An Investigation of the Role of Cavitation in Low-Frequency Ultrasound-Mediated Transdermal Drug Transport

Hua Tang,[1] Chiao Chun Joanne Wang,[1] Daniel Blankschtein,[2,4] and Robert Langer [3]

Received April 22, 2002; accepted May 1, 2002

Purpose. Low-frequency ultrasound (20 kHz) has been shown to increase the skin permeability to drugs, a phenomenon referred to as low-frequency sonophoresis (LFS). Many previous studies of sonophoresis have proposed that ultrasound-induced cavitation plays the central role in enhancing transdermal drug transport. In this study, we sought to definitively test the role of cavitation during LFS, as well as to identify the critical type(s) and site(s) of cavitation that are responsible for skin permeabilization during LFS.

Methods. Pig full-thickness skin was treated by 20 kHz ultrasound, and the effect of LFS on the skin permeability was monitored by measuring the increase in the skin electrical conductance. A high-pressure LFS cell was constructed to completely suppress cavitation during LFS. An acoustic method, as well as chemical and physical dosimetry techniques, was utilized to monitor the cavitation activities during LFS.

Results. The study using the high-pressure LFS cell showed definitively that ultrasound-induced cavitation is the key mechanism via which LFS permeabilizes the skin. By selectively suppressing cavitation outside the skin using a high-viscosity coupling medium, we further demonstrated that cavitation occurring outside the skin is responsible for the skin permeabilization effect, while internal cavitation (cavitation inside the skin) was not detected using the acoustic measurement method under the ultrasound conditions examined. Acoustic measurement of the two types of cavitation activities (transient vs. stable) indicates that transient cavitation plays the major role in LFS-induced skin permeabilization. Through quantification of the transient cavitation activity at two specific locations of the LFS system, including comparing the dependence of these cavitation activities on ultrasound intensity with that of the skin permeabilization effect, we demonstrated that transient cavitation occurring on, or in the vicinity of, the skin membrane is the central mechanism that is responsible for the observed enhancement of skin permeability by LFS.

Conclusions. LFS-induced skin permeabilization results primarily from the direct mechanical impact of gas bubbles collapsing on the skin surface (resulting in microjets and shock waves).

KEY WORDS: drug delivery; mechanisms; ultrasound; sonophoresis; cavitation.

[1] Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue Cambridge, Massachusetts 02139-4307.

[2] Department of Chemical Engineering, Room 66-444, Massachusetts Institute of Technology, 77 Massachusetts Avenue Cambridge, Massachusetts 02139-4307.

[3] Department of Chemical Engineering, Room E25-342, Massachusetts Institute of Technology, 77 Massachusetts Avenue Cambridge, Massachusetts 02139-4307.

[4] To whom correspondence should be addressed. (e-mail: dblank@mit.edu)

INTRODUCTION

Systemic as well as topical delivery of drugs via the transdermal route is limited by the low skin permeability which is attributed to the stratum corneum (SC), the outermost layer of the skin. The SC consists of disk-like dead cells (keratinocytes) containing keratin fibers and water, surrounded by densely-packed lipid bilayers. The ordered structure of the lipid bilayers confers a highly impermeable character to the SC. A variety of physical and chemical approaches have been proposed to enhance transdermal drug transport. These include: (i) ultrasound(1–4), (ii) chemical enhancers (5–7), and (iii) electric fields (8,9). Ultrasound at frequencies between 20kHz and 16 MHz has been shown to transiently enhance the skin permeability in a process referred to as sonophoresis (1–4).

Because of the widespread application of ultrasound in medical practice, the effects of ultrasound on biologic tissues have been studied extensively over the years. Ultrasound can interact with biologic tissues (such as the skin) through various mechanisms. In general, the biophysical modes of ultrasonic action on biologic tissues can be classified into two categories (10): (i) thermal mechanisms, and (ii) nonthermal mechanisms. Of the variety of nonthermal effects of ultrasound in biologic tissues that have been reported, acoustic cavitation is believed to be the most important one (11). Acoustic cavitation is defined as the acoustically induced activity of gas-filled cavities (involving nucleation, oscillation, and collapse). There are two types of cavitation activities. The first is noninertial (or stable) cavitation, where the radius of the bubbles oscillates about an equilibrium value without collapsing over a considerable number of acoustic cycles. The second is inertial (or transient) cavitation, where the bubbles grow rapidly within one or two acoustic cycles before collapsing violently during a single compression half cycle (12,13).

In previous studies of sonophoresis, acoustic cavitation has most often been suggested to be the key mechanism responsible for the observed skin permeability enhancement due to ultrasound. However, this hypothesis that cavitation is the key mechanism responsible for sonophoresis was based on indirect experiment observations, such as the appearance of "holes", or of irregularities, in the skin tissue (14–17). In addition, the inverse dependence of the sonophoretic enhancement on the ultrasound frequency [that is, the observed dependence of the biologic effects of ultrasound on ultrasound frequency was consistent with a cavitational mechanism (4)].

The main objective of this paper was to conduct a systematic investigation of the role of cavitation during low-frequency sonophoresis (LFS), a technique that is capable of facilitating the delivery of large protein drugs via the transdermal route (18). Specifically, the objectives of this study are (a) to obtain direct experimental evidence to test the role of cavitation during LFS, and (b) to investigate the detailed mechanisms via which ultrasound-induced cavitation permeabilizes the skin membrane during LFS (that is, to identify the critical type(s) and site(s) of cavitation that are responsible for the observed skin permeability enhancement during LFS). Specifically, three questions are addressed: (i) does cavitation occur inside the skin membrane during LFS, (ii) which type of cavitation activities—transient or stable—plays the key role

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in enhancing the skin permeability during LFS, and (iii) what are the key mechanisms via which cavitation bubbles modify the skin structure.

MATERIALS AND METHODS

Measurement of the Skin Permeabilization Effect during LFS

Yorkshire pig FTS was prepared following the previously published method (19,20).

The skin electrical conductance (the reciprocal of the skin electrical resistance) is a sensitive indicator of the skin barrier function (21). Because the skin electrical conductance/resistance can be measured nearly instantaneously and with less effort than the skin permeability, in this study, we utilized the skin electrical conductance as an indicator to quantify the skin permeabilization effect induced by ultrasound (20,22). The electrical conductance of *in vitro* pigskin was measured using methods described by Tang *et al.* (19) and Mitragotri *et al*. (20). The enhancement of the skin electrical conductance due to LFS was calculated by taking the ratio of the skin electrical conductance measured before and after the ultrasound exposure.

Ultrasound Application

Twenty kilohertz of ultrasound was generated by an ultrasonic transducer (VCX 400, Sonics and Materials, Inc., Newtown, CT, USA), and was applied in a pulsed mode (0.1 s ON, 0.9 s OFF) to minimize thermal effects. The ultrasound probe was positioned 0.8 cm above the skin inside the donor compartment of the Franz diffusion cells (Permegear Inc., Riegelsville, PA, USA) during ultrasound application. The ultrasound intensities applied were between 1.6 and 33.5 W/cm^2, depending on the experiment [ultrasound intensities were measured using methods described by Mitragotri *et al* (20)]. The typical ultrasound application time for the skin conductance enhancement experiments was 2 h. The temperature in the diffusion cells during the 2-h ultrasound treatment was recorded continuously using a fine rigid wire thermocouple and a digital thermometer (Digithermo, VWR Scientific, Houston, TX). During ultrasound treatment, the temperature of the receiver compartment of the diffusion cells was constant (23 ± 1°C). On the other hand, the temperature of the PBS solution in the donor compartment increased as a function of the ultrasound treatment time. When the ultrasound intensities utilized were greater than 14.4 W/cm^2, the temperature in the donor compartment exceeded 37°C after about 10 min of ultrasound treatment. As a result, during the skin conductance measurement experiments, we replaced the coupling PBS solution in the donor compartment with fresh PBS at room temperature as needed to maintain the skin surface temperature ≤40°C [recall that skin lipids undergo a phase transition at temperatures of about 40°C (23)].

Cavitation Detection Using an Acoustic Pressure Spectrum Analysis

The acoustic method of cavitation detection involves the detection of cavitation bubble activity by measuring the pressure spectrum (acoustic emissions) of an acoustic field using a pressure transducer (hydrophone) (10). When cavitation occurs, features associated with the bubble response appear in the measured pressure spectrum, while such features do not appear in the absence of cavitation (24,25). For example, if the driving acoustic field is a continuous wave of frequency f, the acoustic pressure field scattered by the cavitation bubbles contains special components of harmonic frequencies ($2f$, $3f$, etc.), subharmonic frequencies ($f/2$, $f/3$, etc.), and ultraharmonic frequencies ($3f/2$, $2f/3$, etc.). As a result, the onset of subharmonic signal (f/2) has been commonly utilized as an indicator of the onset of cavitation bubbles in a biologic system (10,24), and its amplitude used to characterize the magnitude of stable (noninerital) cavitation activity (26). In addition, at higher ultrasound intensities [above the threshold of transient cavitation (13)], bubbles grow rapidly and collapse during a single compression half cycle (a phenomenon referred to as transient cavitation). The occurrence of transient cavitation results in the emissions of white noise (or broadband noise), which raises both the peak acoustic signals and the acoustic emission between the peaks. Consequently, the amplitude of the broadband noise can be measured to characterize the magnitude of transient (inertial) cavitation activity (10,24). In summary, there are certain types of acoustic signals within the pressure spectrum that associated with cavitation activities. One can use the acoustic method to directly detect the onset of cavitation, as well as to quantitatively measure the magnitudes of the two types of cavitation activities (transient vs. stable) in the regions exposed to ultrasound.

In this study, we measured the amplitudes of both the subharmonic (f/2) signal and of the broadband noise emission in the LFS system under various ultrasound application conditions to characterize stable and transient cavitation activities in the LFS system. The acoustic measurements were carried out using a hydrophone (a home-made transducer device donated to us as a gift by Prof. Ron Roy in the Department of Aerospace and Mechanical Engineering at Boston University), which was epoxied to the bottom of the diffusion cell. A schematic diagram of the apparatus used to measure the real-time acoustic spectrum is shown in Fig. 1. The output of the hydrophone was analyzed utilizing a digital oscilloscope (Hewlett Packard, Model Infinium 54810A, Palo Alto, CA), which carries out a fast fourier transform (FFT) and displays the frequency spectra of the measured acoustic field. The frequency spectra data was then fed into a PC through a GPIB board (National Instruments, Austin, TX) to obtain the amplitudes of the f/2 and broadband noise signals. Both signals were measured in units of voltage (the output of the hydrophone), and were subsequently utilized to represent the magnitudes of stable and transient cavitation activities in the LFS system (see Results and Discussion). Note that the voltage data measured by the hydrophone can be converted into pressure amplitudes by utilizing a calibrated hydrophone. In this study, for simplicity, we report the results in units of voltage. The amplitudes of the acoustic signals (f/2 and broadband noise) measured during the 2 h LFS experiments were averaged to obtain an average representation of the cavitation activity during each individual LFS experiment. The efficiency of the hydrophone during the course of the study was checked using a calibrated Bruel and Kjaer hydrophone (Type 8103), and was found to be approximately constant.

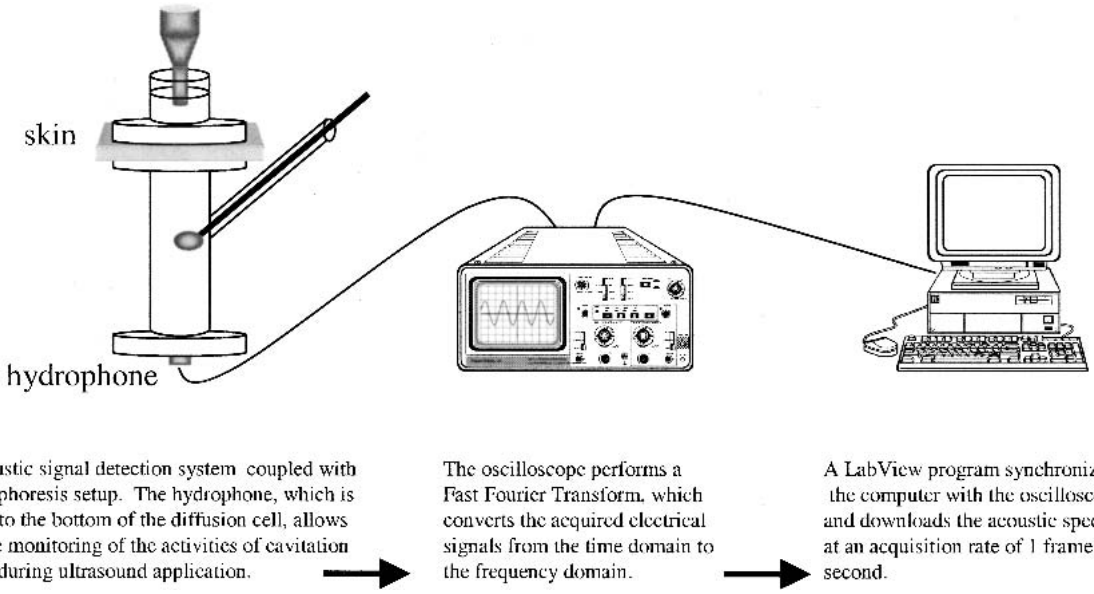

Fig. 1. Schematic diagram of the apparatus used to measure the real-time acoustic spectra.

High-Pressure LFS Cell Studies

A novel, high-pressure LFS cell was constructed and utilized to assess the role of cavitation during LFS. The rationale behind this experimental approach is discussed later. To induce cavitation in a liquid medium, the medium must experience sufficient tension (negative pressure) (27). This tension can be induced by the passage of an acoustic wave, such as ultrasound. Specifically, to induce cavitation in an ultrasound field, the pressure amplitude (intensity) of the ultrasound field must be sufficiently high, so that during the negative pressure cycle of the acoustic wave, the submicroscopic gas nuclei present in the liquid can grow to appreciable sizes, and as a result, cavitation activity can take place. This minimum ultrasound intensity (pressure amplitude) that is required to induce cavitation in a liquid is referred to as the cavitation threshold. The value of the cavitation threshold in a liquid medium is directly proportional to the ambient hydrostatic pressure (13,27). This is because under high ambient pressures, higher ultrasound intensities (pressure amplitudes) are required to offset the applied high ambient pressure to induce sufficient tension in the liquid medium for cavitation to occur. As a result, by manipulating the ambient pressure and by selecting appropriate ultrasound intensities, one can control the onset of cavitation activities in a LFS system. On the other hand, non-cavitational effects induced by ultrasound in a LFS system, including thermal effects, radiation forces, and macroscopic acoustic streaming, are not a function of the ambient pressure. Therefore, by manipulating the ambient pressure during LFS, one can decouple the ultrasound-induced cavitation phenomenon from the other ultrasound-induced non-cavitational phenomena. The experimental approach utilized here (that is, controlling the onset of cavitation activity in a biologic system by manipulating the ambient pressure) provides a definitive test of the importance of cavitation in inducing the observed biologic effects of ultrasound.

The pressure cell constructed has a designed operation pressure of up to 300 psia, which involves a cylindrical stainless steel tube with a height of 30 cm and an inner diameter of 11.2 cm. The ultrasound source (a flanged Sonics and Materials VCX 400 horn, Sonics and Materials, Newton, CT) is mounted on the top of the pressure cell to sonicate the skin samples inside the pressure cell. A circular groove on the bottom of the pressure cell is utilized to help center the diffusion cell inside the pressure cell. Two sets of LFS experiments were conducted using the high-pressure LFS cell: Case 1 (control)—LFS under atmospheric pressure (where cavitation activity is detected in the LFS system), and Case 2—LFS under elevated ambient pressures (where cavitation activity is completely suppressed). Specifically, 20 kHz ultrasound at 6.5 W/cm^2 was applied to the skin samples in a pulsed mode (0.1 s ON, 0.9 s OFF) for 2 h under both normal (atmospheric) (Case 1: 14.5 psia) and elevated (Case 2: 270 psia) pressures. The ultrasound intensity was selected such that there would be no need to replace the PBS solution in the donor compartment of the diffusion cell during the high-pressure-cell LFS experiments (that is, to ensure that the temperature in the donor compartment did not exceed 40°C during sonication). Acoustic emissions from the LFS system inside the pressure cell were monitored using the acoustic method described in High-Pressure LFS Cell Studies to detect the cavitation activities during LFS, and the corresponding skin electrical conductance was measured simultaneously (see Ultrasound Application) to quantify the skin permeabilization effect due to LFS.

Castor Oil Studies

To test whether cavitation occurs inside the skin tissue during 20 kHz LFS (as well as to assess the importance of cavitation inside and outside the skin), we utilized the well-known fact that the cavitation threshold increases as the viscosity of the fluid medium increases (13,27). We utilized a high-viscosity liquid—castor oil—to replace the PBS solution in the donor and the receiver compartments of the diffusion cell to completely suppress cavitation activity outside the skin, and compared the observed skin permeabilization in the absence of external cavitation with that observed when PBS was utilized as the coupling medium, where cavitation outside

the skin can be detected. This comparison enabled us to assess the importance of external cavitation (cavitation outside the skin) during LFS. In addition, we applied the acoustic method described in High-Pressure LFS Cell Studies to monitor cavitation activities in the LFS system during both the castor-oil and the PBS-mediated LFS experiments in an attempt to directly detect whether cavitation could occur inside the skin tissue during LFS.

In the castor oil studies, two sets of LFS (20 kHz, 1.6 W/cm^2, 0.1 s ON, 0.9 s OFF, application time of 2 h) experiments were conducted: Case 1 (control)—LFS with PBS as the coupling medium, and Case 2—LFS with castor oil (obtained from VWR Scientific) as the coupling medium. The ultrasound intensity was selected such that the following two criteria were satisfied: (i) when PBS is used as the coupling medium, significant cavitation activities are detected during LFS using the acoustic method (see Cavitation Detection Using an Acoustic Pressure Spectrum Analysis), and a significant LFS-induced skin permeabilization effect is observed (therefore, it can serve as a good base case for assessing the importance of external cavitation during LFS), and (ii) when castor oil is utilized as the coupling medium, external cavitation is completely suppressed, as indicated by both visual inspection and by the acoustic method (see High-Pressure LFS Cell Studies). Specifically, to confirm that, under the ultrasound intensities utilized, no cavitation activities take place in the coupling medium outside the skin, we conducted an acoustic measurement experiment using a piece of aluminum foil to replace the skin membrane in the diffusion cell during LFS (to ensure that no internal cavitation occurs), and measured the acoustic emissions from the LFS system to detect whether there are any cavitation signals emitted from the coupling medium (castor oil) outside the membrane (aluminum foil). Our results (data not shown) indicated that at ultrasound intensities greater than 1.6 W/cm^2, external cavitation begins to occur due to LFS with castor oil as the coupling medium. Therefore, a maximum ultrasound intensity of 1.6 W/cm^2 was utilized for the castor oil studies (see Results and Discussion) to assess the importance of external cavitation during LFS.

Because castor oil does not conduct, to evaluate the enhancement of the skin electrical conductance before and after skin exposure to ultrasound during the castor oil studies, at the end of each castor oil experiment (2 h), the skin was removed from the diffusion cell, and the application site was subjected to three washing-blotting dry cycles using a PBS solution and Kimwipe paper (VWR Scientific) to remove any castor oil remaining on the skin surface. Next, the skin sample was remounted in a clean diffusion cell with the donor and the receiver compartments both filled with a PBS solution to allow measurement of the electrical conductance of the skin (see Ultrasound Application). To determine whether there are any artifacts in the measurements of the skin electrical conductance values during the castor oil studies due to the washing-blotting dry cycles of the skin sample, we conducted a separate experiment in which we measured the electrical conductance of a skin sample before and after the addition-removal of castor oil from the skin surface according to the washing-blotting dry procedure described above. Our results showed that there is no detectable difference in the readings of the skin electrical conductance values before and after this procedure.

Cavitation Detection Using a Chemical Dosimeter (Terephthalic Acid)

The chemical assay for cavitation detection is based on the measurements of free radicals and chemical reaction products produced by cavitation. Since free radical production is required for such chemical effects, this assay specifically measures the activities of inertial (transient) cavitation in an aqueous solution exposed to ultrasound (10). Specifically, when transient cavitation occurs in an aqueous solution, the concentrated thermal and mechanical energy associated with the collapsing cavitation bubbles induces dissociation of the water molecules into hydroxyl and hydrogen radicals. These radicals are extremely reactive and short-lived [direct evidence of the existence of these radicals is provided by electron spin resonance (ESR) (10)], and form the basis for the chemical assay of cavitation detection (the amount of inertial cavitation in a solution).

In this study, we utilized terephthalic acid (TA) [benzene-1, 4-dicarboxylic acid] as a chemical dosimeter to quantify the total amount of transient cavitation occurring outside the skin under various ultrasound intensities. TA is a highly specific scavenger of •OH, which has been routinely utilized as a dosimeter of transient cavitation in an aqueous solution (28,29). TA does not fluoresce, but when reacted with •OH, it forms a highly fluorescent compound, hydroxyterephthalic acid (HTA), whose concentration can be determined by fluorescent spectrometry.

Commercial TA was obtained from Aldrich, and was purified by extraction and stored in a freeze drier according to the methods described in Refs. (28,29). A 1.5 mM TA solution (pH 7.4) was prepared by dissolving 249 mg TA and 200 ml NaOH solution (0.137 M) in 1L PBS (0.01 M). The donor compartment of the Franz diffusion cell was filled with the TA solution, and the receiver compartment was filled with PBS. A model membrane, aluminum foil (Reynolds), was mounted onto the diffusion cell. Sonication was performed for 20 s in a pulsed mode (0.1 s ON, 0.9 s OFF), and subsequently, the fluorescence of the liquid sample was measured using a fluorescence spectrophotometer (Model A1010, PTI, Lawrenceville, NJ, USA) with an excitation wavelength at 315 nm and an emission wavelength at 426 nm. For each ultrasound condition examined (intensity ranging from 1.6 to 33.5 W/cm^2), an average of 10 repeats was performed to minimize experimental error.

Cavitation Detection Using a Physical Dosimeter (Aluminum Foil)

Aluminum foil can be utilized as a physical dosimeter to characterize the amount of cavitation activity in a LFS system(20). Specifically, this dosimetry method measures the amount of transient cavitation activity in the vicinity of the membrane (a mechanism referred to as asymmetric inertial cavitation, see Possible Mechanisms for Transient Cavitation-Mediated Skin Permeabilization). Aluminum foil (Reynolds) was mounted onto the diffusion cell in the same manner as the skin membrane. The receiver and donor compartments were both filled with PBS. Sonication was performed for 20 to 60 s in a pulsed mode (0.1 s ON, 0.9 s OFF; ultrasouand ON-time is 2-6 s), and then the aluminum foil was removed from the diffusion cell. The number of indentations on a foil

was counted by visual inspection. Note that the ultrasound exposure time for these experiments was typically much shorter than that for the skin permeabilization measurement experiments (2 h), because a longer exposure to ultrasound results in a high number of pits, thus making the counting difficult. The number of pits on each aluminum foil was normalized by the ultrasound ON-time, and utilized to characterize the magnitude of asymmetric inertial cavitation outside the skin. For each ultrasound condition examined (intensity ranging from 1.6 to 33.5 W/cm^2), an average of 15 repeats were performed to minimize the experiment errors.

RESULTS AND DISCUSSION

High-Pressure LFS Cell Studies to Assess the Role of Cavitation during LFS

Ultrasound induces a wide spectrum of physical phenomena, which can potentially permeabilize the skin barrier, including thermal effects, cavitational effects, radiation forces, and macroscopic acoustic streaming. To test the role of cavitation during LFS, a high-pressure LFS cell was constructed (see High-Pressure LFS Cell Studies), and an acoustic signal measurement method was utilized to detect the onset of cavitation in the LFS system.

Figure 2(a) shows a typical acoustic spectrum measured in Case 1 (without pressurizing the pressure cell). One can see that under atmospheric pressure (Case 1), the ultrasound protocol utilized (20 kHz, 6.5 W/cm^2, 0.1 s ON, 0.9 s OFF) induces significant cavitation activity in the LFS system, as indicated by the presence of the subharmonic f/2 emission—the spike at the frequency of f/2. Figure 2(b) shows a typical acoustic spectrum measured in Case 2 (under elevated pressure, 19 atm) under the same ultrasound protocol. The absence of the f/2 signal in the measured acoustic spectrum in Case 2 indicates that no cavitation activity occurred in the LFS system during Case 2.

Figure 3 shows the corresponding enhancement ratios of the skin electrical conductance due to LFS measured in Cases 1 and 2, along with that of a control experiment (Case 3: skin passively hydrated in PBS for 2 h under atmospheric pressure). Whereas LFS induced about a 6.4-fold enhancement of the skin electrical conductance in Case 1 (in the presence of cavitation), the same ultrasound condition induced only about 10% increase in the skin electrical conductance under the elevated pressure condition (Case 2, in the absence of cavitation). Moreover, the enhancement of the skin electrical conductance in Case 2 (approximately 10% enhancement) is not statistically different ($P > 0.05$) from that in the control case (Case 3), where the skin membrane was passively hydrated in the diffusion cell for 2 h. The approximate 10% increase in the skin electrical conductance observed in Case 2 can be attributed to the effect of skin hydration and not to the effect of ultrasound. Therefore, the results shown in Fig. 2(a), Fig. 2(b), and Fig. 3 clearly indicate that ultrasound-induced cavitation is the key mechanism of skin permeabilization during LFS (the skin permeabilization effect disappears almost completely when the cavitation activity is suppressed).

Assessment of the Role of External Cavitation during LFS (Does Cavitation Occur inside the Skin during LFS?)

During LFS, cavitation may occur both inside and outside the skin. It is well known that the cavitation threshold

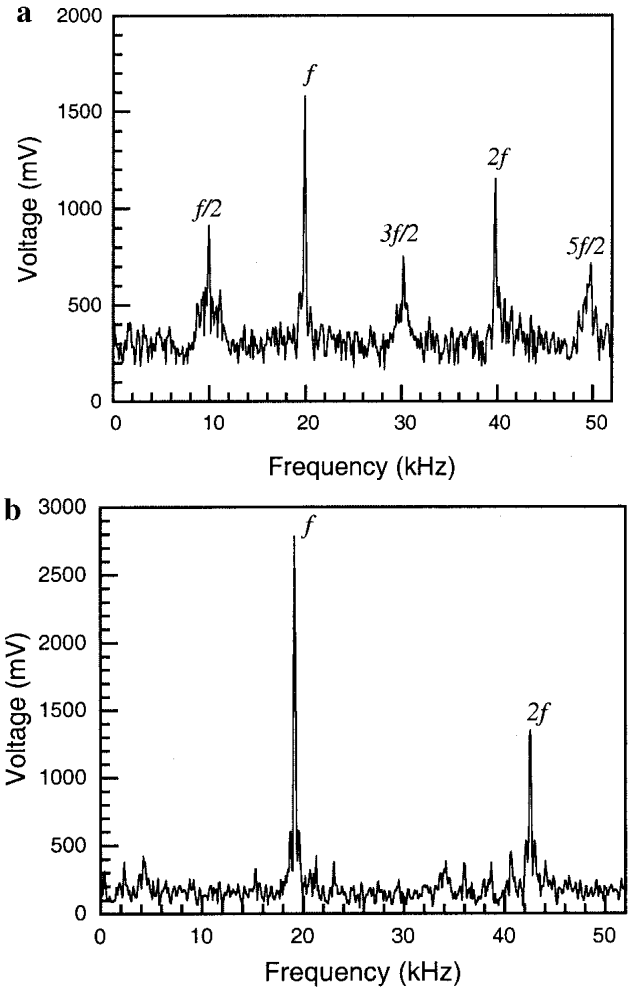

Fig. 2. A typical acoustic emission spectrum (signal amplitude in mV vs. frequency in kHz) measured: (a) in the presence of cavitation (under normal ambient pressure, 1 atm), and (b) in the absence of cavitation (under elevated ambient pressure, 19 atm).

increases as the viscosity of the fluid medium increases. By changing the viscosity of the medium in a particular region of the LFS system, one can selectively suppress the cavitation activity in that region. With this in mind, to evaluate the importance of internal and external cavitation during LFS, as well as to directly monitor whether cavitation occurs inside the skin membrane, we utilized a high-viscosity coupling medium (castor oil) to selectively suppress cavitation activity outside the skin during LFS. Castor oil is a high viscosity fluid (6.3–8.9 poise) possessing a similar acoustic impedance (1.431 $\times$ 10^6 Pa·s/m) to that of water (1.483 $\times$ 10^6 Pa·s/m).[5] Therefore, replacing the water-based PBS solution with castor oil as the ultrasound coupling medium during LFS does not affect the transmission efficiency of ultrasound energy to the skin. In other words, castor oil selectively suppresses cavitation activity outside the skin, while internal cavitation (if it were to occur) should not be affected during the castor-oil-mediated LFS experiments.

[5] The acoustic impedance data were obtained from URL: http://www.ultrasonic.com

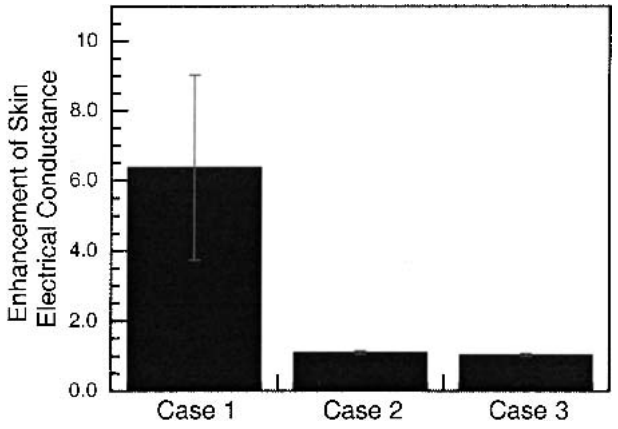

Fig. 3. Enhancement ratio of skin electrical conductance during the LFS or the passive control experiments measured during the high-pressure LFS cell studies. Key: (Case 1) LFS under 1 atm; (Case 2) LFS under 19 atm; (Case 3) skin passive hydration for 2 h under 1 atm. The error bars represent 95% confidence interval (4–8 repeats). 20 kHz ultrasound at 6.5 W/cm^2 was applied to the skin in a pulsed mode (0.1 sec ON, 0.9 sec OFF) for 2 h during LFS.

Figure 4 shows the enhancement of the skin electrical conductance due to LFS measured during the PBS-mediated LFS experiment (Case 1: in the presence of external cavitation, as confirmed by the acoustic method), and during the castor-oil-mediated LFS experiment (Case 2, in the absence of external cavitation, as confirmed by the acoustic method[6]), as well as during a passive control experiment (Case 3, skin immersion in PBS passively for 2 h). Fig. 4 reveals that whereas LFS induces a significant skin permeabilization effect in Case 1 (2.5-fold), when external cavitation is completely suppressed (Case 2), no significant skin permeabilization due to ultrasound is observed (Cases 2 and 3 are not statistically different). Therefore, one may conclude that external cavitation plays a central role during LFS.

[6] Confirmation of the onset and the disappearance of external cavitation activities in the LFS system under the ultrasound conditions utilized (Case 1 and Case 2) was done with a diffusion cell setup with an aluminum foil mounted in the same manner as that of the skin membrane using the acoustic method.

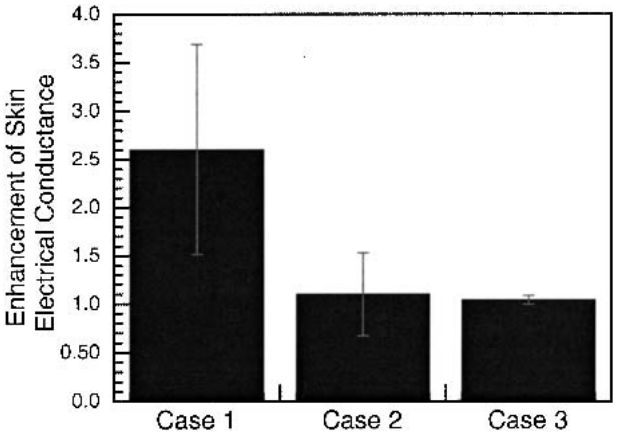

Fig. 4. Enhancement ratio of skin electrical conductance during the LFS or the passive control experiments measured during the castor-oil studies. Key: (Case 1) LFS with PBS as the coupling medium; (Case 2) LFS with castor oil as the coupling medium; (Case 3) skin passive hydration in PBS for 2 h. The error bars represent the 95% confidence interval (4–8 repeats). 20 kHz ultrasound at 1.6 W/cm^2 was applied to the skin in a pulsed mode (0.1 sec ON, 0.9 sec OFF) for 2 h during LFS.

In an attempt to monitor whether cavitation occurs inside the skin (internal cavitation) during LFS, we also measured the acoustic emission from the LFS system during the castor-oil-mediated LFS experiments. Examination of the acoustic emissions from the LFS system in Case 2 revealed that upon suppression of external cavitation, there is no detectable cavitation signal (f/2 emission) in the measured acoustic spectrum of the LFS system (spectra not shown). This result indicates that there is no bubble activity inside the skin membrane (including both the SC and the skin appendages) under the LFS protocols examined.

In the previous studies of sonophoresis, there have been many speculations about the possible occurrence of internal cavitation inside the skin membrane [including the SC lipid region(14) and the corneocytes, (3,4) as well as the skin appendages(2, 30)]. However, to date, no direct experimental measurements have been conducted to support the hypothesized presence of cavitation inside the skin. The experimental technique utilized in this study permit direct detection of cavitation in the skin membrane. Our experimental finding (absence of cavitation bubbles inside the skin during 20kHz-LFS) is consistent with a theoretical calculation. Specifically, under 20 kHz-LFS, the theoretical resonant bubble radius is approximately 150 μm in an aqueous phase, which is much larger than the typical dimension of the skin barrier (thickness of the SC is approximately 10 μm) and than the dimension of the skin appendages [the lumen of a sweat duct has a nominal diameter of about 5 μm (31)].

In addition, based on a theoretical calculation of the resonance size of cavitation bubbles, Simonin (30) suggested that under an ultrasound frequency of 1MHz, cavitation (radius approximately 3 μm) may occur inside the skin sweat ducts, because of the matching sizes of the cavitation bubbles with the skin sweat ducts. Since the resonant bubble size is inversely correlated with the ultrasound frequency, the likelihood of the occurrence of cavitation inside the skin under ultrasound frequencies higher than that considered in this study (20 kHz) awaits future investigation, with the experimental approach utilized in this study providing a valuable method for detecting cavitation activity inside the skin.

Identification of the Critical Types of Cavitation during LFS Using an Acoustic Spectrum Analysis Method

Based on the earlier results, we showed that ultrasound-induced cavitation outside the skin (in the solution on top of the skin) plays the key role during LFS. Ultrasound-induced cavitation fields in a solution are complex. In many cases, when inertial (transient) cavitation is produced in a liquid, non-inertial (stable) cavitation occurs simultaneously (10). The two types of cavitation activities (transient vs. stable) interact with biologic tissues (such as the skin) through different mechanisms (10). Specifically, phenomena associated with inertial (transient) cavitation that can potentially affect the skin permeation properties include direct bubble collapse stresses (high-speed liquid jets and shock waves), and the production of free radicals and other chemically active species. On the other hand, phenomena associated with non-

inertial (stable) cavitation that can potentially lead to skin permeabilization include microstreaming and its related shear stress, and radiation forces exerted by the oscillating bubbles on the neighboring structure. Understanding the relative roles played by the two types of cavitation during LFS not only is of fundamental value, but can also provide guidelines for improving the efficacy of LFS, as well as for achieving better control of LFS. We measured the acoustic signals associated with the two types of cavitation activities (the f/2 signal and the broadband noise) under various LFS conditions (in particular, at different ultrasound intensities) using the acoustic method described in earlier, and correlated the measured acoustic signals with the observed skin permeabilization due to LFS.

We conducted a series of LFS experiments under various ultrasound intensities (1.6, 3.2, 6.5, 10.0, and 14.4 W/cm^2, 3–5 repeats were performed for each ultrasound intensity). During these LFS experiments, skin permeabilization due to LFS, as reflected in the enhancement of the skin electrical conductance, was measured along with the acoustic emissions of the LFS system (which originate solely from the solution outside the skin, since internal cavitation does not occur). We then plotted the measured enhancement of the skin electrical conductance as a function of the measured amplitudes of the broadband signal and of the subharmonic signal in Fig. 5(a) and Fig. 5(b), respectively. Specifically, 21 experiment points are shown in each graph, with each one corresponding to a single LFS experiment. Fig. 5(a) shows that a good correlation exists between the measured skin permeabilization induced by LFS and the measured broadband noise signal ($R^2 = 0.72$). On the other hand, the f/2 signal measured does not show a good correlation with the effect of LFS, as revealed in Fig. 5(b) ($R^2 = 0.39$).

Therefore, based on the comparison of the correlations of the two acoustic signals with the skin permeabilization effect, one may conclude that in the LFS system examined, transient cavitation (occurring outside the skin membrane) plays a more important role in inducing the effects of LFS (skin permeabilization) than stable cavitation. This mechanistic finding can be utilized to aid in the design of clinical LFS systems to achieve better control of the efficacy of LFS. Specifically, measurements of the broadband noise signal can be utilized to provide real-time feedback about the performance of the ultrasound device (or to predict the degree of skin permeabilization during LFS). The ultrasound treatment time can then be optimized to achieve the best drug delivery results.

Similar techniques have been utilized previously in the literature to study the relationship between cavitation activities and the biologic effects of ultrasound. Liu *et al.* (26) studied the cell membrane permeabilization by ultrasound in a bovine red blood cell model system, and found that ultrasound-induced membrane permeabilization in the examined cell suspension system was mediated by both inertial (transient) and non-inertial (stable) cavitation.

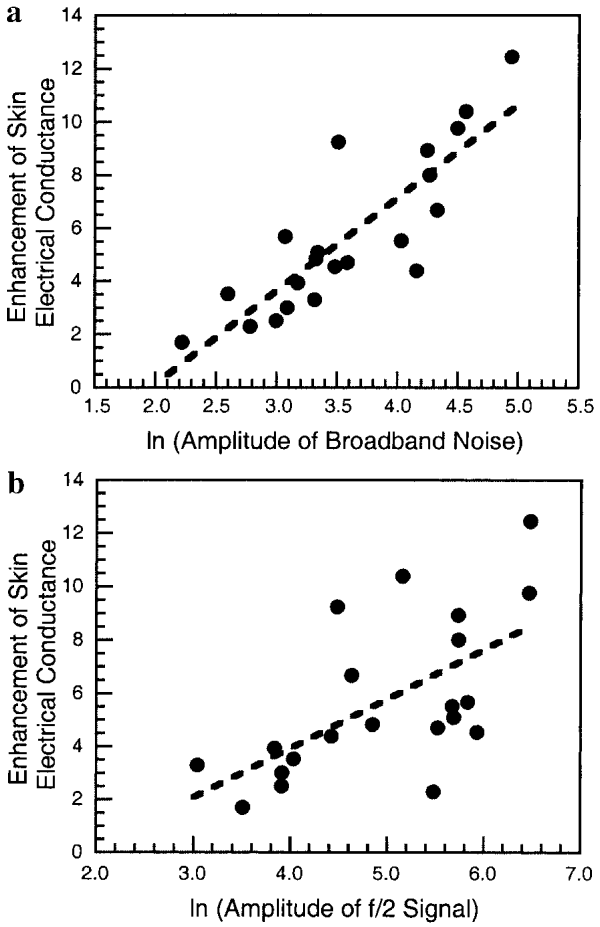

Fig. 5. Relationship between the skin electrical conductance enhancements and the magnitudes of the acoustic signals measured during LFS experiments under different ultrasound intensities (1.6, 3.2, 6.5, 10.0, and 14.4 W/cm^2): (a) broadband noise signal, and (b) subharmonic (f/2) signal. Key: (●) represents a single LFS experiment (N = 21). There is no mechanistic basis for the log-linear functionality (visual inspection indicated that it was the most appropriate fit of the data).

Possible Mechanisms for Transient Cavitation-Mediated Skin Permeabilization

So far, we have shown that transient cavitation outside the skin plays the dominant role in inducing skin permeabilization during LFS. Transient cavitation may occur at different locations in the solution outside the skin, which can then enhance skin permeability via different mechanisms. Specifically, transient cavitation may occur both near the skin surface and in the bulk solution. Collapse of cavitation bubbles in the bulk solution results in the formation of a spherically expanding shock wave (13,32). This shock wave may directly modify the structure of the skin barrier, or act on the nearby cavitation bubbles in the solution, causing them to collapse (33), and hence, to generate mechanical stresses on the skin membrane. In addition, free radicals generated by shock waves in the solution can also potentially interact with the SC. On the other hand, if a collapsing bubble is near the skin surface, in addition to the emission of a shock wave, nonuniformities in the surrounding pressure cause the bubble to collapse asymmetrically, which may lead to the formation of a high-velocity liquid jet directed toward the skin membrane surface. Theoretical calculations have predicted that the microjets formed by bubbles collapsing near a solid wall can reach a velocity of between 50 and 180 m/s (34–37). Accordingly, when these high-velocity liquid jets impinge on the skin surface, the mechanical impact may modify the skin structure,

and thus lead to an increase in skin electrical conductance (that is, to skin permeabilization).

Because cavitation bubble collapses at the two locations in the solution (close to the skin surface and in the bulk solution) occur in fundamentally different patterns (asymmetric vs. symmetric collapses, respectively) (35,38), and both are capable of modifying the skin structure, below, we investigate the relative importance of the two locations of transient cavitation in the LFS system (surface vs. bulk, or asymmetric vs. symmetric transient cavitation), to obtain guidelines for improving the efficacy of the LFS technique (for example, to optimize the cavitation fields in the solution). Specifically, we conducted the following measurement experiments. Two dosimetry techniques of cavitation detection were utilized to characterize the transient cavitation activities in the solution outside the skin. First, a chemical dosimeter [see Cavitation Detection Using a Chemical Dosimeter (Teraphthalic Acid)] was adopted to measure the total amount of transient cavitation occurring outside the skin (specifically, it measures the sum of the bulk and the surface transient cavitation), and second, a physical dosimeter [see Cavitation Detection using a Physical Dosimeter (Aluminum Foil)] was adopted to measure the amount of asymmetric transient cavitation (surface transient cavitation). We measured transient cavitation activities in the two locations of the coupling solution under various ultrasound intensities, and then compared the dependence of the two locations of transient cavitation activities on the ultrasound intensity with that of the observed skin permeabilization effect during LFS on the same ultrasound parameter to elucidate the relative importance of the two locations of transient cavitation in the LFS system (asymmetric vs. symmetric transient cavitation). The results obtained are shown in Fig. 6(a–c).

Figure 6(a) and (b) reveal that the two locations of transient cavitation activities (entire solution volume vs. surface region) measured by the two-dosimetry methods exhibit different dependences on the ultrasound intensity. Whereas the sum of asymmetric and symmetric transient cavitation measured by the chemical assay increases monotonically with increasing ultrasound intensity [Fig. 6(a)], the amount of asymmetric transient cavitation [Fig. 6(b)] measured by the aluminum foil method exhibits a peak value at an ultrasound intensity of about 21.9 W/cm^2. This suggests that asymmetric and symmetric transient cavitation activities have different dependences on the ultrasound intensity in the LFS system examined. Specifically, beyond an ultrasound intensity of about 21.9 W/cm^2, the amount of asymmetric transient cavitation begins to decrease, while the amount of symmetric transient cavitation in the solution continues to increase as the applied ultrasound intensity increases (a discussion of the observed decrease in the activity of asymmetric transient cavitation beyond a certain ultrasound intensity value will be presented below).

Skin permeabilization due to LFS, as reflected in the measured enhancement of the skin electrical conductance, is plotted as a function of ultrasound intensity in Fig. 6(c). Figure 6(c) reveals that the dependence of LFS-induced skin permeabilization on the ultrasound intensity follows a similar trend to that observed for the asymmetric transient cavitation activity shown in Fig. 6(b). Skin permeabilization also exhibits a peak at an ultrasound intensity of about 21.9 W/cm^2, beyond which this biologic effect of ultrasound starts to decrease ($p <$ 0.05). Taken together, the results shown in Fig. 6(a) to (c) indicate that surface transient cavitation (asymmetric bubble collapse) plays the dominant role during LFS, while bulk transient cavitation (symmetric bubble collapse) does not ap-

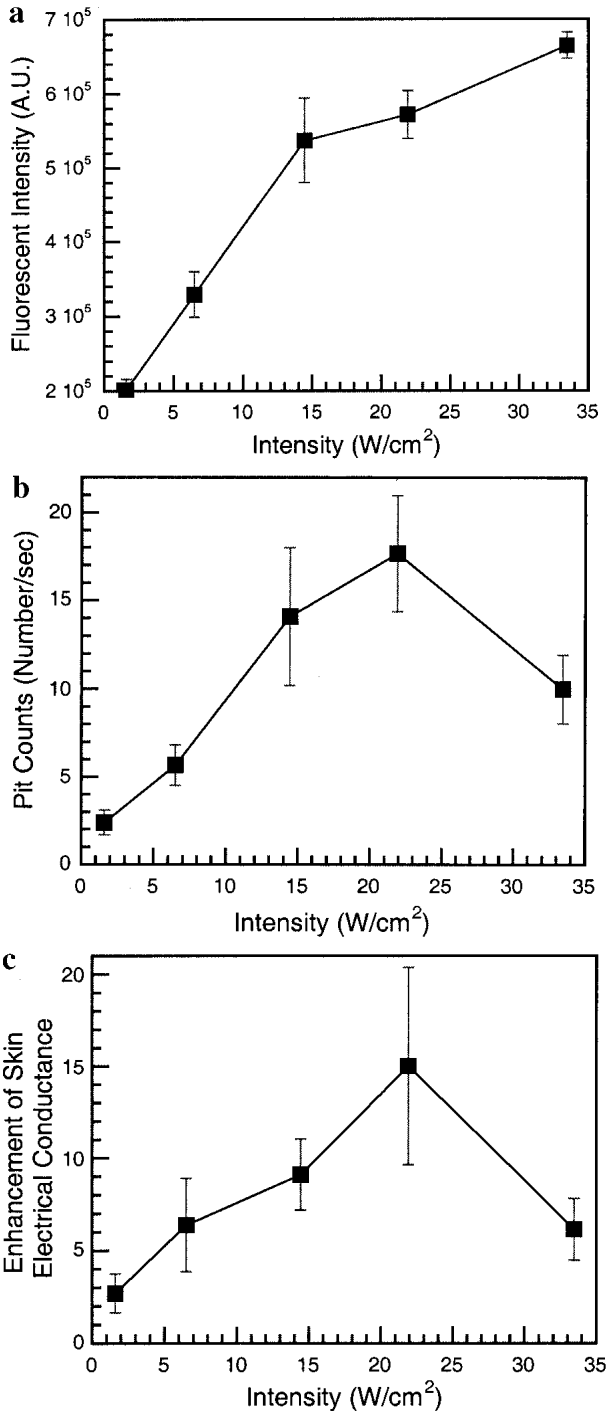

Fig. 6. Dependence of the transient cavitation activities in the solution and of the skin permeabilization on ultrasound intensity: (a) total transient cavitation activity in the solution (both symmetric and asymmetric transient cavitations) measured by the chemical dosimeter (N = 10), (b) asymmetric transient cavitation activity measured by the physical dosimeter (aluminum foil method) (N = 15), and (c) skin permeabilization measured by the enhancement of the skin electrical conductance (N = 8). Each point represents the mean ± 95% confidence interval of N experimental repeats.

pear to contribute significantly to the observed effect of ultrasound during LFS (because beyond an ultrasound intensity of about 21.9 W/cm^2, although the amount of symmetric transient cavitation increases with increasing ultrasound intensity, the effect of LFS decreases). Note that according to the notion of shock wave generation, the activity of symmetric transient cavitation could potentially give rise to more asymmetric transient cavitation activity through bubble-bubble interactions (bubble shattering) (33). Results of our experiment indicate that this effect of symmetric transient cavitation is secondary in the LFS system examined.

The relationship between asymmetric and symmetric transient cavitations (bulk vs. surface) is complex. There are two competing mechanisms of interaction between the two types of cavitation phenomena: (i) shock waves generated by the bulk transient cavitation may cause the nearby cavitation bubbles to collapse (33), and through that, symmetric transient cavitation may trigger more asymmetric transient cavitation, and (ii) because bubbles are good absorbers of ultrasound, if large amounts of bubbles form in the bulk solution in front of the ultrasound transducer, the ultrasound becomes highly attenuated as it passes through this bulk region. As a result, the sonication of the liquid in the vicinity of the skin may be reduced, resulting in less surface transient cavitation activity (less asymmetric transient cavitation) (13). This second mechanism may explain the observed peak in the surface transient cavitation activity, shown in Fig. 6(b). As the amount of bulk transient cavitation increases with applied ultrasound intensity, the surface transient cavitation activity first reaches a peak value and then it starts to fall off at an ultrasound intensity of about 21.9 W/cm^2, because mechanism (ii) begins to dominate.

Because we have shown that the skin permeabilization effect during LFS is primarily mediated by asymmetric transient cavitation, one can speculate that to improve the efficacy of LFS, localization of the transient cavitation activity in the vicinity of the skin membrane is desirable. To diminish the likelihood of bubble formation far from the skin membrane (that is, close to the ultrasound transducer), the pressure amplitude near the transducer face should be kept to a minimum. However, high-pressure amplitude must still be produced in the solution close to the skin to generate cavitation at that site. Both of these requirements may be met if the ultrasound field can be focused (13).

CONCLUSIONS

A high-pressure LFS cell study provided definitive evidence that cavitation is the key mechanism of skin permeabilization during LFS. Utilizing a direct cavitation detection method (the acoustic signal measurement method), we demonstrated that under an ultrasound frequency of 20 kHz, cavitation occurs solely outside the skin. By correlating the skin permeabilization effects with the measured amplitudes of acoustic signals (f/2 signal and broadband noise signal), which are believed to be associated with stable and transient cavitation activities, respectively, we showed that transient cavitation outside the skin is the key mechanism responsible for LFS-induced skin permeabilization.

Utilizing a physical dosimeter (aluminum foil) and a chemical dosimeter, we also characterized the transient cavitation activities outside the skin membrane. The importance of symmetric and asymmetric transient cavitations for inducing the biologic effect of ultrasound (skin permeabilization) was evaluated. Our findings indicate that asymmetric transient cavitation is responsible for the observed skin permeabilization during LFS, while symmetric transient cavitation does not appear to play an important role (the dependence of the observed skin permeabilization on ultrasound intensity deviates from that of the symmetric transient cavitation activity). The results indicate that LFS-induced skin permeabilization results mainly from the direct impact of bubbles collapsing on the skin surface, and future investigations on the optimization of LFS conditions should be aimed at localizing the transient cavitation activity in the vicinity of the skin membrane.

ACKNOWLEDGMENTS

This research was supported by NIH grant GM 44884, and by a grant from the Centers for Disease Control and Prevention. We thank Professor Ron Roy for providing us with the hydrophone for cavitation detection, as well as for very helpful and illuminating discussions on the high-pressure cell LFS studies.

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