Topical Delivery of Methotrexate Via Skin Pretreated With Physical Enhancement Techniques: Low-Fluence Erbium:YAG Laser and Electroporation

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Background and Objective: The high hydrophilicity and molecular weight of methotrexate (MTX) make it difficult to deliver via the skin route for treating psoriasis or rheumatoid arthritis. The objective of this study was to enhance and optimize the skin permeation of MTX using two physical techniques: an erbium:yttrium-aluminum-garnet (Er:YAG) laser and electroporation.

Methods: In vitro skin permeation was performed using horizontal side-by-side diffusion cells. The animal model utilized nude mice. The skin where epidermal hyperproliferation was reproduced by repeated barrier abrogation was also used as a permeation barrier for MTX delivery.

Results: Application of the laser and electroporation significantly enhanced the permeation of MTX. The enhancing effect was more pronounced after applying the laser. Er:YAG laser pretreatment on the skin produced a 3- to 80-fold enhancement dependent upon the magnitude of the laser fluence. Using electroporation, treatment with 10 pulses resulted in a twofold increase in MTX flux. A combination of laser pretreatment and subsequent electroporation for 10 minutes resulted in a higher drug permeation than either technique alone. However, this synergistic effect was only observed when the lower laser fluence (1.4 J/cm^2) was applied. Hyperproliferative skin generally showed a greater variability of MTX flux and lower permeation.

Conclusion: The results shown in the present study encourage further investigation of laser- and electroporation-assisted topical drug delivery. Lasers Surg. Med. 40:468–476, 2008. © 2008 Wiley-Liss, Inc.

Key words: methotrexate; topical delivery; Er:YAG laser; electroporation; hyperproliferation

INTRODUCTION

Psoriasis is one of the most common human skin diseases. It is characterized by excessive growth and aberrant differentiation of keratinocytes, but is fully reversible with appropriate therapy [1,2]. Methotrexate (MTX) is a folic acid antagonist used for treating all forms of psoriasis. It inhibits DNA synthesis, acting primarily at the S-phase of the cell cycle. MTX acts against psoriasis by blocking epidermal mitosis [3,4]. Other than its use in psoriasis, MTX is frequently employed as an immune suppressant in the treatment of rheumatoid arthritis (RA) [5]. When taken orally, the uptake of MTX by the gastrointestinal tract is limited due to the saturation of the transporter, reduced folate carrier 1 (RCF1). The systemic use of this drug can also cause hepatotoxicity, bone marrow suppression, abdominal distress, and nausea [5,6]. Topical MTX delivery has been proposed to prevent these side-effects.

A major problem with topical MTX is that this drug is water soluble, has a high molecular weight (454.56 Da), and mostly occurs in the dissociated form at a physiological pH. Its capacity for passive diffusion across the stratum corneum (SC) is thus limited [4,7]. To overcome the barrier properties of the SC, techniques such as iontophoresis, electroporation, or the use of vehicles such as microemulsions or liposomes have been explored to enhance transdermal MTX delivery [8]. However, there is still a need to develop novel systems that can be applied to enhance MTX permeation. Partial removal of the SC by mechanical abrasion, tape-stripping, or chemical treatment may significantly increase skin permeation of hydrophilic molecules. These approaches may be limited due to the lack of control and reproducibility, as well as their potential to cause irritation. We recently suggested that the erbium:yttrium-aluminum-garnet (Er:YAG) laser can effectively enhance and precisely control drug delivery via the skin, especially of hydrophilic drugs and macromolecules [9–12]. The use of an Er:YAG laser at low fluences can safely, painlessly, and rapidly enhance drug absorption [10,13].

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The aim of the present study was to assess the feasibility of topical MTX delivery using an Er:YAG laser, electroporation, and their combination. Both techniques are physical methods with the advantages of rapid pretreatment and no interaction with the drug being delivered. The laser can ablate a determined layer of SC and irradiate a photomechanical wave to enhance drug permeation into or across the skin [9,13]. Electroporation involves the application of high-voltage pulses which produce transient pores within the lipid bilayers of the SC, that partially account for the increase in skin permeability [14]. In this study, the effects of laser and electroporation alone and in combination on MTX permeation were evaluated in an in vitro diffusion cell model. The mechanisms underlying the enhanced drug permeation by both techniques are also discussed in the present work. Moreover, hyperproliferative skin was induced and used as a skin barrier for MTX permeation in order to mimic the clinical situation.

MATERIALS AND METHODS

Preparation of Skin Membranes

Female nude mice (ICR-Foxn1nu strain, 8 weeks old) were sacrificed using ether, and full-thickness skin was excised from the dorsal region. To obtain SC-stripped skin, adhesive tape was applied to the skin with uniform pressure and then removed. This procedure was repeated 20 times. Delipidized skin was prepared by pretreating the skin surface with chloroform-methanol (2:1, v/v) for 60 minutes in order to extract the lipids from the SC. The cellulose membrane (Cellu-Sep® T2, with a molecular weight cutoff of 6,000–8,000) used for the permeation experiments was supplied by Membrane Filtration Products (Seguin, TX).

Er:YAG Laser Assembly

The Er:YAG laser (Continuum Biomedical, Santa Clara, CA) used here has a wavelength of 2,940 nm and a pulse duration of 250 microseconds. An articulated arm was used to deliver the laser beam onto the skin. Output energies of 0.55–0.75 J with a beam spot diameter of 7 mm achieved fluences of 1.4–1.9 J/cm^2. The energy of the laser pulse was monitored with an energy meter (Nova Display, Ophir, Israel) before and after treatment. Before the in vitro permeation experiments, the laser hand-piece was located 3.7 cm from the skin surface. After laser irradiation, the skin surface was wiped with a cotton wool swab several times. Black polystyrene target material was positioned on the skin when applying laser irradiation in the experiment to irradiate a pure photomechanical wave from the laser. Thus the ablation effect on SC by this laser was ignored.

Electroporation Assembly

Electroporation was performed using an exponential decay pulse generator (Electro Cell Manipulator 630, Genetronics, San Diego, CA). Platinum electrodes (0.5×1.5 cm) were used, each of which was located 3 cm from the skin in the horizontal diffusion cells. The cathode was positioned in the donor compartment, while the anode was in the receptor compartment. The electroporation protocol consisted of one pulse per 30 seconds, applied for 10 minutes. The pulse voltage was 300 V, and pulse length was 200 milliseconds. Voltages were expressed as a directly applied value, not as transdermal values. Since partial voltages may be consumed when applied across the skin.

In Vitro Permeation of MTX

The in vitro skin permeation experiments were performed using horizontal side-by-side diffusion cells. The donor medium was 8 ml of pH 7.4 citrate-phosphate buffer; and the MTX (Sigma–Aldrich Chemical, St. Louis, MO) concentration in the donor compartment was 0.1% (w/v). The receptor medium was 8 ml of pH 7.4 buffer. The excised skin with or without laser pretreatment was mounted between the donor and receptor compartments. The electroporation pulses were then applied for 10 minutes if necessary. The available diffusion area between cells was 0.785 cm^2. The stirring rate and temperature were kept at 600 rpm and 37°C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh buffer. The amount of MTX was determined by a high-performance liquid chromatographic (HPLC) method.

The amount of MTX retained in the skin was examined at the end of the in vitro experiments (24 hours). The application site on the skin was washed 10 times using a cotton cloth immersed in double-distilled water. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of 0.1 N HCl, and ground for 5 minutes with an electric stirrer (300 rpm). The resulting suspension was centrifuged for 10 minutes at 10,000 rpm. The supernatant was analyzed by HPLC. The weight of each skin piece was used to calibrate the drug amount in the skin in ng/mg.

HPLC Analysis of MTX

The HPLC system included a Hitachi L-2130 pump, a Hitachi L-2200 sample processor, and a Hitachi L-2400 UV detector (Tokyo, Japan). A 25-cm-long, 4-mm inner diameter stainless steel RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile: double-distilled water at pH 2.7 adjusted with phosphoric acid (15:85) at a flow rate of 1 ml/minute. The UV detector was set to a wavelength of 303 nm.

Induction of Hyperproliferative Skin for In Vitro Skin Permeation

Epidermal hyperproliferation simulating psoriasis-affected skin was achieved by a tape-stripping technique [15]. Female nude mice (8 weeks old) were used in this study. The dorsal skin of a mouse was stripped using cellophane tape (3 M Scotch®) twice daily for 5 days. After 5 days, the treated skin was monitored by examining the transepidermal water loss (TEWL) with an evaporimeter (TM300, Courage and Khazaka, Köln, Germany) twice daily. When the TEWL values were below 8–10 g/m^2/hour, the skin was excised for in vitro permeation experiment.

Hyperproliferation of the skin was verified by histology. Each specimen was dehydrated using ethanol, embedded in paraffin wax, and stained with hematoxylin and eosin. For each skin sample, three different sites were examined and evaluated under light microscopy (Eclipse 4000, Nikon, Tokyo, Japan). The digital photomicrographs were then processed with Adobe PhotoDeluxe (Adobe Systems, San Jose, CA), and the epidermal thickness was calculated using ImagePro-plus 4.0 (Media Cybernetics, Silver Spring, MD).

Statistical Analysis

Statistical analysis of differences between different treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. An analysis of variance (ANOVA) test was also used.

RESULTS

MTX Permeation With Low-Fluence Er:YAG Laser Pretreatment

In vitro permeation experiments were performed to evaluate the effect of the low-fluence Er:YAG laser on the topical delivery of MTX. The cumulative amounts of MTX ($\mu g/cm^2$) as a function of time after laser treatment at various energy levels are shown in Figure 1. The slopes of the resulting plots were computed, and fluxes ($\mu g/cm^2/h$) were calculated from the slopes as summarized in Table 1. MTX exhibited very low permeation by passive diffusion (without laser treatment). As expected, the application of the laser considerably increased permeation into the receptor medium ($P<0.05$). An increase in the laser intensity from 1.4 to 1.9 J/cm^2 led to further promotion of MTX permeation. Laser exposure treated a limited portion of 49% of the effective diffusion area of the skin. Extrapolating the original flux data of the laser-irradiated area to 100% exposure (normalized flux) resulted in respective enhancement ratios (ERs) of 2.8–81.1 depending on the fluence used (Table 1). It is assumed that the laser beam showed a uniform irradiation on the skin for simplifying the calculation of the normalized flux. A Gaussian effect is thus neglected. After each in vitro permeation experiment, the drug was extracted from the skin to determine the MTX deposition in the skin. The same as with the flux, the increment of laser fluence increased the drug amount which resided in the skin. However, the magnitude of the enhancement of skin deposition was lower than that of flux enhancement.

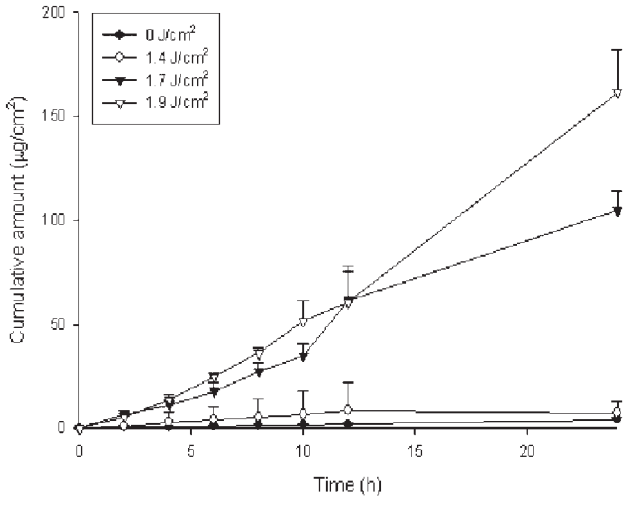

Fig. 1. In vitro cumulative amount-time profiles of the topical delivery of methotrexate (MTX) by Er:YAG laser pretreatment of the skin at fluences of 1.4 and 1.7 J/cm^2. Each value represents the mean ± SD ($n = 4$).

In order to elucidate the mechanisms involved in the topical delivery of MTX by the laser, a permeation study was performed using various skin membranes. Figure 2 shows that the drug flux permeating via SC-stripped skin was 49.2-fold higher ($P<0.05$) than that via intact skin. Delipidized skin also showed greater permeation for MTX; however its ER (30.6) did not reach the level of the SC-stripped group. The diffusion of MTX across the cellulose membrane showed that MTX release was significantly higher ($P<0.05$) than that across SC-stripped skin. No statistically significant difference ($P > 0.05$) was observed for the normalized flux via 1.9-J/cm^2-pretreated skin and the cellulose membrane.

MTX Permeation by a Photomechanical Wave (PW) Generated From the Er:YAG Laser

To explore the mechanisms of the Er:YAG laser on topical drug delivery, a photomechanical wave (PW) was generated by laser ablation of a polystyrene target and then launched into the skin. In this experimental arrangement, the laser's radiation was totally absorbed by the target so that only the PW reached the skin. As shown in Table 1, a PW of 1.7 J/cm^2 did not increase the permeation of MTX compared to the non-treated group ($P > 0.05$). When the laser fluence was raised to 3.7 J/cm^2, there was still no significant difference ($P > 0.05$) between the original flux with the PW and the untreated group. As demonstrated in Table 1, the PWs at 1.7 and 3.7 J/cm^2 increased ($P<0.05$) the skin deposition although they were ineffective in enhancing the flux.

MTX Permeation by Electroporation Pretreatment

In the experiments with electroporation pretreatment, the skin was subjected to 10 or 20 pulses each of 200-milliseconds duration of 300 V. MTX is negatively charged at the physiological pH in skin tissue. The cathode was first positioned in the donor compartment (negative polarity) to examine the effect of electroporation on MTX permeation. As shown in Table 2, applying 10 pulses to the skin with negative polarity resulted in a transport similar to the passive diffusion control ($P > 0.05$). The application of 20 pulses improved the flux by 2.2-fold over passive diffusion of from 0.17 to 0.38 $\mu g/cm^2$/hour ($P<0.05$). Although this enhancement in the MTX flux was relatively lower ($P<0.05$) than that with the Er:YAG laser, electroporation

TABLE 1. Comparison of Methotrexate Fluxes, Enhancement Ratios, and Skin Deposition Via Nude mouse Skin by Pretreatment With Er:YAG Laser

Fluence (J/cm^2)	Original flux (μg/cm^2/hour)[a]	Normalized flux (μg/cm^2/hour)[b]	Enhancement ratio (ER)[c]	Skin deposition (ng/mg)
0 (control)	0.17 ± 0.05	0.17 ± 0.05	1	4.20 ± 1.35
1.4	0.32 ± 0.07	0.48 ± 0.11	2.8	36.70 ± 5.37
1.7	4.57 ± 0.47	9.16 ± 0.94	53.9	42.09 ± 6.93
1.9	6.84 ± 0.97	13.79 ± 1.96	81.1	80.04 ± 14.15
1.7 + filter	0.17 ± 0.04	—	—	33.13 ± 15.99
3.7 + filter	0.13 ± 0.11	—	—	54.37 ± 8.82

—, no significant difference as compared to the original flux of the control (0 J/cm^2).
Each value represents the mean ± SD ($n = 4–7$).
[a]Original flux was calculated directly from the flux across partly laser-pretreated skin.
[b]Normalized flux was calculated from the flux across fully laser-treated skin (100%), which was calibrated by flux of control group (without laser pretreatment).
[c]Enhancement ratio (ER) was normalized flux of laser-pretreated group/flux of control group.

caused greater skin deposition compared to the laser at 1.4 and 1.7 J/cm^2 ($P<0.05$). The influence of the polarity of the electrodes was also examined. No significant enhancement ($P > 0.05$) in MTX flux was induced by electroporation from the anode to the cathode (positive polarity) compared with passive diffusion (Table 2). The skin deposition with positive polarity was lower ($P<0.05$) than that with negative polarity.

MTX Permeation by Combined Laser/Electroporation Pretreatment

Since the enhancement mechanisms for the laser and electroporation differed, higher drug permeation could be expected with laser treatment prior to applying electroporation. Figure 3 depicts the flux and skin deposition of MTX after a combination of laser pretreatment and electroporation. Laser fluences of 1.4 and 1.7 J/cm^2 were used in this experiment, since a fluence of 1.9 J/cm^2 may produce relevant skin disruption. The combination of 1.4 J/cm^2 and 10 electroporation pulses increased the flux 5.0- and 6.6-fold respectively, over transport by the laser and electroporation alone ($P<0.05$). With 20 pulses, laser pretreatment at 1.4 J/cm^2 prior to electroporation consistently yielded a 2.8-fold higher flux compared to electroporation alone ($P<0.05$). This indicates a synergistic effect was achieved with this combination. However, a combination of electroporation and laser at 1.7 J/cm^2 did not deliver greater drug transport than did laser pretreatment alone ($P > 0.05$).

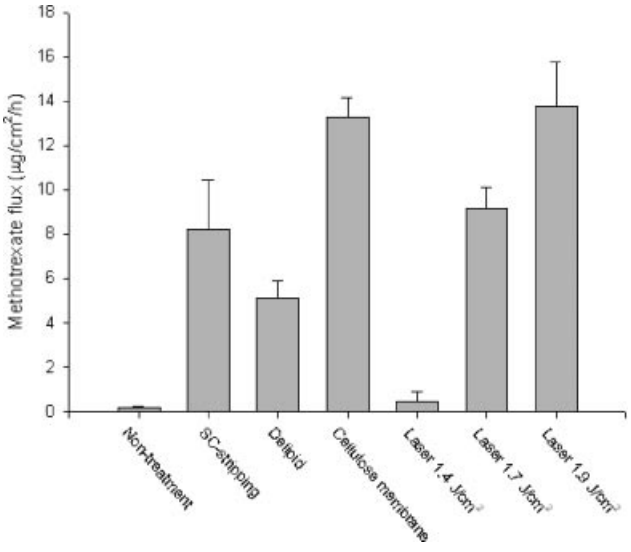

Fig. 2. Methotrexate (MTX) flux via various skin membranes and skin pretreated with an Er:YAG laser at fluences of 1.4 and 1.7 J/cm^2. Each value represents the mean ± SD ($n = 4$).

The combination of the laser at 1.4 J/cm^2 and electroporation increased the amount of MTX remaining in the skin compared to laser pretreatment alone. The same phenomenon was observed with the combination of the laser at 1.7 J/cm^2 and 20 pulses ($P<0.05$). But the skin deposition with the combination still did not reach the level with electroporation alone, although the statistical analysis generally revealed no significant difference ($P > 0.05$).

MTX Permeation Via Hyperproliferative Skin

MTX is extensively used for treating hyperproliferative skin diseases. Figure 4A,B shows representative examples of light microscopic images of vertical skin sections with and without hyperproliferative induction. The control sample showed a well-defined SC, epidermis, and appendages (Fig. 4A). The different layers were clearly visible in the control skin with the SC attached to the epidermis. As shown in Figure 4B, the epidermal thickness was increased by the tape-stripping technique for thickening the epidermis. The epidermal thickness, defined as the distance from the basal layer to the stratum granulosum/SC junction [15], was measured. The estimated thicknesses with and without tape-stripping were 29.6 ± 6.4 and 16.3 ± 3.3 μm, respectively. Routine light microscopy confirmed predominant changes in the psoriasis-like skin. The flux decreased

TABLE 2. Comparison of Methotrexate Fluxes and Skin Deposition Via Nude Mouse Skin by Pretreatment With Electroporation

Pulse number	Electrode direction	Flux (μg/cm^2/hour)	Skin deposition (ng/mg)
10	Cathode → anode	0.25 ± 0.11	86.26 ± 5.15*
20	Cathode → anode	0.38 ± 0.18*	82.04 ± 5.04*
10	Anode → cathode	0.12 ± 0.03	44.25 ± 13.24*
20	Anode → cathode	0.12 ± 0.05	31.20 ± 7.91

Each value represents the mean ± SD ($n = 4–6$).
*$P < 0.05$ as compared to the control (non-treated group).

by a factor of 1.9 compared with normal skin, although no significant statistical difference ($P > 0.05$) was detected.

The normal and hyperproliferative skin samples were exposed to a low-fluence laser at 1.7 J/cm^2 for light microscopic examination (Fig. 4C,D). Some SC fragments remained on the skin surface, although most of the SC layer was ablated by the laser. The histological photographs demonstrate that there were no observable changes in structural features of the epidermis or dermis after laser irradiation. The application of the laser at 1.4 J/cm^2 yielded 7.6-fold higher flux as shown in Table 3. This enhancement was greater than that with normal skin. In contrast to the fluence of 1.4 J/cm^2, psoriasis-like skin treated with 1.7 J/cm^2 produced lower enhancement than did normal skin. An increase in skin deposition ($P<0.05$) was observed after laser application to hyperproliferative skin.

The MTX flux increased 5.9-fold with 10 pulses of electroporation. The same as with the control skin, hyperproliferative skin treated with the combination exhibited synergistic drug flux. Nevertheless, the skin deposition into hyperproliferative skin treated with either the laser or electroporation was significantly lower ($P<0.05$) than that of normal skin.

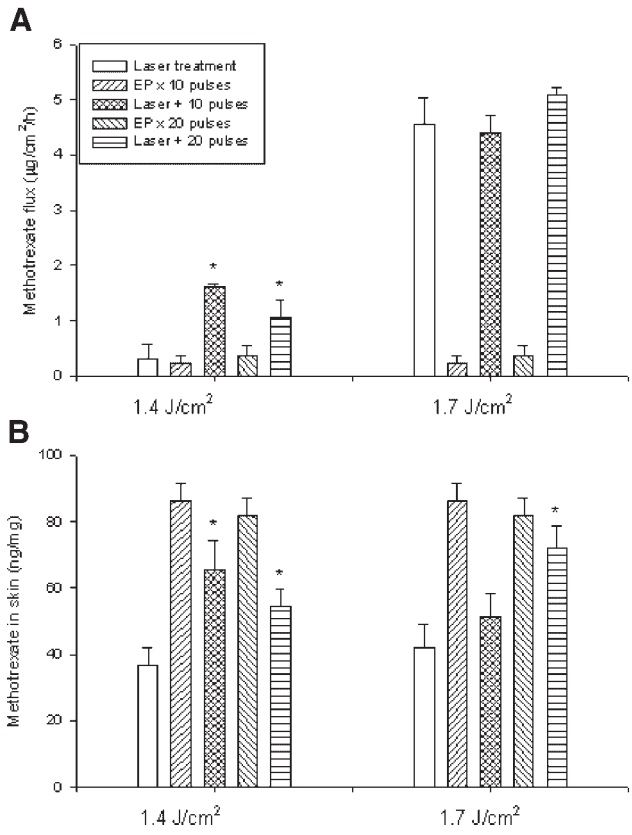

Fig. 3. Methotrexate (MTX) flux (**A**) and skin deposition (**B**) via skin with pretreatment with an Er:YAG laser and electroporation alone or in combination. Each value represents the mean ± SD ($n = 4$). * The value of laser/electroporation combination was significantly higher ($P<0.05$) compared to the value of laser treatment alone.

DISCUSSION

MTX is a polar compound due to the presence of a glutamic acid moiety in its structure. Being water-soluble and negatively charged, MTX is not readily transported into the skin by passive diffusion. The clinical response is also limited when MTX is topically applied [16]. In the present study, we used two physical techniques, a low-fluence Er:YAG laser and electroporation, to enhance the skin permeation of MTX. The results showed that laser and electroporation pretreatment either alone or in combination significantly increased MTX delivery via the skin. These methods were also effective when psoriasis-like skin was used as a permeation barrier, suggesting their future application for in vivo and clinical situations.

The most reliable skin absorption data are collected from human studies. However, such studies are generally not feasible during the initial development of a novel dosage form or system. The availability of such systems is also limited. The skin of rodents is most commonly used for in vitro and in vivo skin permeation studies. There are a number of hairless species (e.g., nude mice and hairless rats) in which the absence of hair coat mimics the human skin better than hairy skin [17]. Hence nude mice were used as an animal model in this study. Although nude mouse skin is more permeable than human skin [18,19], it is still a good model for examining the skin transport of permeants because of the limited variability among individuals and similar hair follicle density to human skin.

The rate-limiting step for MTX uptake into the skin is at the level of the SC. The Er:YAG laser is an ablative tool for the SC with the capability of precise control. Partial ablation of the SC by the low-fluence laser reduced the inherent barrier properties of the skin to MTX and thus enhanced skin permeation. With ablation, laser radiation

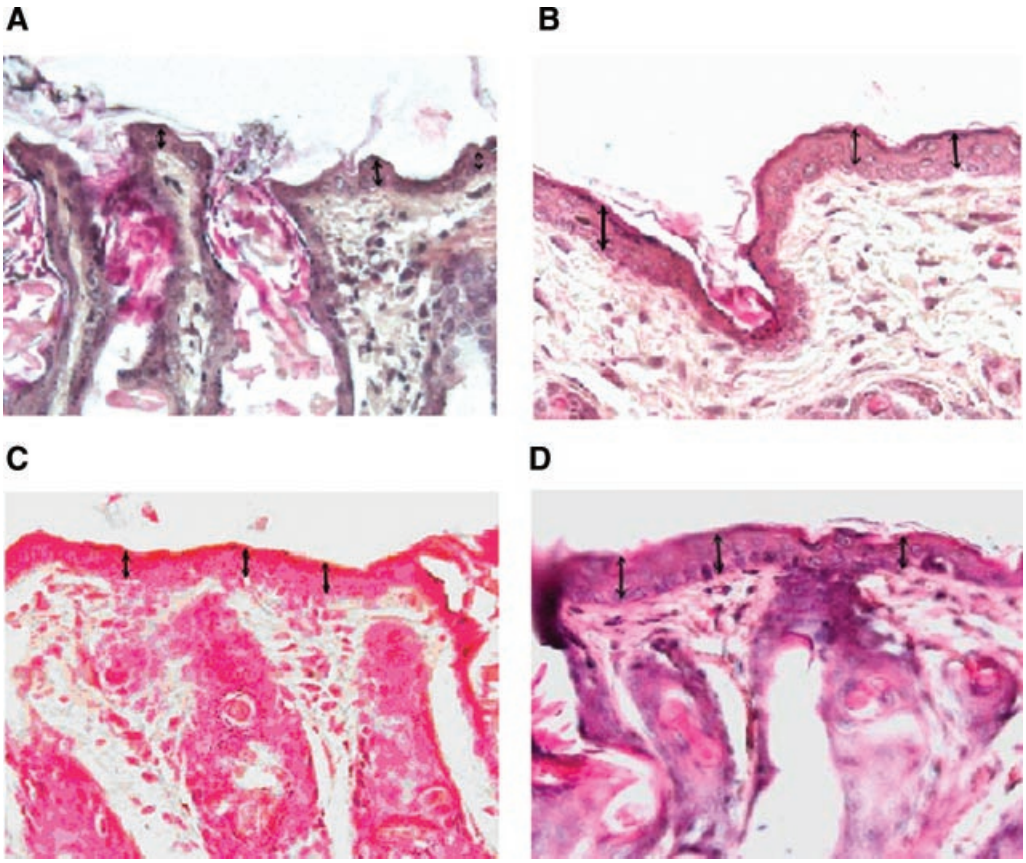

Fig. 4. Histological examination of nude mouse back skin: non-treated control skin (**A**), skin subjected to a tape-stripping technique (**B**), laser-treated control skin at 1.7 J/cm^2 (**C**), and laser-treated hyperproliferative skin at 1.7 J/cm^2 (**D**). Arrows indicate the thickness of the epidermis (magnification 400×).

causes decomposition of the target material into smaller fragments, which move away from the surface of the target at supersonic speed [20]. Significant changes in the skin's structure with the higher laser fluence lead to higher enhancements of MTX permeation. As depicted in our previous studies [10,12], the etched thickness of the SC after laser ablation appears to be proportional (correlation coefficient, $r = 0.95$) to the fluence used.

TABLE 3. Comparison of Methotrexate Fluxes, Enhancement Ratios, and Skin Deposition Via Hyperproliferative Skin by Pretreatment With Er:YAG Laser

Fluence (J/cm^2) or EP pulse number	Original flux (μg/cm^2/hour)[a]	Normalized flux (μg/cm^2/hour)[b]	Enhancement ratio (ER)[c]	Skin deposition (ng/mg)
0 (control)	0.09 ± 0.06	0.09 ± 0.06	1	4.42 ± 3.61
Laser 1.4	0.38 ± 0.19	0.68 ± 0.34	7.6	28.28 ± 9.47
Laser 1.7	1.79 ± 0.32	3.56 ± 0.63	39.6	25.53 ± 3.97
EP × 10	0.53 ± 0.13	—	5.9[d]	15.21 ± 3.21
Laser 1.4 + EP × 10	0.93 ± 0.24	—	10.3[d]	30.85 ± 8.80

—, not determined.
Each value represents the mean ± SD ($n = 4$).
[a]Original flux was calculated directly from the flux across partly laser-pretreated skin.
[b]Normalized flux was calculated from the flux across fully laser-treated skin (100%), which was calibrated by flux of control group (without laser pretreatment).
[c]Enhancement ratio (ER) was normalized flux of laser-pretreated group/flux of control group.
[d]The ER calculated for the group with electroporation was original flux/flux of control group.

The molecular weight cutoff value for the cellulose membrane was 6,000–8,000, and thus drug molecules can freely diffuse across it. The higher flux for MTX through the cellulose membrane demonstrates that the skin indeed exhibits barrier properties in these permeation studies. The passive diffusion of MTX across the membrane was significantly higher ($P<0.05$) than that across SC-stripped skin, indicating that the SC layer as well as viable epidermis/dermis contribute to the barrier function against MTX delivery. Since there are no lipid bilayers present in the SC of delipidized skin, drug transport via delipidized skin should indicate the importance of the barrier property of intercellular pathways rather than non-lipoidal pathways. The flux via delipidized skin was greater ($P<0.05$) than that via intact skin but slightly lower than that of SC-stripped skin. This indicates that lipid bilayer pathways play a predominant role in MTX diffusion, but the role of non-lipoidal (transcellular) routes also cannot be ignored.

The Er:YAG laser at 1.9 J/cm^2 had almost the same effect ($P > 0.05$) on MTX permeation as was seen across a cellulose membrane. This suggests that the laser can totally overcome the barrier function of the skin against MTX. Moreover, the laser at 1.9 J/cm^2 not only ablated the SC layer but also disrupted viable skin. Another observation was that the slopes of the in vitro permeation profile with 1.9-J/cm^2 irradiation gradually increased with time (Fig. 1). This indicates that drug permeation accelerated at the latter stage of application. A possible reason is disruption of the skin after a long application duration. A laser fluence of 1.7 J/cm^2 might not disrupt viable skin since its flux was comparable ($P > 0.05$) to that via SC-stripped skin. Figure 4C confirms that no gross changes in the viable epidermis or dermis were noted with 1.7-J/cm^2 pretreatment. One of the characteristics of an ideal enhancement method is that the skin should recover its normal status following removal of the method. The Er:YAG laser tested in this study used lower energies than those utilized in clinical situations for resurfacing aims. As the SC rapidly regenerates, the skin should quickly recover after laser exposure. In our previous work, the skin recovered to a normal status within 3 days, which was evaluated by the SC thickness and transepidermal water loss [10,21]. Hence although some skin disruption may have occurred in the in vitro permeation experiments, the in vivo and clinical safety of the low-fluence Er:YAG laser can be assured. Of course, caution should be exercised when a fluence of 1.9 J/cm^2 is used, since it can produce more-intense structural changes of the skin.

Three mechanisms, including direct ablation, optical breakdown by the PW, and a photothermal effect, are involved in laser-tissue interactions [20]. The Er:YAG laser emits light with a minimal residual thermal effect because this wavelength corresponds to the main peak of water absorption [22]. The PW is a broadband, unipolar, compressive wave. Previous studies suggested that the PW generated by a ruby laser can enhance transdermal drug delivery [23–25]. The PW induces expansion of the lacunar spaces within the highly tortuous intercellular domains, leading to the formation of transient channels within the SC. Since intercellular pathways are important for MTX permeation, this induction of transient channels may be beneficial for its delivery. The PW generated by 1.4 J/cm^2 did not promote permeation of MTX through the skin. The fluences of the PW generated by a ruby laser in a previous study were 5–7 J/cm^2 [23]. Hence we used a higher fluence of 3.7 J/cm^2 to induce the PW. Limited success in skin deposition was observed compared to the control. This suggests that the enhancing effect of the PW was still far less than that achieved by direct laser ablation. That is, the ablation of the SC is the predominant mechanism responsible for MTX permeation when treated with a low-fluence laser.

For topical drugs used in psoriasis, the site of action is the skin. It should be noted that drug deposition in the skin in an in vitro status cannot represent the drug amount residing within the skin reservoir in the actual situation. In vitro drug permeation always dictates the amount of drug available for absorption into the skin. Drug absorption in the skin can be evaluated by determining the flux value or permeability constant in cases of anti-psoriatic drugs such as MTX, cyclosporine A, and psoralens [8,26–28].

Pretreatment with 20 pulses of electroporation from the cathode to the anode enhanced the drug flux by 2.2-fold. The increase in molecular transport with electroporation can be attributed to the creation of electropores in the lipid bilayers as well as to a local field effect due to electrophoresis/iontophoresis [29,30]. Enhancement of MTX flux was not observed after replacement of the electrode polarity. Negative polarity for MTX caused structural changes in the skin, possibly due to electroporation, as well as more molecules moving across the skin by electrophoresis through both previously existing and newly created pathways. In contrast, a positive polarity for MTX also created transient pathways. But it had no effect on the electrophoretic transport of the negatively charged MTX molecules. The results indicate that electrophoretic movement is important for MTX after electroporation pretreatment. However, electroporation with only 10 pulses was insufficient to induce electrophoretic movement. This is reasonable since the pulse numbers often linearly increase drug transport [30]. Another observation is that the cumulative amount of the drug remained elevated after pulsing until the end of the experiments. This suggests that electroporation produces a large drug reservoir within the skin [31]. Consequently a high skin deposition at the end of the experiments was detected with electroporation pretreatment, especially for the negative polarity group.

The combination of laser and electroporation pretreatment techniques may open new perspectives for topical drug systems. The rationale for such a combination is the difference between their mechanisms of action. As the SC has a much-higher electrical resistance than the underlying skin and deeper tissues, an electrical field applied to the skin will be concentrated in the SC and will be much lower in viable skin [30]. Partial ablation of the SC layer by a low-fluence laser may result in a drop in the electrical resistance of the skin. Thus the threshold for electroporation to open up micropores within the lipid bilayers is

lowered. This explains the synergistic enhancement of MTX permeation by the combination of electroporation and laser at 1.4 J/cm^2. However, this effect was absent when a higher fluence (1.7 J/cm^2) was used for the combination. The higher fluence likely ablated more SC fragments than did the lower one. The main mechanism of electroporation is disruption of lipid bilayers in the SC. The remaining SC after laser pretreatment at 1.7 J/cm^2 might have been insufficient, such that electroporation could not exert its enhancing potency on the disrupted bilayers, resulting in negligible synergism. In the case of 1.7 J/cm^2, the enhancement due to the laser alone was so large that the enhancement due to electroporation was meaningless in comparison. Both the high-fluence laser and electroporation generate some concerns as to safety issues such as skin perturbation and irritation [32,33]. Physical techniques with a lower intensity or fewer pulses may reduce the incidence of irritation responses. It is beneficial to achieve both an enhancement in potency and skin safety by combining the two techniques.

Psoriasis is a disorder triggered when activated immunocytes infiltrate the skin, subsequently inducing prominent epidermal thickening. Two methods can induce psoriasis-like skin in animal models: xenografts in mice and tape-stripping techniques [15,34]. We used the latter method to create hyperproliferative skin because it is a simple process. A reduction in passive MTX permeation in hyperproliferative skin was found. The reduction may have been due to the increment in the epidermal thickness, which created a longer path through which the drug had to pass. However, the statistical analysis showed a P value of >0.05. This is due to inter-subject variations after the tape-stripping treatment, which can be seen from the coefficient of variance (SD/mean) of the flux. The same phenomenon was exhibited with drug permeation by the laser at 1.4 J/cm^2. This result is close to the actual condition of patients with mild to severe psoriasis. Laser pretreatment at a higher fluence (1.7 J/cm^2) reduced the variability according to the MTX flux. The drug permeation via psoriasis-like skin at 1.4 J/cm^2 was comparable to that via normal skin. However, a decline in the flux via hyperproliferative skin with a 1.7-J/cm^2 intensity was detected compared to the normal control, resulting in less enhancement. Since the laser mainly acts on the SC layer, the discrepancy of the enhancing behavior between 1.4 and 1.7 J/cm^2 was possibly due to structural changes of the SC after tape-stripping. Previous studies indicated that psoriasis is characterized by epidermal hyperplasia and abnormalities in keratinocyte differentiation [15,35]. Further investigation is needed to elucidate the actual mechanisms. Although no effect was shown in normal skin, electroporation with 10 pulses increased the drug flux via hyperproliferative skin by 5.9-fold. This confirms the difference in the SC structure of psoriasis-like skin from control skin. A synergistic effect with the laser/electroporation combination was again observed for hyperproliferative skin.

The Er:YAG laser and/or electroporation increased the skin permeation of MTX by 3- to 80-fold in the present study. Other physical methods for MTX transport across skin, including iontophoresis and microneedles, have been reported. The iontophoretic approach allows a 2- to 8-fold increase in MTX flux [36,37]. A synergistic 25-fold enhancement of MTX delivery was observed when a combination of iontophoresis and microneedles was used. The combination of electroporation and anionic lipid enhancers resulted in a 4.4-fold enhancement compared with passive diffusion. Besides the physical techniques, liposomes encapsulating MTX were used to enhance topical delivery. A 26-fold flux enhancement was achieved by MTX inclusion in ethanolic liposomes. Trotta et al. [28] demonstrated a 3- to 4-fold increase in MTX permeation using deformable liposomes as carriers. Contrary to those previous studies, the low-fluence laser used in this study induced a 54-fold increment of permeation at a fluence of 1.7 J/cm^2. It should be noted that the permeation setup and evaluation methods differed in those studies. Comparisons among various enhancing methods should be made with caution. Although direct and fair comparisons are impossible, the low-fluence laser was shown to be a promising technique for enhancing topical MTX delivery.

CONCLUSIONS

The most important problem in therapies for psoriasis is that patients cease to use a drug or look for a rapid treatment response. The long-term aim of this study was to develop an efficient topical system for MTX to treat psoriasis or RA. The results in this study showed that low-fluence laser ablation of the SC greatly increased MTX permeation via the skin. Another physical technique, electroporation, also exhibited moderate enhancement of MTX delivery. The present work suggests that topical MTX delivery was synergistically enhanced by the combined use of electroporation and a laser at a lower intensity. It is important to consider safety issues of these physical techniques. Most research has utilized healthy skin to examine drug permeation, and results from such studies might not be appropriately applied to predict the skin permeation of a drug on disordered skin. The hyperproliferative skin model used in the present study is useful for resolving this problem. The permeation behavior of MTX by laser and electroporation somewhat differed between psoriasis-like skin and normal skin. This work elucidates that the Er:YAG laser and electroporation are promising methods for topical MTX delivery and encourages further investigation.

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