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Evaluation of porcine corneal endothelium after preservation in Eusol-C medium

Avaliação do endotélio da córnea suína após preservação em meio Eusol-C

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Abstract: This study was conducted to evaluate the viability of porcine corneal endothelium after conditioning in the preservation medium Eusol-C. Twelve eyes from six 6-month-old male Large White pigs (Sus scrofa domesticus) were analyzed. Endothelial cell morphology and cell count of all samples were assessed prior storage using specular microscopy to ensure that only healthy corneas were included in the study. The samples were randomly divided into a control group (G1) and a test group (G2). Corneas from G1 were immediately submerged in bottles containing 2.5% glutaraldehyde, whereas corneas from G2 were stored in Eusol-C at 4°C for 14 days. The percentage of endothelial cell loss was evaluated using scanning electron microscopy (SEM). Comparisons between regions were performed using repeated measures Analysis of Variance (ANOVA). Differences were considered statistically significant at $P < 0.05$. Corneal samples from G1 showed no areas of endothelial cell loss. In samples from G2, the average cell loss was 3.01%. In conclusion, the hypothermic medium Eusol-C satisfactorily preserved porcine corneas for up to 14 days.

Key-words: endothelial cells; transplantation; ophthalmology; porcine.

Resumo: O objetivo deste estudo foi avaliar a viabilidade do endotélio corneano suíno após acondicionamento em meio de preservação Eusol-C. Foram incluídos 12 olhos de seis suínos da raça Large White (Sus scrofa domesticus), machos, com seis meses de idade. A morfologia e a contagem de células endoteliais de todas as amostras foram avaliadas antes do armazenamento por microscopia especular de contato, para assegurar que somente córneas hígidas seriam incluídas. As amostras foram aleatoriamente divididas em grupo controle (G1) e em grupo teste (G2). As córneas do G1 foram imediatamente submersas em frascos contendo glutaraldeído a 2,5%. As córneas do G2 foram armazenadas em Eusol-C a 4 °C por 14 dias. A porcentagem de perda de células endoteliais foi avaliada com microscopia eletrônica de varredura (MEV). As comparações entre regiões foram realizadas por meio de análise de variância de medidas repetidas (ANOVA). As diferenças foram consideradas estatisticamente significativas em $P < 0,05$. O G1 não apresentou perda celular. O G2 apresentou perda celular média de 3,01%. O meio hipotérmico Eusol-C preservou satisfatoriamente as córneas suínas por um período de 14 dias.

Palavras-chave: células endoteliais; transplante; oftalmologia; suíno.

1. Introduction

The corneal endothelium is a monolayer of predominantly hexagonal polygonal cells [1]. A decrease in cell density below a critical threshold leads to corneal edema and subsequent loss of corneal transparency [1, 2]. In corneal diseases resulting in loss of transparency, corneal remains the sole therapeutic option for vision restoration [3-5]. Corneal preservation primarily aims to extend the storage time of donor tissues while maintaining endothelial cell viability [6]. The most commonly used corneal preservation methods are hypothermic media and organ culture [6]. Hypothermic media preserves the cornea for 10 to 14 days. Eusol-C is a hypothermic medium developed as an effective alternative to Optisol-GS. The primary advantage of Eusol-C over Optisol-GS is its lower cost, which may enhance its applicability in veterinary medicine. Porcine corneas exhibit biological and morphological characteristics comparable to those of human corneas, which allows comparison between the results of previous studies with the human cornea [7, 8]. Porcine corneas have been used as study models in hypothermic preservation media to assess antibiotic use, compare different media, and observe storage performance and safety [9-11]. However, studies evaluating the Eusol-C preservation medium in this species remain recent and limited, primarily focusing on endothelial evaluation using specular microscopy [10, 11].

Scanning electron microscopy (SEM) is widely used to assess endothelial morphology, with numerous studies published on medication toxicity, evaluation post-preservation, and assessments following corneal or intraocular surgical procedures [12-18]. To the authors' knowledge, this is the first study to evaluate, using SEM, the percentage of endothelial cell loss following the preservation of porcine corneas in Eusol-C. Porcine corneas may represent a promising source of donor tissue for both human and veterinary applications [2, 3, 19]. The establishment of ocular tissue banks presents significant potential, and ongoing research on preservation methods in animals is critical for enhancing the safety and success of corneal transplantation procedures [20-22]. This study was conducted to evaluate the viability of porcine corneal endothelium following conditioning in the preservation medium Eusol-C.

2. Material and methods

Twelve eyes from six 6-month-old male Large White pigs (Sus scrofa domesticus) were included in the study. The eyes were donated by an abattoir (Avisui, Santa Maria, Rio Grande do Sul, Brazil). Immediately following slaughter and prior to scalding, the eyes were enucleated. They were then individually placed in a humid chamber containing gauze and saline solution within a thermal box to maintain a temperature of 2ºC to 8ºC. Within 1 h post-enucleation, all eyeballs were examined using slit-lamp biomicroscopy (Portable Slit Lamp SL15; Kowa, Nagoya, Japan), fluorescein staining (1% sodium fluorescein; Allergan Pharmaceuticals, Dublin, Ireland), and a contact specular microscope (Celmax Medical Service®; São Carlos, SP, Brazil).

Only eyes deemed healthy were included in the study. Images were acquired during specular microscopy performed to select healthy eyes before separating the corneas into groups. Subsequently, these images were processed using imaging software (Adobe Photoshop), and the morphology of 50 endothelial cells per image was analyzed. The 12 selected eyes were decontaminated by washing with a 5% povidone–iodine solution (PVP-I; Rioquímica S/A, São José do Rio Preto, SP, Brazil). Subsequently, the corneoscleral buttons were removed and the samples were randomly divided in two groups: the control group (G1) and the test group (G2). Buttons from G1 were immediately submerged in bottles

containing 2.5% glutaraldehyde. Buttons from G2 were placed in bottles containing Eusol-C and remained refrigerated for 14 days. After 14 days, corneoscleral buttons were transferred to bottles of glutaraldehyde. Following fixation in 2.5% glutaraldehyde and storage at approximately 4°C, samples were individually prepared for SEM analysis at the Microscopy and Microanalysis Center of UFRGS (CMM-UFRGS, Porto Alegre, Rio Grande do Sul, Brazil).

Samples were washed in cacodylate buffer and dehydrated through a series of solutions with increasing ethanol concentrations (30%, 50%, 70%, 85%, 90%, and 100%), remaining for 15 minutes at each concentration and placed three times in the 100% concentration. They were then subjected to critical point drying with liquid carbon dioxide. Corneas were placed on 10-mm aluminium stubs with double-sided adhesive tape and coated with gold-palladium. The posterior endothelial surface of each sample was analysed and photographed using a scanning electron microscope (EVO MA10; Zeiss, Oberkochen, Germany) operating at 10 kV. Five electron micrographs were obtained from each sample (1000×) within corresponding regions: I, central; II, superior; III, inferior; IV, lateral; and V, medial. Additional micrographs at 2000× magnification were obtained to provide greater detail of the endothelium. The 1000× magnification images were individually processed with ImageJ software (National Institutes of Health, Bethesda, MD, USA), using a tool that allowed circling portions of the image where endothelial loss occurred. Measurements were obtained in μm^2 and then converted to mm^2. The percentage of cell loss was calculated by subtracting the circled areas from the total image area.

2.1 Statistical analysis

Endothelial parameters obtained via specular microscopy were analysed using repeated measures ANOVA with a significance level of 5%. For SEM analysis, regions I to V of each sample were compared internally and with the corresponding regions across different samples. The median percentage of cell loss, along with minimum and maximum loss values was calculated for each group and each region. Comparisons of cell loss between G1 and G2 were performed using the Mann–Whitney U test, while comparisons across different regions were conducted using the Friedman test. The Dunn–Bonferroni test was applied to identify statistically significant differences between regions. A significance level of 5% was adopted for all statistical analyses.

3. Results

All enucleated eyes evaluated via slit lamp and specular microscopy were included in the study. Contact specular microscopy enabled visualisation of the porcine corneal endothelium, revealing a regular pattern of juxtaposed polygonal cells. The mean endothelial cell density was 1867.8 ± 78.0 cells/mm^2 in the right eye and 1862.2 ± 85.2 cells/mm^2 in the left eye. Considering the morphology of all analysed cells, 53.8% exhibited six sides, 21.5% exhibited five sides, and 17.7% exhibited seven sides. Additionally, 5% of the cells displayed four sides, and 2% displayed eight sides. No significant differences were observed in endothelial cells when comparing right and left eyes. Corneal samples from G1 exhibited no areas of endothelial cell loss (Figure 1). Endothelial loss was observed in samples preserved in Eusol-C preservation medium (Figure 2). In samples from G2, the average cell loss was 3.01%. A significant difference was found in the endothelial loss between G1 and G2. No significant differences were found among the studied regions within G1. However, a statistically significant difference was detected between regions I and IV within G2 (Table 1).

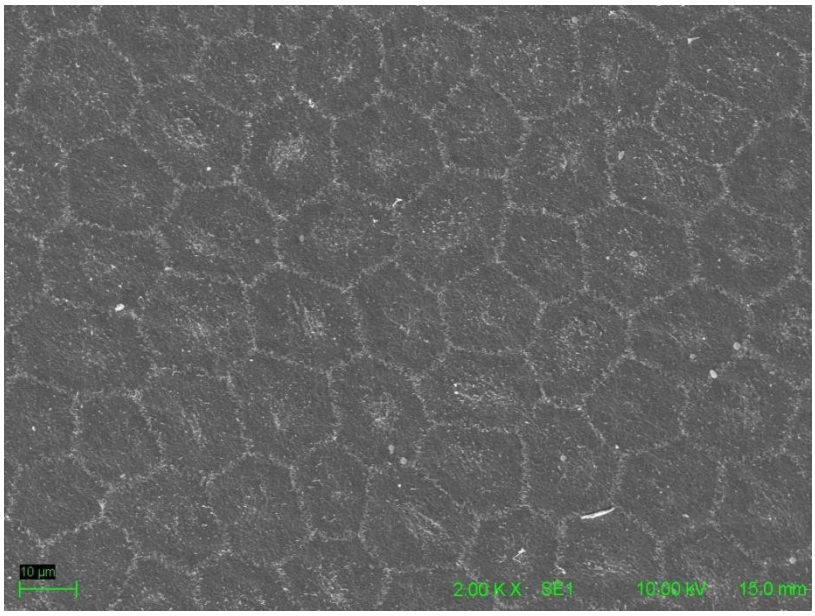

Figure 1. Scanning electromicrograph of the porcine corneal endothelium in G1. A regular corneal endothelial pattern and absence of endothelial damage are observed. Magnification: 2000×. Bar: 10 µm.

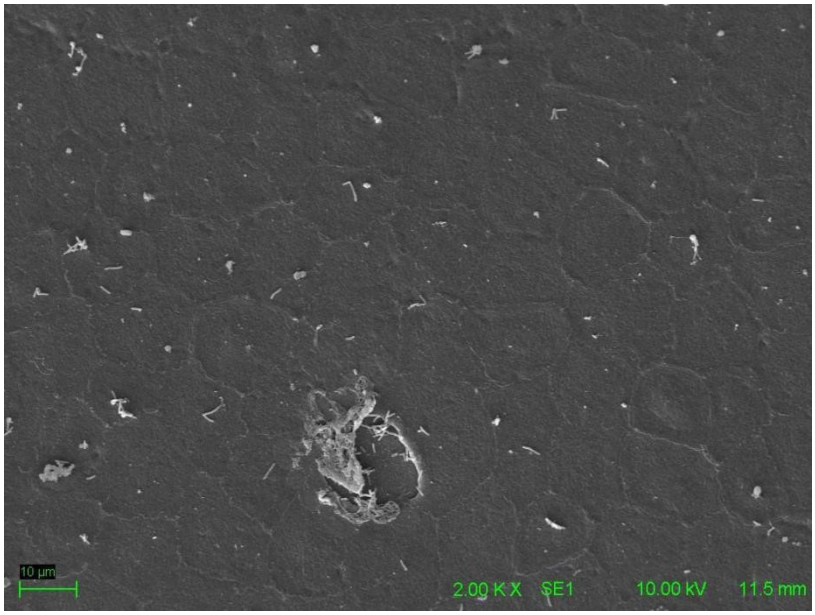

Figure 2. Scanning electromicrograph of the porcine corneal endothelium in G2. Note the area of endothelial loss (arrows). Magnification: 2000×. Bar: 10 µm.

Table 1. Comparison of the percentage of cell loss between groups and among regions.

	G1 n=6	G2 n=6	p between groups*
I - Central	0 (0-0.47)	1.65 (1.55-1.,94)[a]	**0.002**
II – Superior	0 (0-0.56)	2.94 (2.25-4.62)	**0.002**
III – Inferior	0 (0-0)	3.21 (2.25-3.92)	**0.002**
IV - Lateral	0 (0-0)	3.70 (2.83-4.07)[b]	**0.002**
V – Medial	0 (0-0)	3.55 (2.36-4.46)	**0.002**
p among regions**	0.558	**0.007**	

Data presents as median (minimum-maximum); *Mann-Whitney test, **Friedman test followed by Dunn-Bonferroni post hoc test; abdifferent letters denote statistically different groups.

4. Discussion

Few recent studies have analysed corneal endothelial preservation in Eusol-C in either humans [23-24] or in animals [10-12, 25]. Endothelial cell loss was evaluated in equine corneas after 7 and 14 days of storage in Eusol-C using SEM and observed satisfactory preservation for up to 14 days [12]. Satisfactory preservation of porcine corneal endothelium after 14 days of storage in Eusol-C medium was demonstrated [10]. The same group of researchers conducted a comparative study involving human and porcine corneas. After a 14-day period, the authors concluded that corneas from both species exhibited satisfactory quality parameters following conditioning in Eusol-C [11]. Another study evaluating calf corneas that were stored at 2–8 °C for up to 14 days in Eusol-C suggested that bovine and human corneas share comparable preservation requirements under ex vivo conditions [25].

Although a study involving rabbit and human corneas demonstrated the superiority of organ culture medium in preserving corneal endothelial cells for up to 28 days [26], in another retrospective study evaluating the clinical outcomes of corneal transplantation using human donor corneas stored in Eusol-C compared with corneas stored in organ culture, comparable surgical outcomes were obtained with both storage methods when the optimal storage periods for each medium were applied [27]. In comparisons of hypothermic media, Eusol-C has been shown to be as effective as Optisol-GS for corneal preservation for at least five days [28].

Currently, human corneas are the sole donor source for transplantation. This donor source has become increasingly scarce, and this scarcity is the main limiting factor in curing corneal diseases [3-5, 29, 30]. To address this issue, studies have been conducted on corneal xenotransplantation, primarily using porcine species [2, 3, 19]. Although porcine corneas lack Bowman's membrane, their biological and morphological characteristics have proven to be quite similar to those of human corneas [7, 8, 15, 29, 31, 32]. This species has been widely utilized as a study model for ocular diseases and surgical procedures [7, 15, 33, 34].

The authors opted to use eyes obtained from slaughterhouse discard material in the present study to avoid the need for euthanasia. Previous studies conducted within 4-6 hours postmortem, endothelial integrity was maintained [35-38]. Regarding sample size, previous studies under different experimental conditions, the expected percentage of cell mortality ranges from 7,8% (SD= 3,5%) [11] to 3,07% (SD=0,93%) [39]. With 6 animals per group, it is possible to detect differences of 7% and 1,86, considering these variabilities, a power of 90% and a significance level of 0,05. In summary, a sample size of 6 animals allows the detection of a difference of 2 standard deviations with a power of 90%, which is considered a large effect size. This calculation was performed with the help of WINPEPI 11.65 [40].

Corneal specular microscopy was used to assess endothelial health in this study. Specular microscopy is the standard method for endothelial evaluation in both humans and animals and has been employed to analyse corneas from various species [25, 35-38, 41, 42]. The parameters analysed via specular microscopy were the endothelial cell density and hexagonality. In the present study, no significant differences in endothelial density or hexagonality were observed between the right and left eyes. The values found in the present study using specular microscopy were similar to those previously reported [38]. This similarity was attributed to the fact that the animals in both studies were healthy and belonged to the same breed and age group. It has been established that in healthy eyes, no differences exist in corneal endothelial parameters between the left and right eyes [35-38].

In previous studies utilizing Eusol-C medium, corneas were analysed over periods ranging from 7 to 29 days [10, 12, 23, 24]. In the present study, a storage period of 14 days was adopted, corresponding to the storage duration recommended by the manufacturer. A 14-day storage duration has been employed in studies demonstrating satisfactory endothelial preservation within this time frame for Optisol, Optisol-GS, and Eusol-C media [10, 13, 24, 25, 41, 43-45]. Refrigeration of corneas extends endothelial cell viability over prolonged periods(6).

Endothelial viability of preserved corneas can be assessed using different methods, including vital staining, optical microscopy, and SEM, among others [46, 47]. Among the available methods, optical microscopy represents a rapid and cost-effective method for evaluating the corneal endothelium (6). In the present study, SEM was chosen to assess the percentage of endothelial loss following the preservation period. SEM is the most widely used tool for analysing endothelial morphology, with numerous studies published in humans and animals regarding drug toxicity, evaluation after preservation, and assessments following corneal or intraocular surgical procedures [12, 13, 16, 18, 41]. In veterinary medicine, remains in its early stages of development. Grafts typically provide tectonic support, with achievement of an optical purpose still a distant goal [20-22]. However, the lower cost and minimal equipment requirements associated with the Eusol-C hypothermic medium render it a promising option for the establishment of animal eye banks, potentially transforming this reality.

In a recently published study, an ex vivo porcine cornea model has been suggested as an alternative to the human as an alternative to the human cornea for analysing endothelial preservation after storage under hypothermic conditions or organ culture [10]. Images were obtained with a high-resolution camera from light microscopy with trypan blue, and the cell mortality rate and other parameters were assessed with the aid of software. Porcine and human corneas exhibited comparable trends under the investigated storage conditions for up to 14 days. Preservation of human corneas was studied in a new hypothermic preservation medium called Kerasave with Optisol-GS [39]. For this comparison, SEM was not employed, instead trypan blue staining and specular microscopy were used to analyze cell density, mortality, and morphology. On day 14, the mean mortality rate in the central region of the cornea was 0.54% ± 0.40% for corneas in Kerasave and 0.14% ± 0.14% for those in Optisol-GS. In the peripheral regions of the cornea, the mortality rate was 3.07% ± 0.93% for corneas in Kerasave and 3.38% ± 0.78% for those in Optisol-GS. These mortality rates were considered consistent with satisfactory preservation over the analysed period.

In the only published study analyzing porcine corneas preserved in hypothermic medium via SEM [9], porcine corneas were stored in 2.5% chondroitin sulphate for up to 10 days. Cell mortality rates of 12% on day 5 and 26% on day 10 of storage were observed, and these rates were considered similar to those for human corneas in this medium. Rodella and collaborators [10] compared the effects of preservation in Eusol-C and organ culture medium by evaluating cell density and mortality using trypan blue as a cell marker after a storage period of up to 14 days. Mortality rate was estimated using ImageJ software. At the end of the storage period, porcine corneas stored in organ culture medium and Eusol-C showed mortality rates of <10% and <20%, respectively. In a similar study, Giurgola *et al.* [11] investigated the density, morphology, and cell mortality of human and porcine corneas after preservation in Eusol-C for 14 days, washing with cleaning solution, and employing trypan blue and specular microscopy. At the end of storage period, the endothelial mortality rate increased by 3.1% ± 3.3% in human corneas

and by 7.8% ± 3.5% in porcine corneas. Based on these results, the authors concluded that the use of preservation medium and cleaning solution was safe and effective, emphasizing the similar behavior between corneas from both species.

In the present study, the percentage of endothelial loss was 3.01% after 14 days of storage, considering the average losses in the evaluated regions. Compared to previous studies on porcine corneas, the percentage of cell loss obtained in this study was lower than that found using the same storage period [11, 10]. However, the analysis methods employed in these previous studies did not involve SEM. In previous studies of human corneas in which endothelial mortality was established using vital dyes and visualisation under a microscope, cell loss ranged from 4% to 16% after 14 days of storage in preservation media [48-49]. These rates were also slightly higher than those found in our research, although still comparable despite differences in the analysis methods. Although the difference in endothelial loss between G1 and G2 was statistically significant in this study, it was not considered clinically relevant, as a certain degree of loss is expected. This finding is consistent with previously published data in the literature, indicating satisfactory preservation during the observed period. In the current study, the percentage of cell loss was evaluated as a parameter of satisfactory preservation based on the above-mentioned previous studies because the cell density can be significantly different depending on the fixation method used in the sample for SEM. Furthermore, these values may also vary depending on the analysis method utilized [39].

This study evaluated the percentage of cell loss following preservation in Eusol-C medium. Cell density and cell morphology were not evaluated after the preservation period. In a previous study, endothelial density of porcine corneas evaluated by SEM decreased after 10 days of storage in preservation medium [9]. This likely occurred because of cell shrinkage, and it did not follow the same pattern as cell mortality, which increased during both periods. Rodella *et al.* [10] did not observe significant differences in the endothelial density of porcine and human corneas stored in Eusol-C for 14 days. However, endothelial mortality increased significantly over time. These results reinforce that cell density is not a reliable parameter for this type of evaluation. Cellular morphology was assessed only to ensure the inclusion of healthy corneas in the study. No significant changes in cellular morphology are expected in an acute study.

Study limitations include the reduced sample size, the single preservation time, the absence of functional evaluation and the absence of studies designed with SEM, Eusol-C and percentage of cell loss analysis in pigs to compare results more accurately.

This is the first study to associate the evaluation of porcine corneal endothelium preserved in hypothermic Eusol-C medium with SEM, enabling the calculation of the percentage of endothelial cell loss following storage. The results demonstrated satisfactory preservation over the analysed period, with cell loss rates comparable to those reported in previous studies, despite differences in analysis methods. The authors suggest further studies with larger samples and morphological evaluation of preserved corneas after transplantation.

5. Conclusion

The results of this study demonstrated that the hypothermic preservation medium Eusol-C was effective in preserving the corneal endothelium of pigs for up to 14 days.

Conflicts of interest statement

The authors declare no conflict of interest.

Data availability statement

The data will be provided upon request.

Author contributions

Conceptualization: A. F. Silva and J.A.T. Pigatto. Data curation: A. F. Silva and J.A.T. Pigatto. Investigation: A. F. Silva, A. P. Melo., A.M. Pigatto, R.S. Rocha, M.P. Seibel, N.P. Méndez and J.A.T. Pigatto. Project administration: J.A.T. Pigatto. Writing (original draft, review & editing): A. F. Silva, A. P. Melo., A.M. Pigatto, R.S. Rocha, M.P. Seibel, N.P. Méndez and J.A.T. Pigatto.

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