Transdermal Drug Delivery Using Electroporation. II. Factors Influencing Skin Reversibility in Electroporative Delivery of Terazosin Hydrochloride in Hairless Rats

A. SHARMA,[1] M. KARA,[1] F. R. SMITH,[2] T. R. KRISHNAN[1]

[1] School of Pharmacy, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3V6

[2] Department of Chemistry, Memorial University of Newfoundland, St. John's, NF, Canada A1B 3X7

Received 19 October 1999; revised 4 November 1999; accepted 16 November 1999

ABSTRACT: A previous study indicated that the parameters governing the performance of electroporative delivery to the skin, are voltage, pulse length, number of pulses and electrode area.[1] This article describes a study in which the reversibility of the electroporation technique is evaluated with *in vitro* methods. The skin's reversal from an enhanced permeation mode as a result of electroporation to the base level was used as an index to understand the mechanism of drug delivery and also as a preliminary indicator of safety. Maximum delivery of the model drug, terazosin hydrochloride, occurred during the pulsing. Electroporative delivery with a wire electrode (small-area electrode, 0.56 cm^2) using 20 pulses at $U_{skin,0}$ 88 V, and pulse length 20 ms, did not cause any damage to the skin. Increasing the pulse length to 60 ms, while keeping the rest of the parameters fixed, caused a visible change in the external appearance of the skin. However, with the use of a spiral electrode (large-area electrode, 2.74 cm^2) at 60-ms pulse length, there was minimal damage to the skin. This may be attributed to the more uniform flow of current over the whole skin area. The large-area electrode required a smaller electrode voltage, $U_{electrode,0}$ for any given $U_{skin,0}$ and also delivered nearly double the instantaneous power density compared with the small-area electrode. These findings indicate that using shorter pulses and large-area electrodes is a safer technique than large pulses and small-area electrodes when electroporation is used to enhance skin's permeability for drug delivery. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 89: 536–544, 2000

INTRODUCTION

Use of electroporation in improving therapeutic efficacy is being explored in a number of ways; electrochemotherapy in treating certain types of skin cancer,[2] in gene therapy,[3,4] and in transdermal drug delivery.[5–7] This article deals with the latter. Just as any other permeation enhancement technique, electroporation would be useful only when the optimized condition of use is both efficient for drug delivery, as well as safe for the skin. With the use of electroporation pulse to enhance permeation, a rapid structural rearrangement of the lipid bilayer membranes constituting the stratum corneum seems to occur.[8] However, for the technique to be clinically acceptable for use in drug delivery, the process should be reversible and there should be no permanent or long-term damage to the skin. Most of the reported work dealing with the use of electroporation in transdermal drug delivery does not address issues related to the safety of the technique. Two recent articles involving *in vivo* experiments in rats have recently been published by Vanbever et al.,[7,9] in which the safety of the electroporation

Correspondence to: T. R. Krishnan

Journal of Pharmaceutical Sciences, Vol. 89, 536–544 (2000)

was partially addressed. Elsewhere in this journal issue we have described some of the factors influencing electroporative delivery of terazosin hydrochloride in hairless rat skin,[1] and in this article we report on some of the issues concerning the safety of the electroporation technique when used for drug delivery in the skin. This is the first report in which the influence of the electrode area on electroporative drug delivery is described.

EXPERIMENTAL SECTION

Materials and Methods

Terazosin hydrochloride (TRZ) was provided by Abbot Laboratories (Quebec, Canada). Prazosin hydrochloride was obtained from Sigma Chemicals Co. (St. Louis, MO). All other solvents and reagents used were of HPLC and analytical grade. Seven- to 8-week-old hairless rats were procured from Harlan Sprague Dawley (IN).

The experimental setup for performing the electroporation experiments, the HPLC procedure to quantify model probe TRZ, and the treatment of hairless rat's skin before and after the experiment are described in part I.[1]

Reversal of the Skin's Permeability

For the experiments to determine reversal of the skin's permeability, samples of hairless rat's skin were assembled in the diffusion chamber and were subjected to 10 pulses of 20 ms at $U_{skin,0}$ 84 V (using small-area electrodes). $U_{skin,0}$ is the initial ($t = 0$) exponentially decaying voltage drop across the skin. There were six different experimental conditions in which the reversibility of the skin's permeability were examined. These conditions were as follows:

1. Passive control: The donor chamber contained TRZ solution (1.03 mg/mL in PBS) and the receiver chamber contained phosphate-buffered saline (PBS), 0.1 *M* NaCl, 0.02 *M* phosphate, pH 6.4). After allowing 20 min of contact time, the skin was removed and analyzed for TRZ content.
2. The donor chamber contained TRZ solution and the receiver chamber contained PBS solution. Electroporation pulses were delivered (10 min), and the skin was allowed to stay in contact with the drug solution for additional 10 min. Thus, the total contact time between the skin and TRZ solution was 20 min before it was removed and analyzed for TRZ content.
3. Same as in No. 2 but immediately after delivering the electroporation pulses, the TRZ solution from the donor chamber was removed and PBS was added in its place and left for 10 min. The total contact time for the skin with TRZ and PBS was thus maintained at 20 min before removing the skin for drug analysis.
4. Both the donor and the receiver chambers contained PBS solution. Within 20 s after delivering the electroporation pulses, PBS solution from the donor chamber was removed and TRZ solution was added in its place. After allowing a total of 20 min of contact time with TRZ solution, the skin was removed from the assembly and analyzed for TRZ content.
5. Same as No. 4 with the only difference that the TRZ solution was added 5 min after delivery of the electroporation pulses.
6. Same as No. 4 with the only difference that the TRZ solution was added 60 min after delivery of the electroporation pulses. In No. 5 and No. 6 the skin contact time with PBS and TRZ was maintained at 20 min before analysis.

Morphologic and Histologic Changes in Skin with Electroporation

In this study 20 pulses of $U_{skin,0}$ 88 V, at varying pulse lengths (viz., 20 ms, 30 ms, 40 ms, and 60 ms) were delivered to 1.3 cm^2 area portions of freshly excised skin samples. The influence of small-area and large-area electrodes was studied using 20 pulses of 60 ms. The same $U_{electrode,0}$ voltage of 500 V were compared between the small-area electrode ($U_{skin,0}$ = 88 V) and the large-area electrode ($U_{skin,0}$ 116 V). $U_{electrode,0}$ means the initial ($t = 0$) potential difference applied across the delivery Ag, AgCl electrodes. All the studies were done in duplicate. The pHs of the donor and receiver solutions were checked before and after delivering the electroporation pulses.

Two sets of skin samples were chosen for observing changes caused by electroporation pulses. The first set was photographed to observe any gross changes in the morphology, and the second set of skin samples was fixed in glutaraldehyde fixative solution. Sections of skin, 0.5- to 1.0-μm thick were cut with a Cambridge Huxley Ultramicrotome and were stained for 2 min at 70°C

with 1% toludine blue in 1% sodium borate. The slides were viewed using a phase-contrast microscope (Zeiss® IM-35, Thornwood, NY) with 400× magnification and photographed using a 35-mm Pentax® camera (Tokyo, Japan) and a T-Max® b/w 400 film (Kodak, Rochester, NY).

Determination of Current Across the Skin While Delivering the Pulse

The amount of current passing through the skin during the delivery of an electroporation pulse was determined by measuring the voltage drop occurring across a resistance placed in series with the delivery electrodes and was recorded in a storage oscilloscope (Tektronix®-5111, Wilsonville, OR). The external resistance was varied between 0.1 to 5 Ω to enable the oscilloscope to store the pulses at different $U_{skin,0}$ values. This experiment was performed with both small-area and large-area electrodes using a single pulse ranging between 2 and 5 ms at $U_{skin,0}$ between 32 and 116 V. For each determination a fresh piece of hairless rat skin was used with cross-sectional area of 1.3 cm^2.

The initial (t = 0) amount of current I_0 (amperes) passing through the skin during pulsing was calculated using Ohm's law (I = E/R, where *I* is the current in amperes, *E* is the applied voltage ($U_{skin,0}$, in volts), and *R* is a known external resistance (Ω) in series with the diffusion cell. I_0 obtained for each electroporation pulse was divided by the area of the skin to obtain the initial current densities, J_0, amperes per cm^2. The Initial Dynamic Resistance, R_0, can also be calculated by division of $U_{skin,0}$ by the initial current, I_0 (e.g., at $U_{skin,0}$ = 83.6 V, it is 33 ohms for the small-area electrode but for the similar $U_{skin,0}$ = 82 V for the large-area electrode it is only 14 ohms). Multiplying $U_{skin,0}$ by J_0 provides a measure of the initial (instantaneous) power density (watts/cm^2) in Table II. This is the maximum power density. To get some idea of the power dissipated in a pulse, one could consider a pulse to be essentially complete in a few pulse lengths, say 4 τ. Then the average power density over that period will be one eighth of the maximum power density.

The energy density per pulse may be calculated with the aid of the definite integral:

$$\int_0^\infty e^{-ax}dx = 1/a \text{ for } a > 0$$

In this case the energy density is equal to:

$$U_{skin,0} \cdot J_0 \cdot \int_0^\infty (e^{-t/\tau})^2\, dt =$$
$$U_{skin,0} \cdot J_0 \cdot \int_0^\infty (e^{-2t/\tau})^2 \cdot dt = (U_{skin,0} \cdot J_0 \cdot \tau)/2$$

Table II shows the results of such calculations.

RESULTS AND DISCUSSION

Reversal of the Skin's Permeability Enhancement

This part of the study was done specifically to find out how the electroporative permeation enhancement took place in the skin and how long the effect lasted. Answers to these questions would not only furnish information on the safe use of the technique but would also provide an insight into the mechanism of electroporative enhancement. It is realized that reversal of permeability properties of the skin may not necessarily be an absolute indicator of safety, especially because the repair processes may occur *in vivo* that are improbable *in vitro.* The results are presented in the

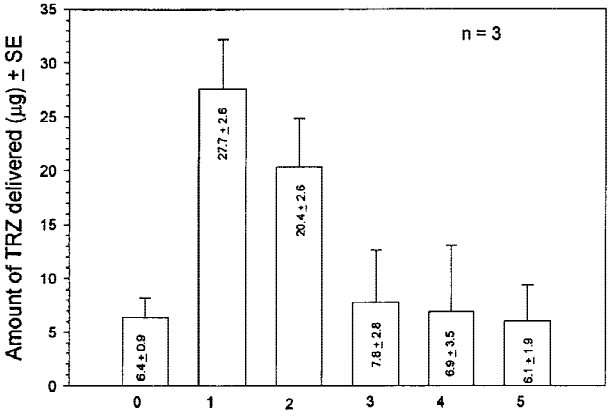
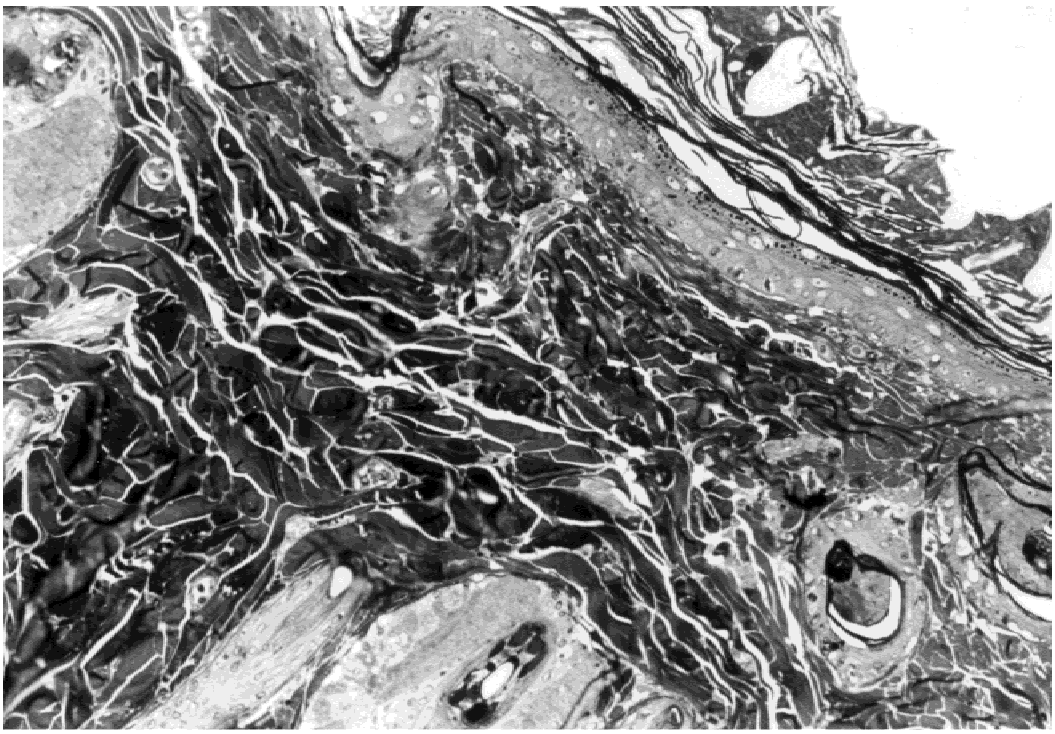
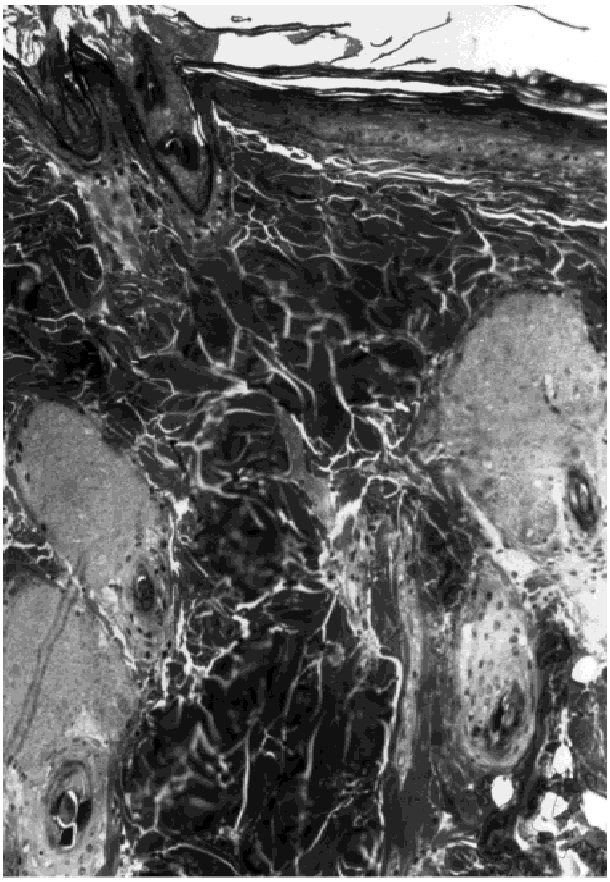


Figure 1. Terazosin hydrochloride (TRZ) delivered in the skin was measured at different experimental conditions to study the reversibility of skin's enhanced permeability. In each instance 10 electroporation pulses of 20 ms at $U_{skin,0}$ 84 V were used. 0, control-passive diffusion; 1, electroporation pulses applied with TRZ as donor solution; 2, same as 1 but TRZ was replaced with PBS immediately after the delivery of the pulses; 3, electroporation pulses delivered with PBS as the donor solution (PBS was replaced with TRZ soon after the delivery of the pulses); 4, same as in 3 except that TRZ was added 5 min after the delivery of the pulses; 5, same as in 3 except that TRZ was added 1 h after the delivery of the pulses.

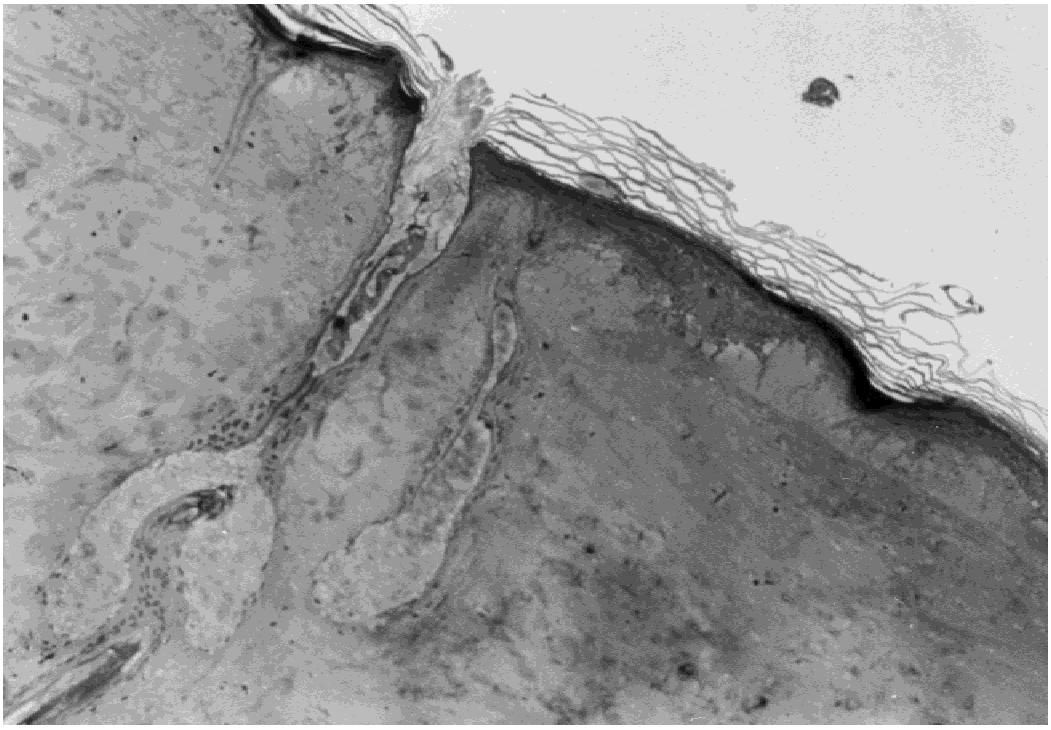


(A)



(B)

Figure 2. A, Microscopic cross-section of untreated skin (control) sample (original magnification, ×400). B, Microscopic cross-section of skin sample subjected to 20 pulses of 88 V ($U_{skin,0}$) and 20-ms pulse length, delivered using small-area electrodes (original magnification, ×400). C, Microscopic cross-section of skin sample subjected to 20 pulses of 88 V ($U_{skin,0}$) and 60-ms pulse length, delivered using small-area electrodes (original magnification, ×400). D, Microscopic cross-section of skin sample subjected to 20 pulses of 88 V ($U_{skin,0}$) and 60-ms pulse length, delivered using large-area electrodes (original magnification, ×400).



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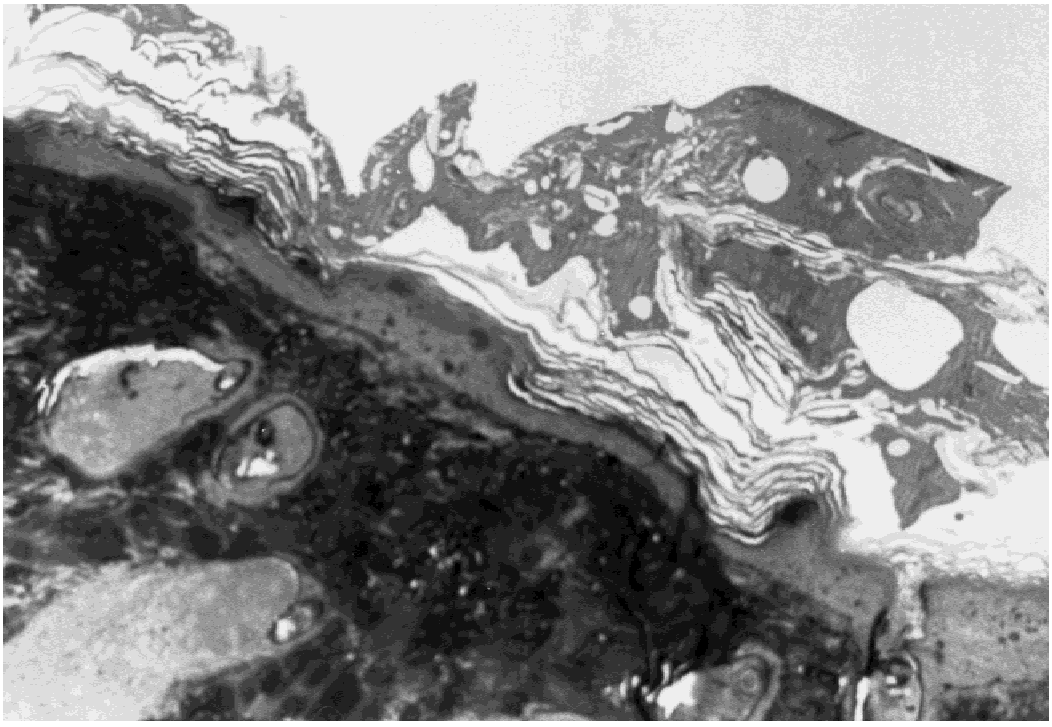

(D)

form of a histogram in Figure 1. The histogram bars labeled 2 and 3 stand out with TRZ quantities 27.7 μg and 20.4 μg, respectively. They represent electroporation pulse delivery with the TRZ solution in the donor chamber. In histogram 2, the TRZ solution was left in contact with the skin during and after the pulse, whereas in histogram 3, the TRZ solution was taken out im-

mediately after the delivery of the pulse. Although these was no statistical difference ($P > 0.05$) between the two deliveries (histogram 2 and 3), the mean TRZ delivered in the case in which the solution was left in contact with the skin during and after the pulse (histogram 2) was 25% larger. The other histogram bars (viz., 4, 5, and 6 that were, respectively, TRZ added immediately after the pulse, 5 min after the pulse, and 1 h after the pulse) were much smaller and statistically not different ($P > 0.05$) from the control. This clearly demonstrates that enhanced transport of TRZ in the skin occurred primarily during the delivery of the electroporation pulses. The electrophoretic component of the driving force that is dependent on the charge and polarity of the electrode might also contribute significantly during the pulse. This would be true for TRZ because it is positively charged at the donor solution pH and is placed in the anode compartment. It is speculated that the permeation enhancement is due to creation of aqueous pores in the stratum corneum.[10] Because adding the TRZ solution immediately after the pulse did not show any significant increase in delivery compared with the control, it appears that such pores close very quickly. This is also in agreement with the reports of Pliquett et al.[11] It should be noted that in this experiment the electroporation parameters (10 pulses, pulse length 20 ms, $U_{skin,0}$ 84 V) were carefully chosen to ensure that the permeation enhancement was achieved using a small $U_{skin,0}$ and pulse length. This was done to ensure that the reversibility of permeation was influenced primarily by time and there was little effect as a result of excessive electrical force that could cause skin damage. Depending on the magnitude of the electroporation condition (voltage, pulse length, current density, etc.), the permeation enhancement may be much more prolonged, but it may not revert to the base level at all. This information could, hence, be used as an indicator of safety for the electroporation technique.

Morphologic and Histologic Changes in the Skin caused by Electroporation Pulse

From the observations in article I it was concluded that with small-area electrodes, $U_{skin,0}$ >88 V and pulse lengths >20 ms caused some visible change in the external appearance to the skin.[1] Attempts were made to find out whether the morphologic and histologic changes in the skin corroborated the preceding results. The photographs show only the area of the skin that was exposed to the drug solution (Fig. 2). In all the test conditions the $U_{skin,0}$ was kept constant at 88 V and the number of pulses was kept at 20. The variables examined were pulse length and area of the electrode. Visual examination with the naked eye of the skin samples indicated that there was no visible change in the external appearance on either side of the skin with the use of 20-ms pulses. As the pulse length was increased above 20 ms, deep red-colored lesions in the skin appeared on the dermal side and a few dark patches were seen on the stratum corneum side, and at 60 ms pulse the lesion was most pronounced. In all the preceding tests small-area electrodes were used. However, it was interesting to find that when the skin was subjected to electroporation pulses of 60 ms, using the large-area electrode (with same $U_{skin,0}$ as for the small-area electrode), the visually apparent damage was much less compared with the skin subjected to 60-ms pulses with the small-area electrode. Also, with the small-area electrode one could see a spark during pulsing, especially at 60 ms pulse, and this was absent with the large-area electrode. Results from previous work showed that similar drug deliveries were obtained with the use of small-area and large-area electrodes at identical electroporation (same $U_{skin,0}$ pulse length, and number of pulses) conditions.[1]

Examination of the cross sections of the skin corrobated the preceding findings. In the control skin sample, the different layers of skin were clearly demarcated [Fig. 2(A)]. In the skin samples subjected to electroporation pluses, as the pulse length was increased above 20 ms, there was progressively increased damage to the skin, and at 60-ms pulse the changes in the histology were more obvious. At one end of the spectrum with a pulse length of 20 ms, the different layers of epidermis and dermis were clearly visible [Fig. 2(B)], whereas at the other extreme with a pulse length of 60 ms, there was degeneration of the basal layer and the collagen in the dermis had an amorphous appearance [Fig. 2(C)]. Although the skin sample subjected to electroporation pulses with the large-area electrode showed some damage [Fig. 2(D)], it was substantially less than that caused with the small-area electrode under the same electroporation conditions. It should be noted that in the *in vivo* studies carried out by Vanbever et al.[7] limited damage was seen; how-

ever, their pulse length of 1.3 ms at 500 V is much shorter than used here.

pH Changes Caused by Electroporation Pulse

The pHs of solutions in the donor and receiver compartments before and after electroporation are shown in Table 1. In the control experiment the pH of the donor and receiver compartments remained unchanged at pH 6.4. In the experiment with a 20-ms pulse length, the donor pH remained unchanged, whereas that of the receiver went up from 6.4 to 8.0. As the pulse length was increased to 30 ms, the pH of the receiver increased to 11. At pulse lengths above 30 ms, the pH of the receiver rose to 12. The change in pH was accompanied by a significant amount of foaming in the receiver chamber (cathode), which was absent in the donor chamber (anode). This was observed particularly after the first three pulses were delivered. When current flows at the reactive electrode, Ag/AgCl in the presence of Cl^- ions, the Ag would be converted to AgCl and no foaming should be expected. However, at the cathode, the AgCl would first be reduced to metallic Ag. Both the donor and receptor compartments in the drug delivery experiments had the same volume (1 mL) and the buffering capacities (0.1 *M* of dihydrogenphosphate and 0.1 *M* of monohydrogen phosphate) were the same. It is possible that insufficient buffering capacity was available in the receiver compartment. However, it is envisaged that the changes occurring in the receiver compartment were the results partly because of exhaustion of the AgCl coating of the current-carrying electrode there. as a succession of long pulses were passed the reaction: $AgCl(s) + e^- \rightarrow Ag(s) + Cl^-$ occurred. On completion of this process (or even in parallel with it because of breaks in AgCl coating), the current would generate hydrogen gas and hydroxide ions from water and the pH would rise in accordance with the reaction:

$$2\ H_2O\ (l) + 2\ e^- \rightarrow H_2\ (g) + 2\ OH^-.$$

This process is not anticipated in the donor compartment where the predominant reaction is:

$$Ag\ (s) + Cl^-\ (\text{from PBS}) \rightarrow AgCl\ (s) + e^-$$

The high currents used here are out of the range of usual electrochemical experience and, hence, the above scenario is quite probable.

To determine the effect of alkaline pH, the skin was exposed to dilute sodium hydroxide (pH 12) for 20 min. Subsequent examination of the skin did not show any damage similar to that seen with the passage of electroporation pulses of 30 and 40 ms. This indicated that exposure to solution with high pH for 20 min alone may not cause sufficient damage to the skin that could be easily visible.

Current Density, Energy, and Instantaneous Power Calculations

The current measurements and calculations done using are shown in Table 2. Because of technical limitations we could determine the currents only at lower pulse lengths, ranging between 2 and 5 ms. However, we believe that the basic information obtained on current density and power etc. are still meaningful and useful in explaining the results obtained by us. During each current measurement the exact pulse length was determined

Table 1. pH Change in Donor and Receiver Chamber as a Result of Electroporation

Electroporation Condition					Donor pH		Receiver pH	
$U_{electrode,0}$ (V)	$U_{skin,0}$ (V)	Pulse Length (ms)	Number of Pulses	Electrode Area	Before	After	Before	After
0	0	0	0	Small	6.4	6.4	6.4	6.4
500	88	20	20	Small	6.4	6.4	6.4	8
500	88	30	20	Small	6.4	6.4	6.4	11
500	88	40	20	Small	6.4	6.4	6.4	12
500	88	60	20	Small	6.4	6.4	6.4	12
500	116	60	20	Large	6.4	6.4	6.4	12

Table 2. Current Density, Power, and Energy with Small-area Electrode (0.56 cm^2) and Large-area Electrode (2.74 cm^2)

$U_{electrode,0}$ V	$U_{skin,0}$ V	Current (Initial) A	Current Density (Initial) A/cm^2 of Skin	Current Density (Initial) A/cm^2 of Electrode	Pulse Length (τ) s	Energy to Skin/Pulse (J/cm^2 of Skin)	Maximum Instantaneous Power Density W/cm^2 of Skin)
				Small-area Electrode			
100	32.4	0.42	0.32	0.75	6.75E-03	0.04	10
200	43.6	1.03	0.79	1.84	5.06E-03	0.09	35
300	65.8	1.82	1.4	3.25	5.12E-03	0.24	92
400	83.6	2.51	1.93	4.48	5.10E-03	0.41	161
500	88.3	3.08	2.37	5.5	4.87E-03	0.51	209
				Large-area Electrode			
100	48.0	1.21	0.93	0.44	3.67E-03	0.08	45
200	57.0	3.35	2.58	1.22	2.48E-03	0.18	147
300	82.0	5.82	4.48	2.12	2.33E-03	0.43	367
400	111.2	9.33	7.18	3.41	2.14E-03	0.85	798
500	116.0	12.8	9.85	4.67	1.76E-03	1.01	1142

and used in arriving at other values reported in Table 2. From this table it is apparent that the use of the large-area electrode increased the current density with respect to area of the skin, although it decreased it with respect to area of the electrode, $U_{electrode,0}$ remaining constant. The large current density increased $U_{skin,0}$ for the same $U_{electrode,0}$; indeed, it might be argued that a key variable for electroporation is the current density over the skin area, provided that $U_{skin,0}$ is sufficiently large. At essentially the same $U_{skin,0}$ the conductance using the large electrode was significantly greater. Comparison of the energy to the skin per pulse at essentially the same $U_{skin,0}$ (e.g., 94 V and 82 V for small-area and large-area electrodes respectively, in Table 2) showed little change. But if the same pulse length had been used because the energy increases linearly with τ, the energy would have been doubled for the large-area electrode relative to the small-area electrode. The other quantities should be unaffected by the pulse length.

The changes in the skin's stratum corneum, caused by electroporation pulses, should normally depend on the area of the skin exposed to the current. We believe the difference in the two cases to be that with the large-area electrode the current was more uniformly distributed, whereas with the small-area electrode it appears to have been localized to the shape of the electrode wire. When two wire electrodes (small-area electrodes) placed parallel to one another in an electrolyte solution are involved, current flows principally between the two wires and widens out like a magnetic iron filings pattern.[12] In our case, because the skin placed between the electrodes was not a good electrical conductor, the current flow may not have been uniform when the small-area electrodes were used, and damage to skin occurred at 60-ms pulse length. With the large-area electrode the current seemed to have been delivered more uniformly, thereby minimizing skin damage [Fig. 2(D)]. In summary, the principal benefits from using a large-area electrode are the more uniform current distribution over the skin and the smaller applied voltage required for a given $U_{skin,0}$.

The preceding findings suggest that using 20 pulses with a $U_{skin,0}$ of 88 V and pulse length of 20 ms would not be damaging to rat skin. Little or no visible effects were seen under these conditions. Use of an electrode with a large area requiring smaller $U_{electrode,0}$ seems to be the safer way to use the technique. The energy deposited in the skin as a result of pulsing depends on voltage, pulse length, and area of the electrode, all of which could be optimized to ensure better safety of the technique.

ACKNOWLEDGMENTS

Financial support for this work was provided by the Memorial University New Research Initiative grant. We are grateful to Abbott Laboratories,

QC, Canada, for the generous gift of terazosin hydrochloride.

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