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# In vivo assessment of skin electroporation using square wave pulses

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**Abstract**

The application of short-duration high-voltage pulses to the skin has been shown to enhance transdermal drug delivery by several orders of magnitude and to transiently permeabilize cells in tissue. Both exponentially decaying (ED) pulses and square wave (SW) pulses have been applied. The latter have also been used for electrochemotherapy. To date, their effect on skin integrity has not been analyzed. The scope of this work was (i) to investigate the effect induced by SW pulses on the stratum corneum and the skin, (ii) to evaluate the safety issue associated with electroporation, (iii) to contribute to the understanding of drug transport. Biophysical techniques (transepidermal water loss, chromametry, impedance and laser Doppler velocimetry or imaging measurement) and histological methods were combined to provide a global picture of the effects. Ten SW pulses applied to the skin induced a mild impairment of the skin barrier function and a dramatic decrease in skin resistance. These changes were reversible. A transient decrease (<5 min) in blood flow was observed. Neither inflammation, nor necroses were observed. These studies confirm the tolerance of the skin to square wave pulses in vivo.


*Keywords:* Electroporation; Electrochemotherapy; Square wave pulse; Skin integrity; Transdermal drug delivery

## 1. Introduction

Transdermal drug delivery has the potential to be a non-invasive, user-friendly method of delivering drugs. However, because transdermal molecular passage is impeded by the barrier properties of the skin, transport of most drugs across the skin is very slow [1]. Different physical and chemical methods have been developed to overcome this barrier and enhance transdermal drug delivery [2].

The application of short-duration high-voltage pulses, i.e., electroporation (i) enhances transdermal drug delivery by several orders of magnitude [3–7] and (ii) transiently permeabilizes cells in tissue [8–12]. The resistance of the stratum corneum, which is the most important barrier of the skin, decreases dramatically within less than 1 μs upon application of high-voltage pulse. This phenomenon is attributed

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to electroporation that involves the creation of transient aqueous pathways by the applied electrical pulses [13,14].

Several studies have investigated the morphological changes or integrity of the skin after exponentially decaying pulses in vitro or in vivo [15,16]. An extended study [Freeze-Fracture electron Microscopy (FFEM), impedance, Fourier transform infrared spectroscopy (FT-IR), X-ray, Differential Thermal Analysis (DTA)] was performed to provide a complete picture of the stratum corneum structure after high voltage pulse application in vitro. Electroporation was reported to induce (i) a disorganisation of the stratum corneum lipid bilayers; (ii) an increase in skin hydration; (iii) a decrease in skin resistance induced by electroporation. These changes were partly reversible [16,17]. Multilamellar vesicles were observed in the intercellular lipid bilayers of the stratum corneum [18]. These studies were supplemented by non-invasive bioengineering methods in vivo. Transient increase in TEWL (transepidermal water loss) was associated with an enhancement in skin hydration and alteration in stratum corneum barrier function. Reversible increase in cutaneous blood flow measured by laser Doppler velocimetry (LDV) and an erythema evaluated by chromametry was also observed [19]. Dramatic decrease in skin resistance or impedance has been reported by many authors (for a review, see Ref. [13]) but to date, there have been no measurements of skin impedance in vivo either during or after pulsing.

Besides transdermal drug delivery, another in vivo application of electroporation is electrochemotherapy, a new therapeutic approach providing delivery into the cell interiors of non-permeant drugs, which have intracellular targets. After intravenous (i.v.) or local injection of a non-permeant drug, e.g., bleomycin, which has a high intrinsic cytotoxicity, local application of short and intense high voltage pulses permeabilize exposed cells. Preclinical trials have shown the efficacy of this new therapeutic modality in various tumor models. Clinical trials have demonstrated its efficacy for the local treatment of cancers. The pulses seem to be well tolerated by patients [20,21].

Both exponentially decaying (ED) pulses and square wave (SW) pulses have been used. In contrast to ED pulses, SW pulses can be set at constant predetermined values of voltage and pulse length. They have been used in clinical trials of electrochemotherapy and are being investigated for transdermal delivery of macromolecules. To date, their effect on skin integrity has not been analysed.

The scope of this work was: (i) to investigate the effect induced by high-voltage pulse application used for transdermal drug delivery and for electrochemotherapy (SW pulses) on the stratum corneum and the skin, (ii) to evaluate the safety associated with electroporation and (iii) to contribute to the understanding of transdermal drug transport. Non-invasive biophysical methods to assess barrier function (TEWL, impedance) and cutaneous blood flow [LDV, Laser Doppler Imaging (LDI), dye diffusion], were combined with histology to provide a global picture of the effects.

## 2. Materials and methods

### *2.1. Animals and chemicals*

Hairless male rats' 8 weeks old were housed in standard cages at room temperature (IOPS mutant from Iffa Credo, France). Standard laboratory food (A04, UAR-France) and water were given ad libitum. Salts for buffer preparation were analytical grade (Merck Eurolab, Belgium).

### *2.2. Rat treatment*

Experiments were carried out in an assigned laboratory room with controlled temperature (20 °C) and relative humidity (30%). Animals were anesthetized before experiments and if necessary, before a measurement session with Thalamonal® (Fentanyl, 50 μg/ml, Droperidol 2.5 mg/ml; Janssen Pharmaceutica, Belgium). A fold of skin was clamped into a custom built clip. The clip was composed of two compartments each containing a platinum electrode with an area of 1 $cm^2$ (99.99% purity, Aldrich, Belgium). The electrodes were at the outer surface of each compartment (not in contact with the skin). The distance between the two electrodes was 6 mm. Both compartments were filled with 100 μl phosphate buffer, pH 7.4 (10 mM). No shift in pH was observed after pulsing. The electrodes were con-

nected to the electroporation device Cytopulse PA-4000 (CytoPulse Sciences, MD, USA) for application of pulses. Cytopulse PA-4000 delivers square wave electric field pulses of variable voltage and duration. During a pulse, the electrical signal was monitored with an oscilloscope (Model 54602B, Hewlett-Packard). The voltage and current were measured directly.

Two pulsing protocols were applied: (i) 10 pulses of 1000 V (applied voltage) of 100 μs duration (10×1000 V—100 μs) and (ii) 10 pulses of 335 V, each with a duration of 5 ms [9,22]. Pulsing caused slight muscle twitches in the rat. Phosphate buffer solution was in contact with the skin for 5 min before and after the electrical treatment (except for impedance measurement, only contact for 5 min before the electrical treatment). A passive diffusion, consisting in applying the clip filled with phosphate buffer for 5 or 10 min, was performed in the control animals. The measures were performed immediately following the electroporation.

TEWL was measured using a Tewameter TM 210 (Germany). The probe was maintained in contact with the skin for 1 min in order to obtain stable TEWL value, the measurement being taken during the last 30-s period. Each measurement (expressed in $g/m^2$ h) is the result of the average of the registered values during the last 30 s.

Impedance was measured according the method described by Kalia et al. [23]. Briefly, the electrical circuit used for making the impedance measurements includes a 2 MΩ resistor in series with the skin. A Macintosh (Apple Computers), equipped with Lab View was used to control a HP8116A Pulse/Function Generator (Hewlett-Packard). A frequency of 1 Hz and an applied voltage of 1.0 V (peak to peak) were used, resulting in a approximately constant current (~0.25 μA). The potential difference across the skin was measured using a Stanford Research Systems 850 DSP lock-in amplifier. All data were analysed using the Microsoft Excel (version 97) software package. The impedance values were normalized with respect to initial pre-electroporation measurement.

### 2.3. Blood flow

Cutaneous blood flow was determined using a laser Doppler velocimeter (Periflux, PF3, Perimed, Sweden). The probe was placed directly on the skin and maintained in contact for at least 1 min. The values were recorded when a constant signal was observed. Each data point (expressed in $10^2$ Hz) is the result of three measurements.

LDI was used immediately after electroporation to visualize the blood flow under the electrodes and in the surrounding tissue. The moving blood cells from the upper 300 μm give rise to a shift in the backscattered light which is directly proportional to skin blood perfusion. The LDI system developed by Moor Instruments (Axminster, UK) is a computer-controlled system that uses a low-power He–Ne laser to scan the tissue. The Doppler components are detected and processed to generate an output signal (within 0–10 V) that is linearly proportional to tissue blood perfusion. When all measurement values have been captured and stored by the system, a color-coded image is displayed on the monitor [24].

One minute after electroporation, a dye injection of 1 ml of a solution containing 50 mg/ml of patent violet blue (Sigma, Belgium) in saline was injected i.v. The distribution of dye in the electroporated and non-electroporated sites was compared. A decrease in blood flow is characterized by a delay in the appearance of dye in the skin whereas an increased blood flow is characterized by a more intense blue color of the skin [25].

### 2.4. Histology

To assess the effect of electroporation on the skin structure, skin samples submitted to high voltage pulses in vivo were fixed in formaldehyde (Merck, Germany) and eosin–hematoxylin stained.

### 2.5. Statistics

The data were validated by the Dixon test. Dunnet's tests were used to compare the different treatments at each time ($P<0.05$).

## 3. Results

The aim of this paper was to investigate the effect of square wave pulses on the stratum corneum and

the skin. Hence, a number of different methods were used in order to provide a global understanding of the effect of electroporation on the skin. The impedance and TEWL evaluated the barrier function of the skin, LDV, LDI and patent blue staining investigated the cutaneous blood flow. The histology assessed skin integrity.

### 3.1. Impedance

Electroporation induces a dramatic decrease in skin resistance, which recovers partially in vitro [26,27]. Previous studies have not investigated the recovery of skin after electroporation in vivo. Normalized impedance values decreased significantly after pulsing as compared to a control site. Impedance value returned to control values within 6 h following the 10×(1000 V—100 μs) protocol application. However, following the 10×(335 V—5 ms) treatment, the impedance values took longer to return to the pre-electroporation values (Fig. 1).

### 3.2. TEWL

A significant increase in TEWL was observed following each electrical treatment. The highest enhancements occurred directly after treatment (at 7 min after electroporation, $P<0.05$). Following either the 1000 or 335 V protocol, TEWL returned to basal values within 35 min ($P>0.05$ at this time) (Fig. 2). These data indicate that SW pulses induce a transient impairment of the barrier function of the skin as already pointed out by the impedance measurements.

### 3.3. Blood flow

Several methods were used to evaluate the cutaneous microcirculation. LDI indicates that the blood flow was strongly decreased straight after pulse application. The blood circulation was similar to the circulation in the skin surrounding the electrodes within 5 min for 335 V (Fig. 3) and 10 min for the 1000 V protocol (data not shown). These data were confirmed by the dye injection method. A similar decrease in blood circulation was detected. The diffusion of the dye in the skin observed approximately 1 min after patent blue injection was delayed at the electroporated site. It increased progressively to reach a color equivalent to surrounding tissue within 6 and 9 min after application of the 335 and 1000 V pulses, respectively.

LDV did not show any modification in blood flow from 7 min to 78 h after electroporation (Fig. 4).

No modification of the redness was observed by chromametry (parameter $a^*$) (data not shown).

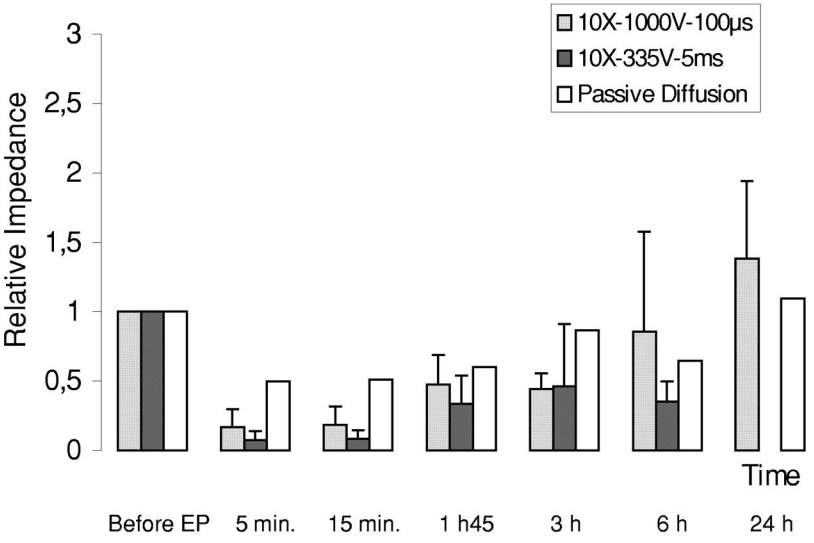


Fig. 1. Relative impedance value after sham control (DP), 10×(335 V—10 ms) or 10×(1000 V—100 μs). $N=6$.

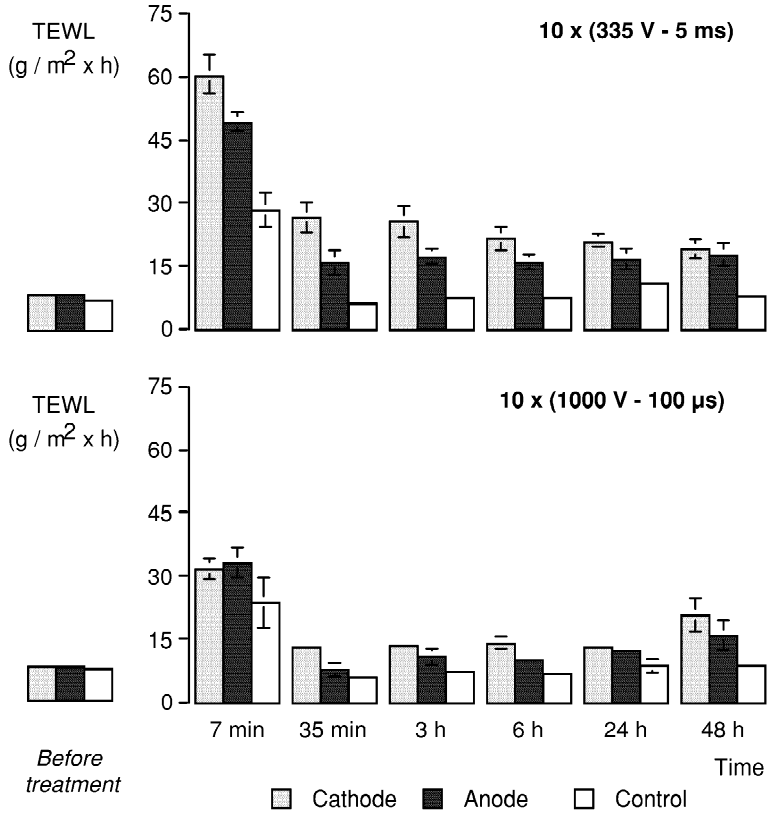


Fig. 2. TEWL ($g/m^2$ h) after skin electrical treatment. Measurements at the cathode, anode and control sites. $N=6$.

### *3.4. Histological studies*

No significant histological changes were detected in the skin subjected to electroporation in vivo. Neither inflammation nor necroses were detected after 5 min, 1, 2, 4 or 7 days after electroporation (Fig. 5).

## 4. Discussion

The scope of this work was: (i) to investigate the effect induced by square wave high-voltage pulse application used for transdermal drug delivery and for electrochemotherapy on the stratum corneum and the skin, (ii) to evaluate their safety and (iii) to contribute to the understanding of transdermal drug transport. Typical electroporation conditions used for transdermal drug delivery or electrochemotherapy were used in this study (for reviews, see Refs. [11,15]).

The effects induced by SW pulses on the skin were relatively mild and reversible.

First, since electroporation is known to induce cell death, we checked if electroporation induced cell death in the skin in vivo. Cell viability as measured by MTT test was not affected. No necrosis or inflammations were observed in histological sections of the skin [28].

These data indicate that the SW pulses used did not induce major cell damage in the skin and suggest that under the experimental conditions used, electroporation permeabilizes the keratinocytes [28–30] or superficial tumor cells [21] without affecting skin viability in vivo. Nevertheless, ultrastructural changes of the stratum corneum, e.g., a disorganisation of the lipid bilayers [17] and the formation of water vesicles [18] are induced by electroporation.

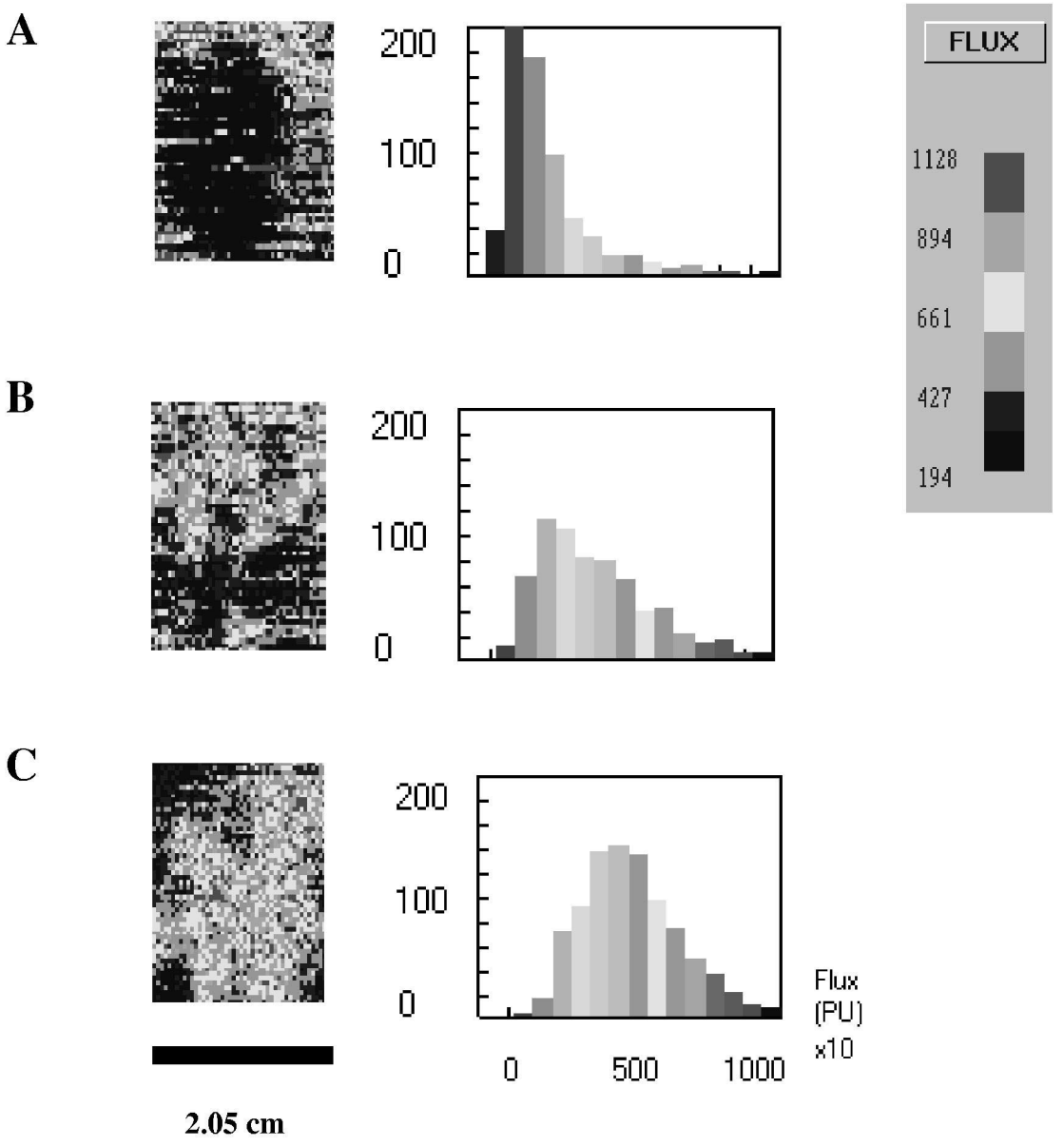


Fig. 3. LDI maps and histograms of blood perfusion in the skin of rat's abdomen 1 min 30 s (A) and 8 min (B) after 10×(335 V—5 ms). Control (C) 3 min 30 s after clipping the buffered skin. $N=3$.

Secondly, the effect of the SW pulses on skin microcirculation was evaluated by different techniques. The data indicate that the blood flow is transiently decreased for a period of 5 min but subsequently recovers and in some cases is slightly increased later on. A similar blockage of circulation was observed by Mir and co-workers in other tissue such as the liver and the muscle [11,25]. Such a decrease in blood flow could be positive for electrochemotherapy in order to prevent the loss of a cytotoxic drug injected directly in the tumor. Whether this blood circulation blockage can affect transdermal drug delivery is still unknown.

Thirdly, SW pulses induced a transient impairment

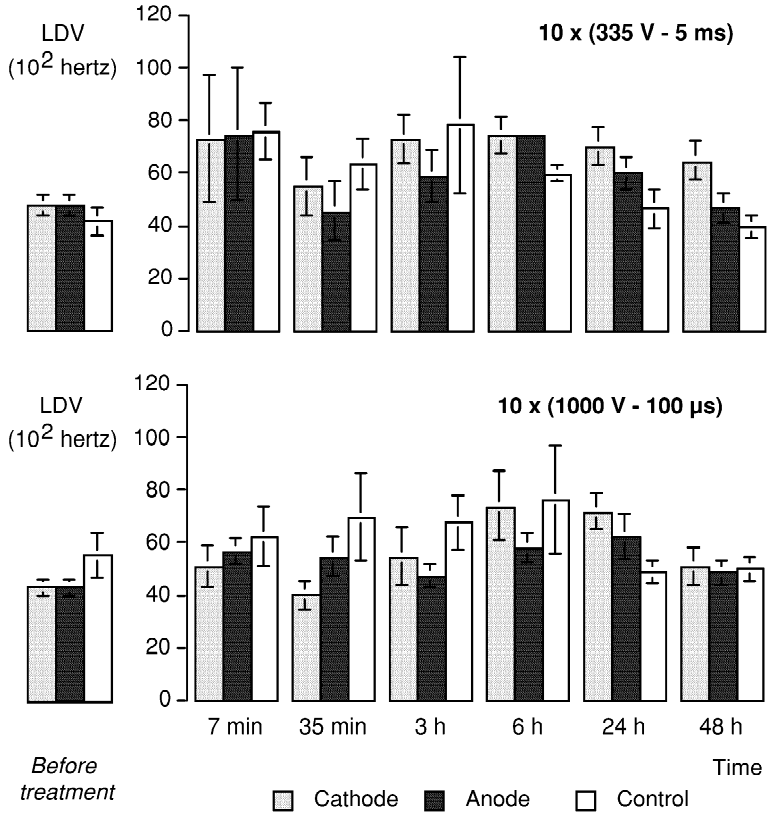


Fig. 4. LDV ($10^2$ Hz) after skin electrical treatment. Measurements at the cathode, anode and control sites. $N=6$.

of the barrier function of the skin. The dramatic decrease in skin impedance reported in all the papers dealing with in vitro skin electroporation [13,26] was also observed in vivo. It is linked with the mechanism of electroporation, which is believed to create new aqueous pathways in the stratum corneum. Skin impedance is an indirect measure of small ion transport. Its decrease indicates an enhanced skin permeability towards ions. Even though high voltage pulses dramatically lowered skin impedance, normal impedance values were achieved within 6 or 24 h, indicating relatively rapid recovery of the increased skin permeability. Similarly, TEWL increased at both the cathode and anode sites after SW pulses. The primary increase in TEWL resulted from water accumulation in the skin and from the electrical treatment itself [17,19]. The TEWL values returned rapidly to basal values. The perturbation of the barrier function of the skin can contribute to the transdermal transport of drug by increasing the penetration by passive diffusion through a permeabilized skin [31].

The effect of two electroporation protocols [10× (335 V—5 ms) and 10×(1000 V—100 μs)] were compared. The modification of the barrier function and the effect on the blood flow were higher for the 335 V protocol than the 1000 V protocol. The higher energy applied and the higher number of charge transferred for the 335 V protocol can easily explain this. Comparison of the SW pulses with the exponentially decaying pulses is difficult because electroporation conditions were different. However, similar findings were reported: a mild and transient impairment of the barrier function measured by TEWL and an erythema.

Using different complementary techniques, we studied the effect of SW pulses on the skin. A mild impairment of the skin barrier function, a decrease in

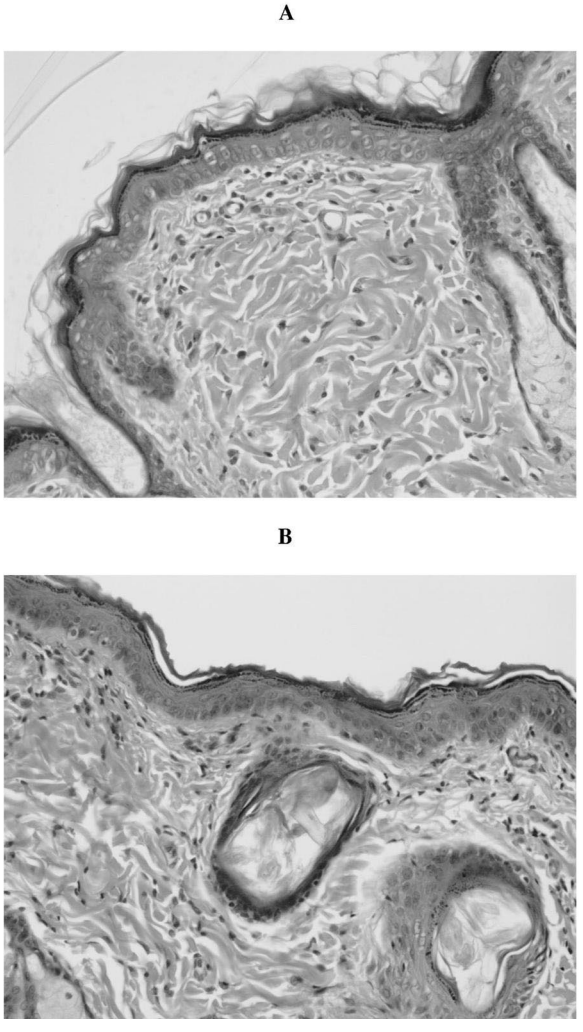


Fig. 5. Hematoxylin–eosin stained slides of control skin (A) and skin 4 days after electroporation 10×(1000 V—100 μs) (B).

skin resistance and a transient decrease in blood flow were induced by electroporation. These changes were reversible and did not alter the viability of the skin. Even though perturbations of the skin were observed, these data confirm the tolerance of the skin to square wave pulses in vivo as suggested by clinical practices showing that the patients submitted to electrochemotherapy tolerate well the 1000 V protocol [20].

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## References

[1] J. Hadgraft, R. Guy, Transdermal Drug Delivery, Development Issues and Research Initiatives, Marcel Dekker, New York, 1989.
[2] C.S. Asbill, El. Kattan, B. Michniak, Enhancement of transdermal drug delivery: chemical and physical approaches, Crit. Rev. Ther. Drug Carr. Syst. 17 (6) (2000) 621–658.
[3] M.R. Prausnitz, V.G. Bose, R. Langer, J.C. Weaver, Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery, Proc. Natl. Acad. Sci. USA 90 (1993) 10504–10508.
[4] R. Vanbever, N. Lecouturier, V. Préat, Transdermal delivery of metoprolol by electroporation, Pharm. Res. 11 (1994) 1657–1662.
[5] J.E. Riviere, N.A. Monteiro-Riviere, R.A. Rogers, D. Bommannan, J.A. Tamada, R.O. Potts, Pulsatile transdermal delivery of LHRH using electroporation: drug delivery and skin toxicology, J. Control. Release 36 (1995) 229–233.
[6] R. Vanbever, U.F. Pliquett, V. Préat, J.C. Weaver, Comparison of the effects of short, high-voltage and long, medium voltage pulses on skin electrical and transport properties, J. Control. Release 69 (1999) 35–47.
[7] C. Lombry, N. Dujardin, V. Préat, Transdermal delivery of macromolecules using skin electroporation, Pharm. Res. 17 (1) (2000) 32–37.
[8] A.V. Titomirov, S. Sukharev, E. Kistanova, In vivo electroporation and stable transformation of skin cells of new-born mice by plasmid DNA, Biochem. Biophys. Acta 1088 (1991) 131–134.
[9] R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, C. Nicolau, In vivo gene electroinjection and expression in rat liver, FEBS Lett. 389 (3) (1996) 225–228.
[10] T. Nishi, K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, I. Kitamura, T. Yoshimura, H. Saya, J. Kuratsu, Y. Ushio, High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation, Cancer Res. 56 (5) (1996) 1050–1055.
[11] L.M. Mir, M.F. Bureau, J. Gehl, R. Rangara, D. Rouy, J.M. Caillaud, P. Delaere, D. Brannelec, B. Shwartz, D. Scherman, High efficiency gene transfer into skeletal muscle mediated by electric pulses, Proc. Natl. Acad. Sci. USA 96 (8) (1999) 4262–4267.
[12] V. Regnier, N. De Morre, A. Jadoul, V. Préat, Mechanisms of

a phosphorothioate oligonucleotide delivery by skin electroporation, Int. J. Pharm. 184 (1999) 147–156.
[13] U. Pliquett, Mechanistic studies of molecular transdermal transport due to skin electroporation, Adv. Drug Deliv. Rev. 35 (1999) 41–60.
[14] J. Weaver, T. Vaughan, Y. Chizmadzhev, Theory of electrical creation of aqueous pathways across skin transport barriers, Adv. Drug Deliv. Rev. 35 (1999) 21–39.
[15] R. Vanbever, V. Préat, In vivo efficacy and safety of skin electroporation, Adv. Drug Deliv. Rev. 35 (1999) 77–88.
[16] A. Jadoul, J. Bouwstra, V. Préat, Effects of iontophoresis and electroporation on the stratum corneum. Review of the biophysical studies, Adv. Drug Deliv. Rev. 35 (1999) 89–105.
[17] A. Jadoul, H. Tanajo, V. Préat, F. Spies, H. Boddé, Electroperturbation of human stratum corneum fine structure by high voltage pulses: a freeze fracture electron microscopy and differential thermal analysis study, J. Invest. Dermatol. Symp. Proc. 3 (1998) 153–158.
[18] S. Gallo, A. Sen, M. Hensen, S. Hui, Time-dependent ultrastructural changes to porcine stratum corneum following and electric pulse, Biophys. J. 76 (1999) 2824–2832.
[19] R. Vanbever, D. Fouchard, A. Jadoul, N. De Morre, V. Préat, J.-P. Marty, In vivo non-invasive evaluation of hairless rat skin after high-voltage pulse exposure, Skin Pharmacol. Appl. Skin Physiol. 11 (1998) 23–34.
[20] L.M. Mir, L.F. Glass, G. Sersa, J. Teissié, C. Domengé, D. Miklavcic, M.J. Jaroszeski, S. Orlowski, D.S. Reintgen, Z. Rudolf, M. Belehradek, R. Gilbert, M.P. Rols, J. Belehradek, J.M. Bachaud, R. DeConti, B. Stabuc, M. Cemazar, P. Coninx, R. Heller, Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy, Br. J. Cancer 77 (12) (1998) 2336–2342.
[21] R. Heller, R. Gilbert, M.J. Jaroszeski, Clinical applications of electrochemotherapy, Adv. Drug Deliv. Rev. 35 (1999) 119–129.
[22] M.P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros, J. Teissié, In vivo electrically mediated protein and gene transfer in murine melanoma, Nat. Biotechnol. 16 (2) (1998) 168–171.
[23] Y.N. Kalia, L.B. Nonato, R.H. Guy, The effect of iontophoresis on skin barrier integrity: non-invasive evaluation by impedance spectroscopy and transepidermal water loss, Pharm. Res. 13 (6) (1996) 957–960.
[24] C. Anderson, T. anderson, K. Wardell, Changes in skin circulation after insertion of a microdialysis probe visualized by laser Doppler perfusion imaging, J. Invest. Dermatol. 102 (5) (1994) 807–811.
[25] J. Gehl, T.H. Sorensen, K. Nielsen, P. Rasmark, S.L. Nielsen, T. Skovsgaard, L.M. Mir, In vivo electroporation of skeletal muscle: threshold, efficacy and relation to electric field distribution, Biochim. Biophys. Acta 1428 (2–3) (1999) 233–240.
[26] U. Pliquett, R. Langer, J.C. Weaver, Changes in the passive electrical properties of human stratum corneum due to electroporation, Biochim. Biophys. Acta 1239 (1995) 111–121.
[27] R. Vanbever, E. LeBoulengé, V. Préat, Transdermal delivery of fentanyl by electroporation I. Influence of electrical factors, Pharm. Res. 13 (4) (1996) 559–565.
[28] N. Dujardin, P. Van Der Smissen, V. Préat, Topical gene transfer into rat skin using electroporation, Pharm. Res. 18 (1) (2001) 61–66.
[29] V. Regnier, V. Préat, Localisation of a FITC-labelled phosphorothioate oligodeoxynucleotide in the skin after topical delivery by iontophoresis and electroporation, Pharm. Res. 15 (1998) 1596–1602.
[30] S. Somiari, J. Glasspool-Malone, J.J. Drabick, R.A. Gilbert, R. Heller, M.J. Jaroszeski, R.W. Malone, Theory of in vivo application of electroporative gene delivery, Mol. Ther. 2 (3) (2000) 178–187.
[31] R. Vanbever, N. Morre, V. Préat, Transdermal delivery of fentanyl by electroporation II. Mechanisms involved in drug transport, Pharm. Res. 13 (9) (1996) 1360–1366.