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Laser irradiation of ocular tissues to enhance drug delivery

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Abstract

Scleral and corneal membranes represent substantial barriers against drug delivery to the eye. Conventional hypodermic needles-based intraocular injections are clinically employed to overcome these barriers. This study, for the first time, investigated a non-invasive alternative to intraocular injections by laser irradiation of ocular tissues. The P.L.E.A.S.E.[®] laser device was applied on excised porcine scleral and corneal tissues, which showed linear relationships between depths of laser-created micropores and laser fluences at range 8.9 – 444.4 J/cm². Deeper and wider micropores were observed in scleral relative to corneal tissues. The permeation of rhodamine B and fluorescein isothiocyanate (FITC)-dextran were investigated through ocular tissues at different laser parameters (laser fluences 0 – 44.4 J/cm² and micropore densities 7.5 and 15%). Both **molecules** showed enhanced permeation through ocular tissues on laser irradiation. Maximum transscleral permeation of **the molecules** was attained at laser fluence 8.9 J/cm² and micropore density 15%. Transcorneal permeation of rhodamine B increased with increasing either laser fluence or micropore density, while that of FITC-dextran was not affected by either parameter. The transscleral water loss increased significantly after laser irradiation then returned to the baseline values within 24 h, indicating healing of the laser-created micropores. Laser irradiation is a promising technique to enhance intraocular delivery of both small and large molecule drugs.

Keywords

Scleral permeability; Corneal permeability; micropores; ocular delivery; optical coherence tomography, laser microporation.

1. Introduction

Drugs which are topically administered to the eye suffer from low ocular bioavailability < 5%, which is attributed to several anatomical and physiological barriers, including poor corneal and scleral permeability. These also include short residence time of drugs in the eye due to rapid tear turnover, nasolacrimal drainage, and conjunctival uptake to the systemic circulation^{1,2}. Intraocular injections using conventional hypodermic needles is routinely used for administration of both small molecules (corticosteroids) and biologics. However, their highly invasive nature causes considerable discomfort, pain, and occasional bleeding^{3,4}. Permeation enhancing strategies such as chemical, physical and mechanical methods can provide an alternative to intraocular injections by increasing drug penetration through the corneal or scleral tissues. In particular, non-invasive methods such as iontophoresis⁵ (e.g., dexamethasone), high-frequency ultrasound/sonophoresis⁶ (e.g., tobramycin), and drug-loaded polymeric microneedles⁷ (e.g., amphotericin) can provide overarching advantages to its counterparts. Although these techniques were primarily investigated to enhance transdermal permeation of drugs^{8,9}, they were extended for ocular applications demonstrating enhanced corneal and scleral permeation of selected drug molecules.

Fractional laser irradiation is a non-invasive technique for enhancement of transdermal drug permeation *via* transient perforation of a localized area in the *stratum corneum*^{10,11}. Nelson *et al.* reported higher permeation of small and large molecule drugs through laser-irradiated skin relative to tape-stripped skin, which implies that laser irradiation increases transdermal permeability by various mechanisms¹². Three mechanisms were reported to be involved in laser-assisted drug delivery, namely, (a) ablation or perforation of surface tissues, (b) generation of photomechanical waves, and (c) photothermal effects, which collectively disrupt the barrier function of the tissues¹³. The predominance of any of these mechanisms depends on laser parameters, including wavelength, power, and pulse duration. For instance, erbium-doped

yttrium aluminium garnet laser (Er: YAG) has a mid-infrared wavelength (2940 nm) which is strongly absorbed by water, resulting in localized water vaporization inside tissues and subsequently confined tissue ablation, with minimum thermal damage to adjacent tissues^{14–16}. Using a similar mid-infrared laser of wavelength 2790 nm, Nelson *et al.* showed that there was a linear correlation between the number of pulses and the depth of the created micropores¹². Pantec Biosolutions developed precise laser epidermal system (P.L.E.A.S.E.[®]), which has wide range of applications in transdermal delivery due to its ability to create micropores with controlled depth, density, and area¹⁷. For example, Bachhav *et al.* reported the presence of linear correlation between the cumulative permeation of diclofenac through porcine ear skin and the laser induced micropores produced by P.L.E.A.S.E.[®] device¹⁸. Similarly, Lee *et al.* investigated the permeability of fluorescein isothiocyanate (FITC)-labelled dextran through mouse and porcine skin after laser application by P.L.E.A.S.E.[®] device and reported an increased fluorescence in the skin after laser treatment compared to the control¹⁹.

On the other hand, controlled laser irradiation is widely used for medical applications in the eye, such as laser photocoagulation surgeries which are used for the treatment of age-related macular degeneration²⁰, open-angle glaucoma²¹, and diabetic retinopathy²⁰. Furthermore, laser ablation is used in vision correction²² and cataract surgeries²³. Laser irradiation was also applied in rabbit eyes to control the release of dexamethasone from intrachoroidal implant²⁴, and for activation of intraocularly injected virus-like particles for treatment of uveal melanoma²⁵.

Although laser irradiation has not yet investigated in ocular drug delivery, the wide ocular applications of laser represent good standing for the technology with respect its safety and feasibility as drug permeation-enhancer in the eye, provided that laser parameters are finely controlled. In this project, for the first time, fractional Er:YAG P.L.E.A.S.E.[®] was investigated for its potential to enhance drug permeation through corneal or the scleral tissues. Rhodamine

B (479 Da, Log P 1.8) was used as a model hydrophobic molecule; FITC-dextran (150 kDa, Log P -7.1) and FITC-labelled bovine serum albumin (BSA) (66.5 kDa) were used as a model for a large molecules.

2. Experimental section

2.1. Materials

Rhodamine B and FITC-BSA was purchased from Sigma-Aldrich (Steinheim, Germany). Phosphate buffer saline tablets (PBS, pH 7.4) was purchased from Oxoid (Hampshire, England). Fluorescein isothiocyanate–dextran Mw 150 kDa was purchased from TdB Consultancy AB (Uppsala, Sweden). Acetonitrile was purchased from Sigma-Aldrich (Gillingham, Dorset). Optimum cutting temperature compound was purchased from Sakura Fine technical (Tokyo, Japan). Other reagents not mentioned were of analytical grade and used as received. All aqueous solutions were prepared with ultrapure water (18.2 MΩ·cm, Millipore).

2.2. Laser irradiation of ocular tissues

Ocular tissues were prepared by removing the adherent muscle tissue from extracted porcine eye bulbs, and circumferentially cutting the anterior segment of the eye behind the limbus. The vitreous humor was removed, and both the anterior sclera and corneal tissues were isolated by removal of the underling tissues. The isolated tissues were kept in phosphate buffer saline (PBS, pH 7.4) containing 0.05% sodium azide and used within 24 h of extraction.

The laser was applied to the extracted corneal and scleral porcine tissues using P.L.E.A.S.E.[®] device (Pantec Biosolutions, Ruggell, Liechtenstein), operated at power 0.9 W and frequency

200 Hz, to produce micropore densities of 7.5 and 15% equivalent to 450 and 900 micropores, respectively. The laser fluences applied were 2.8, 8.9, 44.4, 88.9, 222.2, and 444.4 J/cm².

2.3. Characterization of laser-created micropores in the ocular tissues

Optical coherence tomography (OCT) was used to visualize the micropores created within 1 h after laser treatment. The swept-source Fourier domain OCT system has a laser center wavelength of 1305 ± 15 nm, facilitating real-time high-resolution imaging of the upper ocular tissue layers (7.5 μ m lateral and 10 μ m vertical resolution). 2D cross-sectional scans were done for the ocular tissues at frame rate of 15 scans/s and scan width of 2.0 mm. The micropores were characterized in terms of their depths and widths, using ImageJ[®] software. The results were reported as mean $\pm$ SD (n = 10). Furthermore, the laser-treated ocular tissues were imaged under scanning electron microscopy (SEM) at magnification power 100X.

Scleral and corneal tissues were also visualized using a Leica MZ6 dissection microscope fitted with a Nikon Coolpix 950 digital camera and TM3030 table top scanning electron microscope in order to evaluate the tissue effects of laser treatment.

2.4. In vitro permeation studies through ocular tissues

The *in vitro* permeation studies were conducted on the excised corneal and scleral tissues before and after laser irradiation using modified Franz-type diffusion cells in five replicates. *In vitro* permeation of Rhodamine B and FITC-dextran (150 kDa) were investigated through non-irradiated and laser-irradiated corneal and scleral tissues at fluences of 2.8, 8.9, and 44.4 J/cm² with a micropore density of 7.5%. Furthermore, the effect of micropore density (7.5 and 15%) was studied at a fixed laser fluence of 8.9 J/cm². The transscleral permeation of FITC-BSA was investigated through the scleral tissues before and after laser irradiation at

selected parameters i.e., laser fluence of 8.9 J/cm² and micropore density of 15%. The receptor compartments of the Franz cells were filled with 5 mL PBS containing 0.05% sodium azide, and stirred at 50 rpm and 37°C. The ocular tissues were hydrated in PBS at 37°C for 2 h, then mounted on the receptor compartments. After temperature equilibration, 200 µL solutions of either rhodamine B, FITC-Dextran, or FITC-BSA were added to the donor compartments, and the sampling arms and donor compartments were sealed using Parafilm[®]. After 24 h, samples of 200 µL volume were removed from the receptor compartments and analyzed for the concentrations of the permeated **molecules** using fluorescence plate reader. For assay of rhodamine B, the excitation and emission wavelengths were set at 544 and 620 nm, while they were set at 485 and 520 nm, for assay of FITC-dextran and FITC-BSA. Drug permeation was calculated as % of the amount of drug added to the donor compartments.

2.5. Drug distribution within ocular tissues

The scleral tissues that were exposed to laser of fluence 8.9 J/cm² and micropore density 15% were selected for further investigation of drug distribution relative to non-irradiated tissues. After completing the *in vitro* permeation studies (24 h), the selected tissues were placed into sample blocks containing optimum cutting temperature compound and were snap-frozen with liquid nitrogen to -20°C. The tissue samples were then sectioned into 50 µm thick pieces using a cryostat microtome and examined by confocal laser scanning microscope (CLSM).

2.6. Ocular tissues recovery

The recovery of the ocular tissues following laser irradiation was assessed by measuring the transscleral water loss (TSWL) or transcorneal water loss (TCWL) of the ocular tissues irradiated at laser fluences 8.9 and 44.4 J/cm² and micropore density 7.5% relative to the non-irradiated ones. Six replicates of each of the laser-irradiated and non-irradiated ocular tissues

were hydrated in PBS at 37°C for 2 h, and then mounted on the receptor compartments of modified Franz-type diffusion cells filled with 5 mL PBS / 0.05% sodium azide and stirred at 50 rpm and 37°C. Water loss was measured at time points of 0, 6, 24, and 48 h by mounting VapoMeter (Delfin Technologies; Kuopio, Finland) on the ocular tissues for 20 s. TCWL and TSWL were expressed as g/m².h and was plotted vs. time.

The scleral tissues recovery was also investigated by OCT measurement of micropores depths and diameters after laser-irradiation at fluence of 44.4 J/cm² and micropore density of 7.5%. The laser-irradiated scleral tissues were soaked in PBS / 0.05% w/v sodium azide, and kept in a shaking incubator at 50 rpm and 37°C. At time points of 0, 24, 48 and 72 h, the scleral tissues were removed from the soaking medium and subjected to OCT imaging using the same abovementioned procedure. Micropores depths and widths were measured using ImageJ® software, expressed as mean ± SD (n =10), and plotted vs. time.

2.7. Statistical analysis

GraphPad Prism® version 5.0 GraphPad Software was used to perform statistical analysis. Where appropriate, a Kruskal-Wallis test with *post-hoc* Dunn's test was used for comparison of multiple groups, with a Mann-Whitney U test performed for comparison of two groups. Data were expressed as means ± standard deviation. In all cases, statistical significance was defined at the standard 5 % level.

3. Results

3.1 Characterization of laser-created micropores in the ocular tissues

OCT and SEM imaging showed pore formation in the ocular tissues at laser fluences of 8.9 and 44.4 J/cm², whereas exposure to laser of fluences ≥ 88.9 J/cm² led to severe disruption of the ocular tissues (**Fig. 1-2**). Both micropores depths and widths formed in the scleral tissue were greater than those formed in the corneal tissues at each applied fluence. The depths of the created micropores within the ocular tissues increased linearly with increasing laser fluence in the range of 2.8 – 444.4 J/cm² at micropore density of 7.5% (**Fig. 3**), while the widths of the created micropores were linearly correlated to the log laser fluence.

3.2 In vitro permeation studies through ocular tissues

In the non-irradiated ocular tissues, the % permeation values of rhodamine B were 4.5 and 15.6-fold those of FITC-dextran in scleral and corneal tissues, respectively (**Fig. 4**). Comparing the permeation of **the molecules** through non-irradiated corneal and scleral tissues, rhodamine B showed higher permeation through the corneal tissues, whereas FITC-dextran exhibited higher permeation through scleral tissues. At micropore density of 7.5%, rhodamine B showed its maximum transscleral permeation ($45.8 \pm 9.1\%$) at laser fluence 8.9 J/cm², which was significantly higher than the non-irradiated scleral tissue ($16.1 \pm 3.8\%$) at $p \leq 0.05$. Increasing the laser fluence > 8.9 J/cm² did not increase rhodamine B % transscleral permeation. Increasing the micropore density until 15% at laser fluence 8.9 J/cm², resulted in significant increase of rhodamine B transscleral permeation to $77.5 \pm 15.8\%$ ($p \leq 0.05$). Similarly, FITC-dextran showed its maximum transscleral permeation ($21.9 \pm 6.9\%$) at laser fluence 8.9 J/cm² and micropore density 15%, yet, we did not detect significant increase in permeation on increasing micropore density from 7.5 to 15% ($p > 0.05$). However, the transscleral permeation of FITC-dextran did not show any significant improvement on increasing micropore density from 7.5 to 15% ($p > 0.05$). FITC-BSA did not show detectable permeation through the non-irradiated scleral tissues within 24 h, whereas it showed 4.6%

permeation through the scleral tissues irradiated with laser of fluence 8.9 J/cm² and micropore density 15%.

On the other hand, at micropore density of 7.5%, rhodamine B showed its maximum transcorneal permeation ($41.2 \pm 7.6\%$) at laser fluence 44.4 J/cm². Exposing the corneal tissues to laser of micropore density 15% and fluence of 8.9 J/cm² resulted in rhodamine B transcorneal permeation of $42.3 \pm 2.6\%$, which was significantly higher than that obtained using laser of micropore density 7.5% at the same fluence ($p \leq 0.05$). In contrast, the transcorneal permeation of FITC-dextran did not show significant increase on increasing the laser fluence > 2.8 J/cm² or increasing the micropore density $> 7.5\%$ ($p > 0.05$). The maximum transcorneal permeation of FITC-dextran was obtained at laser fluence 8.9 J/cm² and micropore density of 15% ($6.4 \pm 3.1\%$).

3.3 Drug distribution within ocular tissues

The CLSM showed higher permeation of rhodamine B, FITC-dextran, and FITC-BSA in the laser-irradiated scleral tissues relative to the non-irradiated ones (**Figure 5**).

3.4 Ocular tissues recovery

As shown in **Figure 6**, TSWL increased significantly ($p \leq 0.05$) by 44 – 51% after laser irradiation at fluences 8.9 and 44.4 J/cm². TSWL returned to baseline within 24 h, with associated decrease in micropores depths and widths measured under OCT (**Fig. 7**). On the other hand, TCWL showed a significant increase ($p \leq 0.05$) by 70 – 78% after laser irradiation at fluences 8.9 and 44.4 J/cm², yet, water loss from corneal tissues did not return back to the baseline values after 48 h (**Fig. 6**).

Discussion

This study showed that laser-irradiation enhanced permeation of both small and large molecules through corneal and scleral tissues. Permeation of **the molecules** through ocular tissues was affected by several factors including the physicochemical properties of the molecules, type of the tissue (cornea vs. sclera), as well as laser parameters. For instance, the small molecule hydrophobic rhodamine B showed higher permeation through both corneal and scleral tissues relative to the large molecule hydrophilic FITC-dextran. Furthermore, the permeation of rhodamine B and FITC-dextran through non-irradiated corneal tissues relative to scleral tissues complied with the findings of Prausnitz and Noonan literature analysis, which showed that small molecule hydrophobic drugs tended to show higher permeation through corneal tissues, whereas large molecule hydrophilic drugs generally showed higher permeation through scleral tissues²⁶. At same laser parameters, the micropores created in the scleral tissues were deeper and wider than those created in the corneal tissues, which may be attributed to the more ordered arrangement of the collagen fibrils in corneal tissues (Fig. 8) and the subsequent higher resistance for laser-induced ablation²⁷.

The transscleral permeation of both rhodamine B and FITC-dextran showed maximum increase at laser fluence 8.9 J/cm^2 , which may be due to the photothermal effects causing blockage of the micropores at higher fluences. On the other hand, increasing micropore density from 7.5% to 15% resulted in increasing the transscleral permeation of rhodamine B but not FITC-dextran, which may be attributed to the higher Mw of the latter. The transcorneal permeation did not follow the same pattern, where rhodamine B showed higher permeation on increasing either laser fluence or micropore density, while the permeation of FITC-dextran was not affected by either. This may be explained by the effect of different laser fluences on different corneal layers. While the corneal epithelium presents permeation barrier to all types of drugs, the underlying stroma and endothelium mainly control the permeation of small

molecule hydrophobic drugs²⁶. At relatively low laser fluences $\leq 8.9 \text{ J/cm}^2$, the corneal epithelium was ablated which led to increased permeation of both small molecule hydrophobic rhodamine B and large hydrophilic FITC-dextran. At higher laser fluences, the laser ablative effect may have extended to deeper layers of the cornea which affected the permeation of rhodamine B to a greater extent. This also can be attributed to the lower response of FITC-dextran permeability to laser parameters due to its large Mw. The lack of recovery of TCWL to baseline within 48 h indicates non-healing of the corneal micropores. In contrast, the recovery of TSWL to baseline within 24 h is evidence of healing of the scleral micropores, which was confirmed by decrease of micropore dimensions observed under OCT.

Several studies in the literature reported analogous results on applying P.L.E.A.S.E.[®] device on skin to enhance transdermal drug delivery. Although the differences in histological structure between *stratum corneum* and ocular tissues invalidate comparison between laser-assisted transdermal and ocular drug delivery, the results obtained from previous transdermal studies can support the conclusions drawn from the current study, particularly in absence of previous similar work on laser-assisted ocular drug delivery. Bachhav *et al.* applied P.L.E.A.S.E.[®] device to enhance the transdermal permeation of lidocaine (Mw 234, Log P 2.4), where the authors reported enhanced transdermal permeation at low laser fluences and micropore densities, yet they did not observe any significant difference in permeation at laser fluences $22.7 - 135.9 \text{ J/cm}^2$ or micropore densities $> 7.5\%$, which was attributed to probable drug depletion¹⁷. Similarly, Lee *et al.* used P.L.E.A.S.E.[®] device to enhance the transdermal permeation of 5-aminolevulinic acid (Mw 131, Log P -1.5) at fluence 2 J/cm^2 which produced partial ablation of the stratum corneum²⁸. The authors reported that increasing the number of laser applications did not increase the permeation significantly and concluded that laser enhanced the transdermal permeation by other mechanisms beside the direct ablation of the superficial skin layer. Similar to TSWL and TCWL, the transepidermal water loss (TEWL) has

been commonly investigated as a measure of the skin barrier function. Machado *et al.* validated the hypothesis that TEWL is directly proportional to the reciprocal of the diffusional permeation path length of the *stratum corneum*²⁹. Lee *et al.* also investigated the TEWL as a measure of the integrity of the skin barrier function after laser application²⁸.

FITC-BSA was used as a model for anti-VEGF proteins conventionally administered as intravitreal injections, hence, FITC-BSA permeability was investigated only in scleral tissues. The transscleral permeation of FITC-BSA was investigated only at laser fluence 8.9 J/cm² and micropore density 15%, as these were the parameters showing highest permeation for both FITC-dextran and rhodamine B. The same laser parameters were also selected for CLSM investigation of distribution of different **molecules** in scleral tissues. Transcorneal and transscleral permeation of rhodamine B and FITC-dextran were investigated at laser fluences ≤ 44.4 J/cm², as higher fluences showed disruption of ocular tissues, and would be patient inconvenient.

In conclusion, both small molecule hydrophobic and large molecule hydrophilic model **molecules** showed enhanced permeation across scleral and corneal tissues upon fractional laser irradiation using P.L.E.A.S.E.[®] device. The optimum laser fluence and micropore density differed according to the physicochemical properties of the drug and the irradiated ocular tissues (cornea vs. sclera). The laser-created micropores showed recovery only in the scleral tissues under the study conditions. Fractional laser irradiation is a promising technique to enhance corneal and scleral permeability of different drugs, yet further *in vivo* preclinical studies should be carried out.

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Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

Figure legends

Figure 1. OCT and SEM images of scleral tissue before and after laser treatment at different fluences (J/cm^2) and at a micropore density of 7.5%. Scale bars for OCT images is 1 mm.

Figure 2. OCT and SEM images of corneal tissue before and after laser treatment at different fluences (J/cm^2) and at a micropore density of 7.5%. Scale bars for OCT images is 1 mm.

Figure 3. (a) Depths and (b) widths of the laser-created micropores at different laser fluences (mean $\pm$ SD, $n = 10$).

Figure 4. Transscleral and transcorneal permeation of rhodamine B and FITC-dextran at (a) different laser fluences at micropore density 7.5% and (b) different micropore densities at laser fluence $8.9 \text{ J}/\text{cm}^2$ (mean $\pm$ SD, $n = 5$).

Figure 5. Transscleral distribution of rhodamine B, FITC-Dextran, and FITC-BSA in non-irradiated and laser-irradiated scleral tissues at fluence $8.9 \text{ J}/\text{cm}^2$ and micropore density 15%.

Figure 6. Water loss from non-irradiated and laser-irradiated (a) scleral and (b) corneal tissues (mean $\pm$ SD, $n = 6$).

Figure 7. (a) Micropore dimensions (mean $\pm$ SD, $n = 10$) and (b) OCT images of scleral tissues after laser treatment at fluence $44.4 \text{ J}/\text{cm}^2$ and micropore density 7.5%. Scale bars for OCT images is 1 mm.

Figure 8. SEM images of collagen fibril arrangement in the (a) cornea and (b) sclera. (27).