

Evaluation of equine (*Equus caballus*) corneal endothelium stored in EUSOL-C® preservation medium

Avaliação do endotélio da córnea de equinos (*Equus caballus*) acondicionado em meio de preservação de córnea Eusol-C®

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Highlights:

Corneal transplantation is an excellent alternative for maintaining vision.

Corneal transplantation is applicable in veterinary medicine.

Eusol-C® allows the maintenance of endothelial cell viability in horses.

Eusol-C® maintained endothelial integrity for up to 14 days.

Abstract

The objective of this study was evaluate the maintenance of the corneal endothelium of horses in cold EUSOL-C® preservation medium over different periods (seven and 14 days) using scanning electron microscopy. A total of 20 pairs of eyes from horses were analysed. The corneas were divided into four groups of 10 corneas each (G1, G2, G3 and G4): G1 - the samples were kept in the preservation medium for seven days; G3 - the samples were kept in the preservation medium for for 14 days; G2 and G4 were formed by the control corneal buttons of G1 and G3, respectively. The average cell loss observed in G1 was 7.62%, in G2 it was 7.04%, in G3 9.12% and in G4 7.16%. No statistically significant differences were observed between the four groups. It was concluded that the Eusol-C® hypothermic preservation medium provided satisfactory preservation of the corneal endothelium in equine species for up to 14 days.

Key words: Corneal transplantation. Horses. Endothelium. Corneal storage. Cell viability.

Resumo

Objetivou-se avaliar a manutenção do endotélio da córnea de equinos em meio de preservação a frio EUSOL-C® em diferentes períodos de acondicionamento (sete e 14 dias) utilizando microscopia eletrônica de varredura. Foram avaliados 40 bulbos oculares de 20 equinos. Os bulbos oculares foram divididos em quatro grupos (G1, G2, G3 e G4), nos quais: G1 foi composto por 10 botões corneais acondicionados em meio de preservação para córnea Eusol-C® durante sete dias; o G3 foi formado por 10 botões corneais acondicionados em meio de preservação para córnea Eusol-C® durante 14 dias. O G2 e G4 foram formados pelas córneas controle armazenadas imediatamente em glutaraldeído. A média

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da perda celular observada no G1 foi de 7,62%, no G2 foi de 7,04 %, no G3 foi de 9,12% e o no G4 foi de 7,16%. Não foram observadas diferenças estatisticamente significativas entre os quatro grupos. Foi possível concluir que o meio de preservação hipotérmico Eusol-C® proporcionou de forma satisfatória a preservação do endotélio da córnea na espécie equina durante o período de até 14 dias.

Palavras-chave: Transplante de córnea. Cavalos. Endotélio. Preservação da córnea. Viabilidade celular.

Introduction

The corneal endothelium consists of a monolayer of polygonal cells with a predominantly hexagonal shape (Abib & Barreto, 2001; Pigatto et al., 2004, 2005a, 2005b). In most species, the endothelium has minimal mitosis activity (Abib & Barreto, 2001; Joyce, 2012). Because of this, severe endothelial damage can lead to loss of corneal transparency (Joyce, 2012).

Corneal transplantation is considered one of the main alternatives for the recovery of vision in cases of corneal lesions or disorders that cause loss of transparency. The goal of corneal preservation methods is to maintain a sufficient number of endothelial cells, which are decisive for functionality and graft survival (Feilmeier, Tabin, William & Oliva, 2010). For corneal transplant to be successful, it is crucial that at least 50% of endothelial cells are viable. Healthy endothelium ensures a thinner cornea, which allows a better pre-surgical evaluation and easier manipulation of donor tissue, at the time of the surgical procedure, resulting in early visual rehabilitation (Yüksel, Uzunel, & Küsbeci, 2016).

Corneal transplantation has been performed in horses for the treatment of corneal ulcers, eye perforations, stromal abscesses, among others (Denis, 2004; Brooks et al., 2008; Plumer, 2009).

There are different methods of corneal storage. Currently, the two main methodologies used are hypothermia and tissue culture media (Lira & Sá, 2012). Hypothermia conservation consists of keeping the corneas refrigerated at temperatures ranging from 2 to 6°C in commercially available media (Rosenwasser et al., 2017). Eusol-C® is a preservation medium widely used in Europe for

medium-term corneal storage (Kanavi, Javadi, Chamani, Fahim, & Javadi, 2014; Yüksel et al., 2016).

Scanning electron microscopy (SEM) is a method widely used in the in vitro evaluation of the morphology and endothelial morphometry of different species (Pigatto et al., 2004, 2005a; Rodrigues, Laus, Santos, Rigueiro, & Smith, 2006; Tamayo-Arango et al., 2009). However, studies evaluating equine corneas kept in preservation media have not yet been published. The aim of this study was to evaluate the endothelial viability of equine corneas stored in cold EUSOL-C® preservation medium using SEM.

Materials and Methods

Forty eyeballs, from 20 horses (*Equus caballus*), male or female, mixed breed and of different ages were studied. These eyes were obtained from a local licensed commercial Brazilian slaughterhouse (Foresta, São Gabriel, RS, Brazil). The research was conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research. Enucleation was performed immediately after humane slaughter. Ophthalmic examination was carried out before the start of the experiment. Immediately after slaughter the eye bulbs were enucleated and transported to the laboratory in a moist chamber containing physiologic saline. Eyes were kept in ice-lined thermocol boxes to maintain temperature at 2-8 °C. The examination consisted of evaluation by slit-lamp biomicroscopy (Portable Slit Lamp SL 15, Kowa, Japan) and fluorescein stain (Fluorescein, Allergan, SP, Brazil), and a contact specular microscope (Celmax, Medical Service,

Brazil) with software for corneal endothelium analysis. Eyes that showed evidence of ocular disease were excluded.

Corneas were divided randomly into four groups with 10 corneas each (G1, G2, G3 and G4): G1 - the samples were kept in the preservation medium for seven days; G3 - the samples were kept in the preservation medium for 14 days; G2 and G4 were formed by the control corneo-scleral buttons of G1 and G3, respectively. Immediately after enucleation, the corneas of groups G2 and G4 were excised and placed in a glutaraldehyde solution.

Eyes that were selected were washed with normal saline and the scleral corneal button removed with a No. 23 blade and corneal scissors leaving about 3 mm from the scleral border around the periphery. Subsequently, the corneoscleral button was positioned with the endothelium facing upwards and with the aid of surgical trephines, corneal buttons of 5 mm in diameter were obtained from the central region of the cornea.

After seven and 14 days the corneas of groups G1 and G2, respectively, were removed from the preservation medium and placed in 2.5% glutaraldehyde. The corneal buttons were washed in a cacodylate buffer and dehydrated through an increasing series of ethanol solutions (30, 50, 70, 85, 90, 95 and 100%) for 15 minutes at each concentration and three times at 100%. After dehydration the corneas were submitted to critical-point drying using liquid carbon dioxide and placed on aluminium 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 15 kV. Five random electron micrographs (25× to 35×) of each sample were taken. Electromicrographs were also performed in 1000× and 1500× magnifications to detail the posterior face of the corneal endothelium.

The morphometric study was performed using Image Tool software, which allowed calculation

of the percentage of cell loss use surrounding areas with no endothelial cells. One measurement was expressed in μm^2 and then converted to mm^2 . In this way, the percentage of endothelial damage could be obtained. The calculation of cell losses was performed by subtracting the total sample area from an area with endothelial loss. The result was then evaluated by Wilcoxon's non-parametric test for paired sizes.

Results

Slit-lamp biomicroscopy allowed assessment of the corneal surface integrity of the equine eye bulbs. Of the 20 pairs of eye bulbs initially obtained and evaluated, six had to be replaced by other pairs due to the presence of a lesion on the corneal surface of the right or left eye. Specular microscopy provided evaluation of the regular horse corneal endothelium, formed by polygonal cells of predominantly hexagonal shape. Regarding the right eye bulb, the average endothelial density was 3182 cells/ mm^2 . With regard to cell pleomorphism, 59% of the cells showed a hexagonal pattern. The mean pachymetry was 812.45 μm . The mean cell density of the left eye was 3178 cells/ mm^2 . With regard to cell pleomorphism, 56% of the cells showed a hexagonal pattern. The mean pachymetry was 809.15 μm .

SEM made it possible to analyse endothelial cell loss. All four groups evaluated showed some degree of cell damage. The average cell loss observed in the corneosclerotic buttons packed in Eusol-C® corneal preservation medium for seven days (G1) was 7.62%. The control group (G2) had an average endothelial cell loss of 7.04%. When G1 cell loss averages were compared to those of G2, there were no statistically significant differences.

The average cell loss observed in the corneal buttons packed in Eusol-C® corneal preservation medium for 14 days was 9.12%. The control group had an average endothelial cell loss of 7.16%. When the averages of cell losses in G3 were compared to those of G4, no statistically significant differences

were observed. The comparison between the mean cell losses of G1 and those of G3 also showed no statistically significant differences. Electromicrographs of G1 (Figure 1a) and G2 (Figure 1b) showed average endothelial losses of

7.44% and 6.88%, respectively. Electromicrographs of G3 (Figure 2a) and G4 (Figure 2b) showed average endothelial losses of 9.49% and 7.06, respectively:

Figure 1a

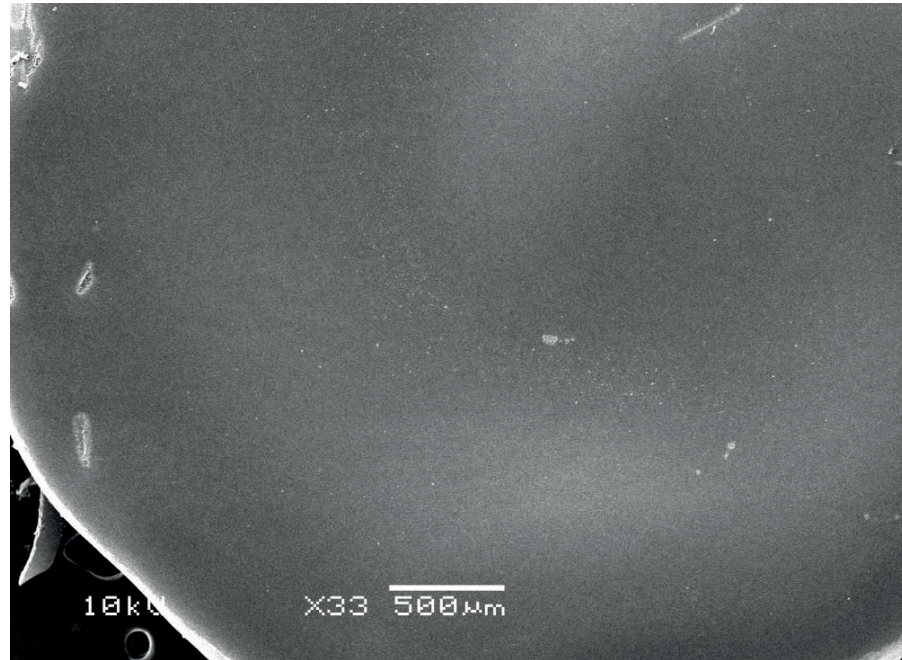
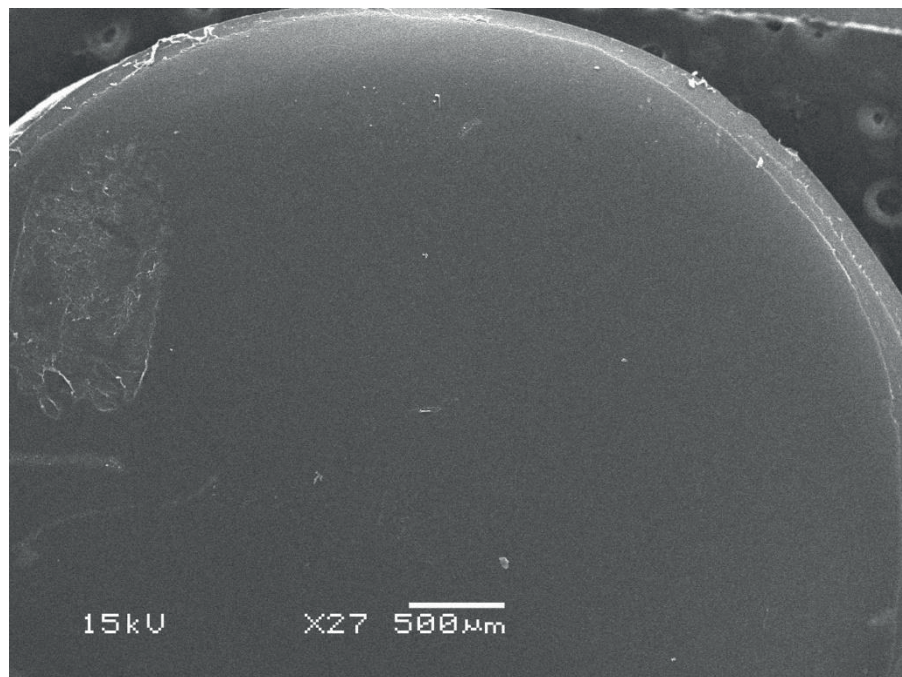


Figure 1b



Figures 1a and 1b. Scanning electromicrographs of the corneal endothelium of horses obtained after storage of the donor button in Eusol-C® corneal preservation medium for a seven-day period (Figure 1a) and of the control group (Figure 1b). Original magnifications of 33× and 27×, respectively. Bar: 500 µm.

Figure 2a

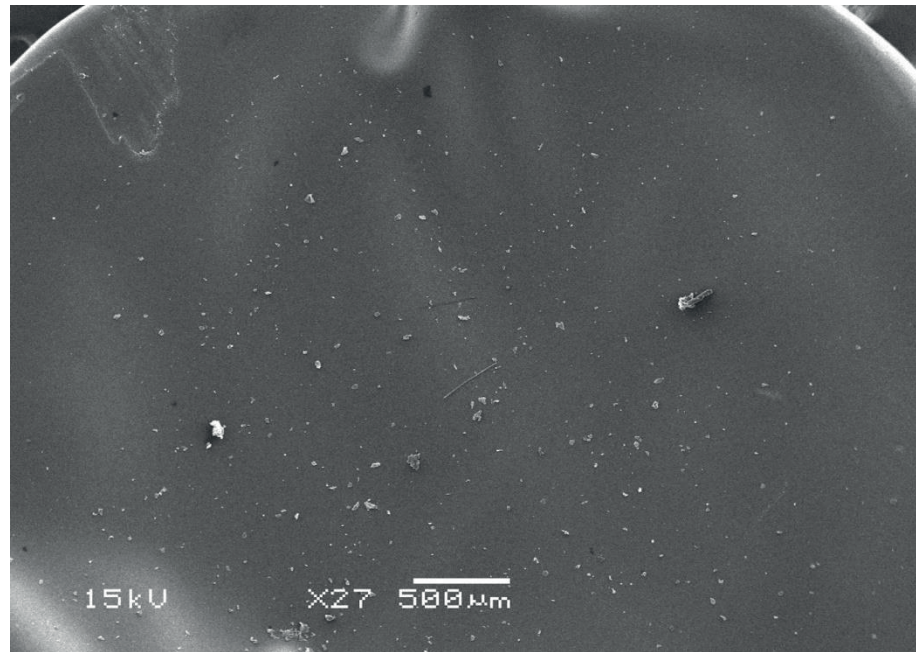
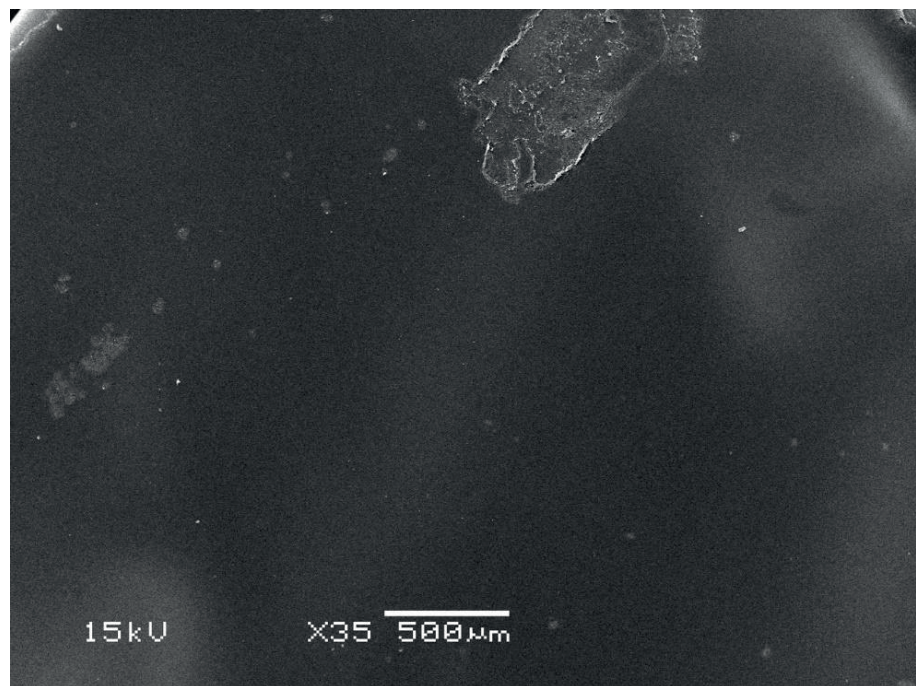


Figure 2 b



Figures 2a and 2b. Scanning electromicrographs of the equine corneal endothelium obtained after storage of the donor button in Eusol-C® corneal preservation medium for a 14-day period (Figure 1a) and of the control group (Figure 1b). Original magnifications of 27× and 35×, respectively. Bar: 500 μm.

Discussion

In humans, transplantation is the main alternative for the recovery of corneal transparency and visual acuity in patients with severe corneal disorders (Feilmeier et al., 2010; Gain et al., 2016). For equine species, Cichocki, Myrna and Moore (2016) refer to posterior lamellar keratoplasty and total keratoplasty as the surgical procedure of choice in the treatment of serious conditions such as deep stromal abscess and eye perforations in horses.

The high frequency of corneal disorders in horses motivated this study to be carried out for this species. In addition, corneal transplantation is already a procedure routinely performed to treat corneal disorders in horses; however, studies evaluating the maintenance of the donor button in corneal preservation medium are not found in the literature.

Success in corneal transplantation depends on a combination of several factors, including the quality and viability of the donor button (Lambert & Chamberlain, 2017). The donor's eye health, the time between death and collection, the collection technique, the storage method and the implant surgical procedure are determining factors for success in transplantation (Feilmeier et al., 2010; Lambert & Chamberlain, 2017). Thus, eye banks are extremely important, as they act at all these stages. The quality of corneal storage medium is essential for the ultimate success of corneal transplantation (Means, Geroski, Hadley, Lynn, & Edelhauser, 1995).

In humans, corneal transplants differ in optical, tectonic, therapeutic and cosmetic aspects. Optical treatments make up the vast majority (Tan, Dart, Holland, & Kinoshita, 2012). Unfortunately, this is different in veterinary medicine, especially in equine species, where most transplants have tectonic purposes due to the inaccessibility of viable fresh tissue (Plumer, 2009; Cichocki et al., 2016). If just the maintenance of the eye bulb is taken into account, the reported success rate of total corneal

transplantation in equine species is around 89.6% (Brooks et al., 2008; Cichocki et al., 2016). Plumer (2009) state that the surgical techniques for total corneal or posterior lamellar transplantation are well established in equine species; however, most transplants do not result in the return of visual acuity, due to the predominant use of cryopreserved corneal buttons. Cichocki et al. (2016) also emphasise the use of cryopreserved allografts in horses, due to the difficulty in obtaining viable donor buttons that provide a clear visual axis. The same authors warn that the preservation of visual acuity in this species should be one of the main concerns, taking into account the risk of maintaining a large blind animal.

The motivation for evaluating the means of corneal preservation in horses in this study is that the authors believe that the difficulty in accessing viable donor tissue in animals makes it difficult to perform optical transplantation in this species. The general emergency nature of surgical procedures for the treatment of corneal ulcers, perforations and lacerations makes it impossible to plan waiting for a hot corneal donor, given the need to use tissue that is immediately available. It is understood that the use of preservation means that it would be possible to increase the number of transplants for visual purposes in horses. Cichocki et al. (2016) also warn that, on many occasions, the lack of access to a viable donor tissue condemns the equine patient to loss of vision. The same authors regret the absence of functional structures such as human eye banks in veterinary medicine.

The decrease in the activity of endothelial cells causes a loss of corneal transparency resulting in loss of vision. The main objective of corneal conservation methods is to maintain endothelial viability to avoid the appearance of corneal oedema during the preservation period (Yüksel et al., 2016). The two methods of preserving the donor button used in human medicine for optical purposes are cell culture and hypothermia (Peels, 1997; Yüksel et al., 2016; Kanavi et al., 2014).

Maintenance in culture medium is the method of choice in European eye banks (Yüksel et al., 2016). Although the effectiveness of this methodology in preserving the human cornea has been proven, the authors of this study believe that its implementation in veterinary medicine is not the best option, due to the need for a laboratory equipped for strict temperature control and sterility, and to require the presence of a laboratory technician to handle the environment, making it bureaucratic and costly.

Another disadvantage of this method is the intense corneal oedema acquired during the maintenance of the donor button (Peels, 1997). Added to this is the fact that the preoperative semiology of the donor button is hampered due to loss of transparency and requires a sophisticated endothelial assessment technique. The authors of this study understand that the need for an equipped laboratory, as well as a laboratory technician, makes the method of preservation in culture medium unattractive for corneal transplantation in veterinary medicine.

The main function of the corneal storage medium is to maintain the viability and functionality of the endothelial cells from collection to use. The hypothermic means of conservation aims to mimic the natural environment of the donor tissue and supply its metabolic needs as long as possible, thus delaying cell death (Means et al., 1995; Kryczka, Chrapusta, Szaflik, Szaflik, & Midelfart, 2014). It consists of maintaining donor tissue in commercially available formulations such as Eusol-C® and Optisol-GS® at an average temperature of 4°C for a period ranging from seven to 15 days (Means et al., 1995; Kanavi et al., 2014; Kryczka et al., 2014; Yüksel et al., 2016).

In this study, we opted for the use of hypothermia as a form of endothelial preservation because it is a practical method that does not require specific apparatus, equipment or facilities for its implementation. It is believed that because it is a methodology of easy application, hypothermia can be implemented in routine veterinary medicine,

which will enable transplants for optical purposes. The only care required is the maintenance of the medium at a low temperature, which can oscillate between 2 and 4°C.

Kanavi et al. (2014) state in their study that hypothermia provides a significant improvement in the preservation of donor tissue. They describe the method as being technically simple, as it does not require expensive installations, laboratories or a specialised technician. Due to the fact that this methodology is easy to apply, is inexpensive, does not require specific training and does not require specialised labour, the authors of the present study believe that hypothermia is a possible methodology to be used in veterinary medicine. It is also believed that this method of preservation will provide a change in the reality of equine corneal transplantation, increasing the numbers of optical, in relation to tectonic, transplants.

A minimum endothelial density is essential for the cornea to maintain its transparency. Andrew, Ramsey, Hauptman, & Brooks (2001) established an average endothelial density in equine species at around 3155 cells/mm². Maintaining a viable endothelium is one of the greatest advantages in using hypothermic preservation media (Means et al., 1995). Since it is a means of preserving the recent cornea, studies evaluating the means of preservation of the human cornea in Eusol-C® are rare. Yüksel et al. (2016) evaluated the survival of endothelium from donated human corneas using Eusol-C® preservation medium through specular microscopy and observed an average loss rate of endothelial density of 24.5% on the eighth day of storage. The corneas were evaluated for a period of 24 days, which allowed the authors to conclude an average loss of 3.1% of endothelial density per day.

Regarding Veterinary Medicine, the literature does not report studies evaluating the use of commercial means such as Eusol-C® to preserve the corneal donor button in animals. In this pioneering study, the average rate of endothelial loss found

after seven days of storage in preservation medium for Eusol-C® cornea was 7.44%, and 9.49% after 14 days of storage.

Kanavi et al. (2014) performed a qualitative and quantitative comparison of corneal buttons stored for seven days in two hypothermic corneal preservation media: Optisol-GS® and Eusol-C®. The authors concluded that there was no statistically significant difference between the mean endothelial density on the first and seventh day of storage. They also reported no statistically significant difference between the two storage media, concluding that both are effective for corneal storage for seven days. Likewise, Wagoner and Gonnah, (2005) attested in their study to the effectiveness of prolonged storage of corneal donor buttons in an hypothermic Optisol-GS® medium. Kryczka et al. (2014) also performed an analysis of the preservation of donor buttons in Eusol-C® medium for up to 14 days and stated that storing the cornea in hypothermic media at 4°C does not negatively affect cell metabolism. In the present study, the option of choosing Eusol-C® was due to the ease of acquisition added to the fact that the cost of Eusol-C® is lower compared to that of Optisol-GS®. The choice to use eye bulbs obtained from a slaughterhouse prevented the euthanasia of healthy animals for experimental purposes alone, coupled with the fact that the use of eyes from refrigerators allows access to a large number of bulbs, allowing the selection of only healthy corneas.

Among the methods used to analyse the corneal integrity are slit-lamp biomicroscopy, the use of vital dyes, specular microscopy and confocal microscopy (Abib & Barreto, 2001; Wagoner & Gonnah, 2005). In the present study, we opted for the use of specular microscopy in addition to slit-lamp biomicroscopy and fluorescein testing, which allowed only healthy eye bulbs to be used. SEM is routinely used to analyse the corneal endothelium in ex vivo studies to determine the endothelial pattern of different species (Collin & Collin, 1998). This methodology is widely used in order to establish the morphology and morphometry of the corneal

endothelium of different species (Pigatto et al., 2005a,b; Rodrigues et al., 2006; Pigatto et al., 2009; Tamayo-Arango et al., 2009; Terzarioli et al., 2016).

Due to the magnification and the possibility of ultrastructural evaluation provided by SEM, the authors of the present study opted for this methodology of endothelial analysis. This method has been chosen by several researchers in the field of ophthalmology. Terry, Hoar, Wall and Ousley (2006) used SEM to compare two different corneal endothelial transplantation techniques. Still within the scope of the transplant, Hwang and Kim (2009) again opted for the use of SEM to analyse the damage caused to the corneal endothelium cells in DSAEK surgery. Villarrubia et al. (2017) also chose SEM with a 100-fold magnification to analyse the stromal receptor bed from three different methods to obtain the donor button.

Conclusion

It was possible to conclude that the hypothermic preservation medium Eusol-C® provided satisfactory preservation of the corneal endothelium in equine species in the period of up to 14 days.

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