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Transdermal iontophoresis of 5-fluorouracil combined with electroporation and laser treatment

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Abstract

The influence of iontophoresis and other physical enhancement methods such as electroporation and erbium:yttrium-aluminum-garnet (YAG) laser on the skin permeation of 5-fluorouracil (5-FU) was examined. Iontophoresis increased the in vitro transdermal transport of both the anionic and non-ionic forms of 5-FU. A combination of electroporation pretreatment and subsequent iontophoresis resulted in a higher permeation of 5-FU than either technique alone. It appeared that electroporation treatment exerted a disruptive influence on the stratum corneum (SC). The SC layers in the skin were partly ablated by the laser, resulting in a great enhancement effect on the skin permeation of 5-FU. Application of iontophoresis further increased the drug permeation across laser-pretreated skin. The laser was consistently the most potent technique to enhance 5-FU delivery among the physical enhancement methods examined in this study.

Keywords: 5-Fluorouracil; Transdermal delivery; Iontophoresis; Electroporation; Erbium:YAG laser

1. Introduction

The delivery of drugs via skin routes has been extensively investigated. Drug transport by the skin offers the advantages of accessibility, noninvasiveness, compliance, safety, and effectiveness. Nevertheless, its clinical application is limited due to the stratum corneum (SC), the predominant barrier of the skin. Many approaches have been used to overcome the barrier presented by the skin, including chemical enhancer modification and physical enhancements. Iontophoresis is a physical method which uses an electrode of the same polarity as the charge of a drug to drive ionic drugs into the body (Banga et al., 1999). Because of the SC's overall hydrophobic character and net negative charge, transdermal delivery of negatively charged hydrophilic drugs is especially challenging (Prausnitz et al., 1996). Hence 5-fluorouracil (5-FU), a hydrophilic and negatively charged molecule, was selected as a model drug in this study of iontophoretic enhancement.

A variety of physical enhancement methods including electroporation and laser irradiation have been successfully used. Skin electroporation takes place at high voltages (=100 V) and is associated with micropore formation in the skin structure (Prausnitz, 1996). Lasers have long been used for medical diagnostic and therapeutic purposes. The laser has been suggested for

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the controlled ablation or removal of the SC (Lee et al., 2001). Pretreatment of the skin provides the ability to dramatically influence the iontophoretic flux of drugs (Riviere and Heit, 1997). The aim of this study was to investigate the influence of electroporation and laser pretreatments on the transdermal delivery of 5-FU across nude mouse skin during iontophoresis.

The transport kinetics of 5-FU was also determined across SC-stripped skin in the presence or absence of physical enhancements to probe the mechanisms of iontophoretic delivery. The electrical behavior of 5-FU transported across the skin was therefore established in this study.

2. Materials and methods

2.1. Preparation of skin membranes

Female nude mice (Balb/c-nu, 6–8 weeks old) were killed by ether, and full-thickness skin was excised from the dorsal region. To obtain SC-stripped skin, adhesive tape (Four-Pillars, Taiwan) was applied to nude mouse skin with uniform pressure and then removed. This procedure was repeated 20 times.

2.2. Iontophoretic instruments and in vitro permeation procedures

The in vitro permeation procedures of iontophoresis were determined using horizontal glass diffusion cells. The receptor phase contained 8 ml of 0.06 M (pH 7.4) citrate-phosphate buffer. The donor compartment of a cell was filled with 8 ml of 0.3% (w/v) 5-FU in buffers with various values. The available diffusion surface area was 0.785 cm^2. The cells were agitated by magnetic stirrers at 600 rpm. A pair of Ag/AgCl wires, having an effective length of 15 mm, was used as electrodes by immersing them in the cell, with the cathode in the donor compartment and the anode in the receptor compartment, unless otherwise noted. The electrodes were connected to a current power supply (Yokogawa, model 7651, Japan). A current density of 0.5 mA/cm^2 was applied for 3 h to stimulate the permeation of 5-FU. Samples of 300 μl were withdrawn from the receptor at regular intervals and immediately replaced by an equal volume of fresh receptor solution. Samples were assayed using HPLC.

2.3. Electroporation protocols

Electroporation was performed using an exponential decay pulse generator (BTX, ECM 630 Electro Cell Manipulator®, USA). Platinum electrodes (0.5 cm × 1.5 cm) were used, each located 3 cm from the skin. The cathode was positioned in the donor compartment, while the anode was in the receptor compartment, unless otherwise noted. The electroporation protocol consisted of 1 pulse per 30 s, applied for 10 min. The pulse voltage was 300 V, and pulse length was 200 ms. Voltages are expressed as applied values, not as transdermal values. After 10 min of electroporation of the skin, iontophoresis was applied in the in vitro experiments if necessary. The time interval between the pre-pulse and switching on of the iontophoretic current application was a few seconds.

2.4. Erbium:YAG laser irradiation protocols

An erbium:YAG (yttrium-aluminum-garnet) laser (Continuum Biomedical, USA) was used to pretreat skin in the present study. The laser has a wavelength of 2940 nm and a pulse duration of 250 μs. An articulated arm was used to deliver the laser beam onto the skin. Output energies of 0.35, 0.45, and 0.55 J per pulse with a beam spot size of 7 mm in diameter achieved fluences of 0.9, 1.2, and 1.4 J/cm^2, respectively. The energy of the laser pulse was monitored with an energy meter (Nova Display, Israel) before and after pretreatment. The laser hand-piece was located approximately 3.0 cm from the surface of the skin. Then the skin was irradiated by various doses of the laser pulse. After laser pretreatment, the skin surface was wiped several times with a cotton wool swab. Then the irradiated skin was mounted in horizontal diffusion cells to perform in vitro permeation experiments with or without iontophoretic application.

Histological changes in nude mouse skin were examined after laser irradiation. Immediately after laser pretreatment, a specimen of the exposed area was taken from a live nude mouse for histological examination. The adjacent untreated skin area was also assessed as the control group. Each specimen was fixed in a 10% pH 7.4-buffered formaldehyde solution for at least 48 h. The specimen was cut vertically against

the skin surface. Each section was dehydrated using ethanol, embedded in paraffin wax, and stained with hematoxylin and eosin. In each skin sample, three different sites were examined and evaluated under light microscopy (Nikon Eclipse 4000, Japan). Photomicrographs of the three randomly selected sites of each skin sample were taken with a digital camera (Coolpix 950, Nikon). The digital photomicrographs were then processed with Adobe PhotoDeluxe (Adobe Systems, USA), and the SC and epidermal thicknesses were calculated with ImagePro-plus 4.0 (Media Cybernetics, USA).

2.5. HPLC determination

The 5-FU content of the various samples was analyzed using an HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7400 UV detector. A 25-cm long, 4-mm inner diameter stainless steel RP-18 column (Merck, Germany) was used. The mobile phase, consisting of a 100% pH 3.1 aqueous solution adjusted with acetic acid, was used at a flow rate of 1 ml/min. The UV detector was set at a wavelength of 265 nm. The retention time of 5-FU was found to be 5.8 min.

2.6. Data analysis

In the in vitro permeation study, the total amount of drug permeating across the unit diffusion surface and into the receptor was calculated and plotted as a function of time. The flux was calculated by the slope of the linear portion of the cumulative amount-time plots for a pseudo zero-order model and expressed as the mass of drug passing across 1 cm^2 of skin over time. In the experiments using laser irradiation, laser ablation pretreated a limited area of SC of 49.02% of the total permeated skin surface area. Consequently, the cumulative amount and flux data of the laser-irradiated area was extrapolated to an area of 100% ablation of the permeated area.

Statistical analysis of differences between different treatments was performed using the unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. The ANOVA test was also utilized in this study.

3. Results and discussion

3.1. Transdermal delivery of 5-FU by iontophoresis

The electric technique of iontophoresis can be used to enhance transdermal drug delivery. During iontophoresis, a small low voltage (typically 10 V or less) and a continuous constant current (typically 0.5 mA/cm^2 or less) are applied to push a drug into the skin. 5-FU behaves as a weak acid, with a pK_a of approximately 8 (Merino et al., 1999). At pH values above 8, the molecule is predominantly negatively charged. Fig. 1 shows the permeated amount of 5-FU ($\mu g/cm^2$) from pH 8.5 buffer as a function of time with or without iontophoresis at 0.5 mA/cm^2 for 3 h. As is evident from the figure, there was no passive permeation (in the absence of iontophoresis) of 5-FU across skin during a 6-h period. Hydrophilic molecules such as 5-FU exhibit very low partitioning into lipophilic environments on the basis of thermodynamics, resulting in the expected low permeation seen for 5-FU. Application of iontophoresis greatly enhanced the transdermal 5-FU transport with fluxes of 0–31.41 $\mu g/cm^2/h$. The permeated amount of drug remained elevated even after the cessation of current density (Fig. 1). This could be due to a drug reservoir within the skin or the alteration of skin structure which might allow 5-FU continuously released from the donor after the end of iontophoresis application. Our previous histological study had indicated that there was almost no change observed in the anatomical structure of skin by the iontophoretic protocol similar as the present study (Fang et al., 1997). This may infer that the continuous permeation of 5-FU after iontophoresis cessation was due to the drug reservoir effect. The observed enhancement in skin permeation of anionic 5-FU can be attributed to the electrical potential gradient resulting from application of iontophoresis.

Passive delivery of 5-FU in pH 8.5 buffer across SC-stripped skin was much higher than that of intact skin, confirming the rate-limiting properties of the SC in the transdermal delivery of 5-FU as shown in Fig. 2. Fig. 2 demonstrates the cumulative amount of 5-FU at the end of in vitro experiment as the representation of the permeability. The application of iontophoresis also significantly increased the apparent permeation of 5-FU after stripping the SC. 5-FU permeated amount

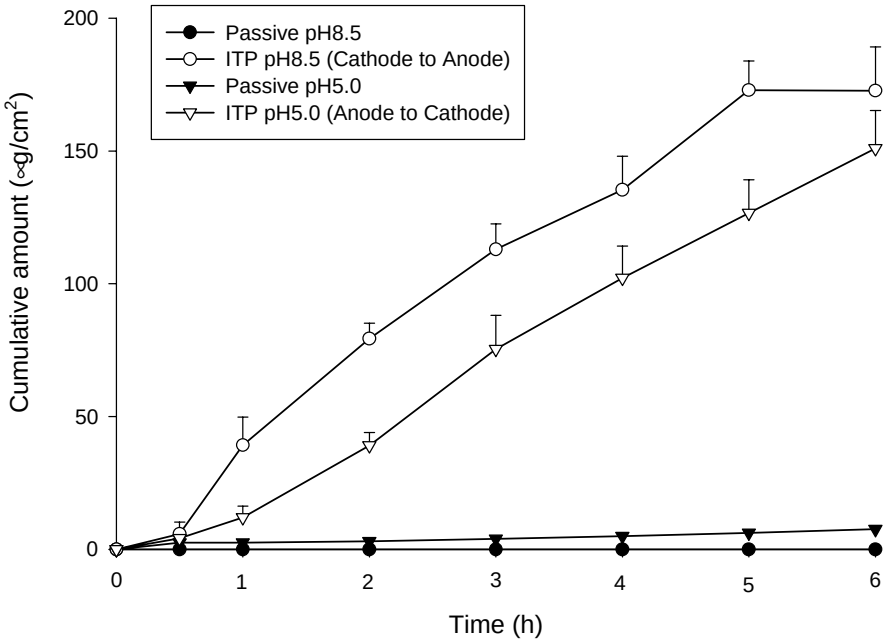

Fig. 1. Cumulative amount of 5-FU detected in the receptor compartment versus time with iontophoresis at 0.5 mA/cm^2 for 3 h from pH 8.5 vehicle (cathode to anode) and pH 5 vehicle (anode to cathode). All data represent the mean ± S.D. of four experiments.

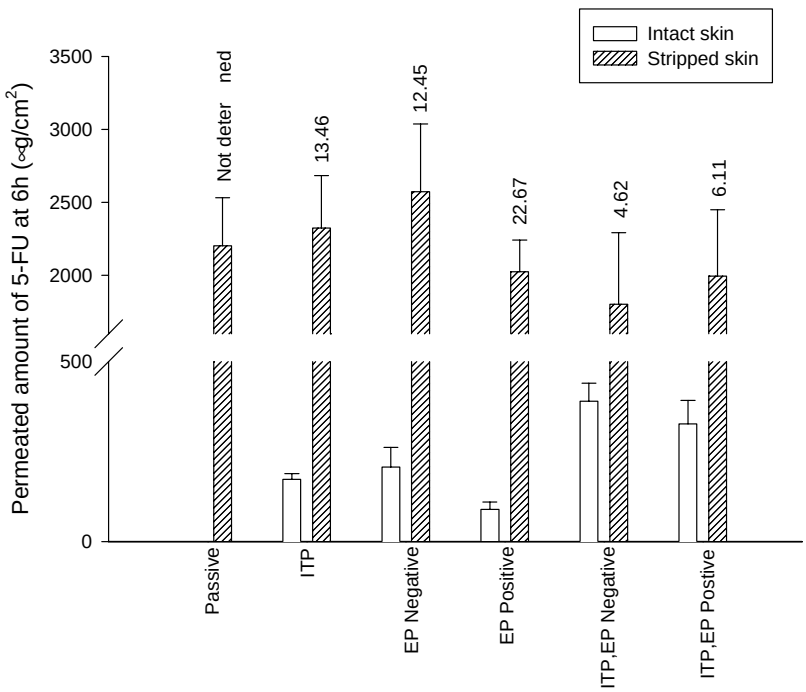

Fig. 2. Skin permeation of 5-FU after treatment with iontophoresis and electroporation across intact and SC-stripped skin. All data represent the mean ± S.D. of four experiments.

across SC-stripped skin were comparable in the condition with or without iontophoresis. SC is the predominant barrier for most of hydrophilic molecules. It might be that the enhancement due to removal of the SC was so large that the enhancement due to iontophoresis was negligible in comparison.

Overall permeation enhancement of ions during iontophoresis is primarily due to the electrochemical potential gradient. However, secondary effects such as electro-osmotic flow may also contribute to permeation enhancement (Sims et al., 1991). Electro-osmosis is the convective movement of solvent that occurs through a charged pore in response to the preferential passage of counter-ions when an electric field is applied (Santi and Guy, 1996). For a porous, net negatively charged membrane such as skin, the solvent flow will be from the anode to the cathode. The electro-osmotic flow in an electric field is therefore opposite that of 5-FU permeation across the skin.

Neutral solutes have been used to determine the electro-osmotic flow since neutral compounds are believed to be transported by this force during iontophoresis (Singh et al., 1995). The proportion of neutral molecules of 5-FU is >99% at pH 5. Passive transport of 5-FU from pH 5 buffer was higher than that from pH 8.5 buffer, indicating that the non-ionized form may partition into and permeate across the skin easier than the ionized form (Fig. 1). Application of iontophoresis from anode to cathode significantly increased the permeation of 5-FU in pH 5 buffer. The increase in permeation relative to passive diffusion of neutral solutes can be interpreted on the basis of electro-osmotic flow.

3.2. Transdermal delivery of 5-FU by electroporation

Electroporation has been demonstrated to be a powerful method for overcoming the SC barrier. Electroporation involves the application of high-voltage pulses which create transient aqueous pathways in the skin and permit transport of drugs across these pathways (Jadoul et al., 1998). In the experiments using electroporation, the cathode was first positioned in the donor phase (negative polarity) to examine the effect of electroporation on 5-FU permeation. As shown in Fig. 3, application of twenty 300-V, 200-ms pulses significantly enhanced 5-FU permeation from pH 8.5 buffer compared to passive diffusion. Electroporation

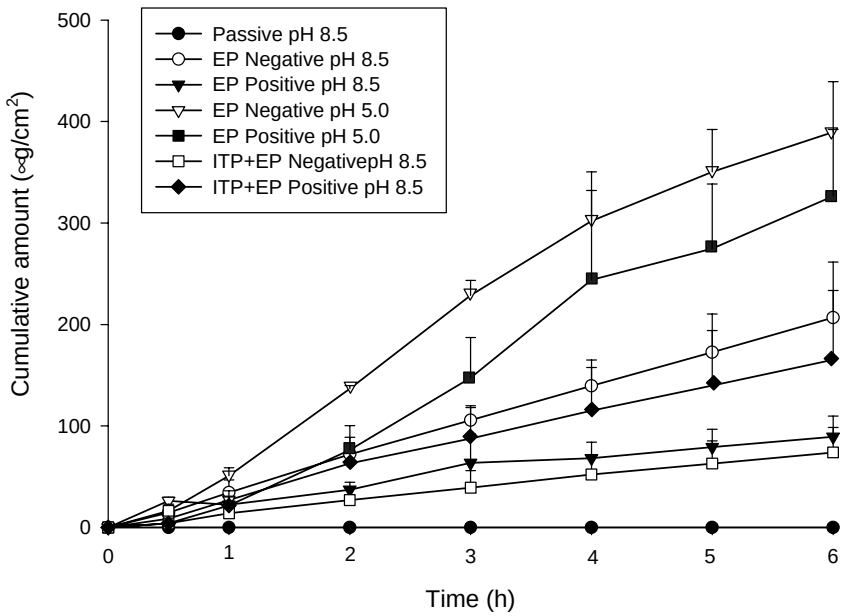

Fig. 3. Cumulative amount of SNA detected in the receptor compartment vs. time using electroporation at 300 V, 200 ms alone or combined with iontophoresis at 0.5 mA/cm^2. All data represent the mean ± S.D. of four experiments.

resulted in a permeation profile similar to that of iontophoresis at the same electrode polarity of cathode to anode. Another observation was that the permeated amount of 5-FU in the first 0.5-h sampling period was significantly higher by electroporation than by iontophoresis (14.44 μg/cm^2 versus 5.82 μg/cm^2, respectively). This may suggest that the rapid transport across highly permeabilized skin occurred during pulsing by the initial 10-min pretreatment with electroporation.

The influence of the polarity of the electrodes was also evaluated. A significant enhancement in 5-FU flux was induced by electroporation from anode to cathode (positive polarity) as compared to passive diffusion (Fig. 3). However, transport of 5-FU into the cathode compartment was significantly lower than that into the anode compartment. Negative polarity pulsing can cause 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 (Prausnitz et al., 1996). In contrast, a positive polarity for 5-FU can also create transient aqueous pathways, the same as the negative-polarity electroporation, in the skin. But it has no effect on the electrophoretic transport of negative 5-FU molecules (Fang et al., 2002). This result indicates that electrophoretic movement is important for 5-FU after electroporation pretreatment. The actual mechanisms of electroporation are further elucidated in the following sections of this study.

As shown in Fig. 3, the permeated amount of 5-FU remained elevated after pulsing until the end of the experiments. This suggests that electroporation produced a drug reservoir within the skin and/or a persistent change in the skin's permeability due to an alteration in the skin's structure (Jadoul and Préat, 1997). In order to verify whether this phenomenon resulted from skin depot or enhancement of skin permeability, the 5-FU solution after pulsing was removed immediately after the last pulse by rinsing and filling the donor with pH 8.5 buffer. Fig. 4 shows that no increase occurred in 5-FU transdermal permeation for either the negative or positive polarities used. It appears that drug permeation after switching off the pulsing did not result from the creation of a skin reservoir which would have progressively released 5-FU afterwards. The altered skin structure created changes in skin permeability which persisted after pulsing. This effect was somewhat different from the inference in the iontophoresis study: the elevated permeation amount after stopping the current density may be due to the skin reservoir effect. In order to verify it,

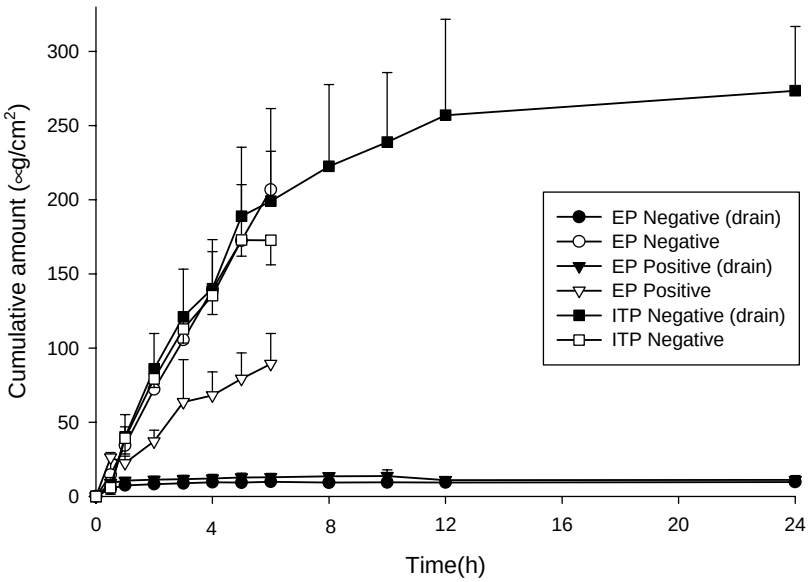

Fig. 4. Cumulative amount of SNA detected in the receptor compartment vs. time using electroporation at 300 V, 200 ms with or without draining the donor compartment after pulsing. All data represent the mean ± S.D. of four experiments.

the drain study was also performed for iontophoresis (from cathode to anode at pH 8.5). As shown in Fig. 4, the permeated amount of 5-FU indeed continuously increased even after emptying the 5-FU in donor after 3-h application of iontophoresis. The transdermal high-voltage pulsing that creates a local transport region (electroporated pores) may disrupt the SC lipid structure in the vicinity of the pores (Prausnitz, 1996; Vanbever et al., 1996; Banga et al., 1999). Lasting effects have been seen after high-voltage pulsing and the increased transdermal flux generally persists for minutes to hours, but is often reversible (Prausnitz, 1996). Based on the findings in Fig. 3, this effect lasted for the 6-h duration of the experiment. The fact that the skin structure did not recover may be attributed to the formation of long-term metastable structures caused by high-voltage pulsing (Takeuchi et al., 2000). Under such circumstances, electric pulse-induced pores are considered to remain open.

There was no significant difference in the permeated amount of 5-FU after negative- and positive-polarity electroporation in the drain experiment (Fig. 4). This suggests that structural changes induced in the skin contribute more significantly to enhanced permeation than does the direct electrophoretic force acting on negative-polarity electroporation. On the other hand, negative-polarity electroporation caused higher 5-FU permeation than did positive-polarity when 5-FU remained in the donor compartment during the experiments (Fig. 4). This implies that greater skin disruption was caused by negative-polarity electroporation than by positive polarity. Further investigation is needed and is in progress to elucidate this effect.

Fig. 2 shows that the permeation of 5-FU by electroporation of negative polarity and positive polarity across SC-stripped skin were 12.39- and 25.39-fold higher, respectively, than that across intact skin; moreover, the permeation of 5-FU permeated across SC-stripped skin by passive diffusion and by electroporation were similar. These observations demonstrate that electroporation had significant effects on the structure of the SC which caused increased 5-FU permeation. Another discussion is that even though the electro-osmotic flow must take place during pulsing, its impact on transport could be low because of the short duration of current application (Vanbever et al., 1998).

Permeation of neutral 5-FU molecules by electroporation of negative or positive polarity was also examined as shown in Fig. 3. Both polarities of electroporation moderately enhanced 5-FU permeation. Permeation in the pH 5 vehicle by negative polarity was higher than that by positive polarity, which also occurred in the pH 8.5 vehicle. Although the vehicle of pH 8.5 buffer showed higher permeation profiles than the pH 5 buffer, the differences were not statistically significant. The elevated transport resulting from electroporation-induced alterations in the skin persisted after pulsing. These results clearly demonstrate that transport of neutral molecules which occurred mainly after pulsing was caused by skin disruption.

Iontophoresis and electroporation produced different influences on and mechanisms of skin permeation by 5-FU. The combined use of these two electric enhancement methods may also produce some interesting results. A combination of electroporation and iontophoresis induced higher transdermal permeation than that induced by either technique alone (Fig. 3). Short pulses of high voltage alter the skin permeability, facilitating subsequent iontophoresis. Negative-polarity electroporation combined with iontophoresis again generated higher enhancement than did positive polarity. This trend was the same as the electroporation treatment alone. It may suggest that combining iontophoresis did not influence the effect of electroporation on the skin and the 5-FU molecules. As shown in Fig. 2, although different permeation enhancements in intact skin were produced by iontophoresis and electroporation, no significant difference was determined among the permeation of SC-stripped skin when iontophoresis and electroporation were applied alone or together. This suggests that the single or combined use of electroporation and iontophoresis acting on the SC layers was the predominant mechanism for the enhanced 5-FU permeation.

3.3. Transdermal delivery of 5-FU by laser treatment

As shown in Fig. 2, the skin barrier to 5-FU permeation can be overcome by stripping of the SC with tape. However, the area and depth of the SC treated by the tape stripping technique cannot be precisely controlled. Few data and references on the safety and recovery rate of skin using this method are available.

The laser has been suggested as a good method for the controlled ablation or removal of the SC. The erbium:YAG laser emits light with a 2940-nm wavelength which corresponds to the main peak of water absorption. This property enables the erbium:YAG laser to ablate the SC with minimal residual thermal damage, thereby potentially minimizing the risks of post-inflammatory hyperpigmentation (Manaloto and Alster, 1999). The erbium:YAG laser is currently used for the resurfacing of rhytides, scars, photodamage, and melasma (Polnikorn et al., 1998).

In order to assess the effect of the erbium:YAG laser on the integrity of the skin structure, nude mouse skin was irradiated by a laser with energy from 0.35 to 0.55 J. The fluences used varied between 0.9 and 1.4 J/cm^2. Histological studies demonstrated that erbium:YAG laser ablation at different energies achieved partial removal of the SC as shown in Table 1. Higher fluences of the erbium:YAG laser generally induced deeper SC/epidermal ablation as determined by calculating the thickness. There were no statistically significant differences in the SC/epidermis thickness after laser treatment using fluences of 0.9 and 1.2 J/cm^2. The laser not only ablated the SC layers, but also removed a part of the epidermal layers at a fluence of 1.4 J/cm^2. This suggests that the threshold fluence for ablation of viable epidermis is about 1.2 J/cm^2.

Table 1 shows the permeation profiles of 5-FU after pretreatment of skin with the erbium:YAG laser. Extrapolating the flux data of the laser-irradiated area to an area of 100% exposure showed greater enhancement of 5-FU permeation than that by iontophoresis and electroporation, indicating that the laser was consistently the most potent enhancement method for 5-FU permeation among the physical enhancement methods examined in this study. Histological alterations of and SC ablation in the skin may have resulted in increased flux after exposure to the laser. The more-significant changes in skin structure with higher fluences may have led to higher enhancement of drug permeation. However, according to the permeation profiles, the effect of enhancement was not proportional to the magnitude of a single pulse of the laser (Table 1). The 5-FU flux increased as the fluence rose from 0.9 to 1.2 J/cm^2, after which the enhancement effect reached a plateau. Improvement in drug partitioning into the SC layers is important for increased 5-FU permeation across the skin (Yamane et al., 1995). Although removal of the SC can reduce the inherent barrier properties of the skin and thus increase skin permeation of 5-FU, the partitioning of 5-FU into the skin should have decreased because of the removal of the SC layers. As a result, increased permeation of the skin due to structural alterations may have been partly offset by a decrease in the partition coefficient between the skin and drug vehicle.

One of the characteristics of an ideal permeation enhancement method is that the skin should recover its normal barrier properties following removal of the enhancement method. Our previous study showed that the depth of the SC could completely recover to a normal range within 4 days (Lee et al., 2001).

Table 1 depicts the flux of 5-FU after combination of laser pretreatment and iontophoresis. A higher effect was observed with a laser exposure of 0.9 J/cm^2 coupled with an electric field as compared to laser treatment alone. As the superficial layers of the SC were removed, there was a gradual drop in the electrical resistance of the skin (Nelson et al., 1991). The enhancement of 5-FU permeation by iontophoresis on laser-irradiated skin decreased following an increase in the fluence. This may suggest that the enhancing effect due to the laser was large compared to that due to iontophoresis. The main mechanism of the laser in enhancing drug permeation is ablation of the SC layers which form the predominant barrier for 5-FU transport

Table 1
SC and epidermal thickness (μm) of nude mouse skin and the flux of 5-FU across the skin after the treatment by erbium:YAG laser

Fluence (J/cm^2)	SC thickness	Epidermal thickness	Laser flux (μg/cm^2/h)	Laser + ITP flux (μg/cm^2/h)	Enhancement ratio (ER)[a]
0.9	6.22 ± 0.19	13.56 ± 0.19	78.66 ± 12.94	125.00 ± 25.8656	1.59
1.2	3.55 ± 0.19	12.33 ± 0.33	130.47 ± 14.61	161.64 ± 34.9132	1.24
1.4	1.78 ± 0.19	8.56 ± 0.38	135.60 ± 18.05	138.59 ± 33.0259	1.02

Each value represents mean ± S.D. ($n = 4$).
[a] Enhancement ratio (ER) = ratio of the flux by laser combined with iontophoresis to the flux by laser treatment alone.

across the skin. Limited enhancement with the combined use of a high-fluence laser and iontophoresis was similar to the result of transdermal iontophoresis across SC-stripped skin.

4. Conclusions

This investigation illustrates the influence of a series of physical enhancement methods, including iontophoresis, electroporation, and an erbium:YAG laser, on the transdermal delivery of 5-FU. The effect of iontophoresis combined with electroporation and laser treatment on 5-FU permeation was also elucidated. The in vitro transport of 5-FU across skin with no enhancement methods was negligible. The permeation of 5-FU extensively increased following application of iontophoresis. The contribution of electro-osmotic flow appeared to be important with the application of iontophoresis. Electroporation using both negative and positive polarities significantly enhanced the transdermal delivery of 5-FU. Skin alteration caused by high-voltage pulsing was related to this electric protocol. SC removal with an erbium:YAG laser can be precisely controlled with a single pulse, and may offer a distinct advantage over the tape-stripping technique, which is both macroscopic and unpredictable. Both SC barrier ablation and drug partitioning into the skin may contribute to the mechanism influencing the permeation of 5-FU molecules across laser-pretreated skin. After comparing the physical enhancement methods tested in this present study, the laser was still the most potent method of enhancing 5-FU delivery across skin. The combination with iontophoresis further increased passive diffusion of 5-FU across laser-pretreated skin.

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