Topical Delivery of Anti-sense Oligonucleotides Using Low-Frequency Sonophoresis

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Purpose. Topical delivery of oligonucleotides, though attractive for the treatment of skin disorders, is limited by the low permeability of the stratum corneum. Herein, we assessed the potential of low-frequency ultrasound (20 kHz, 2.4 W/cm^2) in delivering therapeutically significant quantities of anti-sense oligonucleotides into skin.
Methods. Dermal penetration of oligonucleotides penetration was quantified *in vitro* using radiolabeled oligonucleotides.
Results. Estimated concentrations of oligonucleotides (ODNs) in the superficial layers of the skin ranged from ~0.5% to 5% of the donor concentration after a 10-min application of ultrasound and ODN. Microscopic evaluations using fluorescently labeled oligonucleotides and sulforhodamine B revealed heterogeneous penetration into the skin. Heterogenous penetration led to the formation of localized transport pathways (LTPs), which occupied about 5% of the total exposed skin area. Immuno-histochemical studies using an oligonucleotide that reacts specifically with an antibody also confirmed penetration of ODNs into LTPs. Histologic studies revealed that no gross structural changes were induced in the skin due to ultrasound application.
Conclusions. These results show successful delivery of anti-sense oligonucleotides using low-frequency ultrasound.

KEY WORDS: inflammation; ODN; oligonucleotides; sonophoresis; topical.

INTRODUCTION

Oligonucleotides (ODNs) have been extensively studied as therapeutic agents because they can selectively interfere with gene expression in target cells (1). Pathogenic viral proteins, cellular oncogenes, and cellular proteins associated with diseases are potential targets for oligonucleotides (1). Topical delivery of oligonucleotides is a promising technique, especially for the treatment of local skin disorders including skin carcinoma, melanoma, psoriasis, and viral diseases (e.g., herpes simplex) (2). To produce pharmacodynamic effects, oligonucleotides must traverse across the stratum corneum and reach the underlying tissues. Penetration of relatively large molecular weight, negatively charged molecules across intact stratum corneum (SC) is known to be limited (3,4), suggesting an inherent obstacle which must be overcome for oligonucleotide delivery. SC is a relatively thin (10–15 μm) impermeable membrane that consists of flat, dead cells, which are filled with keratin fibers (corneocytes) that are surrounded by lipid bilayers (5). The highly ordered structure of lipid bilayers confers upon the SC an impermeable character. Different techniques, such as iontophoresis (6–8), electroporation (9,10), chemical enhancers (11), and microprojections (12) have been used to enhance transdermal and topical delivery of antisense oligonucleotides.

Here, we report on the use of low-frequency ultrasound for topical delivery of ODNs. Application of low-frequency ultrasound (20 kHz) has previously been shown to increase skin permeability to macromolecules (low-frequency sonophoresis) (13,14). However, this is the first report on successful delivery of therapeutic quantities of ODNs into skin using low-frequency ultrasound.

MATERIALS AND METHODS

Oligonucleotide Selection

All oligonucleotides used in these studies correspond to second-generation chemistries containing the 2'-*O*-methoxyethyl (MOE) sugar modification. Synthesis and purification was performed as previously described (15,16). Tritium-labeled ISIS 13920 was used for all radiochemical analysis. ISIS 13920 was chosen as an immunohistochemistry marker for these distribution studies since it is easily recognized by a specific antibody as described below. A fluorescent tag (FITC) was attached to ISIS 13920 to enable detection by fluorescence microscopy. The radiolabel was tritium incorporated in the nonexchangeable 5-carbon position of the ribose sugar of one of the 2'-O-methoxy-modified thymidine nucleotides in the 5′ portion of the oligonucleotide. The radiolabel purity of [^{3}H]ISIS 13920 was >99% by HPLC.

In Vitro Experiments

In vitro experiments were carried out with full–thickness pig skin (Yorkshire). The average density of hair on porcine skin used in this study was 45 cm^{-2}. Animals were sacrificed using pentobarbital (100 mg/kg). Skin on the back and the lateral flank was harvested immediately after sacrificing the animal. The underlying fat was removed and the skin (i.e., dermis and epidermis) was cut into small pieces (2.5 cm × 15 cm). Skin pieces with no visible imperfections such as scratches and abrasions were wrapped in aluminum foil and stored in a −80 °C freezer to be used over a period of 12 weeks. Before each experiment, the skin was thawed at room temperature and was mounted on a Franz diffusion cell (Permegear, Hellertown, PA, USA). Only skin having an initial resistivity of 30 kΩ · cm^2 or more was used to ensure that the skin was intact. A 4-mm Ag/AgCl disk electrode (Invivo Metrics, Healdsburg, CA, USA) was introduced in donor and receiver compartments for skin conductivity measurements during the experiment. The receiver compartment was filled with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). The solution has a phosphate concentration of 0.01 M and NaCl concentration of 0.137 M. PBS was prepared using deionized water with ~8 MΩ resistance. A magnetic stirrer bar was added to the receiver compartment.

In the pretreatment-type experiments, the donor compartment was filled with a solution of sodium lauryl sulfate (SLS) (1% weight/volume) in PBS. Ultrasound was applied using methods described later until a skin resistivity of 1

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$k\Omega \cdot cm^2$ was reached. At the end of ultrasound application, the coupling medium was removed and was replaced with a solution of radiolabeled ODN solution (5 μCi/ml) in PBS. In some experiments, permeation of additional solutes including manitol, leutinizing hormone releasing hormone (LHRH), inulin, dextran (ARC, St. Louis, MO, USA) in PBS was also measured. Concentrations of these solutes in the receiver compartment were measured over 24 h. The amount of radiation in the receiver compartment was plotted against time up to 24 h. Slope of the curve was determined at steady state and was used to calculate the flux ($dpm \cdot cm^{-2} \cdot h^{-1}$). The flux was divided by donor concentration (dpm/ml) to calculate permeability. At the end of 24 h, the skin was dissolved in 2% sodium hydroxide solution (Packard, Downers Grove, IL, USA). The amount of radiation in the skin was determined using liquid scintillation analysis (Packard). The scintillation cocktail used for these experiments was Ultima Gold (Packard).

In the simultaneous application type of experiments, solute delivery by a simultaneous application of ultrasound and drug was measured. In these experiments, radiolabeled solutes (mannitol, LHRH, inulin, dextran, and ODN) were added in the coupling medium at a concentration of 3 μCi/ml. Ultrasound was applied using methods described later. Ultrasound was turned off when skin conductivity reached ~1 $(k\Omega \cdot cm^2)^{-1}$. The coupling medium was removed and skin was dissolved in 2% NaOH. Concentrations of radiolabeled solutes were measured using a scintillation counter and analyzed according to methods described above.

Ultrasound Application

A 400-W sonicator (Sonics & Materials, Newtown, CT, USA) operating at a frequency of 20 kHz was used for ultrasound application. Before each experiment, the device was tuned according to the procedures specified by the manufacturer. The horn was placed at a distance of 3 mm from the skin in the donor compartment. A 50% duty cycle was chosen (5 s on, 5 s off). The ultrasonic intensity was 2.4 W/cm^2 as measured by methods described in (17). This value corresponds to a setting of 10% on the sonicator. Each skin was sonicated for a total of 10 min at room temperature (25°C). A typical temperature increase of ~13°C was observed in 2 min when ultrasound was applied with a 50% duty cycle. For this reason, the donor solution (i.e., the coupling medium) was changed every 2 min during the experiment by turning off the ultrasound. We do not anticipate that temperature increase will be a significant issue *in vivo*. Ultrasound increases skin conductivity to desired levels *in vivo* without requiring a change of coupling medium (18).

Imaging of Localized Transport Pathways in the Stratum Corneum

Localization of the permeabilizing effect of ultrasound was assessed *in vitro* using porcine skin. Skin was exposed to ultrasound in the presence of sulforhodamine B or FITC-ODN (concentrations of 10 and 5 mM respectively) as described earlier. Skin was processed in the appropriate manner (see sections below) for further analysis (either immunohistochemistry, histology, or fluorescence microscopy).

Histologic Evaluation

Skin samples were carefully wiped using a wet paper to remove any excess ODN and fixed in 10% neutral buffered formalin for 24 h before transferring to 70% ethanol for dehydration and storage. The tissues were embedded in paraffin and sectioned at 4 μm for analysis. The sections were deparaffinized in xylene and hydrated through graded alcohols for ODN immunostaining and H&E stains. The affinity purified antibody used in this work, 2E1-B5 (Berkeley Antibody Company, Berkeley, CA, USA), is an IgG1 that recognizes a CG or TCG motif in phosphorothioate oligonucleotides (19). Endogenous peroxidase activity was quenched with peroxidase blocking reagent (Dako Corporation, Carpenteria, CA, USA) for 10 min; sections were rinsed with PBS and treated with proteinase K (Dako) for 20 min. After blocking with normal donkey serum, (Jackson Laboratories, Birmingham, AL), sections were incubated for 1 h with 2E1 monoclonal antibody diluted at 1:1000. After rinsing, the antibody was detected using HRP-donkey anti-mouse IgG F(ab)'2 diluted 1:100 (Jackson) for 1 h. DAB (3,3′-diamino-benzidene, Dako) was used as a substrate. The tissue sections were counterstained with hematoxylin, dehydrated and mounted with coverslips. Serial sections of the tissues were stained with hematoxylin and eosin for routine histopathological analysis. Tissue sections were blinded and ISIS 13920 immunostaining was evaluated using a Zeiss Axiolab microscope with a Sony color video camera at various magnifications up to 200×. Because histology was performed on skin samples fixed after treatment with formalin, we expect that ODN distribution will not change during histology.

ODN Distribution by FITC Staining

Skin samples containing FITC-labeled ISIS 13920 were obtained and placed in a labeled cryomold and covered with Optimum Cutting Temperature (O.C.T. Compound VWR cat. no. 25608-930). The mold containing the biopsy was gently submerged in a pre-chilled dewar flask containing liquid nitrogen/dry ice slurry for approximately one minute until OCT compound turned white and opaque.

Tissue was stored in a –70°C degree freezer until sections were cut. Tissue was allowed to come to a –20°C degree temperature before cutting in a Leica CM 3050 cryostat at 5 μm. Sections were picked up on "+" superfrost slides and allowed to air dry for 4 h. Sections were then fixed in 5% neutral buffered formalin for 5 min, rinsed in distilled water to remove formalin, and then mounted in aquamount for fluorescent visualization using a Zeiss Axioskop Microscope with a FITC filter.

RESULTS AND DISCUSSION

Low-frequency sonophoresis (LFS) has been shown to enhance dermal penetration of various macromolecules (20,21). Based on the sequence of ultrasound and drug application, LFS can be classified into two categories: i) simultaneous sonophoresis, that is, simultaneous application of drug and ultrasound to the skin, and ii) pretreatment sonophoresis, where a short application of ultrasound is used to permeabilize skin prior to drug delivery. In this mode, the skin remains in a state of high permeability for several hours. Drugs can be

delivered through permeabilized skin during this period. Use of both modes of LFS was explored for dermal delivery of oligonucleotides.

LFS significantly enhanced penetration of ODNs into porcine skin by both modes of application. In the pretreatment mode, a 10-min simultaneous application of ultrasound (20 kHz and 2.4 W/cm^2) was used to permeabilize the stratum corneum. At the end of ultrasound application, skin conductance increased by about 100-fold. Delivery of ODNs across permeabilized skin was measured over a period of 24 h. Permeability of ultrasonically-treated skin to ODN was measured to be 4.5×10^{-5} cm/hr compared to nearly undetectable values across non-treated skin. With the sonophoretic permeability observed in this study, a transdermal ODN flux of ~4.5 μg/cm^2/hr can be obtained from a donor solution of 100 mg/ml. A significant amount of ODN was also localized in the skin. At the end of 24 h, ~5×10^4 dpm/cm^2 ODN was found in the skin. With this delivery efficiency, a dermal dose of 750 μg/cm^2 can be delivered into the skin from a donor solution containing ODN at a concentration of 100 mg/ml at the end of 24 h.

Greater enhancements of ODN delivery were obtained by simultaneous application of ultrasound and ODN. A 10-min simultaneous application of ODN and ultrasound (20 kHz, 2.4 W/cm^2) resulted in dermal accumulation of 3500 dpm/cm^2 of ODN in the skin. With this efficiency, about 53 μg/cm^2 ODN can be delivered into skin in 10 min from a donor solution containing 100 mg/ml ODN. Distribution of ODN in the skin was not measured. However, experiments performed with FITC-labeled ODN (discussed in the next section) revealed that ODN is largely localized in the superficial layers of the skin. An estimate of local concentration of ODN in the skin can then be performed. Assuming a depth of penetration of 100–1000 μm, the concentration of ODN in the skin at the end of ultrasound application would be about 0.016–0.16 μCi/ml (0.53–5.3% of the donor concentration).

Further investigation of ultrasound-mediated ODN was performed using a simultaneous application of ultrasound and

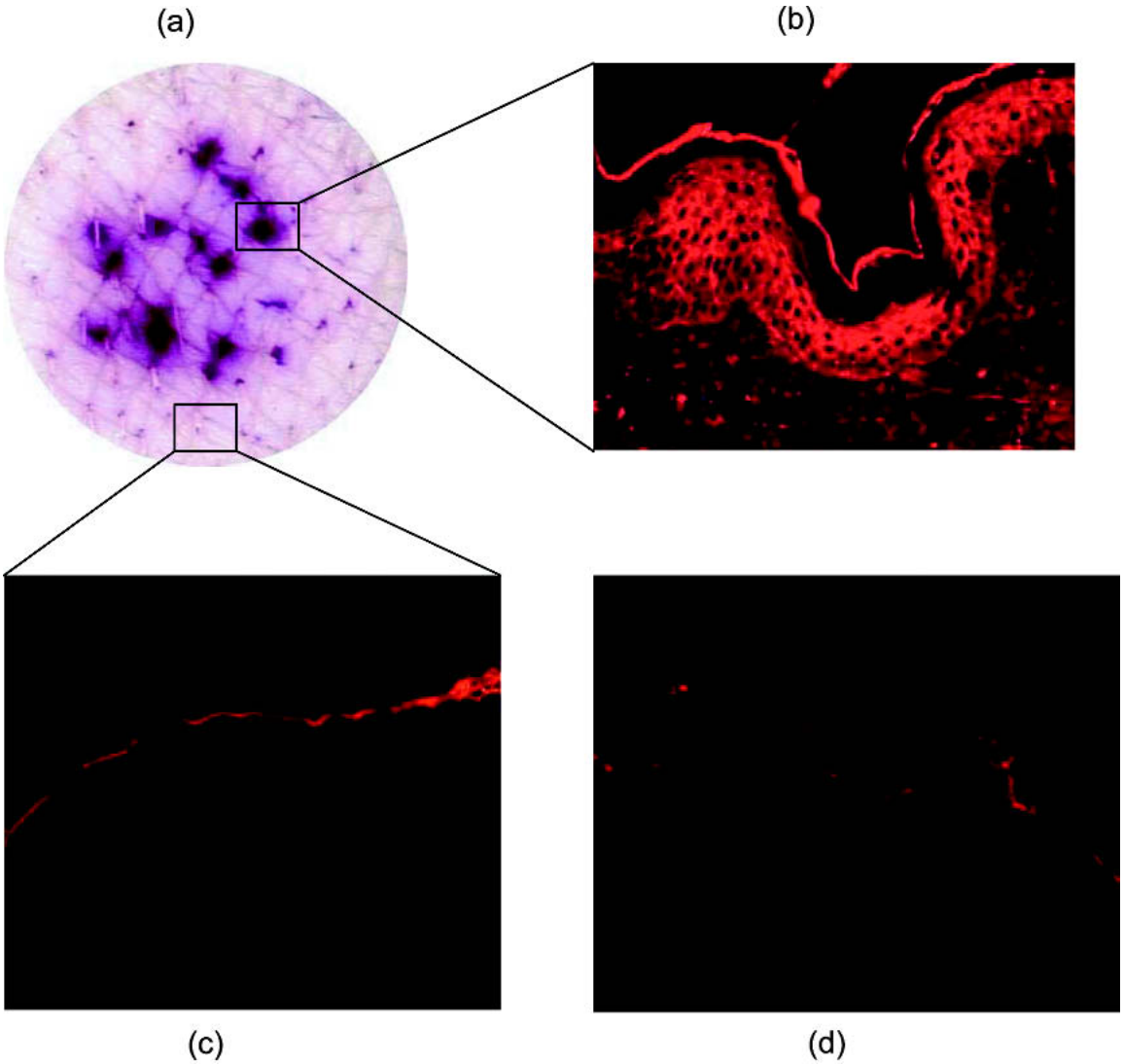

Fig. 1. Penetration of sulforhodamine B (SRB) into porcine skin *in vitro*. (a) Top view of skin exposed to SRB in the presence of ultrasound. Skin conductance was enhanced by about 100-fold. The entire skin diameter is 1.5 cm. (b) Cross-sectional view of an LTP. Extensive penetration of SRB into epidermis can be seen. (c) Cross-sectional view of a non-LTP region. (d) Cross-sectional view of a control skin sample.

ODN. This mode was preferred over the pretreatment mode, as high delivery efficiency was obtained with this mode. Furthermore, the target site of ODN is within a cell and we anticipated that a simultaneous application of ultrasound and ODN will facilitate intra-cellular delivery.

ODN penetration into skin due to LFS was heterogeneous. Heterogeneity of dermal penetration was visualized by monitoring penetration of a dye, sulforhodamine B (SRB) that was incorporated in the coupling medium. SRB penetration clearly indicated 4–5 intensely stained spots (~1 mm in diameter), which were termed as Localized Transport Pathways, LTPs (Fig. 1a). The LTPs collectively occupy about 5% of the exposed skin area. Localized permeabilization of the skin in the presence of low-frequency ultrasound is likely to originate from localization of acoustic cavitation, which is primarily responsible for enhanced transport. Depth of SRB penetration in LTPs was evaluated by cryo-sectioning of the skin followed by microscopic observations. SRB penetrated well beyond the SC in the LTPs (Fig. 1b). Significant staining was observed in the epidermal cells and also in the dermis. No significant penetration could be found in the non-LTP regions (Fig. 1c) or in controls that were exposed to SRB in the absence of ultrasound (Fig. 1d). Experiments were also performed using FITC-labeled ODN to confirm penetration of ODN into LTPs. Figures 2a and 2b show cross sections of the LTP and non-LTP regions of skin exposed to FITC-ODN in the presence of ultrasound. Penetration of ODN in the basal epidermal layer confirms the ability of LFS to deliver macromolecules into the epidermis.

To further ensure that ODN penetrates into skin without losing integrity, skin exposed to ISIS 13920 in the presence of ultrasound was assessed using immunohistochemistry. Figure 3a shows a cross section of the LTP into which ODN has penetrated. Figure 3b shows a cross section of the skin that was exposed to ODN in the absence of ultrasound (control). No visible staining was observed in case of controls. However, the skin treated with LFS was heavily stained suggesting penetration of oligonucleotide delivery. ODN was localized in the epidermis as well dermis. A closer analysis of the epidermal region (Figs. 3c and 3d) suggests that ODN penetrated into epidermal cells. This is a particularly appealing feature since viable epidermal cells are an attractive target for ODN delivery.

The effect of ultrasound on the SC structure was undetectable by histology. Figure 4a shows a section from the LTP of skin that was permeabilized by ultrasound. Conductivity of skin shown in Fig. 4a is about 100-fold higher than that of control (Fig. 4b). Compared to controls, no visible disruption of the SC or epidermis is observed in ultrasound-treated samples. The effects of ultrasound on skin structure appear to exist at a sub-microscopic level. Although the skin is substantially permeabilized by ultrasound application, it still maintains substantial size-selectivity in transport. Figures 5a and 5b show the dependence of dermal delivery on solute hydrodynamic radius. Figure 5a shows data obtained for the simultaneous type sonophoresis, and Fig. 5b shows data for pretreatment type sonophoresis. Closed squares show the amount delivered per unit area of LTPs, and open squares show the amount delivered per unit area into non-LTP regions. Open circles show data for controls (no ultrasound). Penetration of solutes into LTPs and non-LTPs was significantly enhanced. The amount of solute delivered into LTPs by simultaneous application with ultrasound decreased by about 20-fold as the solute hydrodynamic radius increased from 4.4 Å (mannitol) to 26 Å (dextran). Solute delivery into non-LTP regions also exhibited a size-selectivity, although to a lesser extent. Skin permeability measured after ultrasound pretreatment (Fig. 5b) also exhibited similar size-selectivity. Such strong size-selectivity is consistent with no gross structural alterations in the skin (Fig. 4).

The results presented here demonstrate that ultrasound can deliver oligonucleotides into skin. Several methods have been reported in the literature to enhance transdermal absorption of ODNs. Lin *et al.* showed that about 0.48 mg of ODN was delivered in 4 h across 2-cm^2 skin permeabilized with microprojections and iontophoresis from a donor comprising 50 mg/ml ODN (22). This number is generally in the same range as that reported in this paper for ultrasound (53 μg/cm^2 in 10 min from a donor concentration of 100 mg/ml). Iontophoretic permeabilities of ODNs have been reported to be in the range of ~10^{-4} cm/h (23). These permeabilities are higher than those reported in this paper. However, it is diffi-

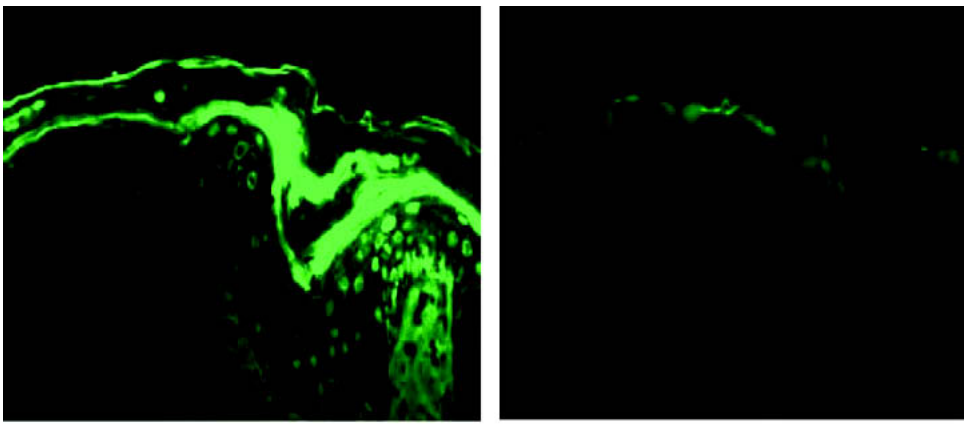

Fig. 2. (a) Penetration of FITC-ODN into porcine skin after ultrasound treatment. The figure shows section of an LTP region. (b) FITC-ODN penetration a non-LTP region.

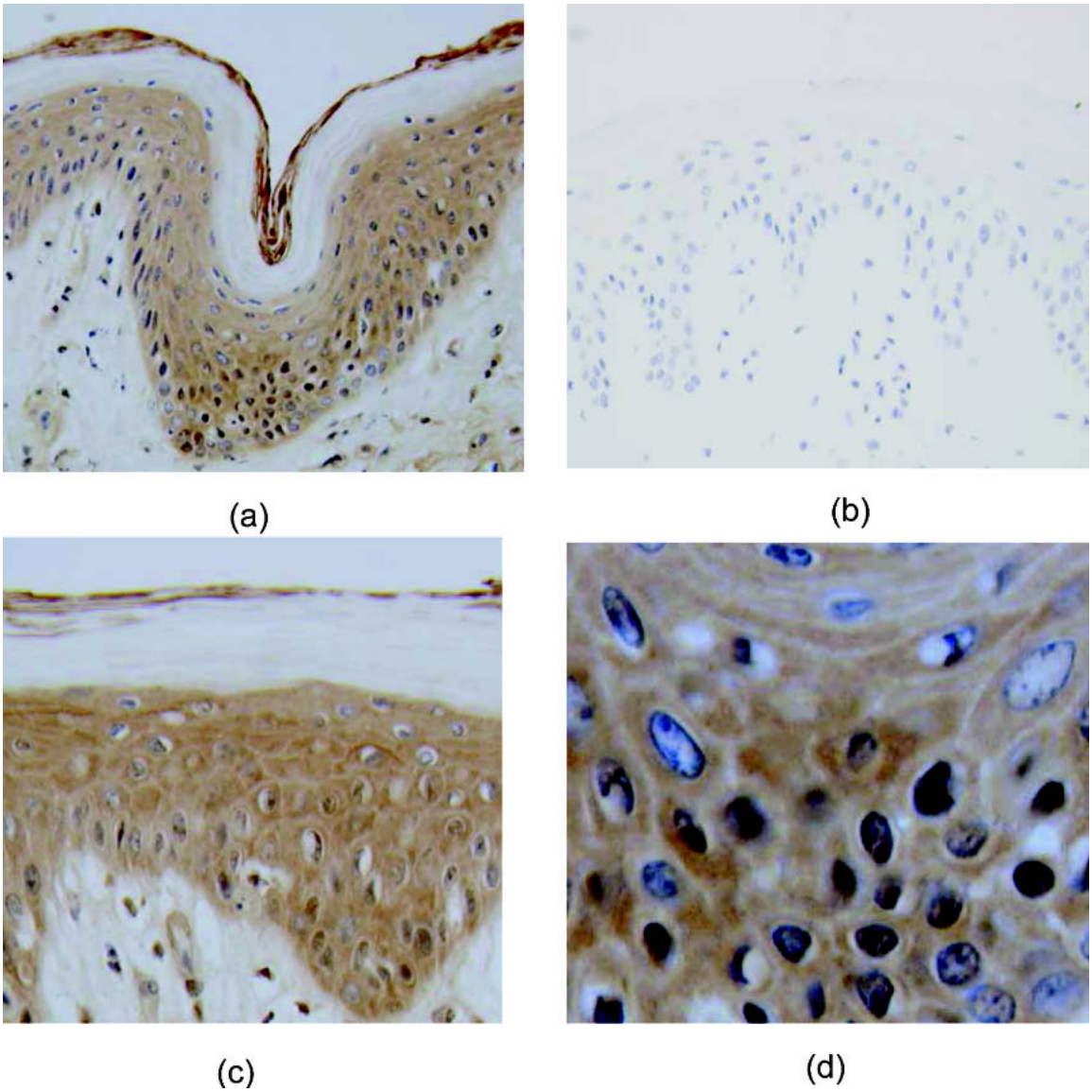

Fig. 3. (a) Penetration of ODN into porcine skin (Immunohistochemical staining) after ultrasound treatment. The figure shows cross section of an LTP region. (b) ODN penetration into control samples (no ultrasound). (c) and (d) Enlarged views of LTPs demonstrating ODN penetration into skin cells after ultrasound treatment.

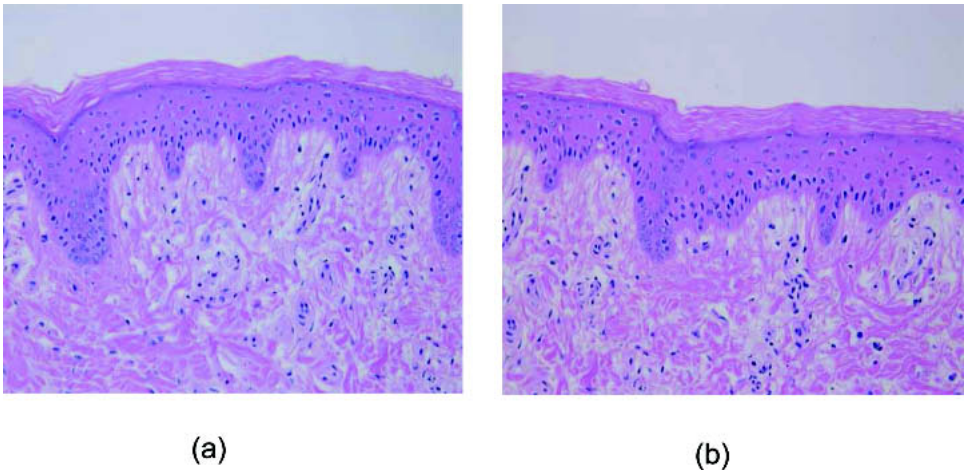

Fig. 4. Results of H&E staining of porcine skin exposed to ultrasound under the conditions where ODN penetration is observed (pretreatment). (a) Section of an LTP region. The skin from which this specimen was collected possessed skin conductivity about 100-times that of control skin. (b) Control samples (no ultrasound).

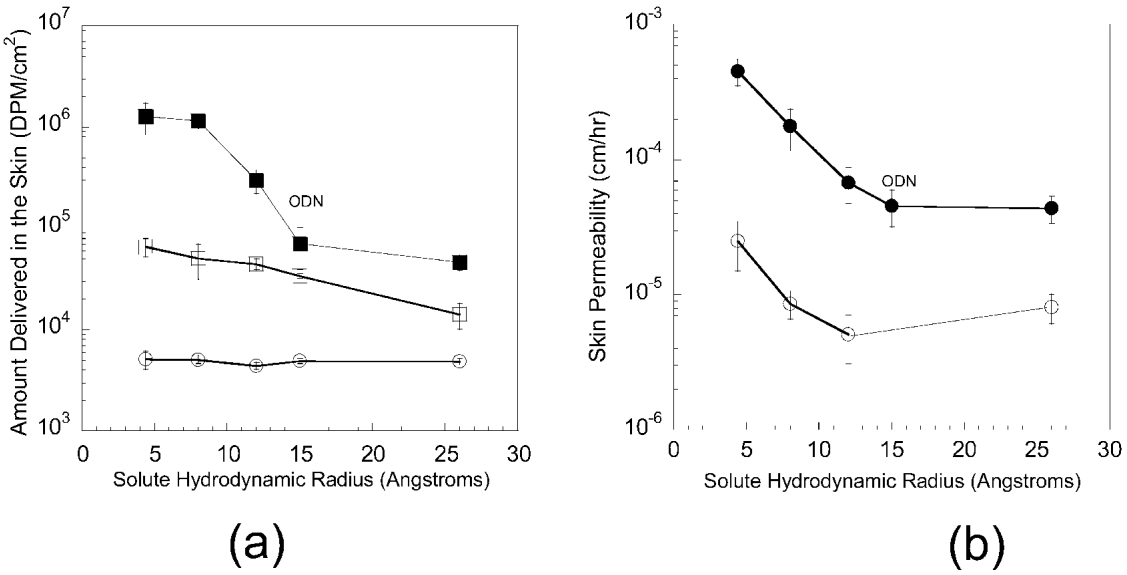

Fig. 5. (a) Dependence of dermal penetration of solutes by simultaneous application of ultrasound and solutes (closed squares: amount delivered per unit area of LTPs; open squares: amount delivered per unit area into non-LTP regions; open circles: controls, that is, no ultrasound). The amount per unit area for LTP and non-LTP regions was determined by first measuring the total ODN in LTP and non-LTP regions and dividing it by respective areas. The area of the LTPs was about 5% of the total exposed area (25). The area of the non-LTP region was determined by subtracting the LTP area from the total exposed area. (b) Dependence of skin permeability when the skin was pretreated with ultrasound prior to the placement of solutes (closed circles: ultrasound treated skin; open circles: control, that is, no ultrasound).

cult to compare these permeabilities with those reported in this study since ultrasound was applied only for 10 min while iontophoresis was applied continuously. Rignier *et al.* reported that electroporation delivered on the order of 100 pmoles/cm^2 into skin in 4 h after exposure to 3.3. μM ODN (24).

CONCLUSIONS

The studies presented here demonstrate that low-frequency ultrasound delivers therapeutically significant quantities of ODNs into skin. Delivery of ODNs is heterogeneous and results in the creation of LTPs. Application of ultrasound did not induce skin damage as judged by histology.

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