S.Y.; TSENG, S.H. *et al.* Comparison of dyes for cataract surgery. Part 1: Cytotoxicity to corneal endothelial cells in a rabbit model. *J. Cataract Refrac. Surg.*, v.31, p.792-798, 2005.
- DADA, V.K.; SHARMA, N.; SUDAN, R. *et al.* Anterior capsule staining for capsulorhexis in cases of white cataract: Comparative clinical study. *J. Cataract Refrac. Surg.*, v.30, p.326-333, 2004.
- DOUGHTY, M.J., BERGMANSON, J.P., BLOCKER, Y. Shrinkage and distortion of the rabbit corneal endothelial cell mosaic caused by a high osmolality glutaraldehyde-formaldehyde fixative compared to glutaraldehyde. *Tissue Cell*, v.29, p.533-547, 1997.
- EGGELING, P.; PLEYER, U.; HARTMANN, C. *et al.* Corneal endothelial toxicity of different lidocaine concentrations. *J. Cataract Refrac. Surg.*, v.6, p.1403-1408, 2000.
- FAGANELLO, C.S.; SILVA, V.R.M.; ANDRADE, M.C.C. *et al.* Morphology of endothelial cells from different regions of the equine cornea. *Cienc.Rural*, v.46, p.2223-2228, 2016.
- HISATOMI, T.; ENAIDA, H.; MATSUMOTO, H. *et al.* Staining ability and biocompatibility of brilliant blue G. *Arch. Ophthalmol.*, v.124, p.514-519, 2006.
- HOLLEY, G.P.; ALAM, A.; KIRI, A. *et al.* Effect of indocyanine green intraocular stain on human and rabbit corneal endothelial structure and viability: an in vitro study. *J. Cataract Refrac. Surg.*, v.28, p.1027-1033, 2002.

- JACOBS, D.S.; COX, T.A.; WAGONER, M.D. *et al.* Capsule staining as an adjunct to cataract surgery. *Ophthalmology*, v.113, p.707-713, 2006.
- LANDRY, H.; AMINIAN, A.; HOFFART, L. *et al.* Corneal toxicity of air and SF6. *Invest. Ophthalmol. Vis. Sci.*, v.52, p.2279-2286, 2011.
- LEDBETTER, E.C.; SCARLETT, J.M. In vivo confocal microscopy of the normal equine cornea and limbus. *Vet. Ophthalmol.*, v.12, Suppl.1, p.57-64, 2009.
- McMULLEN, R.J.; DAVIDSON, M.G.; CAMPBELL, N.B. *et al.* Evaluation of 30- and 25-diopter intraocular lens implants in equine eyes after surgical extraction of the lens. *Am. J. Vet. Res.*, v.71, p.809-816, 2010.
- McMULLEN, R.J.; UTTER, M.E. Current developments in equine cataract surgery. *Equine Vet. J.*, v.37, p.38-45, 2010.
- NELDER, J.A.; WEDDERBURN, R.W.M. "Generalized linear models". *J. R. Stat. Soc.*, n.3, p.135, 1972.
- PANDEY, S.K.; WERNER, L.; ESCOBAR-GOMEZ, M. *et al.* Dye-enhanced cataract surgery - Part 1: Anterior capsule staining for capsulorhexis in advanced/white cataract. *J. Cataract Refrac. Surg.*, v.26, p.1052-1059, 2000.
- PIGATTO, J.A.T.; ABIB, F.C.; PEREIRA, G.T. Density of corneal endothelial cells in eyes of dogs using specular microscopy. *Braz. J. Vet. Res. Anim. Sci.*, v.43, p.476-480, 2006.
- PIGATTO, J.A.T.; ABIB, F.C.; PIZZETI, J.C. *et al.* Análise morfométrica do endotélio corneano de coelhos à microscopia eletrônica de varredura. *Acta Sci. Vet.*, v.33, p.33-39, 2005a.
- PIGATTO, J.A.T.; CERVA, C.; FREIRE, C.D. *et al.* Morphological analysis of the corneal endothelium in eyes of dogs using specular microscopy. *Pesqui. Vet. Bras.*, v.28, p.427-430, 2008.
- PIGATTO, J.A.T.; FRANZEN, A.A.; PEREIRA, F.Q. *et al.* Scanning electron microscopy of the corneal endothelium of ostrich. *Cienc. Rural*, v.39, p.926-992, 2009.
- PIGATTO, J.A.T.; LAUS, J.L.; SANTOS, J.M. *et al.* Corneal endothelium of magellanic penguin (*Spheniscus magellanicus*) by scanning electron microscopy. *J. Zoo Wild. Med.*, v.36, p.702-705, 2005b.
- SAAD, H.S.; TERRY, M.A.; SHAMIE, N. *et al.* An easy and inexpensive method for quantitative analysis of endothelial damage by using vital dye stain and Adobe Photoshop software. *Cornea*, v.27, p.818-824, 2008.
- TAYLOR, M.J.; HUNT, C.J. Dual staining of corneal endothelium with trypan blue and alizarin red S: importance of pH for the dye-lake reaction. *Br. J. Ophthalmol.*, v.65, p.815-819, 1981.
- TERRY, M.A.; SAAD, H.A.; SHAMIE, N. *et al.* Endothelial keratoplasty: the influence of insertion techniques and incision size on donor endothelial survival. *Cornea*, v.28, p.24-31, 2009.
- TERZARIOL, M.; HÜNNING P.S.; BRAMBATTI, G. *et al.* Effects of intracameral brilliant blue on the corneal endothelium of swine: in vitro study. *Pesqui. Vet. Bras.*, v.36, p.775-780, 2016.
- TRIVEDI, R.H.; WERNER, L.; APPLE, D.J. *et al.* Viscoanesthesia. Part I: Toxicity to corneal endothelial cells in a rabbit model. *J. Cataract Refrac. Surg.*, v.29, p.550-555, 2003.
- UDAONDO, P.; DÍAZ-LLOPIS, M.; SALOM, D. *et al.* Brilliant blue G-assisted capsulorhexis: a good help for phacoemulsification surgeons in training. *Arch. Soc. Esp. Oftalmol.*, v.82, p.471-472, 2007.
- VAN DOOREN, B.T.H.; BEEKHUIS, W.H.; PELS, E. Biocompatibility of trypan blue with human corneal cells. *Arch. Ophthalmol.*, v.122, p.736-742, 2004.