Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 308 (2006) 61–68
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Skin targeted DNA vaccine delivery using electroporation in rabbits
II. Safety

Babu M. Medi, Jagdish Singh *

Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State University, Fargo, ND 58105, USA

Received 31 August 2005; received in revised form 18 October 2005; accepted 18 October 2005
Available online 13 December 2005

Abstract

The Achilles heel of gene-based therapy is gene delivery into the target cells efficiently with minimal toxic effects. Viral vectors for gene/DNA vaccine delivery are limited by the safety and immunological problems. Recently, nonviral gene delivery mediated by electroporation has been shown to be efficient in different tissues including skin. There are no detailed reports about the effects of electroporation on skin tissue, when used for gene/DNA vaccine delivery. In a previous study we demonstrated the efficacy of skin targeted DNA vaccine delivery using electroporation in rabbits [Medi, B.M., Hoselton, S., Marepalli, B.R., Singh, J., 2005. Skin targeted DNA vaccine delivery using electroporation in rabbits. I. Efficacy. Int. J. Pharm. 294, 53–63]. In the present study, we investigated the safety aspects of the electroporation technique in vivo in rabbits. Different electroporation parameters (100–300 V) were tested for their effects on skin viability, macroscopic barrier property, irritation and microscopic changes in the skin. Skin viability was not affected by the electroporation protocols tested. The electroporation pulses induced skin barrier perturbation and irritation as indicated by elevated transepidermal water loss (TEWL) and erythema/edema, respectively. Microscopic studies revealed inflammatory responses in the epidermis following electroporation using 200 and 300 V pulses. However, these changes due to electroporation were reversible within a week. The results suggest that the electroporation does not induce any irreversible changes in the skin and can be a useful technique for skin targeted DNA vaccine delivery.

Keywords: DNA vaccine; Electroporation; Skin; Safety

1. Introduction

Cutaneous gene delivery is attractive, as skin is the most accessible somatic tissue (Khavari, 1997). Skin also represents a potential target for DNA vaccine delivery due to the presence of functional bone marrow derived epidermal Langerhan's cells and dermal dendritic cells, which are specialized for induction of immune responses (Tuting et al., 1998). The possibility of using viral vectors for gene/DNA vaccine delivery is limited by the safety and immunological problems associated with the use of viral vectors in humans (Verma and Somia, 1997; Abdallah et al., 1995). The viral vectors are antigenic by themselves and can cause severe inflammatory responses. An alternative approach to genetic immunization is the gene transfer using nonviral methods. Recent setbacks in gene therapy with viral vectors further accelerated the search for efficient nonviral gene delivery systems (Verma, 2000). The nonviral gene delivery methods have significant clinical potential. However, the efficiency of transfection using plasmid/naked DNA as such is low due to extracellular and intracellular barriers (Ma and Diamond, 2001; Herweijer and Wolff, 2003). Following the report of gene expression after direct plasmid DNA injection (Wolff et al., 1990), several studies examined the possibility of vaccination using plasmid DNA coding antigens (DNA vaccines) in vivo (Ulmer et al., 1993; Raz et al., 1994; Lagging et al., 1995). Although direct injections of DNA vaccine do induce immune response in smaller animals, the delivery of the DNA to target cells is not optimal, especially in higher animals (Whalen, 1996; Srivastava and Margaret, 2003). Several chemical and physical methods have been reported to enhance the immunogenicity of DNA vaccines, primarily by increasing the transfection efficiency and thereby the antigen expression (Herweijer and Wolff, 2003). Most of these methods

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

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.10.035

are not suitable for routine use due to the inefficiency, in vivo clearance, toxicity and formulation/manufacturing complexities involved. Furthermore, large amount of genetic material is needed to induce the response with injections. Recently, gene delivery mediated by electroporation has been shown to be efficient (Aihara and Miyazaki, 1998; Glasspool-Malone et al., 2000; Medi and Singh, 2003; Zhang et al., 2002).

Electroporation involves application of controlled, short and high voltage electric pulses to permeabilize the target cell/tissue reversibly for macromolecules such as genes/proteins. Electroporation has been evaluated in animals and humans for the delivery of chemotherapeutic agents with high efficiency (Mir et al., 1998; Sersa et al., 2000). Furthermore, it has been employed in studies involving delivery of plasmid DNA in vivo to different types of tissues with improved transfection efficiency. Most of these studies involve insertion of electrode needles into the tissue after plasmid DNA injection, which may not be feasible for use in humans. To be therapeutically useful, the DNA vaccine must be delivered inside the cells before it can express antigen molecules (Doria-Rose and Haigwood, 2003). This requires efficient membrane permeabilization to allow the DNA vaccines to enter the cells. Cutaneous gene delivery using topical electroporation needs no specialized procedures as the pulses would be applied topically with tweezer type of electrodes, following the injection of plasmid DNA. In a previous study (part I), we demonstrated the efficacy of skin targeted DNA vaccine delivery using electroporation in rabbits (Medi et al., 2005). However, the major factor in the clinical acceptability of electroporation mediated gene/DNA delivery is its effect on the target tissue. The electropermeabilization may leave the target tissue damaged depending upon the electrical parameters associated with the electroporation (Lefesvre et al., 2002). The technique to be clinically acceptable for use in gene/DNA delivery, there should be no permanent damage to the skin. The detailed report on the effects of electroporation on skin safety is lacking. In the present study, we address the issues of skin safety from different electroporation parameters in vivo in New Zealand White (NZW) rabbits.

2. Materials and methods

2.1. Materials

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). 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. Animals

New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*), 10 weeks old and about 2.0–3.0 kg body weight, were used in the study. The animals were housed in North Dakota State University (NDSU), Department of Veterinary Technology Animal Care Facilities and cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, 1996). All the animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee of NDSU.

2.3. Effect of electroporation on skin viability

The hair on the back of the rabbits was closely clipped using an electric clipper carefully without any damage to the skin, 24 h prior to the beginning of the study. At the beginning of the experiment, the rabbits were anesthetized using 30 mg/kg pentobarbital sodium (Nembutal®) given intraperitoneally. An area of the skin was swabbed with 70% (v/v) isopropyl alcohol. Electroporation site corresponding to cathode and anode was marked. The marked site was pinched and clamped with stainless steel tweezer electrode (10 mm × 5 mm, CUY 663B, NEPA Gene Co, Chiba, Japan) and electric pulses were applied using a square-wave electroporator (CUY21 EDIT Version, NEPA Gene Co, Chiba, Japan). The treatment conditions tested were control (no pulses), five pulses of 100 V and 10 ms, five pulses of 100 V and 30 ms, five pulses of 200 V and 10 ms, five pulses of 300 V and 10 ms. The interval between each pulse was 1 s. The electroporation treatment conditions tested were based upon the previous study on DNA vaccine delivery (Medi et al., 2005). The effect of electroporation pulses on the skin viability was investigated by taking skin samples from rabbits by punch biopsy immediately (0 h) and 24 h after electroporation along with control using MTT assay. The viability of the skin samples was assessed at both cathodal and anodal sites. The samples were weighed immediately and incubated with 2 mg/ml of MTT prepared in DMEM containing 5% FBS for 3 h at 37 °C under 5% CO_2 in a 24-well plate. The water-insoluble formazan was extracted using DMSO for 1 h under shaking and the absorbance of the samples was measured by spectrophotometer at 540 nm. The results were expressed as percent viability calculated using the following equation:

$$\%\ \text{skin viability} = \frac{\text{absorbance of the test sample} \times 100}{\text{absorbance of the control}}$$

2.4. Effect of electroporation on macroscopic skin barrier function and skin irritation

The electroporation pulses were applied to the skin as described in the previous section (*Effect of electroporation on skin viability*). The effect of electroporation on macroscopic skin barrier function was studied by measuring the transepidermal water loss (TEWL). TEWL can be considered a determinant indicative of the functional state of the cutaneous barrier (Maibach et al., 1984) and provides a method for assessing macroscopic changes in the barrier properties of the stratum corneum (SC) (Abrams et al., 1993). TEWL was measured quantitatively with a Tewameter™ (Courage and Khazaka, Cologne, FRG), before electroporation (baseline) and

after electroporation at different time points, using the method of vapor pressure gradient at both cathodal and anodal sites. The probe was held on the skin until a stable TEWL value was established (∼1 min). The data was analyzed by generalized linear model using SAS.

The visual scoring system, to evaluate primary skin irritation, described by Draize et al. (1944) was used to grade erythema: very slight erythema barely perceptible # 1; well defined erythema # 2; moderate to severe erythema # 3; severe erythema, beet redness to slight eschar formation, injuries in depth # 4; and edema: very slight edema, barely perceptible # 1; slight edema, edges of area well defined by definite raising # 2; moderate edema, area raised approximately 1 mm # 3; severe edema, raised more than 1 mm, and extending beyond area of exposure # 4.

2.5. Microscopic changes in the skin structure due to electroporation

The electroporation pulses were applied to the skin as described in the previous section (*Effect of electroporation on skin viability*). The microscopic changes in the skin structure at both cathode and anode sites, following electroporation, were studied up to 7 days. Skin samples were excised immediately, 1, 2, and 3 days following electroporation and 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 (Olympus) and photographed using an attached digital camera (SPOT RT, Diagnostic Instruments, Sterling Heights, MI). A stage micrometer was used to calibrate the object and the scale bar was inserted on to the micrograph.

3. Results

3.1. Effect of electroporation on skin viability

The changes in the viability of skin, following topical electroporation were investigated to assess the safety of the technique. Fig. 1A and B shows the percent viability of the skin samples after electroporation compared with the control at cathodal and anodal sites, respectively. The viability of the skin was tested immediately and 24 h following electroporation. Percent viability was calculated by taking the value of control as 100%. Any of the electroporation pulses tested did not affect the viability of the skin at both cathodal and anodal sites. The results of the viability studies indicate that the electroporation parameters tested in this study can be used for DNA vaccine delivery without affecting the skin viability.

3.2. Effect of electroporation on macroscopic skin barrier function and skin irritation

The measurement of TEWL shows the effect of electroporation on macroscopic skin barrier property. Fig. 2A and B shows the TEWL values measured at different time points at cathodal

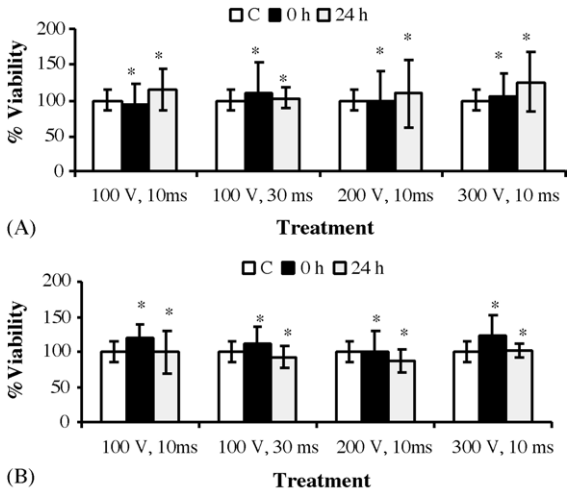

Fig. 1. Effect of different electroporation parameters on skin viability in vivo in NZW rabbits: (A) cathode; (B) anode. Key: C, control; 0 h, immediately following electroporation; 24 h, 24 h after electroporation. Percent viability was calculated by taking the value of control as 100%. All the values are shown as mean ± S.D. ($n = 3$). (*) Skin viability is not significantly different from that of the control.

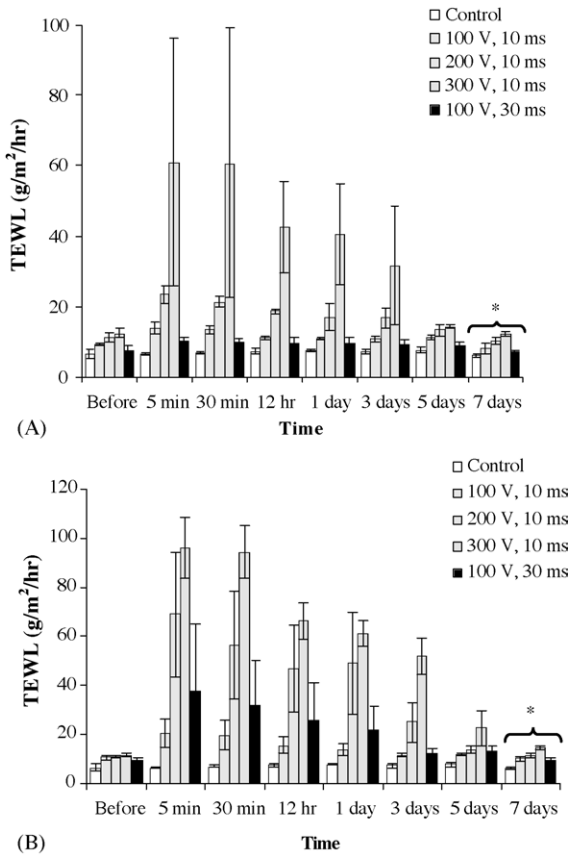

Fig. 2. Effect of different electroporation parameters on TEWL in vivo in NZW rabbits: (A) cathode; (B) anode. Key: five pulses of the specified voltage and pulse length were applied with 1 s interval between each pulse. All the values are shown as mean ± S.D. ($n = 3$). (*) TEWL values were not significantly different ($p > 0.05$) from those of TEWL values obtained before treatment.

Table 1
Draize scores for erythema at different time points following electroporation in vivo in NZW rabbits

	Erythema (Draize scores)				
	30 min	1 day	3 days	5 days	7 days
Cathode					
200 V, 10 ms					
Rabbit 1	1	1	1	1	0
Rabbit 2	1	1	1	1	0
Rabbit 3	0	1	0	0	0
300 V, 10 ms					
Rabbit 1	1	1	1	1	0
Rabbit 2	0	1	1	1	0
Rabbit 3	1	2	1	1	0
Anode					
200 V, 10 ms					
Rabbit 1	1	1	1	0	0
Rabbit 2	1	1	1	0	0
Rabbit 3	1	1	0	0	0
300 V, 10 ms					
Rabbit 1	1	1	1	1	0
Rabbit 2	1	2	1	1	0
Rabbit 3	1	2	1	1	0

Table 2
Draize scores for edema at different time points following electroporation in vivo in NZW rabbits

	Edema (Draize scores)				
	30 min	1 day	3 days	5 days	7 days
Cathode					
200 V, 10 ms					
Rabbit 1	1	1	1	1	0
Rabbit 2	1	1	1	1	0
Rabbit 3	0	1	0	0	0
300 V, 10 ms					
Rabbit 1	0	1	1	1	0
Rabbit 2	1	2	1	1	0
Rabbit 3	1	1	1	1	0
Anode					
200 V, 10 ms					
Rabbit 1	1	1	1	1	0
Rabbit 2	0	1	1	0	0
Rabbit 3	1	1	2	1	0
300 V, 10 ms					
Rabbit 1	0	1	1	1	0
Rabbit 2	1	2	2	1	0
Rabbit 3	1	1	2	1	0

and anodal sites, respectively. The corresponding baseline values were measured before electroporation at the same site. Immediately following electroporation, we found significantly higher ($p < 0.05$) TEWL values in comparison to the control with all the electroporation treatments tested. However, the TEWL values recovered almost to control levels within a week and were not significantly different ($p > 0.05$). Higher TEWL values observed following the electroporation pulses indicate the perturbation of skin barrier property. The recovery of TEWL values indicate that the skin barrier perturbation associated with the electroporation is reversible. No erythema or edema was observed with the 100 V electroporation pulses. However, erythema (Table 1) and edema (Table 2) was observed with 200 and 300 V electroporation pulses that disappeared by 7 days following electroporation. This shows that the mild skin irritation caused by electroporation pulses is reversible.

3.3. Microscopic changes in the skin structure due to electroporation

Light microscopy provides visual evidences of the microscopic changes in the skin. Fig. 3 shows the light micrographs of skin samples treated with electroporation pulses of 100 V amplitude and 10 ms length, along with the control. The different layers of epidermis and dermis were clearly visible in the control skin with SC tightly attached to the epidermis (Fig. 3A). We found detachment of the SC layers immediately following electroporation pulsing (Fig. 3B), which indicates perturbation of SC barrier properties. Degeneration of the basal layer and the breakdown of collagen fibers were observed at 24 and 48 h following electroporation (Fig. 3C and D) with an amorphous appearance in the dermis region. However, the SC recovered well to a normal state, and the microscopic structure was comparable to control skin at the end of 1 week (Fig. 3E). When the pulse amplitude was increased to 200 and 300 V at 10 ms pulse length (Figs. 4 and 5), epidermal thickness was increased at 24 h (Figs. 4C and 5C) and 48 h (Figs. 4D and 5D) following electroporation. These changes were observed in addition to the changes seen with the 100 V electroporation pulses. The changes in the histology were more obvious at 24 and 48 h (Fig. 3C and D) with inflammation in the epidermis. However, by 7 days (Fig. 3E) the SC recovered well to a normal state and the microscopic structure was comparable to control skin.

4. Discussion

The gene therapy works by expressing the encoded protein in vivo. Hence, the viability of the target tissue is important in the case of gene/DNA vaccine delivery in order to express the transgene in vivo. If the gene transfer method is detrimental to the tissue, the technique is not useful for gene transfer. The electropermeabilization may leave the target tissue damaged depending on the electrical parameters associated with the electroporation (Lefesvre et al., 2002). The skin is the largest organ of the human body functioning as a barrier to exogenous harmful influences besides maintaining the homeostasis. Hence, it is important to understand the skin barrier function, irritation and microscopic changes following the application of electroporation pulses. There is not yet an adequately validated in vitro model available to predict skin irritation of topical chemicals and drug delivery systems. Details of the current state of development of these assays are summarized by Rougier et al. (1994). The standard way to forecast skin irritation is by so called predictive tests on man or animals.

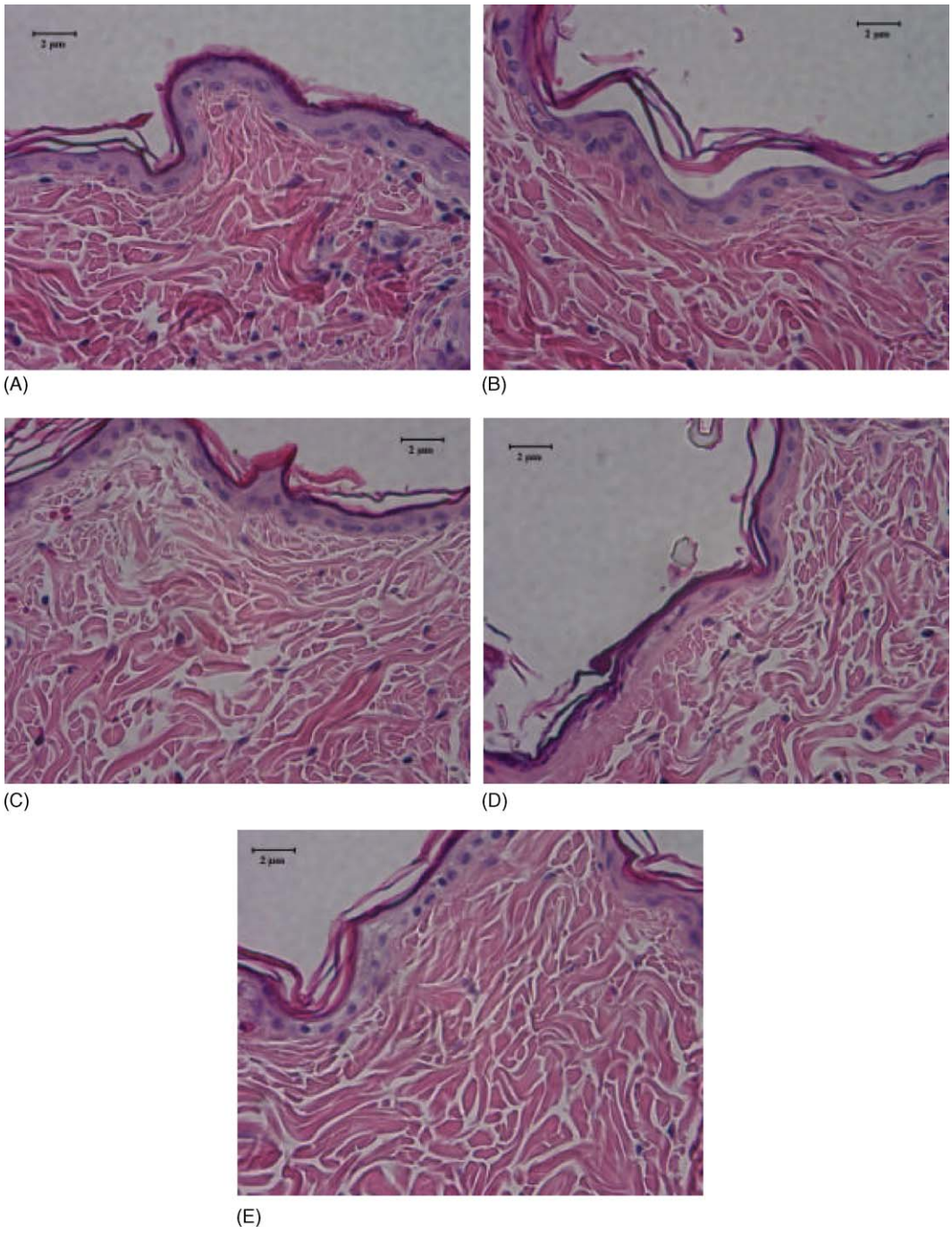

Fig. 3. Light micrographs of skin showing histological changes at different time points following electroporation using five pulses of 100 V, 10 ms with 1 s interval between each pulse. Key: (A) control; (B) immediately following electroporation; (C) 24 h following electroporation; (D) 48 h following electroporation; (E) 7 days following electroporation (scale bar = 2 μm).

The most widely used test for predicting potential skin irritants to man, using animal models, was published by Draize et al. (1944). The rabbit Draize test, properly performed, is highly valuable.

The outermost layer of skin, SC, is regarded as the primary barrier to the external environment. The SC also acts as a barrier to avoid the loss of internal body components, particularly water (Roberts and Walters, 1998). TEWL is regarded as an indicator of skin barrier function as high TEWL generally indicates barrier perturbation (Singh et al., 2001; Sekkat et al., 2002). TEWL has been used in relation to the assessment of either the effects of penetration enhancers (Tanojo et al., 1998) or the irritation (Loffler et al., 2001) on the skin. Higher TEWL values observed following the electroporation pulses indicate the perturbation of skin barrier property. The recovery of TEWL values indicate that the skin barrier perturbation associated with

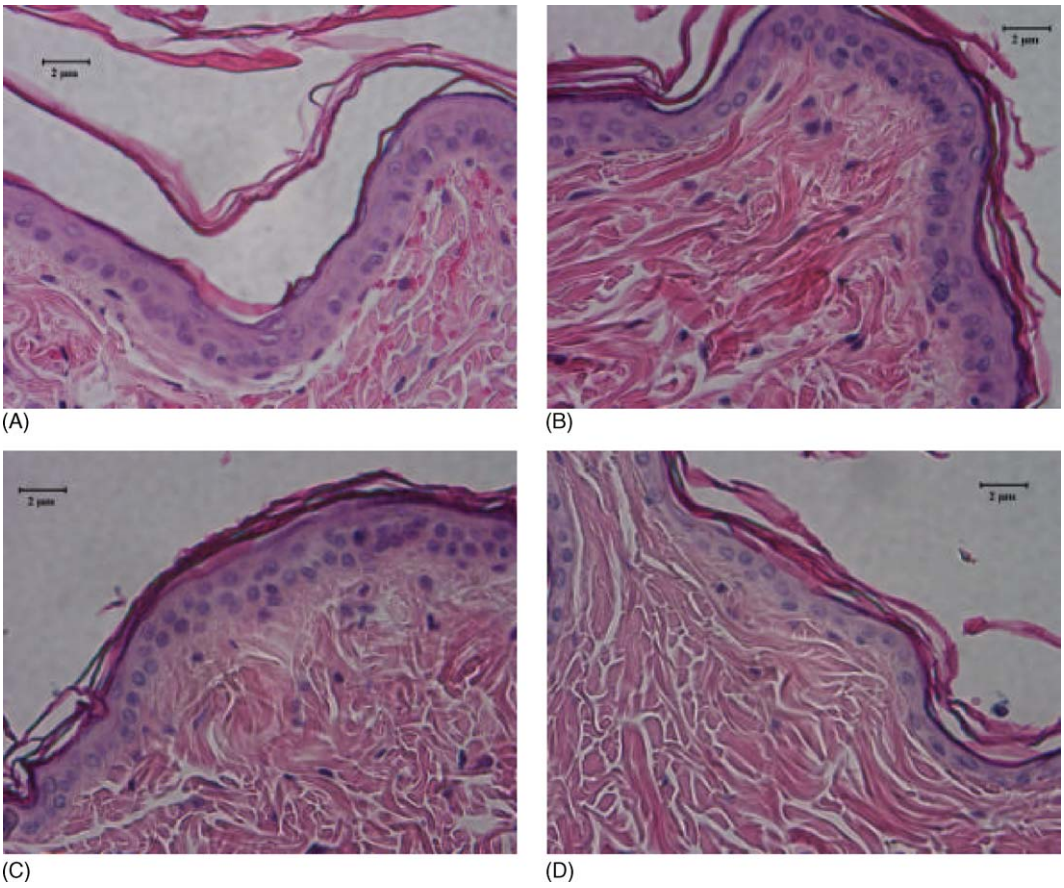

Fig. 4. Light micrographs of skin showing histological changes at different time points following electroporation using five pulses of 200 V, 10 ms with 1 s interval between each pulse. Key: (A) immediately following electroporation; (B) 24 h following electroporation; (C) 48 h following electroporation; (D) 7 days following electroporation (scale bar = 2 μm).

the electroporation is reversible. Transient skin irritation was also observed with 200 and 300 V pulses, which is in accordance with the TEWL results. Irritation tends to reduce the efficiency of stratum corneum barrier function and results in an increase in TEWL. The skin irritation mechanism involves the release of inflammatory mediators and their migration to the exposed area. When the inflammatory mediators enter extracellular fluids, vasodilation results and cause visible erythema and increased vascular permeability that leads to edema.

With all the electroporation pulses tested, the SC was detached immediately following electroporation. An increased detachment in the SC layers with increasing electroporation voltages from 100 to 300 V was observed. The detachment of the SC layers following electroporation pulsing shows the skin barrier perturbation. This is in accordance with the TEWL results discussed previously. The degeneration of basal layer and breakdown of collagen fibers were seen in microscopic studies at 24 and 48 h following electroporation. However, the skin viability was not affected at 0 and 24 h following electroporation. The degeneration of basal layer and collagen fibers breakdown may not lead to cell death, and therefore the mitochondrial dehydrogenase activity (by MTT assay) was not affected. The affected basal layer and collagen fibers have recovered to normal by 7 days as seen in microscopic studies. Furthermore, the skin barrier perturbation, which is mainly attributed to changes in dead cell layers of SC, may not affect the skin viability.

With the higher voltages (i.e. 200 and 300 V), the epidermis assumed amorphous nature. The increased epidermal thickness indicates an inflammatory response. These inflammatory responses may be due to the physical stimulation of skin cells by electroporation and the secretion of proinflammatory cytokines. The disruption of skin barrier can initiate the production of a host of cytokines by keratinocytes (Williams and Kupper, 1996). The types of cytokines produced due to the barrier disruption by electroporation are not known to date. Keratinocyte activation may also induce the release of proinflammatory cytokines from other cell types. This could be useful in the case of DNA vaccine delivery as some of the cytokines help in accelerating/potentiating the immune responses (Chattergoon et al., 2004; Chow et al., 1997). The inflammatory responses as a result of electroporation are unlikely to cause long-term histological changes or pain, since systematic studies on skin did not show any long lasting side effects. Previous studies using bupivacaine to enhance immune responses to DNA vaccines (Davis et al., 1995) suggested that muscle damage/inflammation was important for enhancing immune responses. Thus, the mechanisms by which electroporation enhance immune responses to DNA vaccines may be a combination of increased gene

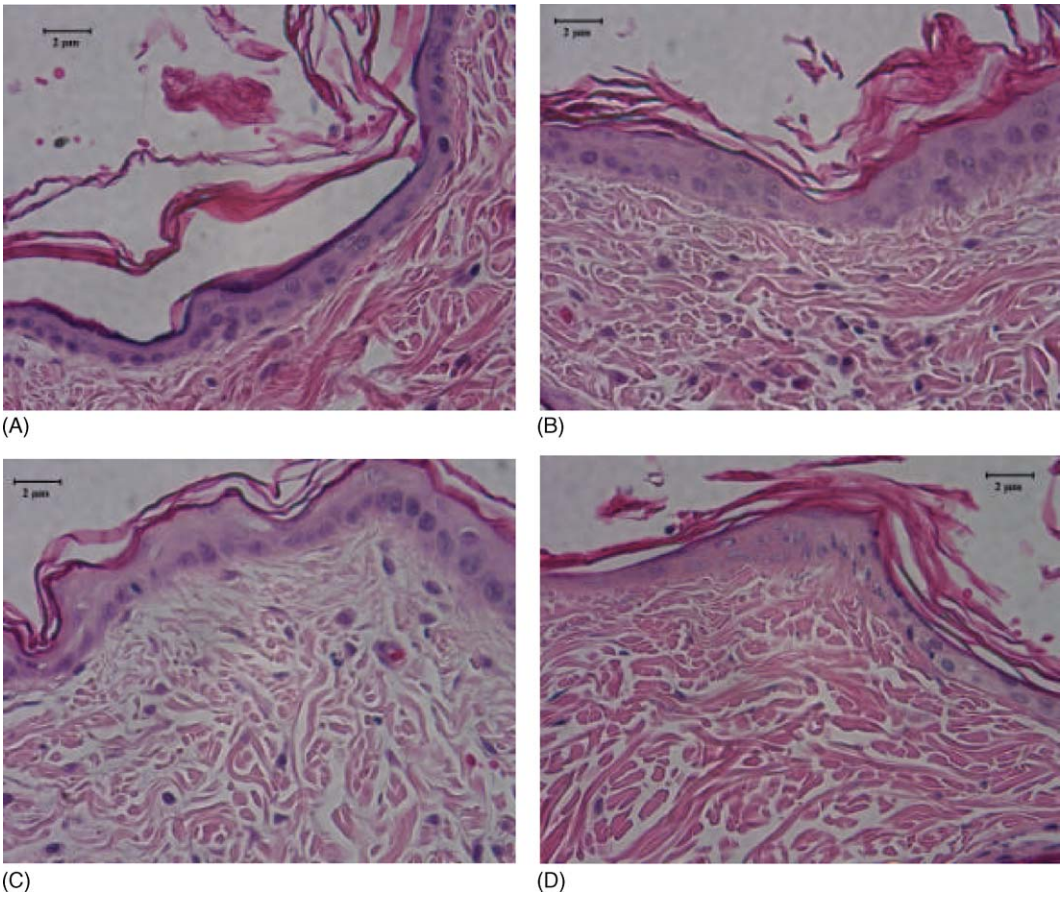

Fig. 5. Light micrographs of skin showing histological changes at different time points following electroporation using five pulses of 300 V, 10 ms with 1 s interval between each pulse. Key: (A) immediately following electroporation; (B) 24 h following electroporation; (C) 48 h following electroporation; (D) 7 days following electroporation (scale bar = 2 μm).

expression, inflammation (possibly by cytokine) and cellular infiltration.

5. Conclusions

The development of techniques that could enhance the delivery of genes/DNA vaccines into target cells with minimal toxic effects is one of the widely pursued areas of research in gene-based therapy. Lack of safe and effective methods for delivering DNA vaccines may be the main reason for the lower efficacy of these agents observed in higher animals and humans. This could be overcome by developing effective delivery methods that can improve the transfection and expression of DNA vaccines in vivo safely. The present study demonstrates the safety of electroporation for skin targeted DNA vaccine delivery, which could be developed as an alternative method for genetic immunization.

Acknowledgements

We acknowledge the financial support from National Institutes of Health grant # HD 41372 and the Presidential Doctoral Fellowship to BMM from North Dakota State University, Fargo, ND 58105. We would also like to thank Thomas R. Gustad for his help in skin viability studies, Sibao Chen and Khaled Al-Tahami for their help during in vivo experimentation.

References

Abdallah, B., Sachs, L., Demeneix, B.A., 1995. Non-viral gene transfer: applications in developmental biology and gene therapy. Biol. Cell. 8, 1–7.

Abrams, K., Harvell, J.D., Shriner, D., Wertz, P., Maibach, H., Maibach, H.I., Rehfeld, S.J., 1993. Effect of organic solvents on in vitro human skin water barrier function. J. Invest. Dermatol. 101, 609–613.

Aihara, H., Miyazaki, J., 1998. Gene transfer into muscle by electroporation in vivo. Nat. Biotechnol. 16, 867–870.

Chattergoon, M.A., Saulino, V., Shames, J.P., Stein, J., Montaner, L.J., Weiner, D.B., 2004. Co-immunization with plasmid IL-12 generates a strong T-cell memory response in mice. Vaccine 22, 1744–1750.

Chow, Y.H., Huang, W.L., Chi, W.K., Chu, Y.D., Tao, M.H., 1997. Improvement of hepatitis B virus DNA vaccines by plasmids coexpressing hepatitis B surface antigen and interleukin-2. J. Virol. 7, 169–178.

Davis, H.L., Michel, M.L., Whalen, R.G., 1995. Use of plasmid DNA for direct gene transfer and immunization. Ann. N. Y. Acad. Sci. 772, 21–29.

Doria-Rose, N.A., Haigwood, N.L., 2003. DNA vaccine strategies: candidates for immune modulation and immunization regimens. Methods 31, 207–216.

Draize, J.H., Woodard, G., Calvery, H.O., 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 82, 377–390.

Glasspool-Malone, J., Somiari, S., Drabick, J.J., Malone, R.W., 2000. Efficient nonviral cutaneous transfection. Mol. Ther. 2, 140–146.

Herweijer, H., Wolff, J.A., 2003. Progress and prospects: naked DNA gene transfer and therapy. Gene Ther. 10, 453–458.

Khavari, P.A., 1997. Therapeutic gene delivery to the skin. Mol. Med. Today 3, 533–538.

Lagging, L.M., Meyer, K., Hoft, D., Houghton, M., Belshe, R.B., Ray, R., 1995. Immune responses to plasmid DNA encoding the hepatitis C virus core protein. J. Virol. 69, 5859–5863.

Lefesvre, P., Attema, J., Bekkum, D.A., 2002. Comparison of efficacy and toxicity between electroporation and adenoviral gene transfer. BMC Mol. Biol. 3, 12.

Loffler, H., Pirker, C., Aramaki, J., Frosch, P.J., Happle, R., Effendy, I., 2001. Evaluation of skin susceptibility to irritancy by routine patch testing with sodium lauryl sulfate. Eur. J. Dermatol. 11, 416–419.

Ma, H., Diamond, S.L., 2001. Nonviral gene therapy and its delivery systems. Curr. Pharm. Biotechnol. 2, 1–17.

Maibach, H.I., Bronaugh, R., Guy, R., Turr, E., Wilson, D., Jacques, S., Chaing, D., 1984. Noninvasive techniques for determining skin function. In: Drill, V.A., Lazar, P. (Eds.), Cutaneous Toxicity. Raven Press, New York, pp. 63–97.

Medi, B.M., Singh, J., 2003. In vivo cutaneous gene delivery using electroporation. Proc. Contr. Rel. Soc., abstract # 611.

Medi, B.M., Hoselton, S., Marepalli, B.R., Singh, J., 2005. Skin targeted DNA vaccine delivery using electroporation in rabbits. I. Efficacy. Int. J. Pharm. 294, 53–63.

Mir, L.M., Glass, L.F., Sersa, G., Teissie, J., Domenge, C., Miklavcic, D., Jaroszeski, M.J., Orlowski, S., Reintgen, D.S., Rudolf, Z., Belehradek, M., Gilbert, R., Rols, M.P., Belehradek Jr., J., Bachaud, J.M., DeConti, R., Stabuc, B., Cemazar, M., Coninx, P., Heller, R., 1998. Effective treatment of cutaneous and subcutaneous malignant tumors by electrochemotherapy. Br. J. Cancer 77, 2336–2342.

Raz, E., Carson, D.A., Parker, S.E., Parr, T.B., Abai, A.M., Aichinger, G., Gromkowski, S.H., Singh, M., Lew, D., Yankauckas, M.A., Baird, S.M., Rhodes, G.H., 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. Proc. Natl. Acad. Sci. U.S.A. 91, 9519–9523.

Roberts, M.S., Walters, K.A., 1998. The relationship between structure and barrier function of skin. In: Roberts, M.S., Walters, K.A. (Eds.), Dermal Absorption and Toxicity Assessment. Marcel Dekker, New York, pp. 1–42.

Rougier, A., Goldberg, A., Maibach, H.I., 1994. In vitro skin toxicology: irritation. In: Phototoxicity and Sensitization. Mary and Liebert, New York.

Sekkat, N., Kalia, Y.N., Guy, R.H., 2002. Biophysical study of porcine ear skin in vitro and its comparison to human skin in vivo. J. Pharm. Sci. 91, 2376–2381.

Sersa, G., Stabuc, B., Cemazar, M., Miklavcic, D., Rudolf, Z., 2000. Electrochemotherapy with cisplatin: clinical experience in malignant melanoma patients. Clin. Cancer Res. 6, 863–867.

Singh, J., Gross, M., Sage, B., Davis, H.T., Maibach, H.I., 2001. Regional variations in skin barrier function and cutaneous irritation due to iontophoresis in human subjects. Food Chem. Toxicol. 39, 1079–1086.

Srivastava, I.K., Margaret, A.L., 2003. Gene vaccines. Ann. Inter. Med. 38, 550–559.

Tanojo, H., Boelsma, E., Junginger, H.E., Ponec, M., Bodde, H.E., 1998. In vivo human skin barrier modulation by topical application of fatty acids. Skin Pharmacol. Appl. Skin Physiol. 11, 87–97.

Tuting, T., Storkus, W.J., Falo, J.D., 1998. DNA immunization targeting the skin: molecular control of adaptive immunity. J. Invest. Dermatol. 111, 183–188.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745–1749.

Verma, I.M., Somia, N., 1997. Gene therapy-promises, problems and prospects. Nature 389, 239–242.

Verma, I.M., 2000. A tumultuous year for gene therapy. Mol. Ther. 2, 415–416.

Whalen, R.G., 1996. DNA vaccines for emerging infectious diseases: what if? Emerg. Infect. Dis. 2, 168–175.

Williams, I.R., Kupper, T.S., 1996. Immunity at the surface: homeostatic mechanisms of the skin immune system. Life Sci. 58, 1485–1507.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P.L., 1990. Direct gene transfer into mouse muscle in vivo. Science 247, 1465–1468.

Zhang, L., Nolan, E., Kreitschitz, S., Rabussay, D.P., 2002. Enhanced delivery of naked DNA to the skin by non-invasive in vivo electroporation. Biochim. Biophys. Acta 1572, 1–9.