

# Permeability of Human Amniotic Membrane to Ofloxacin In Vitro

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**PURPOSE.** The aim of this study was to develop a model to investigate the permeability of the amniotic membrane (AM) to ofloxacin eye drops, a widely used topical antibiotic in ocular surface disease after AM transplantation.

**METHODS.** AM pieces on cellulose acetate filter membranes were mounted in a vertical Franz-diffusion cell system equipped with an autosampler. In vitro release of 300 mg of 3% commercially available ofloxacin ophthalmic solution was determined by quantitative absorbance measurement carried out with a UV spectrophotometer (wavelength, 287 nm). Freshly prepared and cryopreserved AMs were compared. Filter membranes without AM served as positive controls.

**RESULTS.** Ofloxacin was detectable in the acceptor phase 1 minute after instillation, and a gradual increase of concentration could be detected in a period of 90 minutes in all groups. At 30 minutes  $3.35\% \pm 2.23\%$  of ofloxacin penetrated the freshly prepared AM,  $4.35\% \pm 1.8\%$  through cryopreserved AM compared with  $17.52\% \pm 3.91\%$  filter membrane alone. At 90 minutes, penetration rates of ofloxacin were  $5.04\% \pm 1.11\%$ ,  $5.26\% \pm 3.21\%$ , and  $27.91\% \pm 3.05\%$ , respectively. Difference ( $P > 0.05$ ; *t*-test) was not significant between freshly prepared and cryopreserved AMs. Compared with control, both membranes showed significant differences ( $P < 0.05$ ; *t*-test) at all time points.

**CONCLUSIONS.** The in vitro model of the Franz-diffusion cell system was found to be applicable for drug permeability studies of human amniotic membranes to water-based solutions. The filter membrane and AM were permeable to a water-based solution of ofloxacin. Significant barrier function of the AM could be measured in ofloxacin permeability. Cryopreservation did not influence the permeability of the AM. (*Invest Ophthalmol Vis Sci.* 2010;51:1024-1027) DOI:10.1167/iovs.09-4254

Amniotic membrane (AM) is the innermost avascular layer of the placenta consisting of an epithelium, a basement membrane, and a stromal layer.<sup>1</sup> AM transplantation has become frequently used in ocular surface surgery and has been found to be beneficial in a number of ocular surface diseases including persisting epithelial defects, perforating or nonper-

forating corneal ulcers,<sup>2-4</sup> alkali burns,<sup>5</sup> pterygium,<sup>6</sup> and band keratopathy<sup>7</sup> and after excimer laser keratectomy.<sup>8</sup>

In all cases topical antibiotic and anti-inflammatory treatment is essential after amniotic membrane transplantation. Ocular penetration of topically administered medicaments are known; for example, the concentration of ofloxacin was measured in corneal tissues<sup>9</sup> and aqueous humor.<sup>10-12</sup>

Amniotic membrane, especially in cases of multilayer transplantation, creates a barrier for topically administered drugs to reach the corneal tissues. The pharmacokinetic impact of amniotic membrane, however, has not exactly been explored yet. The aim of our study was to develop a model to investigate the permeability of amniotic membrane with eye drops already routinely used in clinical ophthalmologic practice or under development. To test the pharmacokinetic capability of our model, our objective was to examine the transamniotic pharmacokinetics of ofloxacin, a frequently used broad-spectrum topical antibiotic in ocular surface disease.<sup>13</sup>

## METHODS

### Amniotic Membrane Preparation

The research was approved by the Institutional Human Experimentation Committee and adhered to the tenets of the Declaration of Helsinki. Amniotic membrane obtained by elective cesarean section was separated from the chorion as soon as possible, 1 hour after delivery at the latest, by blunt dissection and was rinsed with PBS (pH 7.24). Amniotic membrane pieces of 25 mm in diameter (with the epithelial side up) were placed on cellulose acetate membrane filters of the same size (Porafil; Macherey-Nagel GmbH & Co. KG, Düren, Germany) with pore diameters of 0.45  $\mu\text{m}$ . Two groups were created according to the preservation technique of the amniotic membrane, as follows: with fresh amniotic membranes (no preservative), amniotic membranes with membrane filters were used within 6 hours of preparation; with cryopreserved amniotic membranes, AM pieces on filter membranes were frozen in PBS (pH 7.24) at  $-20^{\circ}\text{C}$ , and neither antibiotic nor preservative was added to the medium.

### In Vitro Drug Permeability Studies

In vitro permeability studies were performed with a vertical Franz-diffusion cell (Fig. 1) system (Microette Topical and Transdermal Diffusion Cell System; Hanson Research, Chatsworth, CA) containing six cells.<sup>14-16</sup> In both groups AM on membrane filters were mounted to Franz diffusion cells. The donor phase contained 300 mg of 3% ofloxacin eye drops (FloXal; Dr. Mann-Pharma, Berlin, Germany), which was placed on the amniotic membrane. The effective diffusion surface was 1.767  $\text{cm}^2$ . PBS was used as an acceptor phase. Rotation of the stir-bar was set to 450 rpm. Experiments were performed at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  water bath. Position and condition of AM was continuously checked.

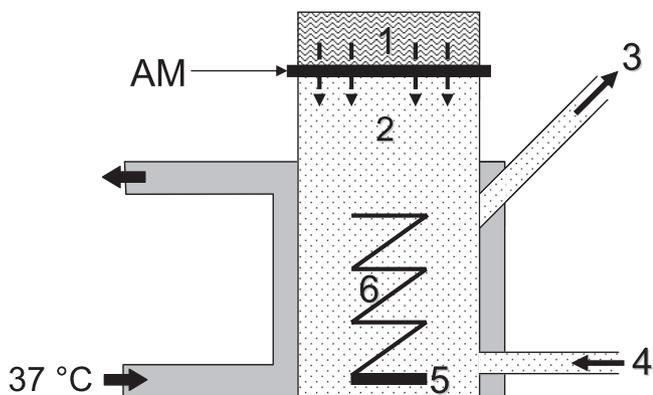
Samples of 0.8 mL were taken from the acceptor phase by the autosampler (Microette Autosampling System; Hanson Research) after 1, 10, 15, 20, 25, 30, 40, 50, 60, and 90 minutes and were replaced

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**FIGURE 1.** Schematic drawing of amniotic membrane mounted in the Franz cell. The donor compartment (1) above contains ofloxacin. The compartment below is the acceptor phase (2), from which samples are taken through the sampling port (3), to the acceptor phase replacing port (4). The acceptor compartment is surrounded with a water jacket kept at 37°C. At the bottom of the acceptor phase, a stir-bar (5) and a helix mixer (6) are rotated magnetically.

with fresh receiving medium. From each group, 10 Franz cells were set.

In vitro release of samples containing 300 mg of 3% ofloxacin eye drops was determined by quantitative absorbance measurement carried out with a UV spectrophotometer (Thermospectronic UV spectrophotometer, v 4.55; Unicam Helios, Cambridge, UK) at a wavelength of  $\lambda = 287$  nm. Before quantitative ofloxacin UV-spectrophotometry calibration was performed, ofloxacin solution was prepared using PBS buffer solution (pH 7.24). This solution was scanned over a range of 200 nm to 500 nm in the spectrum mode. On the absorption diagram (Fig. 2), the highest peak from spectra at wavelength 287 nm was selected for the measurements of ofloxacin. For the quantitative measurements of ofloxacin, different concentrations in the range of 1.0 to 16.0  $\mu\text{g}/\text{mL}$  solutions were prepared with PBS buffer solution. The UV-spectrophotometric calibration curve was constructed by plotting the absorbance values at 287 nm versus concentration of the solution. The calibration curve was found to be linear with the correlation coefficient ( $r$ ) 0.9999; the regression equation was  $y = 0.07978x$ .

### Statistical Analysis

Drug permeabilities of freshly prepared and cryopreserved amniotic membranes were compared with each other and with controls. Filter membranes without amniotic membrane served as positive controls. Negative control meant adding 300 mg PBS without ofloxacin in the donor compartment. Independent sample  $t$ -tests were performed applying spreadsheet software (Excel; Microsoft, Redmond, WA). Differences were regarded as significant, with  $P \leq 0.05$ .

## RESULTS

### Model

Vertical Franz-diffusion cells provided sufficient fixation of the amniotic membranes; significant displacement of the amniotic membrane was observed in two cases, and no filter membrane decentration was found (these cases were excluded). Amniotic membranes were intact at the beginning and at the end of the experiment.

### Drug Release

Filter membrane alone created a barrier for the penetration of ofloxacin. Ofloxacin was detectable in the acceptor phase as early as 1 minute after instillation, when  $5.98\% \pm 2.23\%$  of the

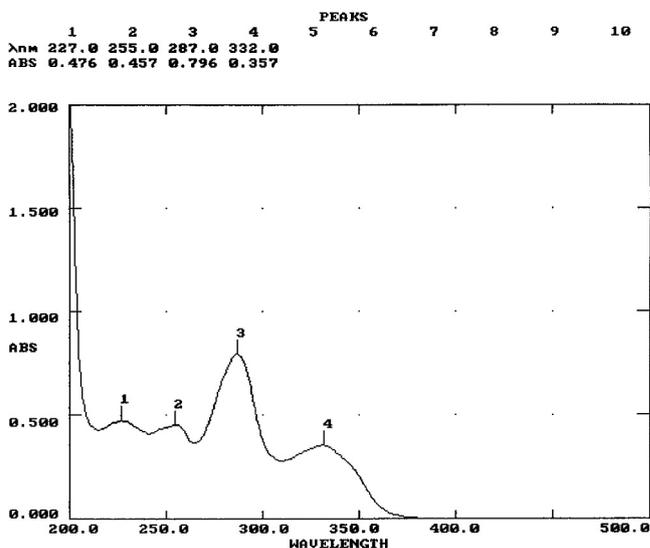
original concentration was measured. A gradual increase of concentration could be observed within 90 minutes, when  $27.91\% \pm 3.05\%$  ofloxacin concentration could be detected in the acceptor phase. Table 1 summarizes the cumulative amount of the penetrated ofloxacin, and Figure 3 depicts the percentages (mean  $\pm$  SD) of penetrated ofloxacin in the acceptor phase in groups 1 and 2 and in positive controls (in negative controls, no ofloxacin could be detected.)

In fresh and cryopreserved amniotic membranes, the percentages of penetrated ofloxacin were lower than in positive control. Compared with control, both membranes showed significant differences ( $P < 0.05$ ,  $t$ -test; Table 1) at all time points. At 30 minutes,  $3.35\% \pm 2.23\%$  of ofloxacin penetrated freshly prepared amniotic membrane, and  $4.35\% \pm 1.8\%$  penetrated cryopreserved amniotic membrane compared with  $17.52\% \pm 3.91\%$  penetrance in the filter membrane alone. At 90 minutes, the penetration rates of ofloxacin were  $5.04\% \pm 1.11\%$ ,  $5.26\% \pm 3.21\%$ , and  $27.91\% \pm 3.05\%$ , respectively. The difference ( $P > 0.05$ ,  $t$ -test) was not significant between freshly prepared and cryopreserved amniotic membranes at any time point.

## DISCUSSION

Ofloxacin is one of the most commonly used commercial preparations of topical fluoroquinolones.<sup>13</sup> In ocular surface disease, topical broad-spectrum antibiotic administration is essential, and the use of amniotic membrane with appropriate topical antibiotic treatment may induce faster wound healing and less corneal scarring.<sup>2</sup> Transcorneal penetration of ofloxacin was investigated in healthy and pathologic corneas as well.

According to the in vivo examinations of Beck et al.,<sup>17</sup> who examined healthy corneal permeability, ofloxacin achieved in aqueous humor the minimum inhibitory concentration ( $\text{MIC}_{90}$ ) values of the frequently occurring Gram-positive and Gram-negative bacteria. Beck et al.<sup>17</sup> examined aqueous samples of 224 patients with healthy corneas undergoing cataract surgery and found good transcorneal penetration after multiple modes of application. Their results were confirmed by Cekic et al.,<sup>18</sup> who also examined the penetration of fluoroquinolones through healthy corneas. It has been shown that the route of ofloxacin administration can influence aqueous concentra-



**FIGURE 2.** Absorption diagram of ofloxacin. The highest peak from spectra at a wavelength 287 nm was selected for quantitative measurement of ofloxacin concentration.

TABLE 1. Summary of Penetrated Cumulative Amounts ( $\mu\text{g}$ ) of Ofloxacin via  $1 \text{ cm}^2$  Amniotic Membrane

Time after Administration (min)	Control (filter)		Fresh AM		Cryopreserved AM		P (fresh AM filter)	P (cryopreserved AM filter)	P (fresh cryopreserved AM)
	Mean	SD	Mean	SD	Mean	SD			
1	30.5	11.3	4.8	6.4	5.6	2.2	0.0105	0.0331	0.9953
10	58.7	10.8	9.0	11.8	13.0	4.7	0.0051	0.0000	0.5795
15	66.5	14.7	10.6	12.4	15.4	6.5	0.0081	0.0001	0.5609
20	74.7	14.8	16.7	19.8	16.9	7.2	0.0305	0.0003	0.9319
25	81.9	15.4	14.5	12.4	18.3	8.0	0.0153	0.0006	0.7005
30	89.0	19.4	17.1	11.4	22.2	9.2	0.0188	0.0014	0.6680
40	97.0	19.0	18.8	9.8	22.0	10.2	0.0267	0.0034	0.8347
50	102.8	16.6	21.1	6.9	23.3	12.3	0.0488	0.0169	0.9486
60	109.0	15.4	22.8	5.1	25.0	13.9	0.0451	0.0429	0.8860
90	142.4	14.5	25.7	5.7	26.8	16.4	0.0311	0.0245	0.7425

Independent sample *t*-test results demonstrate significant differences, where  $P < 0.05$ .

tions.<sup>19</sup> Several modes of application were compared. Some patients received eye drops three times at 2-hour intervals on the day before surgery and three drops at 1-hour intervals on the day of surgery. Other patients received nine drops at 15-minute intervals on the day of surgery only. In all application modes, ofloxacin was detectable in the anterior chamber.

Besides normal corneas, abnormal corneas were also evaluated in a multicenter randomized study by Healy et al.,<sup>10</sup> when 0.3% ofloxacin ophthalmic solution was administered twice (15 and 10 minutes) before penetrating keratoplasty. In corneal stromal tissues and aqueous humor samples, ofloxacin could be detected by high-performance liquid chromatography (HPLC). Transcorneal penetration was examined in corneas with different noninflammatory abnormalities. It was demonstrated that ofloxacin penetration also offers a sufficient concentration in the anterior chamber in healthy and pathologic corneas as well.

Ofloxacin quantitative concentration analysis can be performed by UV-spectrophotometry and HPLC. Both methods were found equally accurate in the case of ofloxacin.<sup>20</sup> There was no significant difference between the two techniques. UV-spectrophotometry is also an accepted method, specifically

in quantitative ofloxacin measurements by Srividya et al.<sup>21</sup> and Fegade et al.<sup>22</sup>

UV-spectrophotometry is a less expensive technique than HPLC and is proven to be same exact.<sup>23</sup> Other authors have found slightly different values of absorption maximum: Srividya et al.<sup>21</sup> at 290 nm, Fegade et al.<sup>22</sup> at 300 nm, and Chavanpatil et al.<sup>24</sup> at 291 nm. The absorption curves can change with the pH and with the instrument used. By automated sampling, continuous measurements could be performed. Franz cells seem to be an applicable model for the examination of amniotic membrane permeability.

Amniotic membrane permeability was originally examined by Kovács et al.<sup>25</sup> to investigate fetomaternal transport. Later, when the amniotic membrane was introduced in ocular surface surgery, the impact of amniotic membrane on membrane transport gained a new perspective of interest. Immunohistochemical and electron microscopic examination of corneas after amniotic membrane transplantation surgery demonstrated that amniotic tissues can integrate the corneal tissues.<sup>26,27</sup> It can be supposed that intracorneal amniotic membrane integration can affect the transcorneal pharmacokinetics of topical drugs.

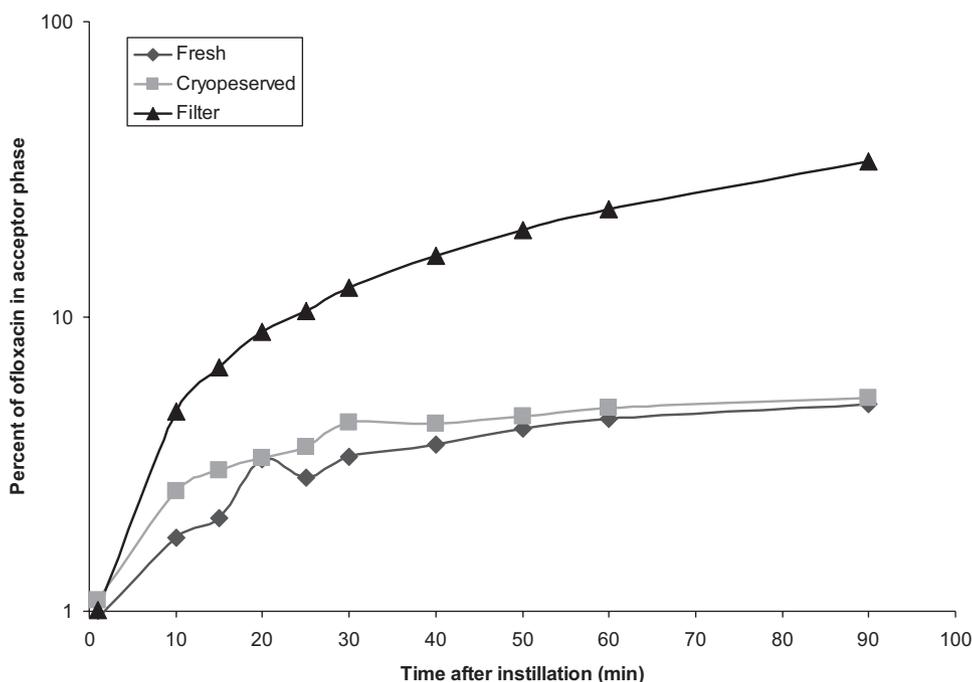


FIGURE 3. Diagram showing the time course of ofloxacin penetrated through amniotic membrane into the acceptor phase when 3% topical ofloxacin eye drops were used. One hundred percent indicates that the total amount was filled into the donor phase of the Franz cell.

Kim et al.<sup>28</sup> evaluated the effect of amniotic membrane on the concentration of ofloxacin in the cornea, aqueous humor, and tears in vivo on the rabbit cornea. They concluded that amniotic membrane transplantation interferes with the ocular penetration of topical ofloxacin in normal rabbit corneas but enhances ofloxacin penetration in corneas with epithelial defects after the administration of ofloxacin four times every 15 minutes. Diamond et al.<sup>29</sup> reported, after four drops of ofloxacin (and three other types of fluoroquinolones) at 2-minute intervals in 12 patients undergoing corneal transplantation, that the corneal concentration of ofloxacin from resected cornea was significantly higher than that of ciprofloxacin and norfloxacin.

In vivo several factors can have impact on transamniotic drug penetration. O'Brien et al.<sup>30</sup> reported that inflammation corneal deepithelialization enhances the ocular penetration of topical antibiotics. Cryopreservation may also affect the amniotic epithelium structure and thus drug permeability. We found, however, that cryopreservation does not have any impact on the permeability of amniotic membranes in vitro.

Healy et al.<sup>10</sup> and Robert and Adenis<sup>31</sup> reported that transcorneal penetration of most drugs, including the fluoroquinolones, occurs primarily by passive diffusion and is correlated in a positive manner with the drug's aqueous solubility and degree of lipophilicity. The thickness variability of fresh or cryopreserved amniotic membranes may explain the SD values in Table 1.

We conclude that the Franz-diffusion cell system provides an applicable model for transamniotic drug release studies for water-based solutions. The barrier effect of amniotic membrane on ofloxacin penetration could be demonstrated and measured by the model. It has been shown that the amniotic membrane reduces ofloxacin penetration and that cryopreservation does not play a significant role in the permeability of amniotic membrane.

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