COMPARISON STUDY ON USING PULSED CURRENT AND DIRECT CURRENT IONTOPHORETIC SYSTEMS IN THE ENHANCEMENT OF METHYLENE BLUE TRANSPORT THROUGH HAIRLESS RAT SKIN

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Abstract. Electric pulses and direct current iontophoresis to permeabilize rat stratum corneum were applied to demonstrate enhanced epidermal transport of methylene blue, a water-soluble cationic dye. Electrodes were placed on the outer surface of excised full-thickness rat skin, and methylene blue was applied to the skin beneath the active electrode (i.e positive electrode). The pulsing square wave electric field used was 10 Volts, with 1, 2, 4, 8 or 16 k pulse/sec. The direct current electric field used was 6 Volts, with 0.2, 0.4, 0.8, 1.6 or 3.2 mA/cm^2. An electric exposure dose lasted for 10 minutes. A comparative study was carried out between these two groups to determine the best iontophoresis conditions of delivering methylene blue in excised full-thickness rat skin. The amount of dye penetrated in a skin sample was determined from the absorbance spectra of dissolved punch skin biopsy sections. Penetration depth and concentration of the dye were measured with light and fluorescence microscopy of cryosections. The maximum absorption for MB is 8 k pulse/sec., and 0.2 mA/cm^2 for direct current.

Key words: Iontophoresis, pulsed square wave iontophoresis, direct current iontophoresis, drug absorption through skin.

INTRODUCTION

Transdermal drug administration has been one of the alternatives to the traditional oral and parenteral routes of drug delivery due to its advantages, such as elimination of first-pass metabolism and limitations associated with gastrointestinal tract absorption, reduction of side effects, better patient compliance, and controlled and programmed rate of delivery. It is of particular interest for drugs that require frequent dosing due to their short elimination half-life or extensive first-pass metabolism [1]. Historically, the skin was viewed as an impermeable barrier, but in recent years [19] it has been increasingly recognized

that intact skin can be used as a port for topical or continuous systemic administration of drugs. The stratum corneum, which is the outermost layer, provides the most resistance to the permeation of drugs through the skin. Due to its excellent barrier properties, a drug has to meet certain requirements – high permeability across the skin, high potency with effective doses in the range of mg/day or those that must be taken several times per day, and no skin irritation – before it is considered a suitable candidate for transdermal administration [19].

To overcome these problems, we have focused on iontophoresis, one of several physical methods. Iontophoresis refers to the use of electrical current to increase skin permeability and enhance the transport of both ionic and neutral polar compounds across the skin. Although this technique has been around for many decades, its use in transdermal systematic drug delivery has been rediscovered and extensively studied in the past decade or so [19]. There are several reasons for the revival of iontophoretic transdermal drug delivery. First, it has been found that this technique can enhance the transport of either ionic and nonionic polar permeants by several-fold, or even more. Also, it is believed that the amount of drug delivered is proportional to the current applied. This dose-current dependence makes it possible to deliver a drug across the skin at a predictable and programmable rate [3, 15, 24, 26]. Iontophoresis drug delivery can be further divided into topical dermatological iontophoresis and systemic iontophoresis.

Topical delivery by iontophoresis refers to the delivery of a molecular drug to the epidermis or deeper layers of the dermis. Dexamethasone sodium phosphate, an anionic molecular drug, has been shown to be effectively delivered locally using iontophoresis in both clinical and animal studies for the treatment of inflamed tissue [12, 14]. Combined iontophoretic delivery of dexamethasone and lidocaine has also been shown to be very efficient and effective in relieving pain and inflammation and promoting healing in patients with infrapatellar tendonitis [20].

Several clinical studies have also shown that iontophoresis delivery of a mixture of dexamethasone, lidocaine, and verapamil provides a novel and effective noninvasive alternative for the treatment of Peyronies disease [16, 22]. Iontophoresis has also been shown to successfully deliver other therapeutic agents topically, such as lidocaine for local anaesthesia [23, 25, 28], and nonsteroidal anti-inflammatory drugs including indomethacin [10], naproxen [21], and diclofenac [6, 8].

We used the phenothiazinium dye methylene blue (MB) in this study because its color allows visual observation of its location, its absorbance and fluorescence properties permit spectroscopic detection, and its cationic nature serves as a model for a number of photosensitizers.

In this study, a comparison between pulsed square wave and direct current (DC) iontophoresis was done using excised rat skin, considering the amount of methylene blue that penetrated into rat skin as determined by a function of the physical parameters that characterize the two ionotophoretic protocols.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines set by the Medical Research Institute, where this study was performed.

Skin

Hairless male rats, 2 months old, weighing 220–310 g, were housed in standard cages at room temperature on a 12 h light and 12 h dark cycle. 110 pieces of abdominal rat skin (full thickness) were cut into equal pieces of 5 cm × 5 cm and kept at 4 °C until use (within 20 hours). Every piece of skin was shaved to remove all hairs, the fat and tissues around the skin were separated, and the skin was momentarily rinsed three times with normal saline followed by distilled water. This method was repeated for every piece of the used skin.

Experimental groups

Three experimental groups were used in this study: the control group, the exposure to pulsing square wave group, and the exposure to direct current group.

The Control group

10 rat skin samples were loaded with MB and no electric field was used.

Pulsing square wave iontophoresis group

This group was subdivided into 5 subgroups. Each one contained 10 rat skin samples subjected to a pulsing square wave with a peak voltage of 10 V and pulse rates of 1 k, 2 k, 4 k, 8 k or a pulse frequency of 16 kHz.

Direct current iontophoresis group

This group was subdivided into 5 subgroups. Each one contained 10 rat skin samples subjected to a direct current of 0.2, 0.4, 0.8, 1.6 and 3.2 mA/cm^2.

EXPERIMENTAL SET-UP

Skin was put dermal side down on ice covered with cling film. Each skin section was used once. The details of the system and used electrodes are shown schematically in Figures 1 and 2. Two Ag/AgCl rings were placed on the skin surface (2 cm apart), and a small piece of cotton soaked with 200 μL of MB (Fisher Scientific, Fairlawn, NJ; 1% in dd H$_2$O, 0.03 M) was placed in the cavity of the active electrode. The two electrodes were fixed to the skin above the two Ag/AgCl rings by adhesive tape. A slight pressure was applied evenly to the

electrodes to ensure good contact with the skin. To overcome the skin's bad conductivity to electricity, these electrodes were supplied with conductive gel to provide good electrical contact between the skin and the electrodes [27]. The Ag/AgCl electrodes were connected to an alternative current (AC) function generator model CA1640 (Yangzhong Ketai Electronic Instrument Co., Ltd., China), for the pulsing current experiments. While for the direct current experiments, a direct current (DC) Power supply (DYNASCAN CORP, Polish made) was used. Pre- and post-iontophoresis, skin resistance was measured using the method described by Yamamoto [29]. All the iontophoretic experiments were carried out for 10 min. At the end of the iontophoresis exposure, skin samples under the MB loaded electrode were collected and rinsed to remove superficial MB dye.
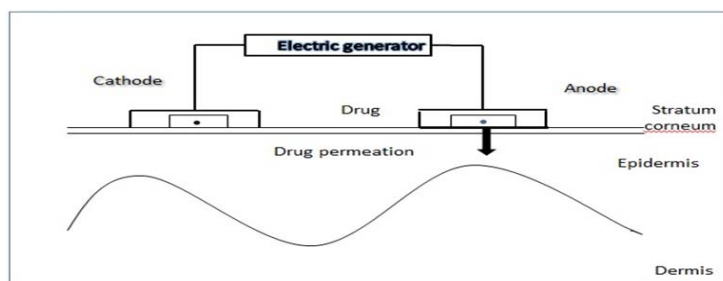

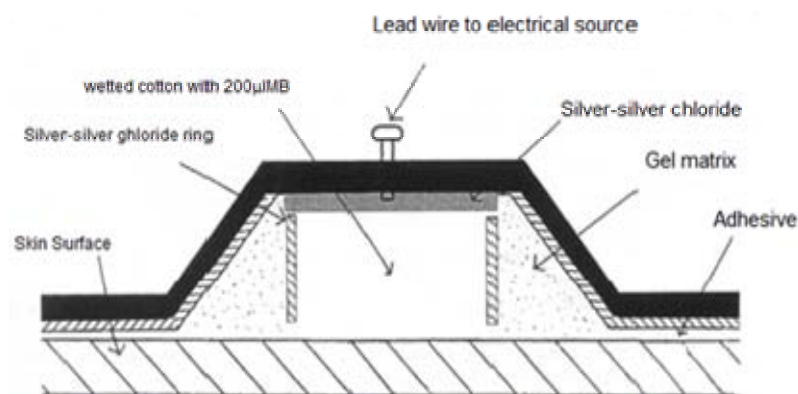Fig. 1. Schematic diagram of the system used to deliver MB using electric field (DC or pulsed).

Fig. 2. The details of the electrode used are shown.

OPTICAL DENSITY OF DISSOLVED SAMPLE

Full-thickness 5 mm punches biopsy samples (mean weight ≈ 38.6 mg) were dissolved in 2 mL NH_4OH at 37 °C overnight. Solutions were centrifuged, and the absorbance spectra of the supernatant fractions were recorded. The MB analytical

curve has a regression line formula $y = 69.737x + 2.5342$, where y is MB absorbance and x is MB concentration in mol/liter. Transport across the skin surface was calculated as microgram dye per cm^2. Average flux (microgram per cm^2 per minute) was calculated as the amount of dye per microgram transported across the area under the active electrode in cm^2 divided by the iontophoretic exposure time t, *i.e* the treatment duration. Experimental results are presented as mean ± SD unless stated otherwise.

HISTOLOGICAL EXAMINATION

Full-thickness 1 cm^2 biopsies were embedded in Tissue-Tek O.C.T. compound (Miles, Elkhart, IN) and frozen in liquid nitrogen. Photomicrographs of cryostat sections (10 μm) perpendicular to the skin surface were prepared. Images were collected with a Dage MTI CCD72 camera (Dage-MTI Michigan City, IN) with a 615 nm long-pass filter, and processed with Image 1 software (Universal Imaging, West Chester, PA). The dye was quantified by measuring the transmission at wavelengths ≥615 nm for each of 300 pixels along a line normal to the skin surface. Pixel number was converted to depth in μm by calibration with a section of known dimensions; pixel size = 0.20 $μm^2$ (0.45 × 0.45 μm).

RESULTS

EFFECT OF PULSED SQUARE WAVE AND DIRECT CURRENT ON TRANSPORT OF MB

Figures 3 and 4 show the values of MB transported through the skin as function of the applied pulsing frequency and direct current, respectively. Clearly the maximum absorption for MB is at a pulse frequency of 8 kHz and 0.2 mA/cm^2 for direct current.
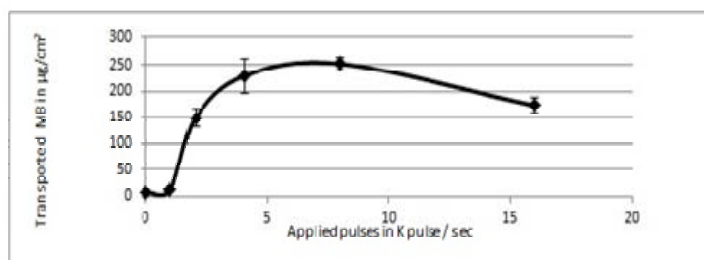

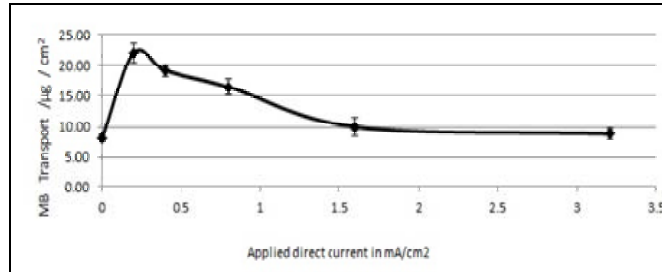Fig. 3. Effect of applied pulsed current on the MB transported in rat skin.

Fig. 4. Effect of direct current iontophoresis on the MB transported in rat skin.

EFFECT OF PULSED CURRENT AND DIRECT CURRENT ON FLUX RATE OF MB AND
IMPEDANCE MEASUREMENT

Figures 5 and 6 show the average amounts of absorbed MB (μg) in 1 cm^2 of rat skin as a function of the exposure time (2, 6 and 10 minutes). The results are tabulated in Tables 1 and 2. Rat skin impedance was measured by an oscilloscope connected to the pulsed and direct current iontophoresis system. Figures 7 and 8 show how the skin resistance changes with applied current.
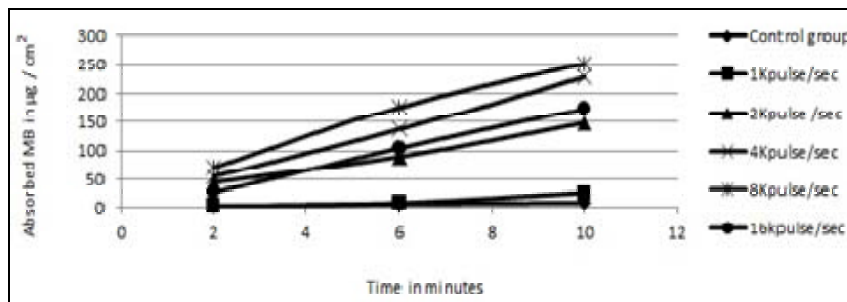

Fig. 5. Average amount of absorbed MB (μg) in 1 cm^2/min of rat skin.
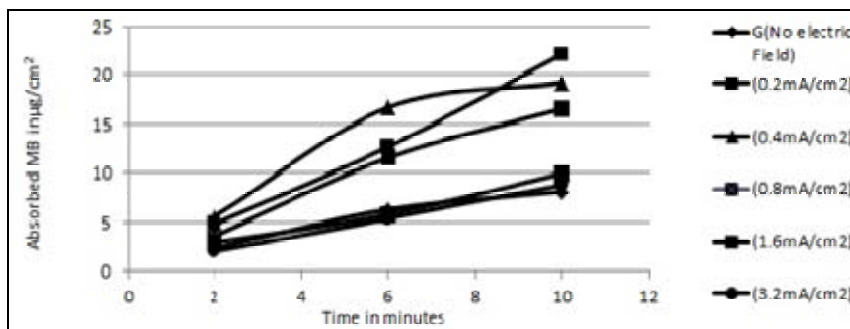

Fig. 6. The effect of varying exposure times in the direct current iontophoresis,
on the absorbed amount of MB.

Table 1

Relation between flux rate and time at different pulse rates

Group	Pulsed field value in k pulse/second	Trend line equation for each group
Group G	0	$y = 0.7163x + 1.3125$
Group G I	1	$y = 2.7875x - 4.9817$
Group G II	2	$y = 12.943x + 16.458$
Group G III	4	$y = 21.676x + 9.9347$
Group G IV	8	$y = 22.68x + 30.24$
Group G V	16	$y = 18.488x - 9.425$

Table 2

Relation between flux rate and time at different direct currents, mA/cm^2

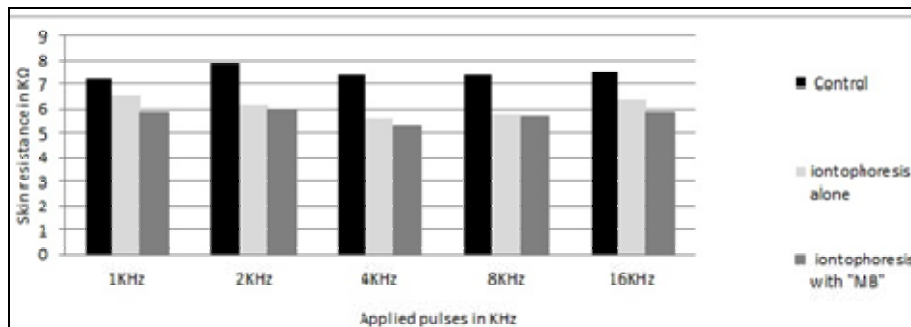| Group	Direct current values (mA/cm^2)	Trend line linear equation
G	0	$y = 0.7163x + 1.3125$
GIa	0.2	$y = 2.1538x + 0.2808$
GIIa	0.4	$y = 1.7x + 3.6293$
GIIIa	0.8	$y = 1.6138x + 0.9092$
GIVa	1.6	$y = 0.8725x + 1.025$
GVa	3.2	$y = 0.85x + 0.3267$

Fig. 7. Variation of skin resistance as the pulsing rate values of the applied current.
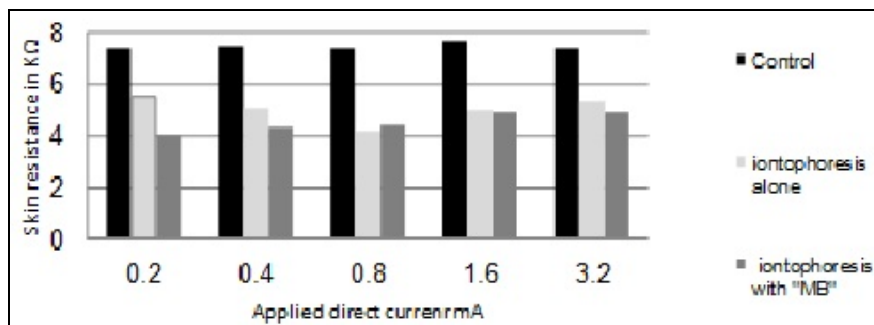

Fig. 8. Variation of skin resistance as the direct current values.

HISTOLOGICAL MEASUREMENT OF MB PENETRATION DEPTH IN RAT SKIN

Skin samples were exposed to pulsing square wave current at different pulse rates (1, 2, 4, 8, a pulse frequency of 16 kHz) and DC at different mA/cm^2 ranging from 0.2 to 3.2 mA/cm^2 in the presence of methylene blue except for control samples as shown in Figures 9 and 10. It is clear that, by increasing the pulsing rate, an increase in methylene blue penetration depth through the skin was observed, reaching to a maximum depth at 8 k pulse/sec while 0.2 mA/cm^2 was the most effective for DC. Figures 11 and 12 show the relation between MB transported amount and penetration depth in rat skin due to applying the pulsed current and direct current respectively.
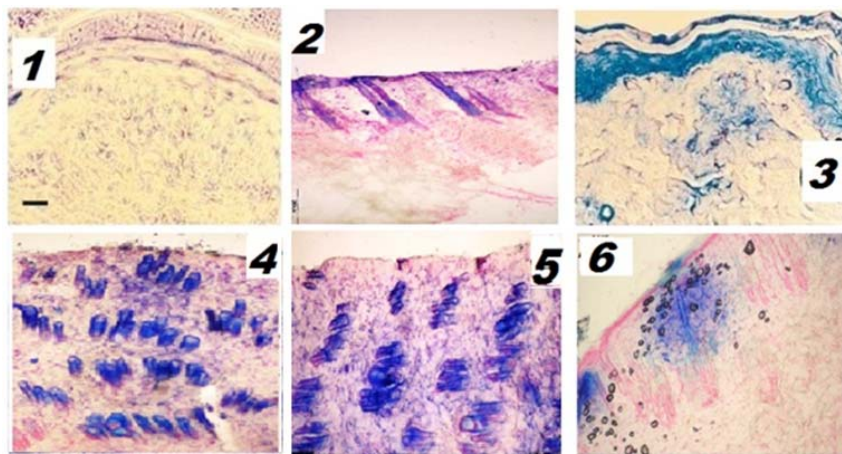

Fig. 9. The effect of the applied pulsed current on the penetration of MB of rat skin (control, 1, 2, 4, 8 and a pulse frequency of 16 kHz, respectively).
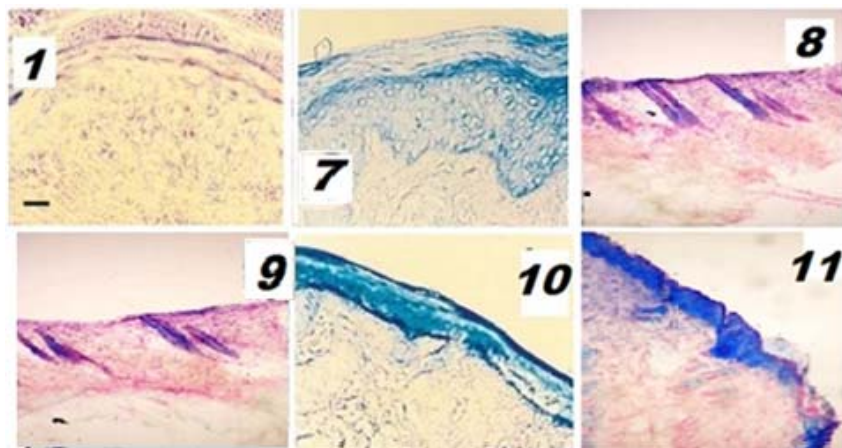

Fig. 10. The effect of the applied direct current electric field on the penetration of MB of rat skin (control, 0.2, 0.4, 0.8, 1.6 and 3,2 mA/cm^2, respectively).
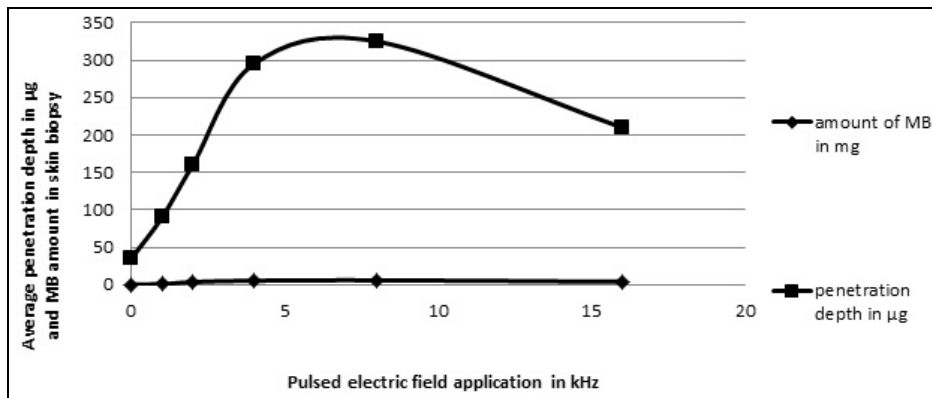
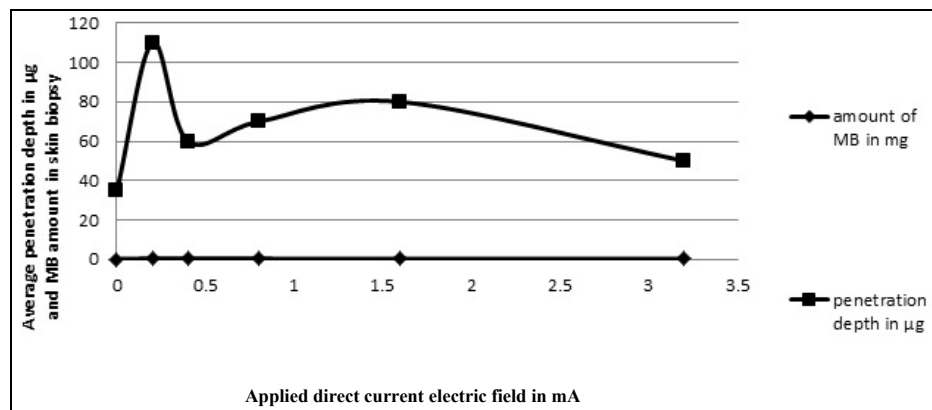Fig. 11. Relation between transported amount and penetration depth of MB.

Fig. 12. Relation between transported amount and penetration depth of MB.

DISCUSSION

Epidermal delivery of MB is enhanced by the application of square pulses. Even at low electric exposure doses, MB transport in pulsing current iontophoresis was ≈8 orders of magnitude greater than that seen using direct current iontophoresis or passive diffusion (Figures 3 and 4). The sharp increase of MB transport in direct current iontophoresis shows a steep jump at a threshold dose of 0.2 mA/cm^2, and is less obvious between 0.4 and 0.8 mA/cm^2 (Fig. 4). This may be explained by the fact that the sizes of pores are related to the direct current values. Therefore, a steep jump might be expected to appear when the average pore size becomes large enough to accommodate the molecule in question (MB) and diffusion and electromotive transport through long-term openings begins to contribute to the total transport.

Taken together, our results support the view [2, 4] that, under pulsing frequency conditions, molecular transport takes place through a combination of forces. An initial permeabilization is required for transport to begin. (Electroporation refers to a decrease of membrane resistance, accompanied by an increase in permeant fluxes). Above that threshold, continued pulsing results in enhanced transport, probably due to electromotive forces (electrophoresis and electroosmosis). Electrophoresis refers to the movement of ionic species due to electrical potential gradient, and it is shown to be very important to the delivery of ionic compound. Electroosmosis refers to the field induced convective solvent flow [18]. As shown in Figure 5, this may be due to changes in skin cell pores caused by the pulsed electric field. Further interpretation is needed using the analytical electron microscope to confirm this hypothesis.

The importance of electropermeabilization as a factor in this process is emphasized by the relatively poor penetration by DC iontophoresis. Furthermore, transport cannot be attributed to ''pulsing iontophoresis'' (i.e., electromotive processes) alone, because penetration of MB, applied to the skin only after pulsing, was enhanced due to diffusion of the drug through persisting electropores (Fig. 6).

Skin impedance is critical in determining the efficacy of electrically assisted transdermal transport techniques. Because living skin impedance greatly varies according to different factors [5, 29], we studied the effect of the entire treatment on skin impedance. The results reported in Figure 7 and 8 demonstrate that rat skin impedance after shaving was about 7.5 kΩ. This value is compatible with the age of the rats used in these experiments, given that skin resistance in rats increases with aging [17], and it is also compatible with values determined in Wistar male rats where abdominal hair was clipped [11].

Tables 1 and 2 show that there is a good correlation between the MB amount penetrated in rat skin biopsies in case of square wave pulsing electric field (corr. coefficient = 0.97) while in DC electric field it was smaller (corr. Coefficient = 0.7). This gives evidence that pulsing frequency plays a crucial role in MB transport and penetration through rat skin biopsies.

Histological measurements demonstrate MB penetration depths enhanced after pulsing square wave and direct current (Figures 9–12). Our results correlate well with the various assays found in the previous studies of Gupta *et al.*, Li SK *et al.*, and Pikal *et al.* [7, 13, 18]. Results shown in this study prove that there is a relation between applied iontophoretic frequency and the penetration depth of the drug inside the skin. In case of *in vivo* studies this will help the drug to reach the systematic blood circulation of the rat.

In future studies, it will be suitable to test the systematic penetration of the iontophoretic drug by taking blood samples from rat tail to analyze the blood content of the drug, and then correlate the drug concentration and the applied iontophoresis frequency.

CONCLUSION

This work indicates that transport induced by electric pulse iontophoresis was more than 8 orders of magnitude greater than that seen following direct current iontophoresis. Our studies indicate that dye penetration was optimal in electric pulse iontophoresis, at a pulse frequency of 8 kHz, whereas in DC iontophoresis dye penetration reached its maximum at a current density of 0.2 mA/cm^2. We believe that the enhanced cutaneous delivery of methylene blue is due to a combination of permeabilization of the stratum corneum by electric pulses, passive diffusion through the permeabilization sites, and electrophoretic and electroosmotic transport by the electric pulses.

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