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Ultrasound-Enhanced Delivery of Antibiotics and Anti-Inflammatory Drugs into the Eye

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Abstract

Delivery of sufficient amounts of therapeutic drugs into the eye is often a challenging task. In this study, ultrasound application (frequencies of 400 KHz to 1 MHz, intensities of 0.3–1.0 W/cm^2 and exposure duration of 5 min) was investigated to overcome the barrier properties of cornea, which is a typical route for topical administration of ophthalmic drugs. Permeability of ophthalmic drugs, tobramycin and dexamethasone and sodium fluorescein, a drug-mimicking compound, was studied in ultrasound- and sham-treated rabbit corneas *in vitro* using a standard diffusion cell setup. Light microscopy observations were used to determine ultrasound-induced structural changes in the cornea. For tobramycin, an increase in permeability for ultrasound- and sham-treated corneas was not statistically significant. Increase of 46%–126% and 32%–109% in corneal permeability was observed for sodium fluorescein and dexamethasone, respectively, with statistical significance ($p < 0.05$) achieved at all treatment parameter combinations (compared with sham treatments) except for 1-MHz ultrasound applications for dexamethasone experiments. This permeability increase was highest at 400 kHz and appeared to be higher at higher intensities applied. Histologic analysis showed structural changes that were limited to epithelial layers of cornea. In summary, ultrasound application provided enhancement of drug delivery, increasing the permeability of the cornea for the anti-inflammatory ocular drug dexamethasone. Future investigations are needed to determine the effectiveness and safety of this application in *in vivo* long-term survival studies. (E-mail: mnabili@gwu.edu)

Keywords

Therapeutic ultrasound; *In vitro*; Drug delivery; Cornea; Ocular diseases; Sonophoresis

Introduction

Millions of people suffer from variety of ocular diseases, which in some cases lead to vision impairment and eventually blindness (Clark et al. 2003; Friedman et al. 2002). Delivery of drugs at therapeutic levels in treatment of various ocular diseases is a challenge because of specific structure, defense mechanisms and physical barriers of the eye (Short 2008). Common approaches for drug administration to the eye include but are not limited to systemic administration, intravitreal injections, ocular implants and topical administration (Gaudana et al. 2010; Short 2008). Systemic drug delivery is inefficient because of different

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eye-blood barriers, including the barriers that prevent delivery of compounds into the anterior chamber of the eye (Davis et al. 2004; Gaudana et al. 2010) and into the posterior or back of the eye (Ali et al. 2008; Davis et al. 2004). Furthermore, systemically applied ophthalmic drugs can carry a risk of severe adverse effects as the drug enters the systematic circulation (Davies 2000; Gaudana et al. 2010). The intravitreal injection is the most direct and effective way of ocular drug delivery; however, it carries a potential risk of severe adverse effects including cataract, retinal detachment and ocular hemorrhage (Bartlett et al. 1984; Gaudana et al. 2010; Janoria et al. 2007; Short 2008). The easiest way to deliver the drug inside the eye is via topical administration (Cheung et al. 2010), which is used to treat both the eye surface and intraocular conditions (Reddy et al. 1996) because of its noninvasive nature and high patient compliance (Gaudana et al. 2010). This method has several advantages: therapeutic effects are localized and unwanted systemic effects are significantly reduced, and it is a fairly convenient and painless method of drug administration (Davies 2000). However, drug delivery using this method is adversely influenced by eye drop immediate spillage from the eye, tear removal, and ocular barriers such as cornea and sclera barriers (Ali et al. 2008; Gaudana et al. 2010) that cause limited penetration of the drug into the eye (Short 2008).

The cornea is a preferred route for topical administration of drugs (Ahmed et al. 1987b; Doane et al. 1978) and consists of three main layers: epithelium, stroma and endothelium (Gaudana et al. 2010; Gwon 2008). Unfortunately, corneal layers represent a major barrier for delivering drugs, which makes it difficult for therapeutic compounds to reach the target ocular tissues (Davies 2000). The epithelial layer is the main barrier for hydrophilic drugs, whereas the stroma mostly acts as a barrier for lipophilic drugs (Davies 2000; Ke et al. 1999). In most cases, the amount of drug that can penetrate through the cornea is less than 10% (Geroski and Edelhauser 2000; Ke et al.1999; Schoenwald 1997; Short 2008), and achieving a twofold to threefold increase in trans-corneal drug delivery is considered clinically significant (Sasaki et al. 1995).

The objective of our study was to investigate ultrasound enhancement of the delivery of ocular drugs (the antibiotic tobramycin and the steroid dexamethasone) through the cornea in a rabbit eye model *in vitro*. The corneal permeability of sodium fluorescein, a drug-mimicking compound, was also investigated using a limited set of parameters for comparison with published studies. Ultrasound has been shown to enhance the delivery of lytic agents into thrombi and anticancer drugs into cells (Abe et al. 2002; Lawrie et al. 1999; Mitragotri 2005; Tachibana and Tachibana 2001). It has also been used for gene delivery (Kowalczuk et al. 2011) into a variety of cells, such as myocardial and endothelial cells (Kodama et al. 2006; Miller et al. 2002; Taniyama et al. 2002). Furthermore, the application of ultrasound for delivery of drugs into the skin has been one of extensively investigated research areas with promising results (Mitragotri et al. 1995; Tang et al. 2001). Barrier properties of the cornea have also been shown to be modified by the application of ultrasound (Cherkasov et al. 1974; Gvarishvili and Dushin 1999; Tsok et al. 1990). The enhancement of drug delivery through the cornea by ultrasound (phonophoresis) was used clinically in the treatment of eye diseases (Cherkasov et al. 1974; Filippenko and Tretiak 1989; Marmur et al. 1979; Tsok 1979) in which phonophoresis was shown to have a positive effect on the outcome of the diseases of the eye anterior segment, such as keratitis and corneal opacities. Phonophoresis also caused faster healing of corneal ulcers and wounds, and faster resolving of corneal inflammation in patients (Egorov et al. 1995; Iakimenko et al. 1989; Marmur et al. 1979; Tsok et al 1979).

Results of our previous study *in vitro* showed that the exposure of the cornea to 880-KHz ultrasound increased the corneal permeability for sodium fluorescein, a small hydrophilic dye (Zderic et al. 2004a). In the study reported here, our goal was to test clinically relevant

compounds that are currently used in the treatment of corneal infections and inflammations (*i.e.*, tobramycin and dexamethasone) and to test a range of frequencies and intensities to find the ones that might provide effective and safe drug delivery through the cornea.

Materials and Methods

Compounds used in our experiments included the ophthalmic drugs tobramycin and dexamethasone sodium phosphate, and the drug-mimicking compound sodium fluorescein. This drug-mimicking compound was used in the initial stages of the study at specific parameters for comparison with our previously published results.

Tobramycin ophthalmic solution 0.3% (Bausch and Lomb Inc., Tampa, FL, USA) is a topical ophthalmic antibiotic formulation prepared specifically for therapy of external infections. This drug is a clear solution and is highly hydrophilic (DiCicco et al. 2003), with a molecular weight of 467.52 D according to the data sheet for this drug. Sodium fluorescein (Sigma-Aldrich, St. Louis, MO, USA) is an orange hydrophilic dye with a molecular weight of 376.27 D (according to the data sheet for this compound) and is used as a model for drugs that penetrate poorly through the cornea (Ke et al. 1999). Sodium fluorescein was used to make a 0.25% solution in Dulbecco phosphate-buffered saline (DPBS, D4031, Sigma-Aldrich), which is a balanced salt solution with inorganic ions and glucoses. Dexamethasone sodium phosphate 0.1% (Bausch and Lomb Inc.) is a topical steroid solution used to suppress inflammatory responses. This drug is a clear solution with hydrophilic properties and a molecular weight of 516.41 D.

Excised eyes of adult New Zealand White rabbits were used in our experiments. Because of their relative similarity to human eyes, rabbit eyes have been used as the standard model for ophthalmic research (Cheung et al. 2010; Gwon 2008; Ke et al. 1999). The size of the eyeballs in rabbits is smaller in comparison to human eyes with anteroposterior size of 16–19 mm in rabbits and 24 mm in humans. The thickness of a rabbit cornea is 0.3–0.4 mm in center of the cornea and 0.45 mm in its periphery (Gwon 2008). These measurements in human eyes are 0.5 mm and 0.7–0.10 mm, respectively. Rabbit corneal epithelium is ~30–40 μm in thickness (Gwon 2008), which is thinner than the 50–60-μm corneal thickness of a human eye (Snell and Lemp 1998). The rabbit cornea consists of one row of columnar basal cells, two rows of polygonal, and up to six rows of wing-shaped and squamous cells on the external surface (Gwon 2008). A stroma in the rabbit cornea is approximately 0.24 mm thick and consists of collagen fibrils. In the human cornea, the thickness of the stroma is approximately 90% of the cornea's thickness (Pavan-Langston 2008), which is thicker compared with those in rabbit eyes. In both rabbit and human cornea, the endothelium consists of a single layer of flattened hexagonal cells (Gwon 2008), which covers the inner surface of the cornea. In general, the cornea has a higher permeability for lipophilic drugs than for hydrophilic drugs (Prausnitz and Noonan 1998).

The excised New Zealand White rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR, USA). The eyes were stored in Dulbecco modified Eagle medium (DMEM) no more than 24 hours after harvesting. There were no preservatives used in the medium. The eyeballs stored in DMEM were kept in wet ice by the vendor and shipped to our laboratory. Before each experiment, the rabbit eyes were visually examined to ensure they were free of abrasion, and the ones with observed corneal damage were not used. The corneas were dissected and placed in DPBS and kept at room temperature for no more than 10 min before the start of ultrasound or sham treatment.

The jacketed Franz diffusion cell (PermeGear, Hellertown, PA, USA) used in our experiments had a spherical joint that helped with preserving the natural shape of the cornea

(Fig. 1). The orifice diameter of the diffusion cell was 9 mm, and the volume of receiver compartment was 5 mL. The cell opening of 9 mm ensured that only cornea (and not sclera) was exposed to ultrasound. The donor compartment of the diffusion cell with the volume of 25 mL was designed such that an ultrasound transducer could be completely submerged inside the drug solution. The receiver compartment was filled with DPBS, and the donor compartment was filled with the drug of interest. The receiver compartment was stirred at 380 rpm using a magnetic stir bar. The dissected cornea was placed between the donor and receiver compartments of the diffusion cell, with the epithelial layer facing the donor compartment.

The transition point from the near field to the far field (d_{ff}) is the location of the furthest maximum pressure for the unfocused ultrasound transducer (Christensen 1988). The ultrasound transducer was positioned in the donor compartment such that the cornea was located at the distance d_{ff} from the transducer to ensure optimal energy delivery. Unfocused circular ultrasound transducers (Sonic Concepts, Bothell, WA, USA) with 15-mm active diameters at frequencies of 400 KHz, 600 KHz, 800 KHz and 1 MHz were used, and d_{ff} was calculated to be 1.5, 2.25, 3.0 and 3.75 cm for each of these frequencies, respectively (Christensen 1988). The driving unit consisted of a function generator (33250; Agilent, Santa Clara, CA, USA) connected to the power amplifier (150A100B; Amplifier Research, Souderton, PA, USA), which was connected to the ultrasound transducer via an electrical power meter (Sonic Concepts). Ultrasound intensity at different input settings was measured using a reflective radiation force balance (Ultrasound power meter; Ohmic Instruments, Easton, MD, USA).

The specific setup for this study consisted of three diffusion cells that were placed on a holding rack. The entire setup was placed in the water bath with an immersion circulator. The corneas were kept at the constant temperature of 34°C, because the physiologic temperature of the rabbit cornea is 34.3 ± 0.7°C (Efron et al. 1989). The drugs were also kept in the heating bath at 34°C for 20 minutes before starting the experiment. Each cornea was exposed to the drug solution for 60 min, including the 5 min of ultrasound exposure. In case of the sham treatments, experimental duration was the same with no ultrasound application, and the cornea was exposed to the drug solution for 60 min. The temperature of the donor compartment solution was measured (Dual Thermometer; Fisher Scientific, Atlanta, GA, USA) during ultrasound application at intervals of 30 s.

A 3-mL solution sample was collected through the sampling port of the receiver compartment after 60 min. The absorbance of the sample was measured using a spectrophotometer (UVmini-1240; Shimadzu, Columbia, MD, USA) at the specific wavelength observed from the standard calibration curves. To generate the calibration curve for each drug, a serial dilution was performed using DPBS as a baseline. The maximum absorbance for sodium fluorescein was at 490 nm, tobramycin at 278 nm, and dexamethasone at 242 nm. No background absorbance was observed at these wavelengths. The calibration curve of the compound concentration versus absorption was used to calculate the compound concentration in the receiver compartment. The number of different ultrasound and sham treatment experiments was 5–18 per same condition.

The increase in permeability of the cornea can be approximated as the ratio of the receiver compartment concentrations in the treatment and control case. Based on Fick's law of diffusion, the average corneal permeability, P in cm/s, can be calculated using the following equation (Ahmed et al. 1987a; Fick, 1855):

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

where V is the volume of the receiver compartment (cm^3), ΔC_r is the change in the drug concentration in the receiver compartment (mg/mL), A is a cross-sectional area of the cornea (cm^2), Δt is the duration of the cornea exposure to the drugs, and C_d is the drug concentration in the donor compartment (mg/mL). The cumulative amount of the compound transferred to the receiver compartment should be less than 5% of the donor amount in order for this expression to be valid (Ahmed et al. 1987a), which was valid for the drugs used in this study at all possible parameters (<0.02%). The expression in equation (1) can be modified to show the corneal permeability after 1 h of drug exposure as:

$$P = \frac{V(C_{r,t} - C_{r,o})}{A(t - t_L)C_d} = \frac{VC_{r,t}}{A(t - t_L)C_d} \quad (2)$$

where P is permeability in centimeters per second, t is the duration of the experiment (~3600 s), $C_{r,t}$ is the drug concentration in the receiver compartment at the end of experiment, and t_L is the corneal lag time in seconds.

The lag time is the minimum time necessary for a drug to pass through the cornea, and it was measured to be 8.5 min for sodium fluorescein, 20 min for tobramycin, and 35 min for dexamethasone. The lag time was obtained from a set of experiments where the cornea was exposed to the drug for 5, 10, 15, 20, 25, 30, 35, and 60 min with no ultrasound application, as described previously (Sasaki et al. 1995). The number of samples in each case in these lag time measurement experiments was three to five.

After the treatment, the corneas were fixed in formalin (Protocol; Fisher Scientific, Kalamazoo, MI, USA) and prepared using hematoxylin and eosin staining procedures (Histoserv, Inc., Germantown, MD, USA). Semi-thin sections of the cornea (~1 μm) were used in the histology slides. The series of corneal images from each slide were obtained at ×20 magnification using a light microscope (ZeissAxio Imager; Carl Zeiss, Inc., Oberkochen, Germany). The images were used to investigate the structural changes in epithelial cells, stroma, and endothelium of the cornea. The changes in the epithelial layer were classified into four classes based on the level of damage. Each class was given a value of 0, 1/3, 2/3 or 1 for the purposes of semi-quantitative analysis. For class 1 (0) there was no epithelial disorganization (all cells appeared intact, the layers of epithelium were well organized). Class 2 (1/3) had minor epithelial disorganization (some of the cells appeared necrotic, some cells were missing or the first layer of epithelium was removed). For Class 3 (2/3) a more severe epithelial disorganization was observed (more cells appeared necrotic or missing, cells in the two to three layers of epithelium appeared damaged). Class 4 (1) had the most severe epithelial disorganization (majority of the cells were ruptured, all the epithelial layers were absent or severely damaged). Figure 2 shows histologic samples that correspond to different classes of damage.

There were two classes of stromal damage; the stromal fibers either appeared well organized (0) or were partially disorganized (1). In the case of endothelium, the categories were either the endothelial cells appear intact (0) or necrotic or absent (1). The apparent damage owing to histology processing artifacts was also considered. The corneal damage at each combination of ultrasound parameters was correlated with the corresponding change in the corneal permeability.

Results

Figures 3, 4 and 5 show the comparison between permeability of the ultrasound-treated and sham-treated corneas for sodium fluorescein, tobramycin, and dexamethasone, respectively. In the case of sodium fluorescein (Fig. 3), ultrasound application for 5 min at 1 W/cm^2 produced permeability increase of 126% at 400 kHz (n = 9), 121% at 600 kHz (n = 13), 47% at 800 kHz (n = 9) and 65% at 1 MHz (n = 12) compared with sham-treated cases (n = 9). Statistically significant ($p < 0.05$) increase in permeability was observed for all cases. For tobramycin (Fig. 4), the increase in corneal permeability ranged 14%–46.9% depending on ultrasound parameter combination, with no statistical significance achieved in all cases.

For dexamethasone (Fig. 5), the increases in corneal permeability at 0.3 W/cm^2 were 43% for 400 kHz (n = 6) and 46% for 600 kHz (n = 6). The increase in permeability at 0.5 W/cm^2 was 76% for 400 KHz (n = 6), 47% for 600 KHz (n = 8), 50% for 800 KHz (n = 6) and 46% for 1 MHz (n = 5). At 0.8 W/cm^2, the increase in corneal permeability was observed as 107% for 400 KHz (n = 6), 51% for 600 KHz (n = 8), 62% for 800 KHz (n = 6), and 63% for 1 MHz (n = 6). The increase in permeability after application of ultrasound at 1.0 W/cm^2 was 109% for 400 KHz (n = 6), 55% for 600 KHz (n = 9) and 72% for 800 KHz (n = 8). In the case of dexamethasone, statistically significant ($p < 0.05$) permeability increase was observed for all parameters tested, except for the application of 1-MHz ultrasound.

Temperature of the solution in the donor compartment was measured during ultrasound application. The temperature at the end of 5 min of ultrasound treatment was 2–3°C higher compared with the temperature before the treatment.

Figure 6 shows the comparison in epithelial changes between ultrasound-treated and sham-treated corneas. Histologic observations indicated that the ultrasound application produced structural changes in the corneal epithelium. These changes included missing cells from different layers of epithelium, and in some cases partially detached cell layers of the epithelium. Stroma and endothelium appeared normal in most observed samples; however, there were cases in which the endothelial layer was detached in both control (sham-treatment) and ultrasound-treatment cases, potentially because of processing artifacts (Silverman et al. 2001; Tegtmeyer et al. 2001).

Discussion

The cornea is the main route for ocular drug delivery to the eye (Ahmed et al. 1987b; Doane et al. 1978); however, the drug penetration through the cornea is insufficient because of its physiologic barrier properties (Davies 2000). In our previous studies, we showed that ultrasound exposure of the cornea increased the corneal permeability for sodium fluorescein, a small hydrophilic dye (Zderic et al. 2004a). In the present study, we tested clinically relevant compounds that are currently used in the treatment of corneal infections and inflammations (tobramycin and dexamethasone). Although the increase observed for tobramycin was not statistically significant, a statistically significant increase in the corneal permeability was achieved for dexamethasone. The variability in our results reflects the challenges of ultrasound-enhanced drug delivery, which is dependent on a number of factors, including drug size, hydrophilicity, control over ultrasound parameters and mechanisms for barrier opening.

Cavitation is thought to be a key mechanism in the enhancement of drug delivery (Mitragotri et al. 1995). “Cavitation” is defined as the formation and activity of gas bubbles (Leighton 1994; Paliwal et al. 2006; Saroha et al. 2011). Cavitation has been shown to enable the penetration of drugs and macromolecules to the cell (Guzman et al. 2002; Lawrie et al. 1999; Liu et al. 1998; Paliwal et al. 2006). In the case of stable cavitation, the sheer

stresses and micro-streaming around bubbles that are oscillating in the ultrasound field can cause the rupture in the cell membrane (Miller 1987). These bubbles can collapse, in the case of inertial cavitation, and generate pits in the surface of the membrane as a result of high-speed liquid jets in a small region (Leighton 1994). The inertial cavitation was shown to be a cause of an alteration in barrier properties and the structure of the stratum corneum (Tang et al. 2002; Tezel et al. 2003). We have also shown cavitation to be involved in drug delivery across the cornea (Zderic et al. 2004b), albeit in a variable manner. We believe that the variability in our current results is in part due to stochastic nature of cavitation.

Ultrasound also causes bulk fluid streaming and micro-streaming (Cui et al. 2007; Lighthill 1987). It has been shown that enhancement of drug delivery through the skin using ultrasound can be due to streaming (Mitragotri et al. 1995; Tang et al. 2001). Our previous study has also indicated that streaming is a contributing factor in ultrasound enhancement of trans-corneal drug delivery (Zderic et al. 2004a). In the current study, streaming may be responsible for drug delivery enhancement, but we did not measure this effect on previous studies, ultrasound at frequencies of 470–880 kHz and intensities of 0.2–0.3 W/cm^2 applied for 5 min in a continuous mode produced up to a 10-fold increase in the corneal permeability for hydrophilic compounds in a rabbit model *in vivo* (Nuritdinov 1981; Panova et al. 1995; Tsok et al. 1990). The mechanism of ultrasound action appeared to be cavitation-induced reversible erosion of the epithelium (*i.e.*, production of pits), which healed within 90 min to 6 h (Nuritdinov 1981; Zderic et al. 2004b). Formation of pits owing to cavitation activity would be the biologic mechanism for enabling the change in the corneal permeability and drug penetration into the eye.

In our current experiments, the structural changes were observed only in the corneal epithelium. In some cases, cells from the surface layer of the epithelium were completely removed. Furthermore, some cells from inner layers of the epithelium appeared lighter in color, which indicated cell membrane rupture. The histologic observations from our previous *in vivo* and *in vitro* studies also showed that ultrasound-induced damage was more obvious in the first layer of epithelium, and only a small number of cells from inner layer were damaged (Zderic et al. 2004a, 2004b). Similar to our previous studies, it appeared that ultrasound did not cause any changes in stroma and endothelium. The absence of endothelium in both control and treated cases could be due to sample processing artifacts and not related to ultrasound application (Silverman et al. 2001; Tegtmeyer et al. 2001).

Thermal effects of ultrasound have also been corroborated for enhanced drug delivery. Mitragotri et al. (1995) indicated that the increase in skin temperature owing to ultrasound might increase the diffusion coefficient of the skin and skin permeability. *In vitro* study using sonophoresis at 150 KHz and 2 W/cm^2 showed an increase in skin permeability to hydrophilic drugs, mainly owing to thermal effects (Ueda et al. 1995). The change in the corneal permeability and increase in drug delivery could also be affected by the increase in corneal temperature. For example, the corneal permeability to water increased as the temperature of the cornea changed from 23°C to 37°C (Green and Downs 1976). In our experiments, a temperature increase of up to 3°C was observed, which might have had an effect on the corneal permeability.

A rise in temperature and overheating of sensitive eye structures is a concern in the clinical application of ultrasound for drug delivery into the eye (Barnett et al. 2000; Boucaud et al. 2001; Kowalczuk et al. 2011; Polat et al. 2011). The absence of blood flow in the cornea and lens, which are avascular, would exacerbate the potential harmful effects of increased temperature because of a decreased ability for heat dissipation (Cucevic et al. 2005; Kowalczuk et al. 2011). The maximal corneal temperature in our experiments was 37°C,

which is less than the hyperthermia levels (41–43°C); therefore, functional changes in the corneal epithelial cells resulting from heat were unlikely (Yamaguchi et al. 1990).

The specific characteristic of the cell membrane makes it easier for lipophilic molecules to cross the lipid portion of the membrane compared with hydrophilic molecules (Martini and Bartholomew 2007). The epithelial layer of the cornea represents the main barrier, especially for hydrophilic drugs (Hughes and Mitra 1993; Nishida 2005). Passage of drugs to deeper layers of the cornea is difficult because of the presence of junctional complexes between corneal epithelial cells (Nishida 2005). Because all the compounds tested in our experiments were hydrophilic in nature, no observations could be made at this point regarding the effect of drug lipophilic or hydrophilic properties on the ultrasound-enhanced trans-corneal drug delivery.

Conclusions

To our knowledge, this report is the first document whether ultrasound can be effective in increasing trans-corneal delivery of clinically relevant compounds that are currently used in the treatment of corneal infections and inflammations, namely an anti-inflammatory drug (dexamethasone) and an antibiotic (tobramycin). Our results showed up to a twofold increase in the delivery of dexamethasone, with statistical significance. Ultrasound appeared to be most effective at lower frequencies (400–600 kHz) and higher intensities (0.8–1.0 W/cm^2). For tobramycin, ultrasound application appeared to lead to some increase in the trans-corneal delivery with no statistical significance. Because the compounds tested in our experiments had relatively similar molecular properties, further study is needed to find optimal ultrasound parameters for ophthalmic drugs of different molecular sizes and hydrophilicity. Some structural changes in the epithelial layer of the cornea were observed at all applied ultrasound parameter combinations. Therefore, our future studies will also focus on investigations of the safety aspect of this method *in vivo*, to determine reversibility of structural changes in the cornea, recovery of corneal barrier properties, and long-term safety of ultrasound exposure in different eye tissues.

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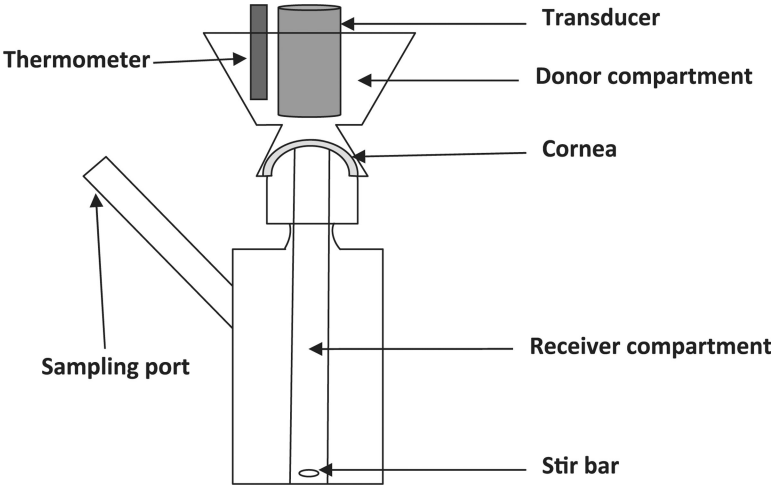

Fig. 1.

Diffusion cell setup. The dissected cornea was placed between donor and receiver compartments, with the epithelial layer facing the donor compartment. The receiver compartment was filled with DPBS, and the donor compartment was filled with a drug solution. The transducer was positioned at different distances from the cornea (at d_{ff}) based on the frequency of the transducer. Ultrasound was applied for 5 min, and the cornea was exposed to drug solution for 60 min.

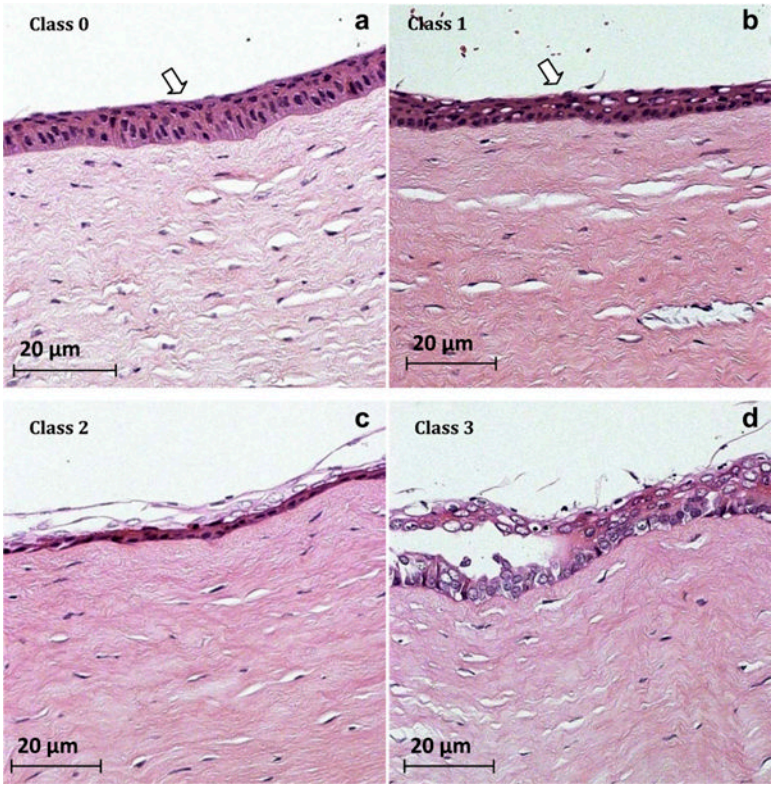

Fig. 2.

Histologic observations. Examples from semi-quantitative analysis study of histologic slides for investigation of corneal damage. (a) Class 0: sham-treated cornea. The surface epithelial cells appear intact (arrow). (b) Class 1: ultrasound application at 1 MHz and 1 W/cm^2. Minor structural changes are present in the surface of the epithelium (arrow), with some cells missing. (c) Class 2: ultrasound application at 400 KHz and 1 W/cm^2. Cells in two layers of epithelium are damaged, missing, or both. (d) Class 3: ultrasound application at 600 KHz and 1 W/cm^2. Severe epithelial damage is observed.

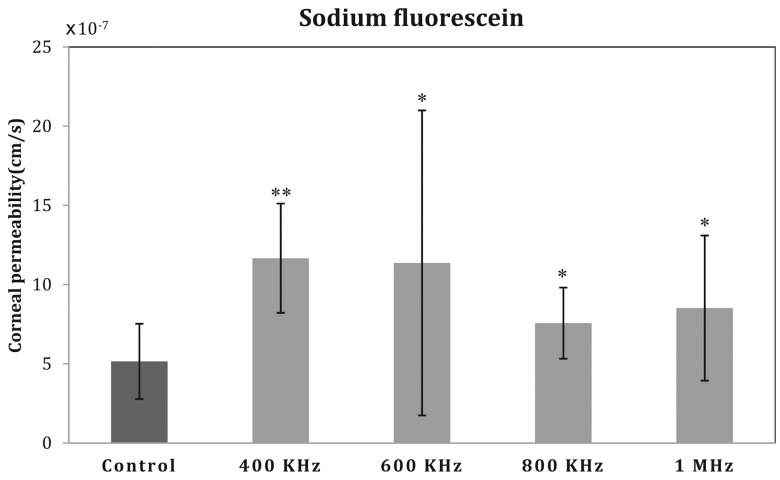

Fig. 3.

The effects of ultrasound on the corneal permeability in the case of sodium fluorescein. Control (sham treatment) represents the corneal permeability with no ultrasound treatment, compared with the corneal permeability at 400 KHz to 1 MHz and 1 W/cm^2 with ultrasound exposure of 5 min. The mean and standard deviation are shown from 9 to 13 experiments per condition. The permeability of ultrasound-treated corneas was up by 126% compared with sham-treated corneas ($p < 0.05$). *$p < 0.05$; **$p < 0.001$.

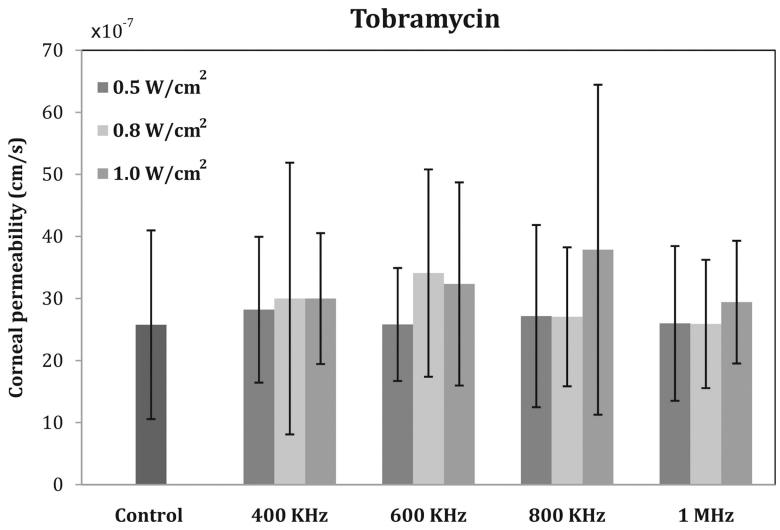

Fig. 4.

The effects of ultrasound on the corneal permeability in the case of tobramycin. Control (sham treatment) represents the corneal permeability with no ultrasound treatment; different shades of gray represent the corneal permeability at intensities of 0.5–1.0 W/cm^2 and frequencies of 400 KHz to 1 MHz, and ultrasound exposure of 5 min. Number of experiments per experimental condition was 9–18. Data are given as mean ± standard deviation. No statistically significant permeability increase was detected for this drug.

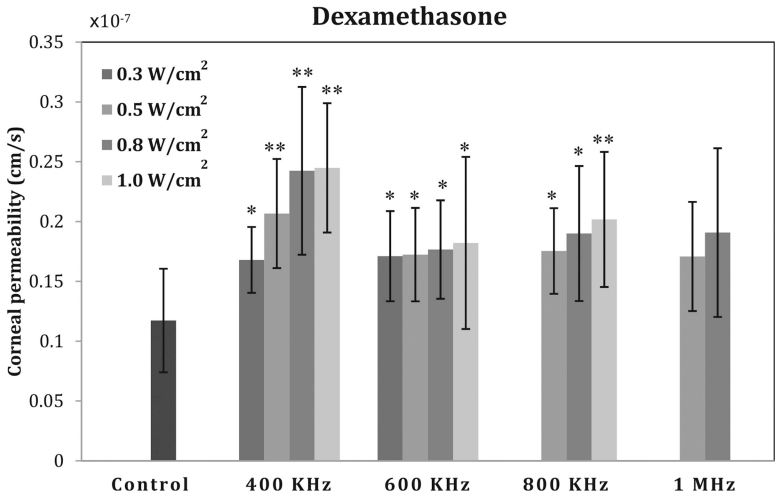

Fig. 5.

The effects of ultrasound on the corneal permeability in the case of dexamethasone. Control (sham treatment) represents the corneal permeability with no ultrasound treatment; different shades of gray represent the corneal permeability at intensities of 0.3–1.0 W/cm^2 and frequencies of 400 KHz to 1 MHz and ultrasound exposure of 5 min. The number of experiments per experimental condition was five to nine. Data are given as mean ± standard deviation. Statistically significant ($p < 0.05$) permeability increase was observed at all tested parameters, except for ultrasound application at 1 MHz. *$p < 0.05$; **$p < 0.001$.

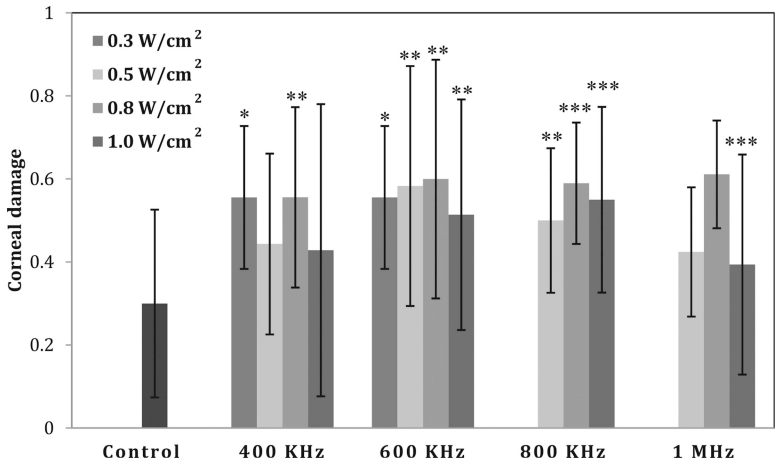

Fig. 6.

Corneal changes resulting from ultrasound application compared with control (sham-treated) corneas. Control shows the corneal changes with no ultrasound treatment; different shades of gray represents the corneal damage resulting from ultrasound application (intensities of 0.5–1.0 W/cm^2, frequencies of 400 KHz to 1 MHz, exposure duration of 5 min). Data are shown as mean ± standard deviation (n = 6–33). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.