Ultrasound-Enhanced Transcorneal Drug Delivery

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Purpose: Ultrasound has been shown to enhance, by up to 10 times, the corneal permeability to different compounds such as β-blockers and fluorescein. Here, we report on our investigation of the mechanisms of ultrasound-enhanced drug delivery through the cornea using light and electron microscopy.

Methods: Enhancement of permeability for a hydrophilic compound, sodium fluorescein, in rabbit cornea in vitro was achieved using ultrasound at a frequency of 880 kHz and intensities of 0.19–0.56 W/cm^2 with an exposure duration of 5 minutes. Light and electron microscopy (transmission and scanning) were used to observe ultrasound-induced structural changes in the cornea.

Results: The permeability increased by 2.1, 2.5, and 4.2 times when ultrasound was applied at 0.19, 0.34, and 0.56 W/cm^2, respectively ($P < 0.05$). The surface cells of corneal epithelium exposed to ultrasound appeared swollen and lighter in color (indications of membrane rupture) as compared with the control cells. Some of the surface epithelial cells were absent. The cells in the inner layers of the epithelium were occasionally lighter in color. Also, holes 3–10 μm in diameter were observed on the epithelial surface. No structural changes were observed in the stroma.

Conclusion: Ultrasound enhancement of drug delivery through the cornea appears to result from minor structural alterations in the epithelium. Careful investigation of the recovery of cornea structure and barrier function after the ultrasound application, in vivo, is needed.

Key Words: ultrasound, drug delivery, cornea, sonophoresis, phonophoresis

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The cornea is the main route for penetration of ophthalmic drugs, even though it is an inefficient pathway because of its low permeability.[1,2] Short residence time of the drugs on the corneal surface and small surface area contribute to poor penetration through the cornea.[2,3] Fewer than 10% of all topically applied drugs can penetrate through the cornea into the anterior chamber, with virtually no penetration into the back of the eye.[1,4] Most topically applied drugs that penetrate the conjunctiva and sclera are carried away by the systemic circulation before diffusing into the inner structures of the eye.[5] Drug delivery by systemic administration is also ineffective because of blood–aqueous and blood–retina barriers.[6] In some cases, intraocular injection of drugs is used clinically, but this method is invasive and not very efficient.[7] We are investigating the use of ultrasound to increase the permeability of the corneas.

ULTRASOUND ENHANCEMENT OF DRUG DELIVERY

It has been shown that the barrier properties of the skin can be modified using low-frequency ultrasound (20 kHz) to enhance the efficiency of transdermal drug delivery.[8] Improvement of as much as 1000-fold was achieved in the delivery of hydrophilic and/or large compounds without long-term damage to the barrier properties of the skin.[9] These results motivated us to investigate the use of ultrasound to enhance drug delivery through the cornea. However, the skin barrier layer, stratum corneum, is a dead structure, and ultrasound effects on the barrier function of live corneal epithelium may be significantly different. In preliminary studies, we found that 20-kHz ultrasound applied at intensity of 14 W/cm^2 resulted in a 4-fold increase in the corneal permeability for β-blocker drugs (atenolol, carteolol, timolol, and betaxolol) in a rabbit model in vitro, with significant disorganization of the epithelium.[10] The cells from the outer layers of the epithelium were absent, and the cells from the inner layers appeared swollen and ruptured. Thus, the epithelial healing was expected to be slow (within 5 days) with the possibility of complications such as corneal haze and infection.[11]

Ultrasound application (phonophoresis) at medium frequencies (470–880 kHz) and intensities of 0.2–0.3 W/cm^2 has been used in the USSR and Russia for transcorneal drug delivery, to improve treatment of corneal inflammation, wounds, and retinal dystrophy.[12–17] However, the Russian research on ocular phonophoresis is widely unknown in the United States, Europe, and Japan. Ultrasound application was reported to produce up to a 10-fold increase in the corneal permeability to

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hydrophilic compounds.[18–20] The mechanism of ultrasound action was thought to be the erosion of corneal epithelium, which was observed with an ophthalmoscope to heal within 6 hours.[19]

The goal of our study was to investigate the mechanisms of ultrasound enhancement of drug delivery through the cornea in a rabbit model, in vitro, using light and electron microscopy (transmission and scanning). Some histologic observations of ultrasound-induced changes in the cornea have been reported previously in the literature. For example, Saito et al[21] performed histology of the corneal endothelium after phacoemulsification, and Rutzen et al[22] analyzed lesions produced in the cornea after application of high-intensity focused ultrasound. Nuritdinov[19] observed changes in the cornea after ocular phonophoresis only with an ophthalmoscope at low magnification, with no light or electron microscopy observations. Our current study has been, to the best of our knowledge, the first histologic investigation of the corneal changes after ultrasound application at parameters used for ocular phonophoresis.

MATERIALS AND METHODS

All animals (New Zealand white adult rabbits) were cared for in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research. The rabbit eyes were enucleated within 15 minutes of euthanasia. The eyes were visually examined, and those with abrasions such as scratches on the cornea were discarded. The corneas were dissected, placed in a storage medium [Dulbecco phosphate-buffered saline (DPBS, D6650, Sigma), pH 7.3, a balanced salt solution that contains inorganic ions and glucose, essential for normal cell metabolism][23] and kept at 4°C until the experiment (randomized between 30 minutes and 4 hours after euthanasia).

The cornea was placed between the donor and receiver compartment of a vertical glass diffusion cell (PermeGear, Bethlehem, PA) such that the epithelial layer was facing the donor compartment. The natural shape of the cornea was not preserved because of flattening in the diffusion cell. The orifice diameter of the diffusion cell was 1.1 cm. The donor compartment was filled with 3 mL of sodium fluorescein solution (an orange dye), and the receiver compartment was filled with 12 mL of DPBS. Sodium fluorescein (Sigma Chemicals, St Louis, MO) is a hydrophilic dye that can be used as a model for small hydrophilic drugs.[1] The sodium fluorescein solution of 0.25% was made in DPBS and kept in a dark place until the experiment. The receiver compartment was stirred at 700 rpm using a magnetic stir bar.

Ultrasound Application

The ultrasound instrument used for transcorneal drug delivery was commercially available in Russia (UZT-1.04, Ultramed, Moscow, Russia). The instrument was characterized using radiation force and needle hydrophone methods[24] for determination of the ultrasound intensity. The transducer (diameter of 1.1 cm) was placed in the donor compartment at a distance of 0.3 cm from the cornea. Ultrasound was applied to the cornea at a frequency of 880 kHz and intensity (I) of 0.19, 0.34, or 0.56 W/cm^2 for 5 minutes in continuous mode. The intensities corresponded to ultrasound pressures of 0.08–0.13 MPa and mechanical indices[25] of 0.08–0.14 at the cornea surface. Ultrasound application started within 2 minutes after the dye solution was placed in the donor compartment. The cornea was exposed to the dye solution for 60 minutes in both control and ultrasound-treatment experiments. The treatment group had an initial ultrasound application of 5 minutes followed by 55 minutes of exposure to the dye solution. A 3-mL sample was then taken from the receiver compartment through the sampling port for spectrophotometric analysis. The number of experiments (n) at different levels of ultrasound intensity was 6–7. The number of control experiments (with no ultrasound application) was 6.

The temperature of the donor compartment solution was measured using a temperature probe (type-K, 80TK, Fluke, Everett, WA) connected to a scopemeter (105 B, Fluke, Everett, WA) via a thermocouple module. The donor compartment solution was at room temperature before the experiment. The thermocouple was continuously present in the donor compartment during the last 30 seconds of ultrasound exposure to determine maximal temperature increase. The temperature increased by up to 9°C as a result of ultrasound application. After the ultrasound exposure, the diffusion cell was placed in the water bath with an immersion circulator (Model 1112, VWR, West Chester, PA), and the cornea was kept at 34 ± 1°C. The temperature behavior of the ultrasound treatment experiments was mimicked in the control experiments by adjusting temperature of the water bath in which the diffusion cell was submersed. In the control experiments, the temperature changed from room temperature to 34°C over the initial period of 5 minutes and was kept at 34 ± 1°C for additional 55 minutes.

Spectrophotometry

The absorbance of sodium fluorescein in the sample was measured with a UV-visible spectrophotometer (UV-1601, Shimadzu, Columbia, MD) at 490 nm. Standard calibration curves were used to calculate the dye concentration in the receiver compartment from the measured absorbance.

The corneal permeability, P, was calculated using the Fick law for diffusion[26]:

$$P = \frac{V\Delta C_r}{A\Delta t C_d} \quad (1)$$

where V is the volume of the receiver compartment (cm^3), A is the cross-sectional area of the cornea (cm^2), ΔC_r is the change in the dye concentration in the receiver compartment (mg/mL), C_d is the dye concentration in the donor compartment

(mg/mL), and Δt is the duration of the cornea exposure to sodium fluorescein (seconds). This expression is valid when the cumulative amount of the compound transferred to the receiver compartment is less than 5% of the donor amount (it was less than 0.04% in our experiments). Equation 1, for the corneal permeability after 60 minutes of dye exposure, could be written as

$$P = \frac{V(C_{r,t} - C^{0}_{r,0})}{60A(t - t_L)C_d} = \frac{VC_{r,t}}{60A(t - t_L)C_d} \qquad (2)$$

where P is given in centimeters per second (cm/s), t is 60 minutes, A is approximately 1 cm^2, $C_{r,t}$ is the dye concentration in the receiver compartment after the time t, and t_L is the cornea lag time (minutes). In one set of experiments, the receiver compartment was sampled at different time points (5, 10, 15, 20, 30, and 60 minutes) after the cornea was exposed to the sodium fluorescein solution to determine the lag time. The lag time of 8.5 minutes based on our measurements in the control experiments agrees fairly well with the previously reported value of 9.9 minutes.[27]

Light and Transmission Electron Microscopy

The corneas were fixed in Karnovsky (glutaraldehyde-paraformaldehyde) half-strength solution,[28] postfixed in osmium tetroxide, stained with uranyl acetate, passed through graded alcohol series, and embedded in Spurr Epoxy (Ted Pella, Redding, CA). Semithin sections (1 μm) of the cornea were poststained with Richard stain (methylene blue/azure II) and observed with light microscope. The number (n) of control corneas observed under light microscope was 2. The number of observed ultrasound-treated corneas was 3, 1, and 2 at 0.19, 0.34, and 0.56 W/cm^2, respectively. The overlapping series of images of light micrographs were taken at ×40 magnification and used to count the damaged epithelial cells. Thin sections (~100 nm) were poststained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Phillips CM100). One control and 2 ultrasound-treated corneas were observed under TEM.

Scanning Electron Microscopy

The corneas were fixed in Karnovsky half-strength solution, postfixed in osmium tetroxide, and passed through graded alcohol series. The samples were then exposed to hexamethyldisilazine for 10 minutes and air-dried overnight. After drying, the samples were placed on SEM specimen stubs and sputter coated with Au/Pd. The specimens were imaged using a JEOL 840A scanning electron microscope operated at 10 kV. Approximately one third of the cornea area was prepared for the SEM observations. No quantification of the damaged cells per area was performed. Three control and 9 treated corneas were observed.

RESULTS

The exposure of rabbit cornea to ultrasound at intensities of 0.19, 0.34, and 0.56 W/cm^2 resulted in 2.1-, 2.5-, and 4.2-fold increase in the permeability of sodium fluorescein, respectively (Fig. 1). A statistically significant difference ($P < 0.05$) was obtained between the corneal permeability of the control and ultrasound-treatment groups at all applied intensities.

Figure 2 shows light micrographs of the control (Fig. 2A) and ultrasound-treated epithelium and stroma (Fig. 2B). The surface epithelial cells exposed to ultrasound were lighter in color and swollen (Fig. 2B, black arrow) as compared with the control cells (Fig. 2A, arrow). The numbers of damaged epithelial cells were 2 and 4 per millimeter of the 2 observed control corneas. In the observed ultrasound-treated corneas, the number of damaged epithelial cells per millimeter of cornea was 0, 22, and 52 at 0.19 W/cm^2 (n = 3), 11 at 0.34 W/cm^2 (n = 1), and 41 and 50 at 0.56 W/cm^2 (n = 2). No structural changes were observed in the stroma (Fig. 2B, white arrow). Endothelium was absent in both control and ultrasound-treated samples.

TEM micrographs of the control (Fig. 3A) and treated epithelium (Fig. 3B) also show that, after treatment, the surface epithelial cells were lighter in color and swollen (Fig. 3B, black arrow) as compared with the control cells (Fig. 3A, arrow). In addition, the cells were occasionally absent from the surface epithelial layer exposed to ultrasound (Fig. 3B, asterisk). Some of the cells in the inner layers of treated epithelium were also lighter in color (Fig. 3B, white arrow) as compared with the control cells.

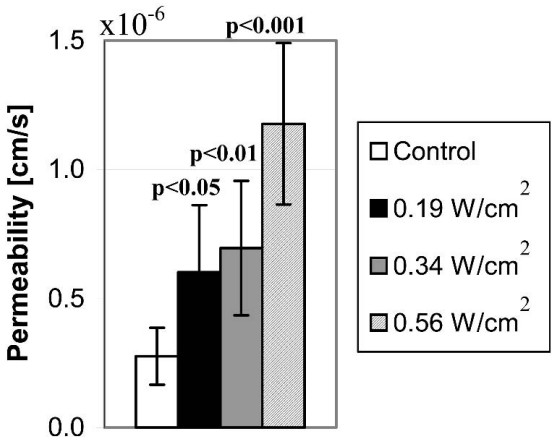

FIGURE 1. Permeability of control and ultrasound-treated corneas (ultrasound intensity of 0.19, 0.34, and 0.56 W/cm^2, ultrasound exposure of 5 minutes). The permeability of ultrasound-treated corneas was up to 4.2 times higher than that of control corneas. Number of experiments per experimental condition (n) was 6–7. The corneal permeability is given in the units of centimeters per second (cm/s). The data are given as mean ± SD. The statistical significance ($P < 0.05$) was calculated for each intensity as compared with the control group.

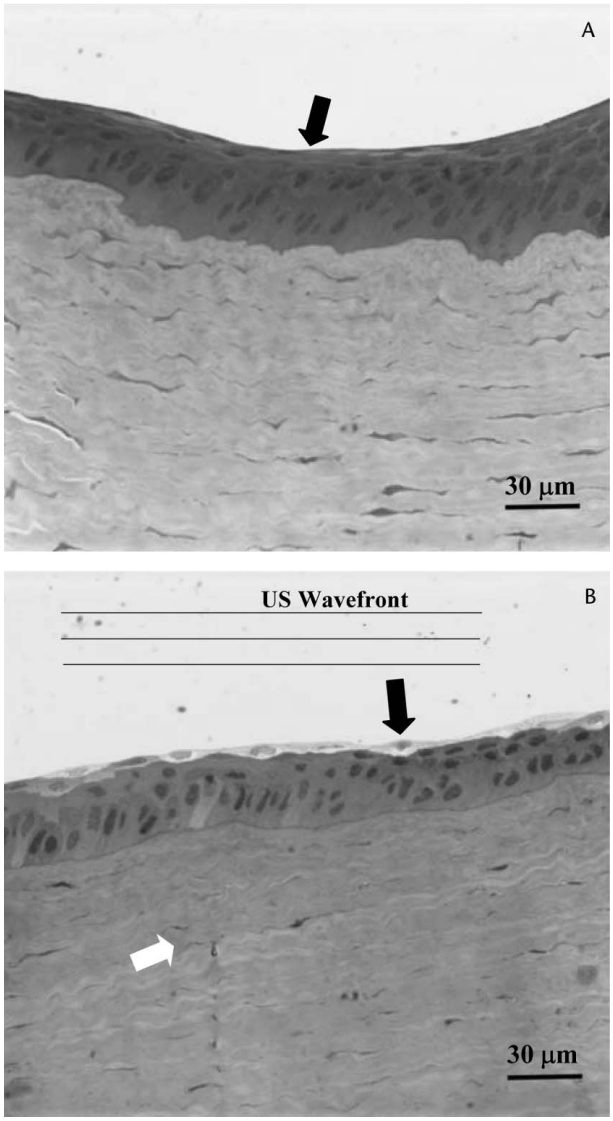

FIGURE 2. Light microscopy observations. A, Control cornea. The surface epithelial cells appear intact (arrow). B, Application of 880-kHz ultrasound (I = 0.34 W/cm^2) produced structural changes in the surface epithelial cells (black arrow). No changes in the stroma (white arrow) were observed. Parallel lines show the propagation of ultrasound (US) wavefront.

Figure 4 shows SEM micrographs of the control (Fig. 4A) and ultrasound-treated epithelial surface (Fig. 4B–D). The control epithelium (Fig. 4A) appeared normal, consisting of both light (white arrow) and dark (black arrow) polygonal flat cells, as reported previously.[29] Figure 4B shows the absence of some of the surface epithelial cells in the cornea exposed to ultrasound (arrow). The holes 3–10 μm in diameter (Fig. 4C, arrow) and sloughing of the surface cells (Fig. 4D, arrow) were also observed in the ultrasound-treated epithelium. The holes in the epithelium were observed under SEM in 8 of 9 ultrasound-treated corneas and in none of the 3 control corneas.

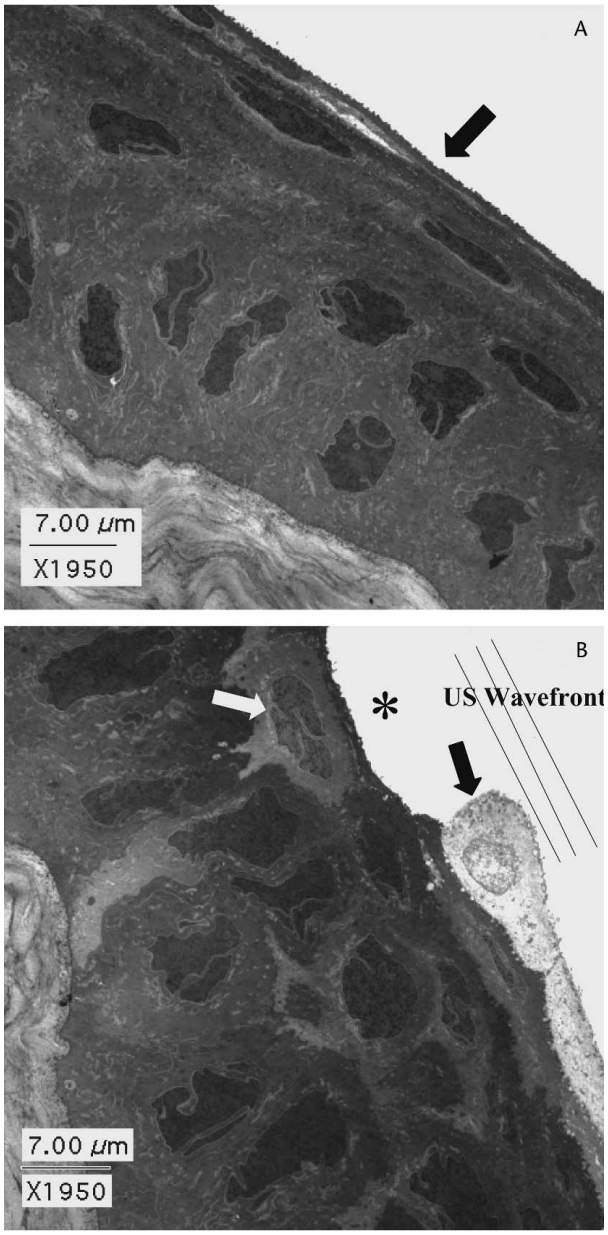

FIGURE 3. TEM observations. A, Control epithelium appears intact (arrow). B, Structural changes in the surface layer of epithelium produced by 880-kHz ultrasound (I = 0.56 W/cm^2). The surface epithelial cells were lighter in color (black arrow) and occasionally absent (asterisk). Some of the cells in the inner layers of epithelium also appeared lighter in color (white arrow). Parallel lines show the propagation of US wavefront.

DISCUSSION

In recent years, ultrasound has been investigated as a tool for controlled delivery of therapeutic compounds such as thrombolytic agents, anticancer drugs, and genes.[30–32] Ultrasound-enhanced transdermal drug delivery has also been heavily investigated and showed an increase in the skin permeabil-

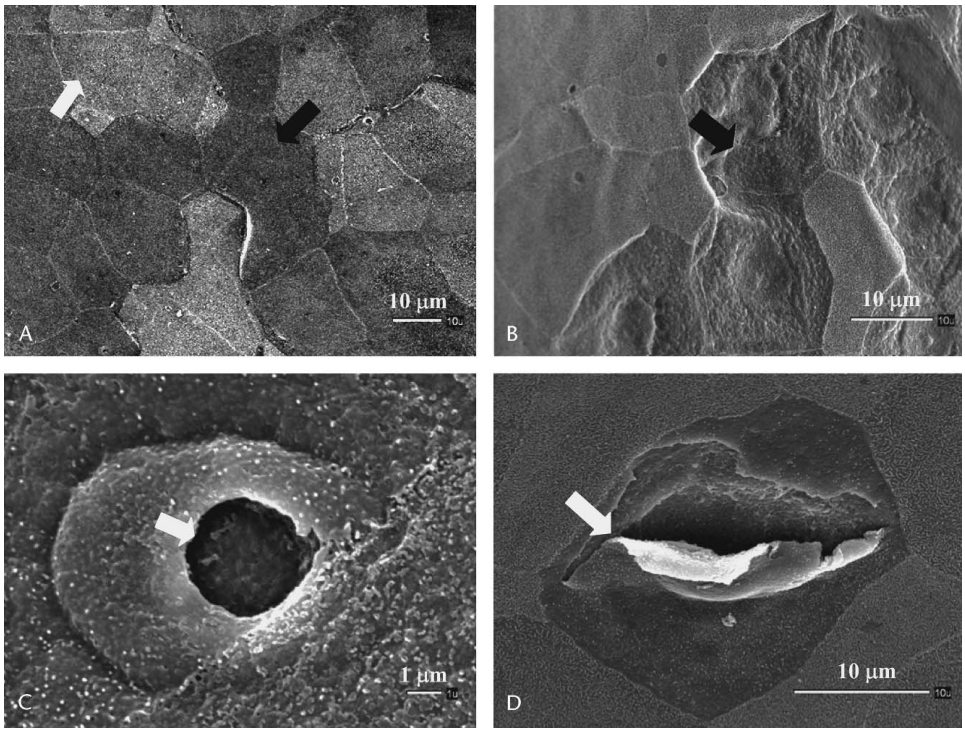

FIGURE 4. SEM observations. A, Control epithelium appeared normal with both light (white arrow) and dark cells (black arrow) present. B, Removal of the surface epithelial cells as a result of ultrasound application (I = 0.19 W/cm^2) (arrow). C, Hole in the epithelium (arrow, diameter 3 μm) after ultrasound exposure (I = 0.34 W/cm^2). D, Sloughing of the epithelial cell (arrow) in the ultrasound-treated cornea (I = 0.19 W/cm^2).

ity to various compounds of different hydrophilicities and molecular weights by 3 to 5000 times.[8] The mechanisms of the ultrasound enhancement of drug delivery are thought to be cavitation activity and streaming.[33,34] Cavitation, defined as the formation and activity of bubbles,[35] is thought to be the primary mechanism of drug delivery and has been shown to increase the penetration of drugs into a thrombus[33] and to enable drugs, DNA, and macromolecules to enter cells.[31,36,37] Cavitation is believed to cause ruptures in the cell membranes as a result of shear stresses and microstreaming around bubbles oscillating in an ultrasound field (stable cavitation).[38] In addition, bubble collapse (inertial cavitation) can result in high-speed liquid jets in a small region (within 1 μm^3), capable of causing pits in the surface of biologic membranes.[35] A recent study by Tang et al[39] showed that the dominant mechanism for skin permeability enhancement was the bubble collapse against the skin surface exposed to the drug solution. In addition, cavitation activity was shown to produce pits in the external epithelia of fish skin (a stratified, metabolically active, squamous epithelium), increasing its permeability, when 1-MHz ultrasound was applied for 30–90 seconds at an intensity of 1 W/cm^2.[40]

In our experiments, ultrasound application produced structural changes in the surface layer of the corneal epithelium consistent with cavitation-induced structural alterations previously observed in the fish epithelium.[40] Some of the cells were completely removed from the surface layer of the cornea, and sloughing of the cells was also observed. The remaining surface cells appeared swollen and lighter in color, which indicated ruptures in the cell membranes and cell necrosis.[40] The cells in the inner layers of epithelium occasionally appeared lighter in color and therefore may have been necrotic. The cavitation activity (eg, bubble collapse and shear stresses) in the dye solution would first impact the surface epithelial layer, and the inner layer may have been affected only after the surface layer was ruptured. The absence of endothelium in both control and ultrasound-treated samples may be related to sample processing and not the result of an ultrasound effect on the endothelium.

The two surface layers of the epithelium, with tight junctions,[41] represent the barrier for hydrophilic compounds such as sodium fluorescein.[42] Therefore, any damage of the first surface layer is expected to significantly enhance penetration of sodium fluorescein through the cornea. Mitragotri et al[43] proposed that the skin permeability increased through ultrasound-induced defects in lipid bilayer structures of stratum corneum. In our experiments, the membranes of cells in the first surface epithelial layer appeared to be affected by ultra-

sound, resulting in the enhancement of the dye penetration through this barrier layer.[44] In addition, ultrasound exposure may have opened tight junctions in the epithelium. The reversible opening of tight junctions was observed previously in the endothelial cell layer of blood–brain barrier exposed to ultrasound.[45] Finally, the holes present in the surface epithelium of ultrasound-treated corneas may have provided additional pathways for the dye delivery into the cornea. The cavitation-induced erosion of the epithelium has been reported previously to be responsible for the enhancement in corneal permeability after application of 880-kHz ultrasound.[19] A 5-fold increase in the permeability of fluorescein was achieved after the application of 880-kHz ultrasound at 0.2–0.3 W/cm^2 for 5 minutes to the rabbit cornea in vivo.[19] The permeability increases in our experiments were on the same order of magnitude as this previously reported value.

Ultrasound-induced streaming of the drug solution is thought to provide convective transport of the drugs across the skin[34,43] and into thrombi.[33] Furthermore, we reported previously that no enhancement in the penetration of betaxolol across the cornea was achieved when ultrasound and the drug solution were applied separately, as compared with 4-fold enhancement when they were applied together.[10] The lack of enhancement after the separate application of ultrasound and the drug solution indicated that streaming may have been an important mechanism that could have also contributed to the increase in the corneal permeability to sodium fluorescein.

The corneal permeability was shown previously to be dependent on temperature.[46] In our experiments, temperatures of control and ultrasound-treated corneas were kept the same to minimize the thermal effect of ultrasound on the change in permeability. In addition, the temperature of the cornea was below or at physiological temperature of 34°C[47] during the experiments to prevent hyperthermia-induced changes in the cornea tissues. In clinical situations, the cornea temperature during phonophoresis may reach hyperthermia levels of 41–43°C if the current ultrasound parameters are used. Although it has been shown that several minutes of exposure to temperatures of 41–42°C can result in functional changes in the cornea epithelial cells (such as production of heat shock proteins),[48] after 5 minutes of hyperthermia (at 43°C) the necrosis of cornea cells is unlikely.[49]

Ultrasound-induced heating of the cornea and lens is a concern because these structures are largely composed of collagen (an efficient absorber of ultrasound energy) and also represent avascular structures with no heat dissipation via perfusion.[50] For example, cataract was produced in a lens of a guinea pig after hyperthermia at 42.5°C for 60 minutes,[51] and exposure to high-intensity focused ultrasound (frequency of 3.5 MHz, intensity of 25 W/cm^2, exposure duration of 40 seconds) was shown to produce cataract in rabbit lens.[52] Therefore, thermal safety requirements for diagnostic application of ultrasound in the eye are very strict, with the maximal allowed temperature increase of 1°C.[53] In addition, maximal allowed ultrasound intensity levels in ophthalmology (peak intensity of 28 W/cm^2, average intensity of 17 mW/cm^2) and mechanical index of 0.23 are set significantly below the levels for any other clinical diagnostic application.[53] In our experiments, applied ultrasound intensity of 0.19–0.56 W/cm^2 and temperature increase of up to 9°C were above the FDA approved levels. Therefore, a detailed safety study of all eye structures has to be performed to determine the feasibility of clinical application. For example, it would be necessary to determine whether transcorneal phonophoresis may result in necrosis and apoptosis of corneal endothelial cells, degradation of stroma, and cataract production.

It was reported previously that storage of rabbit corneas in various storage media for up to 4 hours could have an effect on the cornea thickness, hydration, and structure,[54] possibly influencing its permeability. In our experiments, the cornea storage times in DPBS were randomized to minimize the influence of storage time on the assessment of difference between the control and treatment permeability and structure. No correlation was observed between the control permeability and storage time (30 minutes to 4 hours) ($R^2 < 0.0063$, number of cornea samples was 6). The permeability of intact cornea in vitro for fluorescein was reported previously to be $0.38 \pm 0.03 \times 10^{-6}$ cm/s in adult rabbits (3–4 years old).[1] The control permeability of $0.35 \pm 0.14 \times 10^{-6}$ cm/s, obtained in our experiments, corresponded well with this previously reported value. However, the alteration of cornea structure from flattening in the diffusion cell may have affected the permeability. The use of a side-by-side diffusion cell[23] that preserves natural curvature of the cornea would have been a better choice in our experiments.

Ultrasound parameters used in our previous study with 20-kHz ultrasound[10] were significantly different from the parameters used in the current study. In the previous study, application of high peak ultrasound intensities of 14 W/cm^2 and long exposure times of 60 minutes (pulsing of 1 second on, 6 seconds off) produced significant disorganization of the corneal epithelium. In addition, the permeability increase of 4 times was achieved for the lipophilic drug betaxolol, whereas the clinical interest lies in corneal permeability enhancement for hydrophilic drugs.[7] Therefore, it was necessary to find a more optimal set of ultrasound parameters that may result in a permeability increase for a hydrophilic compound but with minor changes in the cornea tissues. The current choice of ultrasound parameters (frequency of 880 kHz, intensities of 0.19–0.56 W/cm^2, exposure duration of 5 minutes) appeared to provide a significant permeability increase for a hydrophilic compound, sodium fluorescein, with only minor damage to the surface epithelium.

The long dye exposure time of 60 minutes was chosen because of the low sensitivity of the spectrophotometer that we used. This exposure time is unrealistic for a clinical situation,

and in the future in vivo experiments it will be reduced to a few minutes. On the other hand, the exposure time of 60 minutes may have been too short to achieve steady-state conditions,[1] which are needed for accurate calculation of the corneal permeability.[26] However, the goal of our study was not to accurately measure the permeability of the control and ultrasound-treated corneas but to get an estimation of the relative permeability increase caused by ultrasound exposure at different intensities. In our experiments, the lag time was assumed to be the same for both control and ultrasound-treated corneas. Ultrasound was shown previously to shorten the lag time in the delivery of different compounds through the skin[55] and may have also shortened the lag time for the delivery of sodium fluorescein through the cornea. However, even if the lag times were decreased to less than a minute, the permeability of ultrasound-treated corneas, calculated from Equation 2, would not change drastically (the values of permeability enhancement at different ultrasound intensities would decrease by less than 20%).

Ultrasound has the potential to provide a minimally invasive, efficient method for controlled drug delivery into the eye, for example, in the treatment of resistant bacterial organisms. According to publications from Russia and the USSR, phonophoresis caused faster healing of cornea ulcers and wounds and faster resolution of the cornea inflammation.[12,14,15] Phonophoresis also had a positive effect in reducing cornea opacities that were up to 15 years old.[19] In addition, the combination of phonophoresis and iontophoresis was used in the treatment of retinal dystrophy.[17]

In summary, application of 880 kHz ultrasound resulted in a 4-fold increase in the corneal permeability for sodium fluorescein, with minimal structural changes in the cornea epithelium. In future experiments, we are planning to determine the reversibility of structural changes in the cornea and the increase in the corneal permeability caused by ultrasound using a rabbit model in vivo.

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