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Acute effects of ropivacaine hydrochloride on corneal endothelial cell ultrastructure of horses: *ex vivo* study

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ABSTRACT: *The objective of this study was to evaluate the acute effects of ropivacaine hydrochloride on the corneal endothelium of horses. Forty-eight eyes were obtained from a commercial slaughterhouse and were randomly divided into three groups. In group A, the corneal endothelium was exposed to 0.75% ropivacaine hydrochloride for 60 seconds. In group B, the corneal endothelium was exposed to 0.75% ropivacaine hydrochloride for 15 minutes. In group C, the corneal endothelium was exposed to a balanced saline solution for 60 seconds. Afterwards, all samples were prepared for evaluation with scanning electron microscopy. Random electromicrographs were obtained from each sample. The images were analysed and, with the aid of software, areas with no endothelial cells were measured. The average endothelial loss, expressed as a percentage in relation to the total area, of the samples in group A was 5.28%. The average endothelial loss of samples from group B, expressed as a percentage in relation to the total area, was 20.39%. The damage to the corneal endothelium was significantly greater in group B compared to groups A and C. It was possible to conclude that 0.75% ropivacaine hydrochloride induced acute damage to corneal endothelium cells.*
Key words: *equine, intraocular surgery, cornea, intracameral anaesthesia.*

Efeitos agudos do cloridrato de ropivacaína na ultraestrutura das células endoteliais da córnea de equinos: estudo *ex vivo*

RESUMO: *Objetivou-se avaliar os efeitos agudos do cloridrato de ropivacaína no endotélio da córnea de equinos. Quarenta e oito olhos de equinos foram divididos aleatoriamente em três grupos. No grupo A o endotélio da córnea foi exposto a cloridrato de ropivacaína a 0,75% por 60 segundos. No grupo B o endotélio da córnea foi exposto a cloridrato de ropivacaína a 0,75% por 15 minutos. No grupo C o endotélio da córnea foi exposto à solução salina balanceada por 60 segundos. As amostras foram preparadas para avaliação com microscopia eletrônica de varredura. Eletromicrografias eletrônicas de varredura foram obtidas aleatoriamente de cada amostra. As imagens foram analisadas e, com o auxílio de um programa para morfometria foram medidas as áreas sem células endoteliais. A perda endotelial média foi expressa em porcentagem em relação à área total das amostras do grupo A foi de 5,28%. A perda endotelial média de amostras do grupo B foi expressa em porcentagem em relação à área total, foi de 20,39%. O dano ao endotélio da córnea foi significativamente maior no grupo B, comparado aos grupos A e C. O cloridrato de ropivacaína a 0,75% induziu dano agudo nas células do endotélio da córnea de equinos.*
Palavras-chave: *equinos, cirurgia intraocular, córnea, anestesia intracameral.*

INTRODUCTION

A minimum number of endothelial cells is vital for maintaining the transparency of the cornea (PARIKH & EDELHAUSER, 2003). A loss of corneal endothelial cells always occurs during surgical procedures to remove cataracts. Moreover, the corneal endothelium has minimal capacity for mitosis and high cell losses may lead to vision loss. In order to minimise the loss of endothelial cells and avoid corneal decompensation after intraocular surgery, it is important that solutions that are toxic to the corneal endothelium are not used inside the eye (ISHIKAWA, 2002). Any substance that may cause toxicity to the corneal endothelium must be carefully evaluated before being routinely used inside the anterior chamber. The use of intracameral anaesthesia has become frequent as an alternative to traditional blocks due to its ease of use and analgesic comfort during and after the procedure in humans

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(PARK et al. 2010; WANG, 2013). VÄ LIMÄKI and TÖRNBLOM compared viscoanaesthesia and intracameral anaesthesia with 1% lidocaine in patients undergoing cataract surgery. Patients who are given viscoanaesthesia may be at increased risk for postoperative corneal oedema (VÄLIMÄKI & TÖRNBLOM, 2009). Some studies have already been developed to analyse the effects of intraocular injection of lidocaine on the corneal endothelium. Effect of intracameral ropivacaine on corneal endothelium was studied (ÇAÇA et al, 2006). Cataracts are an important cause of blindness in horses and, at present, the only form of treatment is surgical removal of the opaque lens (EDELMANN et al, 2014; BROOKS et al. 2014; TOWNSEND, 2017). To our knowledge, there are no studies evaluating the toxicity of intracameral local anaesthetics on the corneal endothelium of horses.

The aim of this study was to evaluate the acute ex vivo effects of ropivacaine hydrochloride in the ultrastructure of the corneal endothelial cells of horses.

MATERIALS AND METHODS

Forty-eight corneas from 24 horses, male or female, of different ages were studied. The eyes were obtained from a licensed Brazilian commercial slaughterhouse (xxx). The experiment was carried out according to the standards of the Association for Research in Vision and Ophthalmology (ARVO). Immediately after slaughter, all eyes were examined. With slit-lamp biomicroscopy (Portable Slit Lamp SL 15, Kowa, Japan) the ocular surface, anterior chamber, and lens were examined. Corneal stain with fluorescein (Fluorescein, Allergan, SP, Brazil) was made. Eye bulbs that showed evidence of eye disease were excluded. Immediately after the eye exam, enucleation was performed, and the eye bulbs were kept in a humid chamber until the corneas were collected. The corneoscleral buttons were removed with a scalpel and scissors. Corneas were then excised with an eight mm diameter trephine. All corneas were analyzed within 4 hours of death. Corneas were randomly divided into three groups.

In group A (12 corneas), with a dropper 0.2 ml of 0.75% atracurium besylate (Cristália, São Paulo, Brazil) was dripped onto the endothelium. Three minutes after the sample was rinsed with balanced salt solution (BSS) (Halex Istar, ophthalmic solution, GO, Brazil) to remove the atracurium besylate. In group B (12 corneas), with a dropper 0.2 ml of 0.75% atracurium besylate was dripped onto the endothelium. Fifteen minutes after the sample was rinsed with BSS to remove the atracurium besylate. In group C (24 corneas), with a dropper 0.2 ml of BSS was dripped onto the endothelium. All samples were kept in 2.5% glutaraldehyde, in 0.1M sodium cacodylate buffer and at pH 7.4 for 24 hours. The corneas were removed from the glutaraldehyde solution, washed in sodium cacodylate buffer solution and dehydrated in ascending concentrations of acetone (30, 50, 70, 80, 90 for 10 minutes at each concentration and for 20 min at 90% again). Afterwards the samples were left for 30 min in 100% acetone and were subjected to drying at a critical point with liquid carbon dioxide. The samples were fixed in stubs with adhesive tape and metallized with gold-palladium. The posterior endothelial surfaces were examined with a scanning electron microscope (JSM 6060, JEOL, Tokyo, Japan) operating at 15 kV. From each sample, five electron micrographs were obtained with 950x magnifications. Occasionally, images with a 30x magnification were obtained to have a panoramic view of each sample. The areas devoid of endothelial cells were calculated. The morphometric study was performed using ImageJ software (ImageJ 1.51k), which allowed calculation of the percentage of cell loss use surrounding areas with no endothelial cells. All analyses were performed by the same examiner. The distribution of variables was assessed using the Shapiro-Wilk test, which indicated a non-normal distribution. The Friedman test was used to compare the sum of areas expressed as a percentage of the total between treatments. The Wilcoxon test was used to detect the differences and its P value was corrected by the Bonferroni test, due to the multiple comparisons made. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

With scanning electron microscopy (SEM), it was possible to observe, analyse and obtain images of the corneal endothelium in all samples analysed. In the samples of the control group, there were no areas with endothelial damage and the regular pattern of endothelial cells was observed in all samples (Figure 1). Endothelial losses were observed both in group A and group B samples (Figure 2). The average endothelial loss, expressed as a percentage in relation to the total area, of the samples in group A was 5.28±2.49%. There was a statistically significant difference significant among all compared groups. The average endothelial loss from samples from group B, expressed as a percentage in relation to the

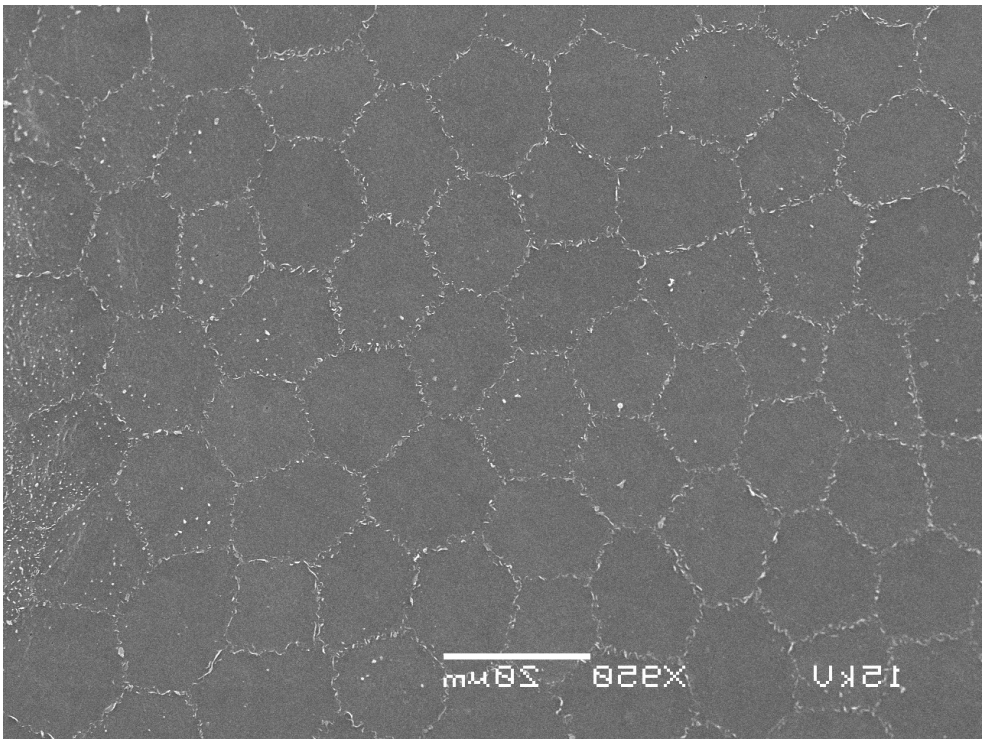

Figure 1 - Scanning electromicrograph of the corneal endothelium of horses of control group. Polygonal cells and regular corneal endothelial pattern and absence of endothelial damage are observed. Magnification:X950. Bar:20µm.

total area, was 20.39 ± 10.46%. The values found in the sum of the areas expressed as a percentage of cell loss were higher in group B when compared with group A (P = 0.006) and group C (P = 0.003). There was also a significant difference between the values found in A in relation to C (P = 0.005).

DISCUSSION

A minimum endothelial density is essential for the cornea to maintain its transparency. In addition to the surgical procedure, numerous other factors, including intraocular anaesthetics and intraocular dyes, can cause endothelial damage. These factors can compromise the function of the endothelium, mainly because in most of the studied species the mitotic activity is limited in this layer (NAUTSCHER et al, 2015). To avoid endothelial decompensation, it is important to choose substances that do not damage the corneal endothelium (KHABAK et al. 2006; BORAZAN et al. 2009; LEE et al, 2016). The loss of a large number of endothelial cells can cause irreversible loss of corneal transparency (PARIKH & EDELHAUSER, 2003). In this sense, studies on the toxicity of drugs in the corneal endothelium are extremely important. Intracameral anaesthesia have been popularised as new techniques for use in cataract surgery in humans. These anaesthetic routes have potential safety advantages over traditional techniques such as retrobulbar and peribulbar anaesthesia (OLMEZ et al, 2004). Regarding intracameral anaesthesia in humans and other animal species, studies were carried out evaluating the toxicity of the corneal endothelium and the comfort of patients (TAN & BURTON, 2000; BORAZAN et al. 2009; PARK et al. 2010). However, there is a lack of research in regard to equine species and intracameral anaesthesia. This, and the importance of the theme, helped to motivate this study. In addition, cataracts are an important cause of blindness in horses and, at present, the only form of treatment is surgical removal of the opaque

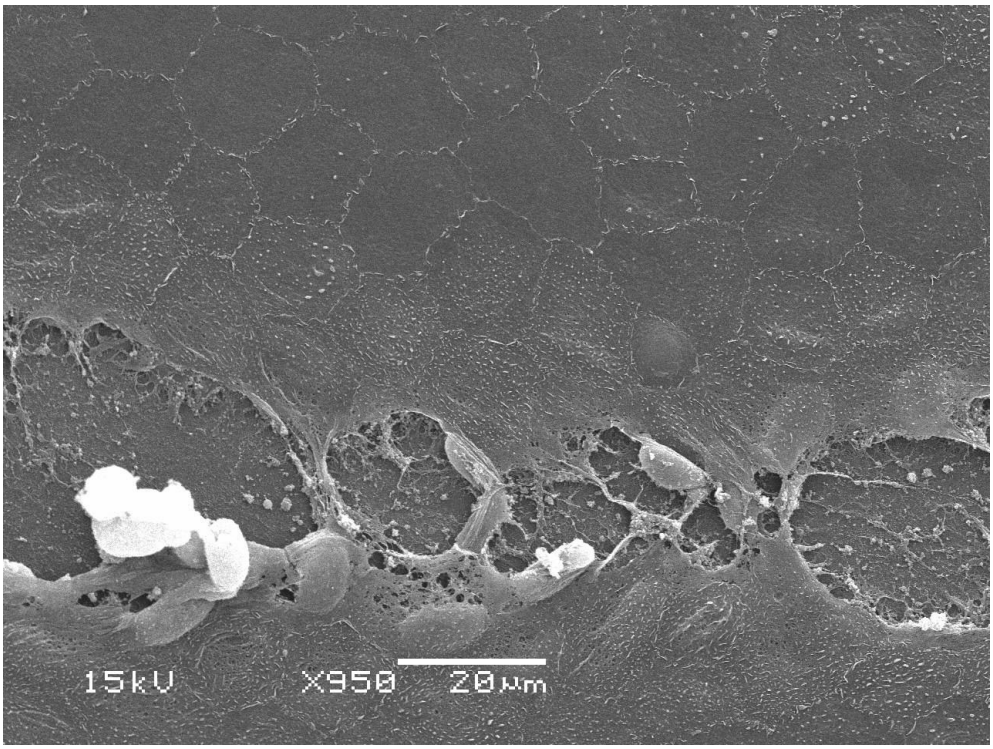

Figure 2 - Scanning electromicrograph of the corneal endothelium of horses of group B. Cell loss and Descemet's membrane exposure are observed. Magnification:X950. Bar:20µm.

lens (BROOKS et al, 2014; EDELMANN et al, 2014; TOWNSEND, 2017). In the present research, an ex vivo study was chosen. Normally, research related to the toxicity of intraocular drugs using live animals is carried out on laboratory animals. Previous studies carried out on animal eyes determined that within six hours after death, the endothelium can be analysed without structural changes occurring in this layer (SIT et al, 2001; PIGATTO et al, 2005; PIGATTO et al, 2009; FAGANELLO et al, 2016). The use of slaughtered animal eyes for endothelial analysis has increasingly been shown to be an alternative to the use of live animals to assess the toxicity of intraocular drugs (PESCOSOLIDO et al, 2011; WEN et al, 2015; TERZARIOL et al, 2016; SILVA et al, 2018; JIANG et al, 2018).

Corneal endothelial toxicity is related to substances that come into contact with the endothelium, based on their chemical compositions, pH and osmolarities (PARIKH & EDELHAUSER, 2003). The final preparation of ropivacaine for clinical use is presented with a pH ranging between 4.0 and 6.0 (RAMOS et al, 2000). Analysis of the corneal thickness data and interpretation of the scanning electron micrograph reveals that outside of the pH range of 6.5 to 8.5, structural and functional alterations occur (GONNERING et al, 1979). Endothelial losses were observed both in group A and group B samples. In the present study, ropivacaine was kept in direct contact with the endothelium and direct contact with the corneal endothelium in order to verify whether it would induce endothelial damage. In previous studies this methodology has already been used with excellent results (ÇAÇA et al, 2006; KHABAK et al, 2006; PESCOSOLIDO et al. 2011; WEN et al, 2015; TERZARIOL et al, 2016; SILVA et al, 2018). In other studies, however, an intracameral injection of the substance was performed, which was analysed (BORAAN et al, 2009). Thus, dilution of the substance in aqueous humour could minimise its toxic effects on the endothelium. In the present study, as we were unaware of the possible damage of the tested substance, corneal trepanation and direct exposure of the anaesthetic to the corneal

endothelium were chosen. In addition, with the intracameral injection of the tested substance it would be difficult to have a homogeneous direct contact between ropivacaine and the corneal endothelium. In the present study, ropivacaine directly contacted with the endothelium across the entire area that was later analysed. Ropivacaine is a local anaesthetic of the amide type with a long duration of action, and its effect occurs through a reversible inhibition of the influx of sodium ions in the nerve fibres. It has a high degree of sensory motor differentiation, which can be useful when motor block is undesirable (KUTHIALA & CHAUDHARY, 2011). Commercially available ropivacaine does not contain preservatives in its formulation. At this concentration, this anaesthetic is widely used in blocking ophthalmology because it promotes analgesia in some patients for up to 12 hours, being superior to lidocaine (KUTHIALA & CHAUDHARY, 2011). In previous studies in humans, 1% ropivacaine was more effective in analgesia and did not promote significant endothelial cell loss when compared to 2% lidocaine (MARTINI et al, 2002; IACOBELLI et al. 2005).

In studies carried out on other animal species, ropivacaine in concentrations between 0.1% and 0.5% was demonstrated to be safe and provide pain relief, but at concentrations of 1%, ropivacaine induced lesions in the corneal endothelium (KLAMT et al, 2003; CACA et al, 2006; BORAZAN et al, 2009). In one study only, 0.75% ropivacaine was observed to induce toxicity in the corneal endothelium of rabbits (KHAZBAK et al, 2006). Among the techniques normally used to prove the toxicity of drugs in the corneal endothelium, optical microscopy associated with alizarin red and SEM stand out (ÇAÇA et al, 2006; SCHELINNI et al, 2007; SEGARRA et al, 2018; SILVA et al, 2018). The use of optical microscopy after staining the endothelium with vital dyes has been shown to be a simple, fast and practical way to detect cell damage as well as to analyse the shape of endothelial cells (FAGANELLO et al, 2016). In the present study, SEM was chosen because it has already been widely used in studies related to corneal morphology, the toxicity of intracameral drugs, the effectiveness of means of corneal preservation and in the evaluation of the endothelium ultrastructure of different species (OJEDA et al, 2001; PIGATTO et al, 2009; TERZARIOL et al, 2016). In the present study, the methodology employed proved to be feasible, allowing images to be obtained, and an analysis and quantification of endothelial cell losses. With SEM, due to the large increase in images, it is possible to delimit the edges of areas with cell loss, thus allowing establishment of the percentage of endothelial loss. It was possible to visualise the acute toxic effects caused by direct exposure of ropivacaine hydrochloride to the corneal endothelium. Similar lesions in the corneal endothelium have been documented in other studies using local intraocular anaesthetics (EGGELING et al, 2000; TAN & BURTON 2000; KHABAK et al, 2006). In humans, the most commonly used anaesthetic within the eye is 1% lidocaine as it has less toxicity compared to other anaesthetics and concentrations (LIOU et al. 2004; BORAZAN et al, 2009; LEE et al, 2016).

CONCLUSION

According to the conditions proposed for this study, it was possible to conclude that ropivacaine 0.75% caused acute damage to the corneal endothelium of horses.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This research was approved by the Research Committee of the xxx, and followed the ethical norms of the Association for Research in Vision and Ophthalmology (ARVO).

DECLARTION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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