

# Effect of Amniotic Membrane Transplantation on the Healing of Bacterial Keratitis

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**PURPOSE.** To study, with the use of an animal model, the efficacy of amniotic membrane (AM) transplantation as adjunctive treatment in corneal healing after bacterial keratitis.

**METHODS.** *Staphylococcus aureus* keratitis was induced in 47 rats by injection of bacteria into the corneal stroma. Treatment was started 48 hours later with one of three randomly assigned protocols: cefazolin drops (50 mg/mL) and AM transplantation ( $n = 16$ ); nonpreserved 0.9% saline drops and AM transplantation ( $n = 15$ ); or cefazolin without AM transplantation ( $n = 16$ ). Cefazolin and saline drops were administered every 30 minutes for 6 hours, then hourly for 6 hours. AM was transplanted 24 hours after termination of cefazolin or saline treatment. Results were clinically assessed 7 days after AM transplantation or at the corresponding time in the nontransplanted animals. The rats were then killed, and their corneas were removed for bacterial counts or histopathologic examination.

**RESULTS.** The best clinical results were observed in the group treated with cefazolin and AM transplantation, manifested by the least corneal haze and neovascularization ( $P = 0.007$  and  $P = 0.014$ , respectively) and minimal bacterial counts (28 colony-forming units [CFU]/mL compared with 160 CFU/mL and 240 CFU/mL, respectively). Histopathologic examination showed that the central corneal vessels from rats treated with cefazolin and AM were smaller and less congested than those from the other two groups.

**CONCLUSIONS.** AM transplantation is a useful adjunctive treatment after bacterial keratitis in this rat model. The transplanted AM improved the healing process, resulting in decreased corneal haze and less neovascularization. (*Invest Ophthalmol Vis Sci.* 2008;49:163–167) DOI:10.1167/iovs.07-1005

Bacterial keratitis continues to be a sight-threatening disease despite the development of potent new antibacterial agents. In spite of intensive antibiotic treatment, corneal damage can occur as a result of keratolytic and inflammatory processes caused by infection or scarring and of neovascularization related to the healing process.<sup>1,2</sup>

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Transplantation of human amniotic membrane (AM) is used for many ophthalmic indications. Reports describe its efficacy in reconstructing a corneal surface severely damaged by chemical agents,<sup>3,4</sup> promoting the healing of persistent corneal epithelial defects with or without ulcerations,<sup>5–9</sup> enhancing the success of corneal surface reconstruction surgery in patients with stem cell deficiency states,<sup>10,11</sup> and substituting for conjunctival autografts after excision of pterygium or removal of conjunctival lesions.<sup>12,13</sup>

Human AM has antibacterial,<sup>14,15</sup> antiangiogenic, anti-inflammatory,<sup>16</sup> and antifibroblastic properties.<sup>17</sup> These characteristics may play a role in the use of AM transplantation in the treatment of infectious keratitis. Studies have shown that human AM transplantation promotes rapid epithelialization and reduces stromal inflammation and ulceration in herpes simplex virus (HSV)-1 keratitis.<sup>18,19</sup> When used with antifungal agents as adjunctive treatment, AM transplantation can enhance epithelialization and prevent corneal perforation in acute fungal keratitis.<sup>20</sup> In a group of patients with various infectious ulcers, AM promoted wound healing, reduced inflammation, and improved visual acuity in those without scarring.<sup>21</sup> In a recent report of patients with severe bacterial keratitis, Gicquel et al.<sup>22</sup> reported immediate pain relief and epithelial healing that they attributed to early AM transplantation combined with topical corticosteroids.

The purpose of the present study was to investigate the effect of human AM transplantation as an adjunctive treatment for bacterial keratitis in a controlled experimental model of methicillin-sensitive *Staphylococcus aureus* (MSSA) keratitis in rats. The induction of experimental keratitis was based on a modification of previous models of keratitis in rats,<sup>23</sup> resembling the induction of experimental keratitis in rabbits.<sup>24</sup>

## MATERIALS AND METHODS

### Animals

Forty-seven male Wistar rats, aged 3 months, were used. All rats were treated and maintained in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the Tel-Aviv University Institutional Animal Care and Use Committee. The sample size was determined after an experimental clinical pilot study.

Before any surgical procedure, each rat was anesthetized by intramuscular injection of ketamine hydrochloride 100 mg/kg and xylazine hydrochloride 8 mg/kg. On conclusion of the study, each rat was killed by intramuscular injection of 1 mL of each anesthetic drug.

### Bacteria

An MSSA ocular strain, isolated from a human corneal specimen, was used to induce experimental keratitis. The bacteria were propagated on Mueller-Hinton agar plates (Hy Laboratories, Rehovot, Israel) and incubated at 37°C for 18 hours. Several bacterial colonies were pooled and suspended in saline to adjust the turbidity to 0.5 McFarland units (equivalent to  $5 \times 10^8$  colony-forming units [CFU]/mL). The suspension was then adjusted to a final concentration of  $10^5$  CFU/mL, as verified by a quantitative bacterial count on Mueller-Hinton agar plates.

## Induction of Experimental MSSA Keratitis

With a 30-gauge needle and a 1-mL tuberculin syringe under a stereoscopic surgical microscope (Wild M690; Wild Heerbrugg, Gais, Switzerland), we injected 0.05 mL freshly prepared MSSA ( $10^5$  CFU/mL) into the stroma at the center of the right cornea. The bacterial infection was allowed to progress undisturbed for 48 hours before initiation of therapy.

## Antibiotics

The appropriate antibiotic for use in this study was selected after a preliminary evaluation was performed in six rats to compare the efficacy of cefazolin (Cefamezin; Teva Pharmaceuticals Ltd., Jerusalem, Israel) and vancomycin hydrochloride (Vanco-Teva; Teva Pharmaceuticals Ltd.) against the MSSA used in the model. In that evaluation, after the induced MSSA keratitis had been allowed to progress undisturbed for 48 hours, the rats were randomly assigned to receive topical treatment with either cefazolin 50 mg/mL or vancomycin 33 mg/mL. Drops were administered at 30-minute intervals for 6 hours and then hourly for 6 hours. One hour after completion of the topical treatment, the rats were killed and corneal buttons were removed aseptically with corneal scissors. The corneal buttons were rinsed and homogenized in sterile phosphate-buffered saline (PBS; 3 mL/cornea) with a homogenizer (Polytron; Glen Mills Inc., Clifton, NJ). Aliquots of the corneal homogenates were serially diluted in the same buffer, plated in triplicate on Muller-Hinton agar plates (100  $\mu$ L/plate), and incubated for 24 hours at 37°C. Mean bacterial counts were  $150 \times 10^2$  CFU/mL in the vancomycin-treated corneas and  $5 \times 10^2$  CFU/mL in the cefazolin-treated corneas. Cefazolin was more effective and was therefore used in this study.

Cefazolin (50 mg/mL) was obtained from the central pharmacy at the Sheba Medical Center (Tel-Hashomer, Israel). All antibiotic solutions were freshly prepared before use and were kept at 4°C.

## Treatment Groups

Rats ( $n = 47$ ) were randomly assigned, 48 hours after the induction of keratitis, to three treatment groups. Rats in the study group (cefazolin-AM;  $n = 16$ ) were treated with cefazolin drops, 50 mg/mL, every 30 minutes for 6 hours and then hourly for 6 hours, and AM transplantation was performed 24 hours after completion of the topical treatment. Rats in the first control group (saline-AM;  $n = 15$ ) received 0.9% nonpreserved saline drops for 12 hours, and AM was transplanted 24 hours after completion of the topical treatment. Rats in the second control group (cefazolin-no AM;  $n = 16$ ) received cefazolin drops applied over 12 hours, as described for the study group, but did not undergo AM transplantation.

## Amniotic Membrane Transplantation

Cryopreserved human AM, kindly provided by Bio-Tissue, Inc. (Miami, FL), was transplanted onto the infected cornea as a patch, with the epithelial side facing down. The membrane was attached to the conjunctiva around the limbus by means of four 10/0 interrupted nylon sutures. To avoid sloughing of the AM, we performed central tarsorrhaphy in all treated eyes using 5/0 polypropylene mesh (Prolene; Ethicon Inc., Piscataway, NJ) sutures. Tarsorrhaphy was also performed in the treated eyes of the second control group, even though they had not undergone AM transplantation.

## Clinical Evaluation

All rats underwent biomicroscopic assessment of corneal abscess formation 48 hours after MSSA keratitis was induced. The investigator, who was blinded to the treatments received by the rats, used a stereoscopic microscope to measure the diameter of the abscess in millimeters.

Seven days after AM transplantation (or, in the cefazolin-no AM group, 8 days after completion of treatment with cefazolin drops), the stitches were removed, and all rats underwent biomicroscopic exam-

ination, again in a blinded manner. Corneal opacity and vascularization were assessed under the stereoscopic microscope and graded on a scale from 0 to 4, based on modification of clinical grading described by Heiligenhaus et al.<sup>18</sup> Corneal opacity was graded according to the diameter of opacity (0, clear cornea; 2, 0.5–1 mm; 3, 1.1–2.0 mm; 3, 2.1–3.0 mm; 4, 3.1–4.0 mm) and vascularization according to the location of neovascularization in the cornea (0, no vascularization; 1, vascularization at the limbus; 2, vascularization to the midperiphery of the cornea; 3, vascularization beyond the midperiphery but not including the center of the cornea; 4, vascularization reaching the center of the cornea).

## Bacterial Quantification

After clinical evaluation, five rats from each of the three groups were randomly selected for bacterial quantification, which was also performed in a blinded manner. The rats were killed, and corneal buttons were excised aseptically with corneal scissors, rinsed, and homogenized in sterile PBS (3 mL/cornea) with a homogenizer (Polytron; Glen Mills Inc.). Aliquots of the corneal homogenates were serially diluted in the same buffer, plated in triplicate on Muller-Hinton agar plates (100  $\mu$ L per plate), and incubated for 24 hours at 37°C. Numbers of CFU/mL per cornea were then counted.

## Histopathologic Evaluation

The remaining 32 rats that were not taken for bacterial quantification were also killed after the clinical examination, and their eyes were enucleated and fixated in 4% formaldehyde. Corneas were sectioned centrally and embedded in paraffin, and 5- $\mu$ m sections were stained with hematoxylin and eosin. Histopathology (corneal fibrosis, corneal inflammatory reaction, and corneal vascularization) in the stained sections was evaluated under light microscopy by a blinded observer, and each parameter was graded on a scale of 0 to 4. Corneal fibrosis was assessed in terms of the numbers of fibroblastic cells detected in the central cornea, and corneal inflammatory reaction was assessed by the presence of neutrophils and mononuclear cells in the central cornea. Corneal vascularization was assessed in the central, midperipheral, and peripheral cornea.

## Statistical Analysis

Individual outcomes in the three treatment groups were compared using a Kruskal-Wallis nonparametric test.  $P \leq 0.05$  was considered significant.

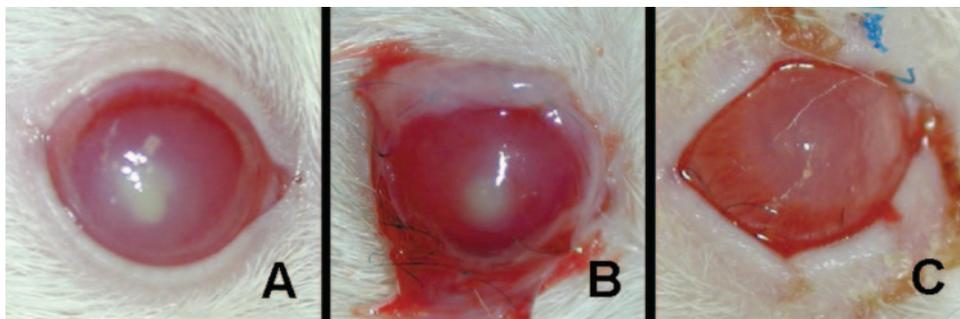
## RESULTS

### Clinical Evaluation

Forty-eight hours after the induction of MSSA keratitis, a corneal abscess ranging in diameter from 1 to 3 mm ( $2.1 \pm 0.8$  mm [mean  $\pm$  SD]) was observed in all eyes. The mean abscess diameter was similar in all three groups:  $2 \pm 0.89$  mm in the cefazolin-AM-treated eyes (Fig. 1A),  $2 \pm 0.82$  mm in the saline-AM-treated eyes, and  $2.27 \pm 0.65$  mm in the cefazolin-no AM-treated eyes. None of the corneas showed vascularization 48 hours after MSSA keratitis induction.

Seven days after AM transplantation (corresponding to 8 days after the completion of cefazolin treatment in the non-transplanted control group), no corneal abscesses could be detected. In all AM-transplanted eyes, the membrane was found to be dissolved (Fig. 1C). Significantly less corneal opacity and corneal vascularization were observed in the cefazolin-AM-treated eyes than in the saline-AM-treated or cefazolin-no AM-treated eyes ( $P = 0.007$  or  $P = 0.014$ , respectively; Table 1).

**FIGURE 1.** Clinical findings in a rat eye in which induced MSSA keratitis was treated with cefazolin drops and AM transplantation (cefazolin-AM). (A) Corneal abscess, photographed 48 hours after MSSA induction. No vascularization is seen. (B) Appearance of the eye after AM transplantation (performed 24 hours after termination of cefazolin administration) and before tarsorrhaphy. (C) Appearance of the eye 7 days after AM transplantation, immediately after removal of the tarsorrhaphy.



### Bacterial Quantification

Seven days after AM transplantation (or 8 days after the cessation of treatment in the cefazolin-no AM control group), mean bacterial counts were  $240 \pm 230$  CFU/mL in corneas from saline-AM-treated eyes and  $160 \pm 219$  CFU/mL in corneas from cefazolin-no AM-treated eyes but only  $28 \pm 63$  CFU/mL in corneas from cefazolin-AM-treated eyes.

### Histopathologic Evaluation

Results of histopathologic examination are summarized in Figure 2. Slightly less corneal fibrosis was observed in the two AM-treated groups of eyes than in the cefazolin-no AM-treated eyes (Fig. 2), but differences among the groups were not significant ( $P = 0.145$ ). Slightly less corneal inflammatory infiltrate was observed in eyes treated with cefazolin (with or without AM) than in saline-AM-treated eyes (Fig. 2), but again the intergroup differences were not significant ( $P = 0.436$ ). Photomicrographs of corneal fibrosis in the cefazolin-no AM-treated control group and of inflammation in the saline-AM-treated control group are shown in Figures 3A and 3B, respectively. Central corneal vessels seen in the cefazolin-AM-treated eyes (Fig. 3C) were markedly smaller and less congested than those in cefazolin-no AM-treated (Fig. 3D) or saline-AM-treated eyes (Fig. 3B). There was less vascularization in the midperipheral and in the peripheral cornea cefazolin-AM-treated eyes than in the other two groups (Fig. 2), but this finding was not significant ( $P = 0.138$  and  $P = 0.225$ , respectively).

### DISCUSSION

MSSA keratitis is one of the commonest forms of bacterial keratitis.<sup>25</sup> An important feature of *S. aureus* organisms is that in addition to fighting off the host's defense mechanisms and destroying healthy corneal tissue, they produce extracellular

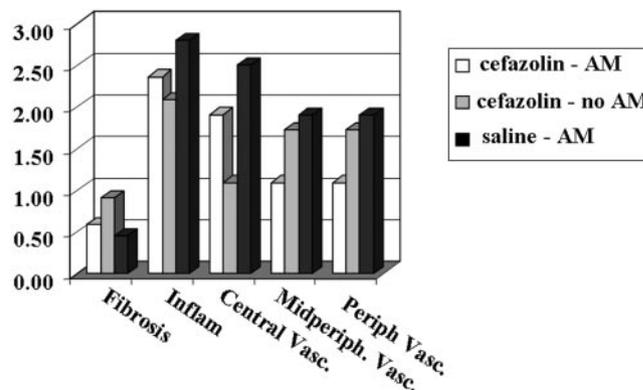
proteins, including enzymes that facilitate their multiplication and dispersion in the corneal tissue.<sup>1</sup> These proteins include hyaluronidase, which degrades extracellular ground substance, thereby creating space for migration and potentiating invasion; fibronectin, which facilitates migration; proteases, collagenases, and nucleases, which enhance bacterial pathogenicity; catalase, which reduces oxidative killing by neutrophils; leukocidin, which damages leukocyte membranes, causing the death of these cells; and coagulase, which helps prevent phagocytosis by macrophages and polymorphonuclear (PMN) leukocytes. Once bacteria have invaded the corneal stroma, an inflammatory response is initiated by the PMN leukocytes, which phagocytize the bacteria and destroy them by secreting proteolytic enzymes. However, these enzymes also produce toxic metabolites that may contribute to progressive destruction of the cornea. Antibiotic treatment initiates a process of repair that, in combination with the host's inflammatory response, usually succeeds in arresting the infectious process. Neovascularization and disorderly collagen resynthesis restore corneal integrity, albeit at the expense of degradation in optical clarity and in the refractive properties of the cornea. Fibrosis and angiogenesis are common causes of reduction in visual acuity.<sup>2</sup>

The rationale for using human AM in the treatment of infectious keratitis is based on several of its properties. Talmi et al.<sup>14</sup> found in vitro that human AM has an antibacterial effect. Kjaergaard et al.<sup>15</sup> demonstrated an inhibitory effect of fetal membranes against a range of bacteria, with the most pronounced effect obtained by both the chorion and the amnion against group A streptococcus, *S. aureus*, and *S. saprophyticus*. In the present study, the results of AM transplantation after

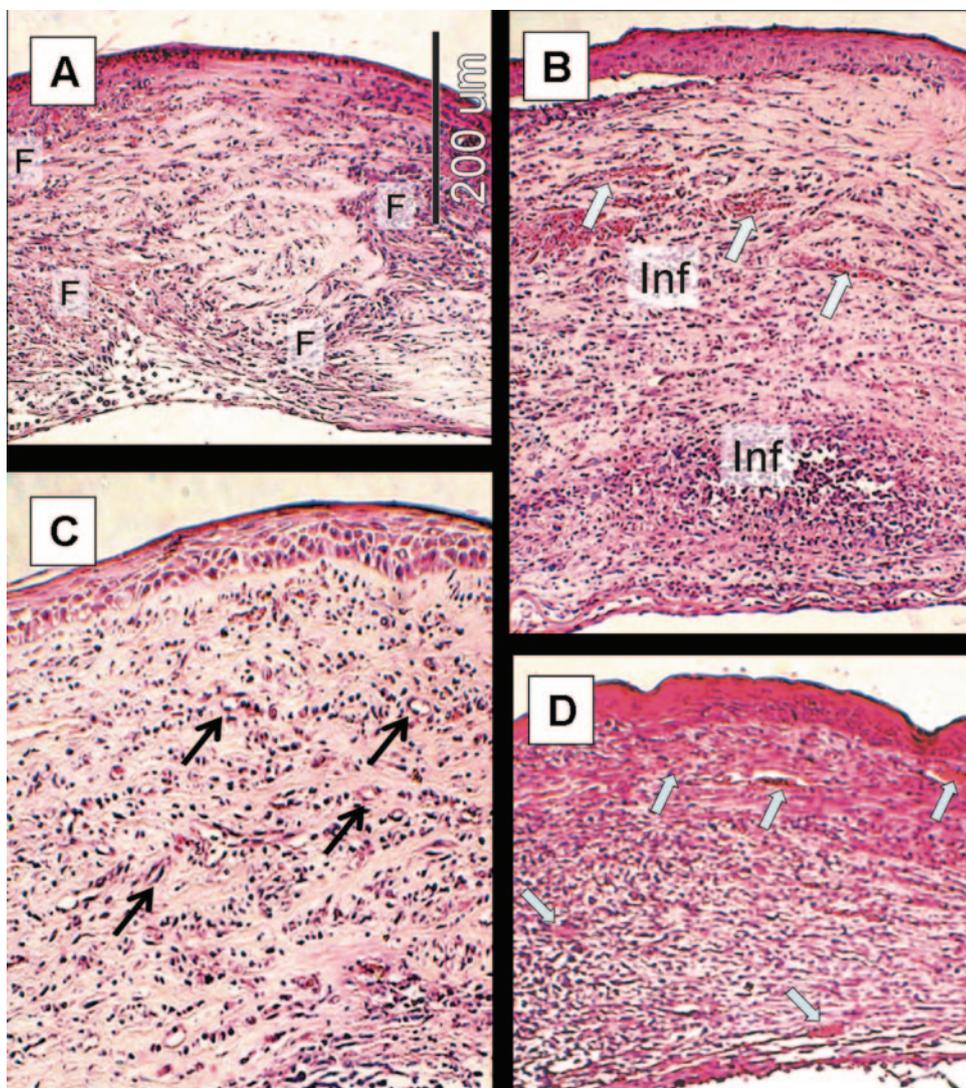
**TABLE 1.** Clinical Findings in the Three Treatment Groups

	Cefazolin-AM (n = 11)	Saline-AM (n = 10)	Cefazolin-no AM (n = 11)
Corneal abscess	None	None	None
Corneal opacity	$0.68 \pm 0.6$	$1.6 \pm 0.81$	$1.64 \pm 0.67$
Corneal vascularization	$0.82 \pm 0.95$	$2.6 \pm 0.99$	$2.2 \pm 1.67$

Results were evaluated 7 days after AM transplantation (8 days after the cessation of cefazolin treatment in the cefazolin-no AM group). Data are recorded as mean grades on a scale of 0 to 4 ( $\pm$  SD) in all groups. For corneal opacity (graded according to opacity diameter): 0, no opacity; 1, 0.5 mm opacity; 2, 1 mm opacity; 3, 2 mm opacity; 4, 3 mm opacity. For corneal vascularization (graded according to the location of neovascularization in the cornea): 0, no growth; 1, at the limbus; 2, at the midperiphery; 3, between the midperiphery and the center; 4, at the center.



**FIGURE 2.** Results of histopathologic examination in the three treatment groups. Data are recorded as mean grades on a scale of 0 to 4 in all groups. Corneal fibrosis was assessed in terms of the numbers of fibroblastic cells detected in the central cornea and corneal inflammatory reaction by the presence of neutrophils and mononuclear cells in the central cornea. Corneal vascularization was assessed in the central, midperipheral, and peripheral cornea.



**FIGURE 3.** Histopathologic findings. (A) Light microscopy of a cornea from the cefazolin-no AM control group, demonstrating large fibrotic areas (F). (B) Light microscopy of a cornea from the saline-AM control group, demonstrating diffuse inflammatory areas (Inf) and large, congested corneal stromal blood vessels (*light arrows*). (C) Light microscopy of a cornea from the cefazolin-AM group, demonstrating small stromal blood vessels (*dark arrows*). (D) Light microscopy of a cornea from the cefazolin-no AM control group, demonstrating large congested corneal stromal blood vessels (*light arrows*).

antibiotic treatment pointed to the synergistic effect of the two modalities in decreasing the bacterial counts compared with the control groups, which had been treated either by antibiotic eye drops or by AM transplantation but not both.

To distinguish the effect of AM transplantation on corneal healing, we performed transplantations 24 hours after termination of the 12-hour regime of intensive antibiotic treatment. Based on a comparison with the two control groups, it was clear that AM transplantation caused significant further reduction in the clinical signs of corneal neovascularization and opacification.

Hao et al.<sup>16</sup> showed that epithelial and mesenchymal cells of human AM express various antiangiogenic and anti-inflammatory proteins, such as IL-1 receptor antagonist, tissue inhibitors of metalloproteinase, collagen XVIII, and IL-10. Tseng et al.<sup>17</sup> demonstrated that human AM exerts antifibroblastic activity by suppressing the transforming growth factor- $\beta$  signaling system, DNA synthesis, and subsequent myofibroblast differentiation for cultured human corneal fibroblasts and limbal fibroblasts. All these potential benefits are useful in a clinical setting, in which intensive antibiotics are initiated immediately on diagnosis, but an additional tool is needed to enhance corneal healing after clinical amelioration of the infection. Gicquel et al.<sup>22</sup> also reported the absence of neovascularization and a decrease in ocular inflammation in a noncomparative case series of patients with severe bacterial keratitis who had un-

dergone AM transplantation at 48 hours and received topical steroids at 72 hours after starting antibiotic treatment.

Our histopathologic analysis, which demonstrated smaller and less congested corneal vessels in the cefazolin-AM-treated rats than in the two control groups, supports the clinical findings of reduced corneal neovascularization in the cefazolin-AM-treated group. The other histopathologic findings, namely fibrosis and inflammatory infiltrate, were also less prominent in that group than in the other two groups. The differences, however, were not statistically significant, possibly because the nylon sutures used to secure the AM to the limbus (but not used for sham purposes in the nontransplanted eyes) might themselves be capable of inducing some inflammation and vascularization. This issue can be addressed in future studies by the use of bioadhesive-coated, freeze-dried amniotic membrane, which does not require suturing.<sup>26</sup>

The AM in our study was transplanted, epithelial side down, in the form of a patch that covered the entire corneal surface. The use of this technique, with the aim of optimizing the limbal and corneal epithelial cell microenvironments, was reported in two clinical studies of eyes with infectious keratitis.<sup>21,22</sup> The AM provides an effective barrier against PMN leukocytes from the tear film. When the AM was used as a patch in a rabbit model of corneal ulcers induced by alkali burn, inflammatory cells adhered primarily to the stromal side of the AM, and corneal opacity was minimized.<sup>27</sup>

In conclusion, the results of this study showed that AM transplantation, when used as an adjunct to antibiotic therapy, has a beneficial effect on the healing process of corneas infected by bacterial keratitis. AM transplantation evidently reduces corneal neovascularization and opacity, thereby improving the clinical outcome.

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