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Microbiological profile of donor corneas stored for tectonic transplantation purposes in rabbits

[*Microbiologia de amostras de bancos de córneas destinadas a transplantes, em coelhos*]

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ABSTRACT

This study aimed to evaluate the microbiota of donor rabbit corneas stored for tectonic transplantation purposes. Swabs from both corneas of 20 rabbits were carefully collected and submitted to microorganism isolation and identification. After this first swab collection, rabbits were euthanized for reasons other than this project and the eyes were enucleated. The corneas were collected and stored to compose the cornea tissue bank. Corneas were stored in a 0.3% tobramycin solution at -20°C. After 30 days, the corneas were thawed at room temperature and removed from the antibiotic. New swabs were obtained from the corneas and submitted to microorganism isolation and identification. Gram positive organisms were predominant in the rabbit corneal flora before storage and the Staphylococcus sp. was the most common microorganism isolated from those samples. No growth was observed on the samples collected after storage. The methods used for collection and storage of the corneas were efficient to constitute a sterile donor corneal tissue bank.

Keywords: rabbit, corneal bank, microbiota, corneal transplant

RESUMO

Analisaram-se córneas armazenadas para transplantes tectônicos usando-se suabes coletados de 20 coelhos, visando ao isolamento e à identificação de microrganismos. Após a coleta das amostras, os coelhos foram submetidos à eutanásia, por razões alheias ao estudo, e enucleados. As córneas foram coletadas e armazenadas a fim de se constituir o banco de córneas. O armazenamento deu-se em solução de tobramicina 0,3% a -20°C, por 30 dias. Após esse período, as córneas foram descongeladas à temperatura ambiente e removidas da solução de antibiótico. Novos suabes foram coletados e submetidos ao isolamento e à identificação dos microrganismos. A flora corneal mostrou-se predominantemente composta por bactérias Gram positivas, sendo o Staphylococcus *sp. o mais identificado. Não se verificou crescimento de colônias bacterianas ou fúngicas nas amostras após o armazenamento. Considerando-se a maneira como a pesquisa foi concebida e as injunções do meio em que ela foi realizada, há como admitir, pela ausência de crescimento microbiano nas amostras armazenadas, que a técnica de armazenamento empregada é segura para a estocagem de córneas destinadas a transplantes.*

Palavras-chave: coelho, banco corneal, microbiota, transplante de córnea

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INTRODUCTION

Disorders such as dystrophy, keratoconus, corneal abscesses, keratomalacia, corneal sequestrum and iris prolapse affecting the cornea are relatively common in humans and domestic animals. Corneal transplantation may be a useful method to manage these abnormalities and the surgeries can be performed for optical, therapeutic, tectonic or cosmetic purposes (Brooks *et al*., 2008).

Corneal transplantation can be performed for different reasons: optical transplants improve or restore vision in cases of corneal opacification; therapeutic grafts are performed to control refractory corneal diseases by removing necrotic or infected tissue; tectonic transplants preserve or restore the structural integrity when corneal tissue is missing; cosmetic grafts improve the appearance of the eye without necessarily improving vision. Corneal transplantation is a routinely performed procedure and is considered the most widely practiced form of organ transplantation in humans. In animals other than horses, the practice is less frequent (Brooks *et al.*, 2008).

The corneal thickness varies between species, breeds and individuals and can range from 0.60 to 1.50mm in horses (Samuelson, 2007). In leporids, the fluctuation ranges from 0.37 to 0.43mm (Chan *et al*., 1983).

A corneal bank that provides sterile and healthy corneal tissue is necessary when considering corneal transplantation as a treatment option. The qualitative control of the donor tissue is imperative. It starts with the use of appropriate collection and preservation techniques and, in some cases, a compatibility evaluation between donor and recipient (Hirai *et al*., 2009).

Since living endothelial cells are not essential for tectonic transplantation, frozen corneal tissue is a good alternative for grafting, and the material can be stored for months or even years (Mueller *et al*., 1966). However, graft contamination may result in suture dehiscence and even lead to a devastating endophthalmitis following penetrating keratoplasty (Andrew *et al*., 1998; Fontana *et al*., 2007). Besides environmental and iatrogenic contamination during conservation and surgery, the germ responsible for the endophthalmitis may come from the graft itself (Robert *et al*., 2002). In that sense, it is essential that the preservation method used is able to inhibit any microorganism present on the tissue.

The objective of this study was to analyze the microbiota of donor corneas stored for tectonic transplantation purposes, in order to evaluate the efficiency of the freeze storage technique on inhibiting the contamination of donor corneal banks.

MATERIAL AND METHODS

The research was conducted in the ophthalmology unit of a Veterinary Teaching Hospital. The study protocol was submitted to and approved by the Committee of Ethics and Animal Welfare of the College of Agricultural and Veterinarian Sciences – UNESP – Jaboticabal (Protocol 007105-06). Bioethics cares followed the guidelines from *Association for Research in Vision and Ophthalmology (National Institutes of Health Publications No 85-23: Revised 1985).*

Twenty healthy New Zealand white rabbits without ocular diseases were used. For the selection, a routine clinical ophthalmic examination was performed including dazzle and menace responses, pupillary light reflexes, Schirmer tear test (Schirmer tear test strips – Ophthalmos Ltda), slit-lamp biomicroscopy (Slit Lamp SL-14 – Kowa Company Ltda), tonometry (TonoVet J1000, Icare.), gonioscopy (Koeppe medium diagnostic lens 18mm – Ocular®), fluorescein staining (Fluorescein strips – Ophthalmos Ltda.) and indirect binocular ophthalmoscopy (Binocular indirect ophthalmoscope FOH-5 – Eyetec S.A.).

One day after the selection, samples were collected using a sterile cotton swab from both eyes of all rabbits (one individual swab for each eye), smoothly touching the ocular surface while avoiding contact with the eyelids and skin. Swabs were individually stored in tubes containing brain heart infusion broth medium (Broth brain heart infusion, Himedia) and were incubated at 37°C for 24 hours. The samples obtained from the right eye were kept under anaerobic conditions (screw cap tubes) and those collected from the left eye were kept under aerobic conditions. The samples kept under

anaerobic conditions were inoculated onto blood agar (Prepared with firming agar (Biolog), BHI and horse blood (7%), free of hematological diseases) and BHI agar (Prepared with BHI and bacteriological agar) and incubated in an anaerobic jar under a mixture of CO_2, H_2 and N_2 at 37°C for 48 hours. Those samples kept under aerobic conditions were then inoculated onto blood agar, MacConkey agar (MacConckey agar, No 3, Oxoid), mycosel agar (Mycosel Agar, BBL) and potato agar plates (Prepared with boiled potato broth, glucose (Bacto-Dextrose, DIFCO), yeast extract (Extrato de levedura, OXOID) and bacteriological agar, adjusting the pH with hydrochloric acid. The first two were incubated at 37°C for 24 hours. Plates containing mycosel and potato agar were incubated at 25°C for up to 5 days. The evaluation of all media was performed regularly at 24-hr intervals. After the growth of any colonies, slides were prepared to evaluate the morphology and Gram staining (Set for Gram stain, New Prov) characteristics.

The rabbits were euthanized for reasons other than this project and then the corneal bank was prepared. The corneas were excised around the limbus and rinsed with 10% povidone-iodine and saline solutions. Corneas were stored frozen at -20°C for 30 days in 0.3% tobramycin solution (Tobramicin 0.3% – Biosintética, Aché). After 30 days the corneas from the corneal bank were thawed at room temperature. New swabs were performed from the samples and incubated in BHI solution. Corneal swabs from the right eyes were kept under anaerobic conditions and those from the left eyes were kept under aerobic conditions. Following incubation, the samples were inoculated and processed as previously described.

Fisher's exact test was used to compare the presence of microorganisms on the samples before and after 30 days of storage ($P<0.05$).

RESULTS

The ocular surface microflora obtained from samples before storage was composed primarily of gram-positive bacteria. *Staphylococcus sp.* was the most common isolate from both aerobic and anaerobic conditions. Frequently more than one microorganism was isolated from each eye (Table 1).

Table 1. Microorganisms isolated from the ocular surface of rabbits prior to storage, incubated in blood agar, brain heart infusion (BHI) agar and kept under aerobic and anaerobic conditions

Microorganism	Number of samples			Porcentage		
	Blood agar aerobic conditions	Blood agar anaerobioc conditions	BHI agar anaerobioc conditions	Blood agar aerobic conditions	Blood agar anaerobioc conditions	BHI agar anaerobioc conditions
Staphylococcus sp.	12	8	5	60%	40%	25%
Bacillus sp.	5	0	3	25%	0	15%
Streptococcus sp.	0	3	0	0	15%	0
Corynebacterium sp.	4	2	1	20%	10%	5%
Gram negative rods	1	1	1	5%	5%	5%

For those plates cultivated in blood agar and kept under aerobic conditions, growth was observed in 18 samples. *Staphylococcus sp.* was the most frequent bacteria isolated and was present in 60% of the samples, followed by *Bacillus sp.* (25%), *Corynebacterium sp.* (20%) and Gram negative rods (5%). Two samples (10%) exhibited no microbial growth. The Gram negative rods exhibited hemolytic activity and were transferred to a Rugai-lysine culture medium, but no changes in the color were observed. No growth was observed in any sample cultivated in MacConkey agar. Colony growth was observed in two plates of potato agar and in only one plate of mycosel agar. Yeast colonies were observed in both cases.

For those plates cultivated in blood agar and kept in anaerobic conditions, growth was observed in 14 samples. *Staphylococcus sp.* was the most common isolate (40%), followed by *Streptococcus sp* (15%), *Corynebacterium sp* (10%) and Gram negative rods (5%). For those plates cultivated in BHI agar and kept under anaerobic conditions, *Staphylococcus sp.* was identified in 25% of the samples, followed by *Bacillus sp.* (15%), *Corynebacterium sp.* (5%) and Gram negative rods (5%). No growth was

observed in any sample collected after 30 days of storage.

There was a significant difference in the number of microorganisms isolated between samples collected before and after storage ($P<0.01$) in all eyes.

DISCUSSION

According to previous studies, thirty percent of the processed corneas in corneal tissue banks are not used for transplantation in humans. The main reasons for discarding corneas are inadequate endothelium before or after preservation, and positive serology for diseases and contamination (Delbosc and Boissier, 1999). Among these, the last one is the easiest to manage.

Robert *et al*. (2002) and Araujo and Scarpi (2004) reported the role of povidone-iodine solution in reducing the microorganism population in human donor corneas. Donor age, cause of death and time between donor decease and cornea donor excision showed no correlation with corneal donor contamination. In our study, the povidone-iodine solution was used to rinse the corneal tissue after collection, which helped to reduce the bacterial contingent of the samples.

Staphylococcus sp., *Bacillus sp*. and *Corynebacterium sp*. were the most common bacteria isolates in the present study, previous to storage. Variations on the microbiota may occur due to habitat, season, sampling methods and other factors (Urban *et al*., 1972; Murphy *et al*., 1978; Eichenbaum *et al*., 1987; Moore *et al*., 1988; Andrade *et al*., 2002; Hendrix, 2007).

The smaller contingent of Gram negative bacteria on the ocular surface may be partially explained by their morphological characteristics. These microorganisms have a thinner cell wall compared to Gram positive bacteria, composed by lipopolysaccharids and other substances. Therefore, the action of the pre-corneal tear film enzymes, such as lysozyme, lactoferrin and β-lysine, eliminates the Gram negative bacteria much easier from the ocular surface (Urban *et al*., 1972; Murphy *et al*., 1978; Trindade *et al*., 2000). In the present study it was not possible to identify the Gram negative rods isolates as enterobacteria since no color change was observed after inoculation in the Rugai-lysine medium.

Our findings are in agreement with studies by Cooper *et al*. (2001), which found that the most common isolates found in eyes of healthy rabbits were *Staphylococcus sp*. (40%), *Micrococcus sp.* (18%) and *Bacillus sp*. (13%). The authors showed no difference between genders or ages. According to Whitley and Gilger (1999), *Staphylococcus spp.* (39%), *Streptococcus spp*. (25%), *Pseudomonas spp*. (9.4%), *Escherichia coli* (4.7%), *Corynebacterium spp.* (3.9%), and *Bacillus cereus* (2.4%) are among the bacteria more commonly isolated from eyes coursing with external diseases. In our study, under aerobic conditions, most isolates of *Corynebacterium sp*. (75%) showed hemolytic activity. Hemolysis was also observed on *Staphylococcus sp.* and *Bacillus sp*. isolates.

The bacterial contingent found under anaerobic conditions was smaller when compared to those under aerobic conditions. Considering that the cornea is constantly exposed to air, the proliferation of such organisms is impaired. Although not statistically significant, Santos *et al*. (1999) showed that the presence of fungi on the ocular surface is higher in immunosupressed individuals. In our study, only two samples exhibited fungi growth.

Tobramycin is a broad spectrum aminoglycosides and has been used in the control of superficial and deep eye infections for a long time (Wilhelmus *et al*, 1987). The drug acts by preventing the mRNA transcription and is especially effective against *Pseudomonas sp.* and *Klebsiella sp*. (Whitley and Gilger, 1999; Uesugui *et al*, 2002). In our study, the association of tobramycin and freezing was effective in eliminating the microorganisms from the donor corneas, and also in controlling the contamination of the corneal buttons during storage. According to Moeller *et al.* (1999), tobramycin exhibits *in vitro* activity significantly higher than gentamicin and results in a smaller number of resistant microorganisms. Its access ease, affordable price and effectiveness make it a good alternative for the preservation of donor corneal buttons for tectonic purposes.

CONCLUSIONS

The freeze storage technique used in this study was efficient on eliminate the initial microflora and inhibit the contamination of donor cornea banks for tectonic transplantation purposes, since no microorganisms were found on the tissue 30 days after storage.

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