**ABSTRACT**

**Purpose/aim of study:** Drug delivery to the ocular posterior segment is of importance, but it is a challenge in the treatment of irreversible blindness disease, such as age-related macular degeneration. Although some methods (i.e. intraocular injection, sustained release by polymer and iontophoresis) have been applied, some technical drawbacks, such as slow rate and damage to the eye, need to be overcome for wide use.

**Materials and methods:** In this study, the feasibility of high-intensity focused ultrasound (HIFU) to enhance the transsclera drug delivery was tested for the first time. One-hundred HIFU pulses with the driving frequency of 1.1 MHz, acoustic power of 105.6 W, pulse duration of 10–50 ms and pulse repetition frequency of 1 Hz were delivered to the fresh *ex vivo* porcine sclera specimen.

**Results:** In comparison to the passive diffusion (control), 50-ms HIFU can increase the penetration depth by 2.0 folds (501.7 ± 126.4 µm versus 252.4 ± 29.2 µm) using bicinchoninic acid assay and Rhodamine 6 G fluorescence intensity by 3.1 folds (22.4 ± 12.3 versus 7.1 ± 4.1) and coverage area by 2.6 folds (40.4 ± 9.1% versus 15.8 ± 2.9%). No morphological changes on the sonicated sclera samples were found using a surface electron microscope.

**Conclusions:** In summary, pulsed-HIFU may be an effective modality in the transsclera drug delivery with a high transporting rate and depth. *In vivo* studies are necessary to further evaluate its performance, including the drug penetration and its possible side effects.

**Keywords:** Fluorescence, high-intensity focused ultrasound, penetration, pulse duration, transsclera drug delivery

**INTRODUCTION**

The posterior eye segment (i.e. retina, vitreous and choroid) is an important therapeutic target with unmet medical needs. Severe vision loss from age-related macular degeneration (AMD), diabetic reino-pathy, glaucoma and retinitis pigmentosa accounts for most cases of irreversible blindness worldwide. Approximately 1.7 million Americans over the age of 65 years suffer from AMD, and there are estimated 200,000 new cases per year. Topical administration of eye drops or ointments is the easiest way to deliver ocular drugs. However, the drugs are rapidly drained and turned over from the ocular surface. So the time for drug absorption is only a few minutes, and bioavailability is very low (i.e. less than 5%) to intraocular tissues. The major portion of the instilled dose is absorbed systemically through the conjunctiva, the highly vascular conjunctival stroma, the lid margin vessels and the nasolacrimal duct. The blood–retinal barrier (BRB) is formed by the inner barrier that is composed of the endothelial of retinal capillaries and the outer barrier that is composed of the retinal pigment epithelium. Tight junctions in BRB restrict the influx of drugs from systemic blood circulation to the retina and from the vitreous to the blood stream. As a result, only less than $1/10^5$ of protein drug may reach the back of the eye due to long diffusion distance, counter-directional intraocular convection, lacrimation and corneal...
impermeability to large molecules.\(^2\)-\(^4\) Therefore, effective methods of posterior segment therapies with improved ocular drug bioavailability and prolonged duration of activity with minimum risk of ocular complications for the outpatient are of great interest.\(^5\)-\(^7\)

Several approaches have been employed to deliver drugs to the posterior segment: systemic, intraocular and periocular (including subconjunctival, subepithelial and retrobulbar).\(^8\) The most direct and effective practice is intravitreal injection. However, this invasive approach has the risk of cataract, retinal detachment, vitreous hemorrhage and endophthalmitis.

In the case of chronic eye disease, multiple injections are required but are not well tolerated by patients. Systemic administration must overcome the blood–aqueous barrier and BRBs, so larger doses are necessary. More importantly, systemic delivery of biologically active molecules is potentially hazardous. In the case of growth factors and their inhibitors, cardiac and reproductive function can be adversely affected. The human sclera accounts for 95% of the total surface area of the globe and provides a significantly larger avenue for drug (i.e. neuroprotective molecules, antioxidants or angiostatic agents) diffusion – compared, for example, to the cornea – to the inside of the eye or specific regions of the retina.

Furthermore, the permeability of sclera is higher than the cornea to a wide range of solutes and does not seem to be correlated with age (9 days to 87 years).\(^9\)

Drug delivery across the sclera is governed partially by the transient diffusion that typically occurs over a course of minutes, unless some type of sustained-release formulation or device is used. Although polymer implants can easily enase drugs with molecular weights less than about 100 Da and can last for months, chronic diseases may require repetitive insertion.

The transscleral iontophoresis is safe, well tolerated and easily applied to the treatment of severe ocular inflammation to reduce the systemic side effects of corticotherapy.\(^10,11\) In the iontophoretic treatment of methylprednisolone using 1.5 mA for 4 min, 88% of treated eyes demonstrated a complete reversal of the corneal graft rejection processes with no significant side effects.\(^12\) Key factors determining the amount of drug delivered by iontophoresis are current density, duration of treatment, drug concentration, pH and the permeability of the tissue for the drug molecule. Hydrophilic drugs with low molecular weight at physiological pH are the better candidates, and negatively charged molecules penetrate deeper than positively charged ones. Initially, the threshold for avoiding ocular toxicity due transscleral iontophoresis was set as current density of 500 mA/cm\(^2\) for 5 min.\(^13\) However, recent studies showed this value may be too high. When 4 mA current was applied, half of the subjects reported a burning sensation, which was resolved after 22 h. Although animals experienced no evident discomfort corresponding to all current densities applied with the maximal voltage of 150 V, histopathological examination showed hemorrhagic necrosis, edema and infiltration by polymorphonuclear cells in the retina, choroid and ciliary body using 254.6 and 127.0 mA/cm\(^2\) for 5 and 10 min, respectively.\(^14\) Even a current density of 200 mA/cm\(^2\) for 10 min induced minimal histopathological damage.\(^15\)

Ultrasound has been developing as a noninvasive modality in the drug delivery to solid tumor\(^16\) or transdermal drug delivery (sonophoresis).\(^17\)

Ultrasound could disrupt the outermost layer of the skin temporarily and increase its permeability. The permeability of rabbit cornea increased by 1.9–4.4 folds for lipophilicity (atenolol, carteolol, timolol and betaxanol) after 60 min of 20 kHz ultrasound exposure at \(I_{SAPA}\) of 14 W/cm\(^2\) \textit{in vitro}; and the differences between the treatment and control experiments became statistically significant after 10–30 min of ultrasound exposure for all four drugs.\(^18\)

Epithelial disorganization and structural changes in the corneal stroma were produced. Focused ultrasound (30 s continuous wave, 1 MHz, 0.05 W/cm\(^2\)) can enhance protein penetration into the rabbit sclera, increasing the diffusivity by 1.6 folds while causing no damage to the retinal tissues and negligible temperature rise (<0.5 °C). This enhancement is temporary since the diffusional resistance was restored 15 min after sonication.\(^2\) Recently, high-intensity focused ultrasound (HIFU) has become an effective and noninvasive approach for the solid tumor ablation by elevating the temperature over 65 °C within seconds for the formation of irreversible coagulation.\(^19\) Pulsed-HIFU, in which the mechanical mechanism dominates, can increase Doxorubicin concentration in tumors by 124% with apparent extravasation in the vasculature of treated tumors and with no deleterious effect.\(^20\)

In this study, the feasibility of pulsed-HIFU on transscleral drug delivery was investigated for the first time. Bicinchoninic acid (BCA) and Rhodamine 6 G fluorescent dyes were used as drug alternatives to illustrate the penetration depth and concentration in the porcine sclera samples. Pulse duration of HIFU varied from 10 ms to 100 ms at a constant pulse repetition frequency (PRF) of 1 Hz. It is found that 100 HIFU pulses with pulse duration of 50 ms could achieve 2.0-fold increase in the penetration depth, 3.2-fold increase in the fluorescence intensity and 2.6-fold increase in the distribution area in comparison to the topical administration, respectively \((p<0.05\) in all cases). Surface electron microscope (SEM) images of the sonicated sclera specimen did not show significant morphological changes. However, 100-ms HIFU pulses may induce erosion on the tissue surface occasionally. Altogether, it shows that pulsed-HIFU can significantly enhance the transsclera drug
delivery. Parameter optimization and in vivo trials are required to evaluate its performance and potential for clinical translation.

**MATERIALS AND METHODS**

**Ex Vivo Tissue**

The use of animal tissues was approved by the local animal ethics committee: Agri-Food Veterinary Authority of Singapore. Fresh porcine eyes were purchased from a local abattoir (Primary Industrial Pvt. Ltd., Singapore) within two hours of sacrifice. The eyes were then immersed into a phosphate buffered saline (PBS) solution and put into a coolant filled with ice for transportation. In the laboratory, each eye was washed with a PBS solution at room temperature to remove the blood from the surface, and all the periorcular tissue (i.e. conjunctiva, muscles and orbital fat) was carefully removed using surgical tweezers and scissors to expose the eye ball. Then the cornea was cut, and the iris and lens were removed. The sclera was cut in half along the equator and immersed in PBS to maintain hydration. The total preparation took about 10–15 min.

**Experimental Setup**

An annual HIFU transducer (H-102, outer diameter = 69.94 mm, inner diameter = 22.0 mm, \( F = 62.64 \text{ mm}, \ f_0 = 1.1 \text{ MHz}, \) SonicConcepts, Bothell, WA) was driven by sinusoidal bursts produced by a function generator (AF3021B, Tektronics, Beaverton, OR) together with a 55 dB power amplifier (150 W, A150, ENI, Rochester, NY) and an impedance match unit. The acoustic pressures of the HIFU transducer with the peak-to-peak voltage of 1 V from the function generator were \( p^+ = 46.8 \pm 1.3 \text{ MPa} \) and \( p^- = -15.7 \pm 0.8 \text{ MPa} \) at the focus with –6 dB beam size of 0.5 mm x 4 mm measured by a fiber-optic probe hydrophone (FOPH-500, RP Acoustics, Leutenbach, Germany), and the acoustic power was 105.6 ± 7.2 W measured by a radiation force balance (RFB-2000, Onda Corp., Sunnyvale, CA). A customer-built coupling cone filled with degassed water (\( O_2 < 4 \text{ mg/L}, T = 25^\circ\text{C}, \) measured by DO700, Extech Instrument, Waltham, MA) and sealed with kitchen wrap (negligible acoustic attenuation) on the top and was attached to the HIFU transducer. Kitchen wrap was replaced in each experiment to avoid cross contamination. A reservoir was attached firmly to the ex vivo porcine sclera examples that sat on a semi-spherical gel phantom with similar radius of curvature as the eye ball (Figure 1). No leakage was confirmed before the injection of drug solution for the delivery experiment. Afterwards, the coupling cone with HIFU transducer was immersed in the reservoir with its top attached to the surface of the sclera. A LabVIEW program (National Instruments, Austin, TX) in a personal computer was used to control the delivery of HIFU bursts.

BCA reagents (Pierce, Rockford, IL) and Rhodamine 6 G (C_{28}H_{31}N_2O_3Cl, Sigma-Aldrich, St. Louis, MO) were used as the drug alternatives in the experiment. The chelation of copper with protein in an alkaline environment forms a light blue complex, and then the chelation of two molecules of BCA with one cuprous ion generates the intense purple-colored reaction product. Compared with most dye-binding methods, the BCA assay is affected much less by protein compositional differences and provides greater protein-to-protein uniformity. So it is much easier and faster than the classical Lowry method. Rhodamine 6 G is a fluorescent dye with a molecular weight of 479 Da, excitation wavelength of 432 nm and emission wavelength of 566 nm. Rhodamine 6 G was dissolved completely in methanol (Sigma-Aldrich) to reach the concentration of 8%, and then diluted 10 times using methanol (final concentration of 0.8%).

**Drug Delivery Evaluation**

After the experiment, the samples were washed with PBS solution thrice, kept at \(-70^\circ\text{C} (MR-DF-N86, LabFreez Instruments Co., Ltd., Beijing, China) for at least 30 min, and then cut with a cryostat microtome (CM1850, Leica Microsystem, Wetzlar, Germany) into thicknesses of 100 μm. The distribution and intensity of fluorescence in the sliced samples were acquired using a fluorescence microscope (LSM 700, Carl Zeiss Vision GmbH, Aalen, Germany). A band-pass filter...
Surface Electron Microscope

Immediately after sonication, the sclera specimen was washed with PBS solution thrice and fixed by 2.5% glutaraldehyde (Sigma-Aldrich) overnight at 4°C. Samples were washed the next day with PBS to remove glutaraldehyde and were dehydrated with increasing concentration of ethanol from 25% to 100%, dried using a critical point dryer (CPD 030, Bal-Tec, Witten/Ruhr, Germany) and then photographed using an SEM (JSM-5600LV, JEOL, Tokyo, Japan) with an appropriate magnification.

Statistical Analysis

A two-way analysis of variance (ANOVA; two-factor without replication, \( p = 0.05 \)) was performed to test the statistical significance between the treatment protocols using SPSS® Statistics (IBM Software, Somers, NY). The sample size in each testing condition was at least five.

RESULTS

The temperature elevations on the surface of the sonicated sclera specimen in all conditions were less than 1°C as measured by a thermocouple and a thermometer (Omega, Stamford, CT); therefore, the thermal effects induced by HIFU can be ignored. The reaction of BCA reagents in the scleral network after exposure was clearly observed in the sliced sample under the inverted microscope (Figure 2), from which the penetration depth was determined quantitatively (Figure 3). In the control situation (i.e. no sonication), the BCA can penetrate as far as 252.4 ± 29.2 μm. The penetration depth increases with the pulse duration of HIFU (from 276.2 ± 64.3 μm at 10 ms to 501.7 ± 126.4 μm at 50 ms), and the difference in comparison to the control becomes significant when the HIFU pulse duration is longer than 20 ms (\( p < 0.05 \)). Although the BCA can completely pass through the sclera samples at the HIFU duration of 100 ms, erosion on the surface was also found after sonication (Figure 2e), which is due to the subcellular tissue fragmentation by bubble cavitation that occurred at the interface of tissue and water.\(^{23}\)

HIFU in Transsclera Drug Delivery

Histotripsy therapy using high-pressure (i.e. peak rarefractional pressure >20 MPa) and short pulses (<10 cycles) has been shown to be a versatile treatment for producing well-confined lesions as long as the acoustic pressure is higher than the cavitation threshold.\(^{24}\) Therefore, in order to avoid this side effect during transsclera drug delivery, bubble cavitation induced by HIFU should be monitored and controlled.

Rhodamine 6G distributed in the sclera after topical administration and HIFU sonication (Figure 4). Although some isolated bright dots were observed in the fluorescent images, which may be due to the perfusion through capillary, fluorescein penetrated gradually from the surface. Fluorescence could also be detected, but it is quite weak. The average intensity is 7.1 ± 4.1, and the detected fluorescence covers 8.3 ± 6.0% of the observation region. After HIFU sonication, the penetration depth, distribution area and intensity of Rhodamine 6G could be enhanced. With the increase of pulse duration from 10 ms to 50 ms, there is 2.7- and 2.1-fold increase in the intensity (from 8.3 ± 6.0 to 22.4 ± 12.3) and area (from 19.5 ± 3.3% to 40.4 ± 9.1%), respectively (Figure 5).

The structure of sclera samples was observed and compared with each other using SEM and different magnifications (Figure 6). The scleral stroma has similar structure and composition to corneal stroma, including collagen fibers, a sparse population of fibroblasts, proteoglycans and a few elastic fibers. So the sclera is an elastic and microporous tissue. After 50-ms HIFU pulse sonication, there is no significant morphological change such as the breakage and disorganization of collagen fibrils and the formation of pores inside. However, possible tissue injuries need further in vivo investigation.

DISCUSSION

Transsclera drug delivery is a non-destructive and minimally invasive method to treat ocular disease in posterior segments of the eye. Although the sclera area is much larger than that of the cornea to select an appropriate entry site and the thickness of sclera is also smaller, penetration via the transcleral route is still quite challenging due to the affection of the scleral stroma on pharmacokinetics. Low-intensity continuous ultrasound exposure can increase the diffusivity with no damage to the retinal tissues and negligible effect of ultrasound-induced convection and temperature rise.\(^2\) In this study, ultrasound with higher intensity but shorter pulse duration was conducted and achieved promising results in an ex vivo experiment of transsclera drug delivery. The penetration could be up to 501.7 ± 126.4 μm after 100 HIFU pulses with pulse duration of 50 ms and PRF of 1 Hz. The sclera thickness at the limbus and near the

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equator of the globe is 0.53 ± 0.14 mm and 0.39 ± 0.17 mm, respectively, and increases gradually moving toward the posterior. The ideal location for transscleral drug delivery is said to be near the equator at 12–17 mm posterior to the corneoscleral limbus. It suggests that our approach could completely penetrate most parts of the porcine sclera in a short period of time. No morphological change on the specimen was found after sonication using SEM if the pulse duration is no longer than 50 ms, and temperature elevation was also negligible (<1 °C). In vivo experiments are required to further evaluate the performance of this method, especially the drug concentration in the ocular artery, pathological tissue injury and other side effects associated with HIFU exposure, which will be carried out in the next step; 23.1% of patients with hepatocellular carcinoma (HCC) experienced mild local pain when undergoing HIFU ablation, which worked in continuous wave mode with stepwise increase power of 160–250 W and scanned along pre-determined trajectories. However, the sclera is not very sensitive to pain and hypoxia because the cornea is an avascular and highly innervated tissue.

In the pulsed-HIFU sonication, the thermal effect is negligible because of the low duty factor applied, and no thermal damage was found in the specimen. Therefore, mechanical effects such as bubble cavitation, acoustic radiation force and associated streaming dominate the enhancement. Stable cavitation induced
FIGURE 3 Comparison of the penetration depth observed in the pathological slice by control and pulsed HIFU exposure. *p < 0.05 in comparison to control.

FIGURE 4 Representative fluorescent images showing the distribution of Rhodamine 6G in the porcine sclera by (a) topical administration as control, after 100 HIFU bursts with pulse duration of (b) 10 ms, (c) 20 ms and (d) 50 ms at a pulse repetition frequency of 1 Hz. The scale bar is 100 µm.

FIGURE 5 Comparison of the fluorescence intensity and covered area in the observation window in the porcine sclera slice of control and pulsed HIFU exposure. *p < 0.05 in comparison to control.
by moderate pressure and long durations of acoustic pulses can cause a widespread but mild disruption of the vessel wall. In contrast, microchannels in the direction of HIFU propagation (>1 mm in length and a few cells in width) was found to correlate with inertial cavitation under the sonication with high pressure and short pulses. As a result, extravasation of a macromolecular compound (Ad-Luc) increased dramatically – up to a 60-fold increase in luciferase expression.26 Meanwhile, the transfer of momentum from the ultrasound wave to the medium in the high acoustic field generates a unidirectional, radiation force that is proportional to the acoustic absorption coefficient and the acoustic power delivered but is inversely proportional to the speed of sound in the medium. In the ultrasound-mediated transcutaneous drug delivery (i.e. phonophoresis), it is postulated that cavitation increases the skin permeability, and the radiation force facilitates drug diffusion.27 They work synergistically instead of independently. In the pulsed-HIFU treatment, the situation is similar, but the acoustic radiation force dominates. The acoustic emission signal from bubble oscillation and cavitation will be monitored in real-time in the following work since the strengths of stable and inertial cavitation are represented by the harmonics and subharmonics in the spectrum, respectively.2 Correlation with the drug delivery may shed light on their roles and illustrate new treatment strategies or parameter optimization for higher efficiency and safety.

The eye is a promising target organ for macromolecular and gene therapy (i.e. neuroprotective agents or vectors for the treatment of glaucoma and other chorioretinal degenerations) because of its unique features, such as easy accessibility and direct assessment of visual function as the therapeutic outcome. Scleral permeability decreases exponentially with the molecular weight and radius, and there is an abrupt decline in permeability at larger molecular weights. Changes in the physiochemical properties of the sclera have a relatively weak effect on the diffusion of small solutes. Because the sclera is an aqueous milieu (composed of approximately 70% water), hydrophilic compounds diffuse faster through the sclera than hydrophobic one. For large molecules, increased hydration and transient modification of the scleral extracellular matrix would result in significant improvement. Ultrasound has already achieved promising results in gene transfection, especially in aid of microbubbles.28,29 Sever chronic hindlimb ischemia could be markedly improved in both microvascular blood flow and vessel density by

![FIGURE 6 Representative SEM images of porcine sclera sample at two different magnifications after (a) topical administration as control and (b) 100 HIFU bursts with pulse duration of 50 ms at pulse repetition frequency of 1 Hz. No significant morphological change is found after sonication.](image)
ultrasound-mediated destruction of microbubbles bearing vascular endothelial growth factor-165 (VEGF165) plasmid DNA in rats. The improvement was attributed predominantly to arteriogenesis, and the perfusion peaked at 14 d after treatment and was followed by a partial regression of neovascularization at six weeks. Ultrasound-mediated gene transfer has the advantages of high selectivity (i.e. the only expression in the focused area with undesirable side effects) and noninvasiveness. So its transscleral application is possible.

To evaluate the distribution and elimination of drugs from the posterior eye in vivo, three important mechanisms for drug transport should be considered: loss to the choroidal circulation, active transport by the retinal pigment epithelium (RPE) and loss to the conjunctival lymphatics and episcleral veins. Although choroidal blood flow is high, it constitutes only a minor fraction of the entire circulation in the body. Unlike retinal capillaries, the choroidal vasculature has large fenestrations for the delivery of oxygen and nutrients to the eye, and it was initially thought to be a sink for transsclerally delivered drugs. Active transport by the RPE is significant compared to passive diffusion and convection. Subsequently, the outward permeability (i.e. from the retinal space into the choroidal circulation) is much higher (roughly 100 times) than the inward one. Therefore, drugs easily gain access to the choroidal extravascular space, but distribution in the retina is limited by the RPE and retinal endothelia. As a result, losses in choroidal circulation are not as important of an impeding factor. Active transport could induce periscleral movement of the drug and could result in more rapid distribution and elevated drug concentration in the choroid and sclera. In contrast, clearance via episcleral veins, conjunctival blood and lymphatic flow has a more profound effect on transscleral delivery. Episcleral implants on rabbits showed negligible vitreous concentrations but significant vitreous delivery. Episcleral implants on rabbits showed negligible vitreous concentrations but significant vitreous concentrations in the ex vivo experiments. Hence, design of the drug administrated on the scleral surface needs more attention but not on the choroidal blood flow. High delivery rate of HIFU pulses shown in this study may reduce the loss from the scleral surface and consequently may enhance efficiency.

In conclusion, pulsed-HIFU is an effective approach for transscleral drug delivery in ex vivo experiments. Animal experiments are required to evaluate its performance as well as the feasibility of clinical application. The underlying mechanisms need extensive investigation for the technological improvement and parameter optimization. Other therapeutic agents (i.e. liposome, nano- and micro-particles and macromolecules) may be applicable for HIFU-mediated transscleral drug delivery because ultrasound has no special requirement on the drug properties (i.e. charge or hydrophilicity). However, dependence on particle size may be a major issue for penetration.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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