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International Journal of Pharmaceutics 263 (2003) 25–33
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Electronically facilitated transdermal delivery of human parathyroid hormone (1–34)

Babu M. Medi, Jagdish Singh*

Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State University, Fargo, ND 58102, USA

Received 14 February 2003; received in revised form 27 May 2003; accepted 12 June 2003

Abstract

Electronically facilitated transdermal delivery of human parathyroid hormone (1–34), hPTH (1–34), was investigated in vitro, using dermatomed porcine skin. The effect of iontophoretic current density, electroporative pulse voltages and also electroporation followed by iontophoresis was investigated on the in vitro percutaneous absorption of hPTH (1–34). Iontophoresis at 0.5 mA/cm^2 current density significantly enhanced ($P < 0.05$) the flux of hPTH (1–34) in comparison to passive flux. Electroporation pulses of 100, 200 and 300 V significantly increased ($P < 0.05$) the flux of hPTH (1–34) in comparison with the passive as well as iontophoretic flux at 0.5 mA/cm^2. The electroporative flux of hPTH (1–34) was found to vary linearly ($R^2 = 0.97$) with the pulse amplitude. The principal barrier of the skin, stratum corneum, was found perturbed following the pulses as evident by light microscopy studies. The application of electroporation pulses followed by iontophoresis further increased the flux by several fold. The flux of hPTH (1–34) with the electroporation pulses of 100 and 300 V followed by iontophoresis at 0.2 mA/cm^2 was 10- and 5-fold higher, respectively, in comparison to the flux with corresponding pulses alone. This shows the synergistic effect of iontophoresis in combination with electroporation on skin permeability of hPTH (1–34). The results indicate the possibility of designing controlled transdermal delivery systems for hPTH (1–34) using electroporation followed by iontophoresis.

Keywords: Transdermal drug delivery; Electroporation; Iontophoresis; hPTH (1–34); Porcine skin

1. Introduction

Bioactive recombinant macromolecules, such as proteins and peptides, are poorly bioavailable when delivered orally and therefore administered invasively by intravenous or subcutaneous injections. The need for frequent administration associated with their short plasma half-lives and poor patient compliance demands an alternative drug delivery mode for these molecules. Transdermal protein/peptide delivery is such a mode given its noninvasiveness, ease of administration and better patient compliance in addition to avoiding gastro-intestinal and first-pass degradation in liver. But the highly lipophilic nature of stratum corneum (SC) hampers the passive transport of these charged macromolecules across the skin into systemic circulation (Flynn, 1989). Several investigations delved into this aspect and studied different methods including chemical and physical methods to overcome this primary barrier (Bronaugh and Maibach, 1989; Singh and Singh, 1993). Electrical methods like iontophoresis and electroporation have been studied extensively to enhance the transport of macromolecules across the skin by overcoming the barrier of the SC.

* Corresponding author. Tel.: +1-701-231-7943; fax: +1-701-231-8333.
E-mail address: jagdish.singh@ndsu.nodak.edu (J. Singh).

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doi:10.1016/S0378-5173(03)00337-5

Iontophoresis, a non-invasive technique, is the application of physiologically acceptable mild electrical potential gradient to increase the migration of ions and charged molecules through the skin (Green et al., 1993; Singh and Bhatia, 1996). This approach seems to be promising for peptide/protein delivery (Bhatia and Singh, 1998; Rastogi and Singh, 2001, 2002). Transdermal iontophoresis facilitates the transport of poorly permeable molecules across the skin by electrorepulsion and electroosmosis (Guy et al., 2000). The intercellular and trans-appendageal pathways of SC are shown to be predominant during iontophoretic transport (Monteiro-Riviere et al., 1994).

Electroporation is yet another electrical technique, which involves the application of high voltage electric pulses of very short duration (microsecond–millisecond) to enhance the skin permeability reversibly for macromolecules. Electroporation of cell membrane has been studied extensively and used since 1970's for DNA transfection of the cells by reversibly permeabilizing the cell membranes with the application of brief electric pulses (Auer et al., 1976; Kinosita and Tsong, 1977; Andreason and Evans, 1988). But, the use of electroporation has been shown recently to reversibly permeabilize skin for enhancing the transdermal delivery of several molecules such as calcein (Prausnitz et al., 1993), oligonucleotides (Zewert et al., 1995), cyclosporin A (Wang et al., 1998), calcium regulating hormones (Chang et al., 2000), terazocin HCl (Sharma et al., 2000). Skin electroporation is believed to create new transport pathways, in addition to expanding the existing pathways (Pliquett, 1999).

Human parathyroid hormone (1–34), hPTH (1–34), has been shown to have both anabolic and catabolic effects on bone (Dempster et al., 1993; Uzawa et al., 1995). Daily injections of 20–40 μg of hPTH (1–34) has been recommended in the treatment of osteoporosis (Mitlak, 2002). Osteoporosis is a condition of decreased bone mass affecting mostly post-menopausal women, which causes bones to be more susceptible to fracture. Daily injections are agonizing for patients especially the older persons, in whom the condition is prevalent. The noninvasiveness and the ability to deliver in a controlled, pulsative manner may increase the acceptability of transdermal delivery systems of hPTH (1–34). Although iontophoretic transdermal delivery of hPTH (1–34) was reported in detail (Suzuki et al., 2002) there are no detailed reports on electroporative transdermal delivery of hPTH (1–34), either alone or in combination with iontophoresis. In this study, we investigated the effect of iontophoresis, electroporation and also electroporation followed by iontophoresis on the transdermal delivery of hPTH (1–34). We also studied the effect of electroporation pulses on the microscopic changes in the porcine skin.

2. Materials and methods

2.1. Materials

^{125}I-hPTH (1–34) was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Ten percent neutrally buffered formalin (Accustain®) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Silver wire (0.5 mm diameter, 99.9%) was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). All other chemicals used were of analytical grade. Deionized water obtained with a Barnstead Nanopure Infinity® ultrapure water system (Barnstead, Boston, MA), having resistivity of $\geq$18 MΩ cm was used to prepare all solutions and buffers.

2.2. Preparation of porcine skin

Porcine skin was used in the in vitro transport studies because of its similarity to the human skin (Walker et al., 1997; Wester et al., 1998). Skin from pig and monkey more generally approximates the permeability of human skin (Wester and Maibach, 1989). Histological characteristics of pig and human skin are comparable, in respect to epidermal thickness and composition, pelage density (Montagna and Yun, 1964), lipid content (Gray and Yardley, 1989) and general morphology (Meyer et al., 1978; Monteiro-Riviere, 1986). Porcine ears (Yorkshire marine pigs, male, weighing about 200 lb) were obtained from a local slaughterhouse. After cleaning under cold running water, the external/dorsal skin was dermatomed to 0.5 mm thickness using Padgett Electro Dermatome (Model B, Padgett Instrument Inc., Kansas City, MO, USA), and stored refrigerated at 4 °C in Eagle's minimum essential medium to preserve skin viability (Hurst et al., 1984). For light microscopic studies, full thickness porcine skin was used. Full thickness dorsal/external skin from the pig ear was removed carefully

from the underlying cartilage using a scalpel, and used immediately to study the effect of electroporation on the skin.

2.3. In vitro transdermal delivery

In vitro percutaneous absorption experiments can be very good predictors of in vivo absorption (Bronaugh, 1989). The ability of static and flow through systems to measure in vitro percutaneous absorption of chemicals is reported to be very similar (Hughes et al., 1993; Clowes et al., 1994). Franz diffusion cells modified for iontophoresis were used in all in vitro transport studies. The skin was sandwiched between the donor and receiver compartments of the diffusion cell with the stratum corneum side facing the donor compartment. The surface area of skin exposed to the drug solution was 0.785 cm^2. The donor compartment was filled with 1 ml of 0.2 μCi/ml (specific activity: 1044.3 Ci/mmol; donor concentration: 1.915×10^{-04} nmoles) of ^{125}I-hPTH (1–34) in citrate buffer (pH 6.0). The donor solution consisted of citrate buffer (51 mM) containing 75 mM sodium chloride, with 0.2 μCi/ml of ^{125}I-hPTH (1–34). The receiver compartment was filled with 5 ml of phosphate buffered saline (PBS, pH 7.4). The PBS contained 100 mM phosphate buffer and 154 mM NaCl. The cells were maintained at $37 \pm 0.5\,^{\circ}C$ by PMC Dataplate® stirring digital dry block heater (Crown Bioscientific Inc., NJ). The contents of the receiver compartment was stirred with a small Teflon-covered magnetic bar at 100 rpm.

2.3.1. Iontophoretic delivery

For iontophoretic transport studies, a constant current source (Kepco power supplier, APH 1000 M, Kepco, Flushing, NY, USA) was used to deliver the required current. The isoelectric point of hPTH (1–34) is around 8.0, so at pH 6.0 it was positively charged and was reported to be stable (Nabuchi et al., 1997). Iontophoretic studies were performed using Ag/AgCl (0.5 mm diameter) electrodes. The anode was kept in the donor and the cathode in the receiver compartments (anodal iontophoresis).

2.3.2. Electroporative delivery

In electroporative transport studies, a square wave pulse electroporator (CUY21 EDIT Version, Protech International Inc., San Antonio, TX, USA) was used to generate the electric pulses of different magnitude. Silver wire was used as anode and was placed in the donor. Silver–silver chloride was used as cathode and located in the receiver compartment. The anode was placed 1 cm above the skin in the donor compartment and the cathode was placed 1 cm below the skin surface. For electroporative delivery of hPTH (1–34), 20 pulses of 100 ms pulse length with 1 s interval between each pulse and of different voltage were applied at the beginning. The pulse voltages investigated were 100, 200 and 300 V. Pulses were applied to 0.785 cm^2 area of the skin.

2.3.3. Electroporation in combination with iontophoresis

In the case of electroporation in combination with iontophoresis, pulses of specified magnitude were applied using silver–silver chloride electrode in the beginning followed by iontophoresis at 0.2 mA/cm^2 immediately for 15 h. At specified time intervals, 0.5 ml samples were withdrawn from the receiver compartment and an equivalent amount of PBS was added to maintain a constant volume. All the samples collected were analyzed for hPTH (1–34) using a gamma counter (Gamma 5500 counting system, Beckman Instruments Inc., Irvine, CA, USA) by quantifying ^{125}I-hPTH (1–34).

2.4. Light microscopy

Freshly removed full thickness porcine ear skin was used for light microscopic investigations. The skin was sandwiched between the donor and receiver compartments of Franz diffusion cells with the SC side facing the donor compartment. Phosphate buffered saline (pH 7.4) was filled in both donor and receiver compartments. The donor and receiver compartments contained 1 and 5 ml of PBS, respectively. Electroporation pulses were applied as described in Section 2.3.2. The area of the skin exposed to the pulses was 0.785 cm^2. The electroporation-treated and control (without any pulses) skin samples were fixed immediately in 10% neutrally buffered formalin solution (Accustain®). Sections of 10 μm were cut by a rotary microtome and stained with hemotoxylin and counter stained with eosin. The stained sections were observed under light microscope (Meiji Microscope, Osaka, Japan) with

100× magnification and photographed by Polaroid® Microcam camera on 339 Polaroid Auto Film.

2.5. Data analysis and statistics

The receiver compartment concentration of hPTH (1–34) was corrected for sample removal by using the equation given by Hayton and Chen (1982). The steady-state flux (J_{ss}) was calculated as the slope of the straight-line region from the plot of cumulative amount of hPTH (1–34) permeated per unit skin surface area versus time. The permeability coefficient (K_p) was calculated as (Scheuplein, 1978):

$$K_p = \frac{J_{ss}}{C_v}$$

where, C_v is the donor concentration of hPTH (1–34). Statistical comparisons were made using ANOVA and Student's *t*-test. The probability values of less than 0.05 was considered to be significant.

3. Results and discussion

3.1. Iontophoretic delivery

The effect of iontophoresis on the transport of hPTH (1–34) across the porcine skin was studied. Fig. 1 shows the iontophoretic transport profiles of hPTH (1–34) at 0.2, 0.3, 0.4 and 0.5 mA/cm^2 along with the passive transport. The flux and permeability coefficients of hPTH (1–34) during passive and iontophoresis are given in the Table 1. Iontophoretic current densities of 0.2, 0.3, and 0.4 mA/cm^2 did not produce any significant increase ($P > 0.05$) in hPTH (1–34) flux in comparison to the passive flux. The iontophoretic flux of hPTH (1–34) at 0.5 mA/cm^2 was significantly higher ($P < 0.05$) than the passive flux. The iontophoretic flux has been shown to increase linearly with the current density in iontophoretic studies (Behl et al., 1989). But, our study shows non-linear dependence of iontophoretic flux on current density. Similar non-linear dependence of flux has been shown for calcein (Prausnitz et al., 1996). The above study offers the explanation that at low voltage/current, transdermal pathways pass small ions and molecules, but partially hinder transport of larger molecules whereas at higher

Table 1
The effect of current density on flux and permeability coefficient of hPTH (1–34) through porcine skin

Treatment	Flux ($\times 10^9$ nmol/(cm^2 h))	Permeability coefficient ($\times 10^6$ cm/h)
Passive	1.52 ± 0.46	7.94 ± 2.42
0.2 mA/cm^2	1.74 ± 0.30	9.08 ± 1.58
0.3 mA/cm^2	1.73 ± 0.15	9.06 ± 0.78
0.4 mA/cm^2	1.96 ± 0.13	10.25 ± 0.69
0.5 mA/cm^2	3.06 ± 0.02[a]	16.00 ± 0.10[a]

Values are expressed as the mean ± S.D. of three determinations.
[a] Significantly higher ($P < 0.05$) than passive.

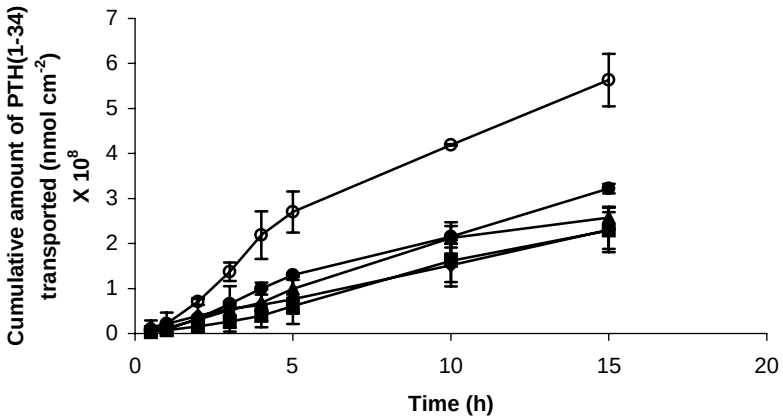

Fig. 1. The effect of iontophoretic current density on in vitro transport of hPTH (1–34) through porcine skin. Each data point is the mean ± S.D. of three determinations. Key: (◆) passive; (■) 0.2 mA/cm^2; (▲) 0.3 mA/cm^2; (●) 0.4 mA/cm^2; (○) 0.5 mA/cm^2.

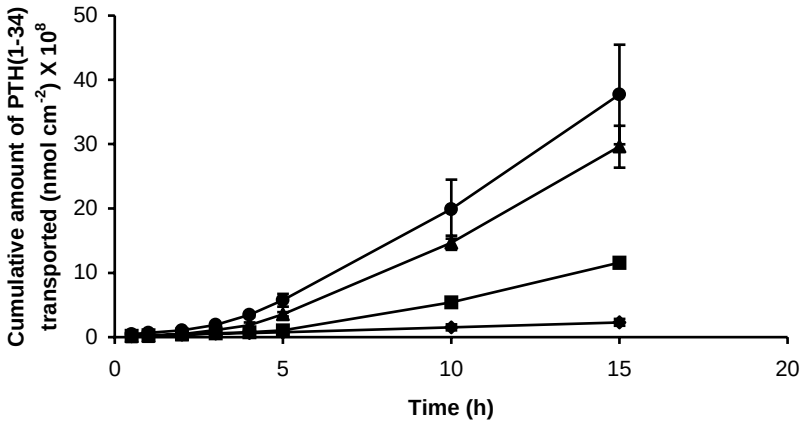

Fig. 2. The effect of electroporation pulse voltages on transdermal transport of hPTH (1–34) through porcine skin in vitro. Each data point is the mean ± S.D. of three determinations. Key: (◆) passive; (■) 100 V; (▲) 200 V; (●) 300 V.

voltages/currents, larger pathways exist, which allows passage of both small as well as larger molecules.

3.2. Electroporative delivery

We also investigated the transport of hPTH (1–34) through porcine skin facilitated by electroporation. The effect of the pulse magnitude was studied by keeping the other parameters (pulse length, pulse interval, number of pulses) constant. Fig. 2 demonstrates electroporative transport profiles of hPTH (1–34) with 100, 200 and 300 V pulses. Electroporation of skin with pulses of 100, 200 and 300 V significantly ($P < 0.05$) enhanced the flux of hPTH (1–34) in comparison to the passive and iontophoretic flux (Table 2). Enhancements in the flux of hPTH (1–34) by $U_{electrode,0}$ (initial voltage applied across the delivery electrodes) of 100, 200 and 300 V electroporation pulses in comparison to

Table 2
The effect of electroporation pulse voltages on flux and permeability coefficient of hPTH (1–34) through porcine skin

Treatment	Flux ($\times 10^9$ nmol/(cm^2 h))	Permeability coefficient ($\times 10^5$ cm/h)
Passive	1.52 ± 0.46	0.79 ± 0.24
100 V	10.49 ± 0.58[a]	5.47 ± 0.30[a]
200 V	25.15 ± 2.57[a,b]	13.13 ± 1.34[a,b]
300 V	31.07 ± 6.74[a,b]	16.22 ± 3.52[a,b]

Values are expressed as the mean ± S.D. of three determinations.
[a] Significantly higher ($P < 0.05$) than the passive.
[b] Significantly higher ($P < 0.05$) than 100 V.

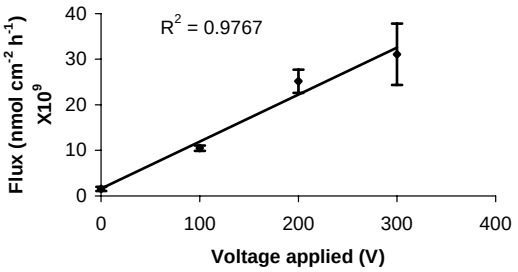

Fig. 3. Effect of electroporation pulse voltages on the flux of hPTH (1–34) through porcine skin. Values are shown as the mean ± S.D. of three determinations.

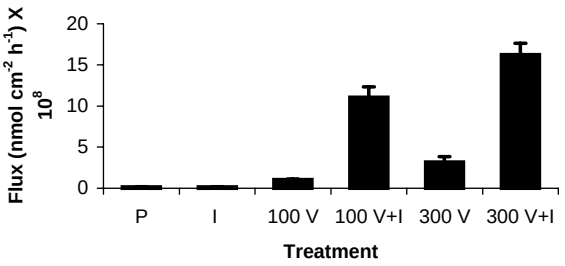

Fig. 4. Flux of hPTH (1–34) with electroporation and iontophoresis through porcine skin. Key: (P) Passive, (I) Iontophoresis at 0.2 mA/cm^2, (100 V) electroporation pulses of 100 V, (100 V + I) electroporation pulses of 100 V followed immediately by iontophoresis of 0.2 mA/cm^2, (300 V) electroporation pulses of 100 V and (300 V + I) electroporation pulses of 300 V followed immediately by iontophoresis of 0.2 mA/cm^2. All the values are shown as the mean ± S.D. of three determinations.

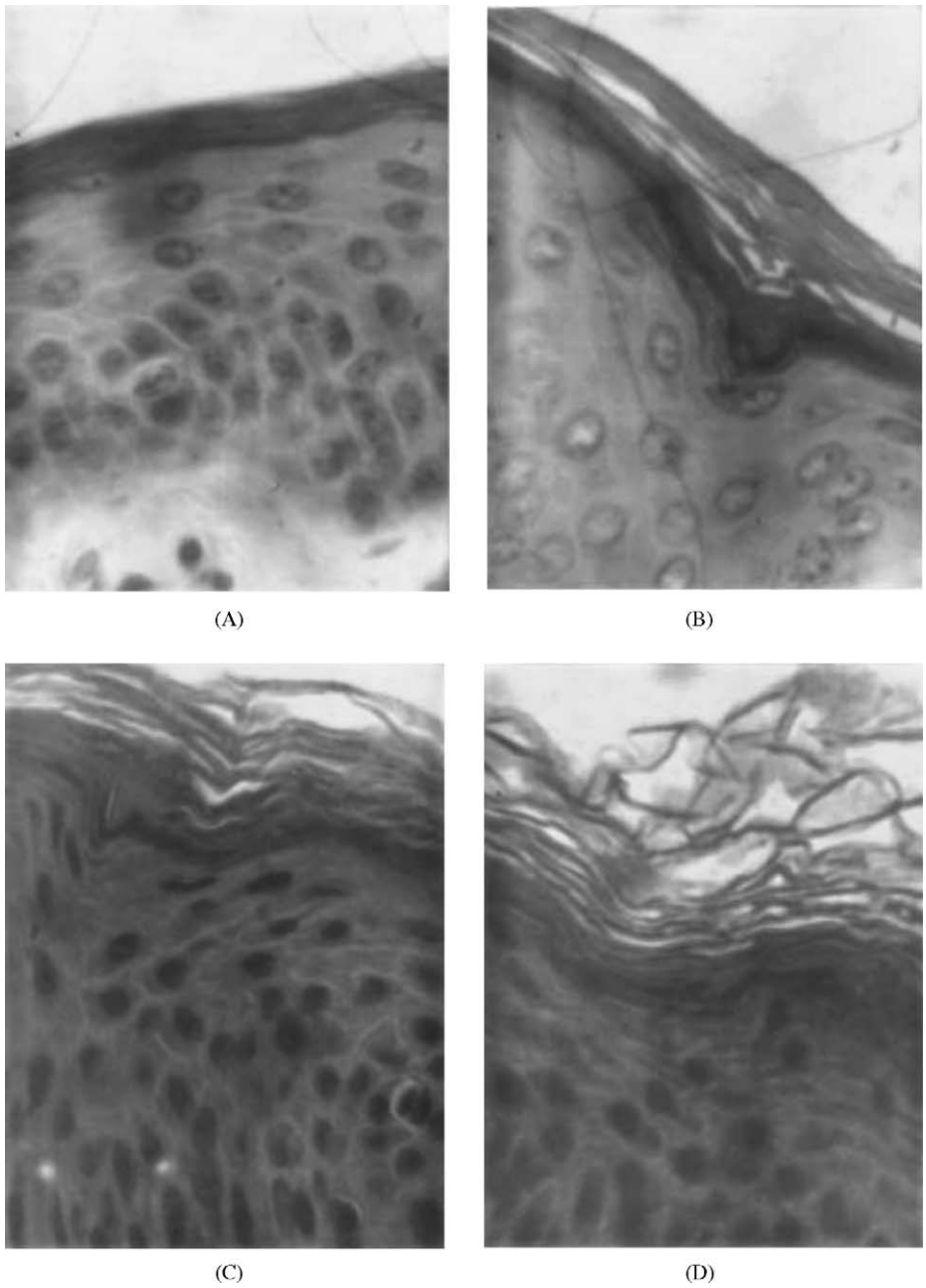

Fig. 5. Effect of electroporation on skin. Key: (A) microscopic section of control porcine skin (without any electric pulses), 100×; (B) microscopic section of skin sample electroporated with pulses of 100 V; (C) microscopic section of skin treated with pulses of 200 V; (D) microscopic section of skin treated with pulses of 300 V.

passive flux were 6.9, 16.5 and 20.4, respectively. The flux increased linearly ($R^2 = 0.97$) with the increasing $U_{electrode,0}$ (Fig. 3). This is consistent with the findings reported by Sharma et al. (2000). They have reported that the flux of Terazosin hydrochloride increased linearly as the voltage was increased, above a threshold voltage. The long-lasting increase in the permeability of the skin observed in this study is likely due to the changes in SC caused by the rise in temperature with the long duration high voltage pulses (Martin et al., 2002; Pliquett et al., 2002). The increase in the flux of hPTH (1–34) with increasing electroporative pulse voltage provides a way for controlled delivery of this hormone.

3.3. Electroporation in combination with iontophoresis

Electroporation followed by iontophoresis further enhanced the flux of hPTH (1–34) by several fold (Fig. 4). The flux was 10- and 5-fold higher with the electroporation pulses of 100 and 300 V, respectively, followed by iontophoresis at 0.2 mA/cm^2 in comparison to the flux of hPTH (1–34) with pulses alone. This shows the synergistic effect of electroporation in combination with iontophoresis. Bommannan et al. (1994) also reported the similar results with LHRH using electroporation of skin followed by iontophoresis. It may be due to the formation of large number of transient pores in the skin by electroporation, which can be used as transport pathways during iontophoresis (Mitragotri, 2000; Prausnitz et al., 2000). Therefore, electroporation followed by iontophoresis immediately, might provide the driving force required for achieving therapeutic levels of hPTH (1–34) transdermally.

The flux of hPTH (1–34) with 100 and 300 V electroporation pulses combined with iontophoresis was found to be 4.63×10^{-4} and 6.78×10^{-4} μg/(cm^2 h), respectively. Assuming linear relationship between the donor concentration and the flux, a donor concentration of 5 mg/ml and a patch size of 10 cm^2 results in a flux of 29 and 42 μg/h, respectively, with 100 and 300 V pulses followed by iontophoresis. The therapeutic dose recommended is 20–40 μg per day (Mitlak, 2002). This shows that electroporation pulses of 300 V followed by iontophoresis at 0.2 mA/cm^2 may result in delivering the desired daily dose of hPTH (1–34) within 1 h. The electrical parameters can be varied along with the drug concentration in order to control the amount of hPTH (1–34) delivered. This shows the possibility of designing electronically controlled transdermal delivery systems for hPTH (1–34).

3.4. Light microscopy

There were no visible changes in the skin immediately after electroporation at all the tested voltages. Fig. 5 shows the light micrographs of control and electroporation-treated porcine skin. The effect of pulse amplitude was examined by keeping the other parameters constant. Fig. 5A shows the light micrograph of control skin where SC was found intact. We found an increased detachment in the SC cell layers with increasing electroporation voltages from 100 to 300 V (Fig. 5B–D). With the higher voltages (i.e. 200 and 300 V) the epidermis assumed amorphous nature. We also observed dramatic decrease in the skin resistance with electroporation pulses (data not shown). This indicates the perturbation of SC barrier properties. This can be correlated to the observed linear increase in the flux of hPTH (1–34) with the electroporation pulse amplitude and also to the flux enhancement by the combination of electroporation and iontophoresis. In the case of electroporation followed by iontophoresis, the large increase in the flux can be explained due to the driving force provided by iontophoresis for the hPTH (1–34) molecules to cross the electroporated SC.

4. Conclusions

In this study, we demonstrated that iontophoresis and electroporation enhanced the transdermal delivery of hPTH (1–34). Electroporation pulses resulted in the perturbation of SC barrier properties. The linear increase in the flux of hPTH (1–34) with increasing $U_{electrode,0}$ along with the iontophoretic current density, might provide a means of controlling the amount of hPTH (1–34) delivered through the skin. Electroporation followed by iontophoresis shows synergistic effect on flux enhancement. More studies to optimize the amount of hPTH (1–34) delivered and to establish the recovery of skin after electroporation are warranted.

Acknowledgements

We acknowledge the financial support from National Institutes of Health grant #HD 41372 and the Presidential Doctoral Fellowship to B.M.M from North Dakota State University, Fargo, ND 58105, USA.

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