The Effects of Low-frequency Ultrasound (35 kHz) on Methicillin-resistant Staphylococcus aureus (MRSA) in vitro

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Abstract
Scanning electron microscopy study results support an in vitro bactericidal effect of low-frequency ultrasound (LFU) delivered at 40 kHz on bacteria, including methicillin-resistant Staphylococcus aureus (MRSA). The purpose of this in vitro study was to determine the effects of LFU delivered at 35 kHz on bacterial viability, cell wall structure, and colony characteristics, including antibiotic resistance on vegetative forms of MRSA. A known MRSA isolate from a patient lower extremity wound was subcultured, plated, and grown on sheep blood agar (SBA). Serial dilutions of the organisms were made and treated with LFU for 30, 60, and 180 seconds. One hundred microliters (µL) of control (untreated organisms) and treated samples were inoculated to SBA in triplicate and three separate experiments were conducted. Using standard microbiological techniques, a reduction in MRSA from 1 million colony forming units (CFU) at baseline to 6 CFU after 30 seconds of treatment with 35 kHz was observed. MRSA plated at 10² CFU and treated with 35 kHz showed a 44.1% viability with flow cytometry, compared to 92.5% viability of untreated control MRSA. Changes in pigmentation, odor, colony size, and hemolysis pattern also were observed in the LFU-treated bacteria. The effect of LFU on methicillin resistance was dose-dependent; the zone of inhibition increased 14.3 mL after 30, 16.7 after 60, and 20.3 after 180 seconds of treatment. The results suggest that, in this in vitro model, 35 kHz LFU reduces CFU of bacteria, punctures and fractures cell walls, and alters colonial characteristics of MRSA, including resistance to the oral form of methicillin. Studies to elucidate the observed effects of LFU on MRSA and evaluate its effect in vivo are warranted.

Key Words: low-frequency ultrasound, antibiotic resistance, 35 kHz, wound therapy

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Antibiotic-resistant micro-organisms are commonly found in open wounds; methicillin-resistant Staphylococcus aureus (MRSA) is of particular concern due to the high morbidity and mortality associated with this infection. Multiple studies, including population surveillance studies, have shown that MRSA infections in hospitalized patients are associated with greater lengths of stay, higher mortality, and increased costs. These statistics have recently been confirmed for patients with lower extremity wounds. Edris and Reed found that MRSA infections of lower extremity wounds are associated with increased lengths of stay and a higher incidence of adverse post-surgical outcomes in hospitalized patients. Unfortunately, the overall incidence of invasive MRSA in the US and internationally has increased steadily since the first MRSA case was diagnosed in 1961. In the past decade, the numbers of cases of MRSA infections in open wounds has continued to increase in different healthcare settings. In 1994, Terpenning et al found high colonization rates (18% of patients with chronic wounds) of skin and soft tissue by MRSA in patients in a long-term care facility affiliated with an acute care Veterans Affairs Medical Center. More recently, Basu et al demonstrated 18% of pathogens isolated from chronic wounds in outpatients were drug-resistant; MRSA was a primary offender.

Likewise, a retrospective study of common burn wound pathogens and their corresponding antibiotic susceptibility patterns occurring over 20 years showed pronounced increases in MRSA isolates. MRSA isolates increased 3% during the initial 11 years (1986 to 1997), followed by a more rapid increase of 16% in the next 3 years. Results from this study also showed an increase in isolates of methicillin-resistant, coagulase-negative strains of Staphylococci, a greater increase than for MRSA.

Results of the first nationwide surveillance study published in 2007 by the Centers for Disease Control and Prevention (CDC) indicated the incidence of invasive MRSA infections in 2005 was 31.8 per 100,000 people. Individuals older than 65 years, men, and blacks exhibited the highest rate of invasive MRSA infection. Further, estimates derived from this study project that more than 90,000 invasive MRSA infections occur per year with an estimated total number of 18,650 deaths. This MRSA infection estimate is three times the rate projected previously and higher than the death rate associated with AIDS (14.561) in 2007. Because of this increased prevalence of MRSA infections as well as other antibiotic-resistant wound pathogens, novel treatment methods have been pursued for local management of open wound infections. These methods include antiseptic preparations and biophysical agents that may prove beneficial because they work through physical mechanisms that microbes may not be able to evade with genetic alterations — ie, MRSA and other antibiotic-resistant microbes may not be able to easily generate resistance to these treatments. Examples of these treatments include using cadexomer iodine, silver dressings, plant-derived antiseptics, and Manuka honey and biophysical agents such as ultraviolet light C (UVC) and low-frequency ultrasound (LFU). Although LFU medical devices using frequencies ranging from 20 to 40 kHz are marketed for wound cleansing and debridement, most research has been conducted using 40 kHz. The purpose of this in vitro study was to determine the effects of a low-frequency ultrasound (35 kHz) on bacterial viability, cell wall structure, and colony characteristics, including antibiotic resistance of vegetative forms of MRSA.

Literature Review
UVC irradiation has been used to treat wounds containing high levels of MRSA. An in vitro study demonstrated 99.9% effective against MRSA in as little as 5 seconds and that once-daily exposure for 1 week completely clears MRSA from acute surgical wounds of rats. In a small case series, Thai et al demonstrated a reduction of MRSA in patients with chronic wounds using an exposure of 180 seconds; longer exposure times were required to decrease MRSA numbers compared to other bacterial species found in the wound bed. Recently, attention has turned to LFU to manage chronic wound infections, including those caused by MRSA. Acoustic energy delivered at frequencies above 20 kHz is thought to be detrimental to bacterial cell growth. An in vitro study examining the effects of LFU delivered at 40 kHz using a fine-particle saline mist demonstrated damage to membranes of MRSA and other types of bacteria. Another in vitro study demonstrated that a 5-minute, 40-kHz LFU treatment produced 33%, 40%, and 27% reduction in live Escherichia coli and E. faecalis bacteria, respectively, as compared to no effect with a sham treatment. Results from this same study indicated that 40-kHz LFU produced little or no effect on MRSA or methicillin-susceptible S. aureus. This study also examined the effects on wound bioburden (colony counts) of 40-kHz LFU in vivo in a porcine wound model comprising four experimental groups — 40-kHz LFU, a silver dressing, sham ultrasound, and moisture control. Results indicated using 40-kHz LFU was marginally better than using the sham or moisture control and similar in effect to the silver dressing group to control porcine wound bioburden (due to a small sample size, statistical analysis was not performed).

The same article also described the results of a small, prospective clinical study examining the effects of 40-kHz LFU on bacterial numbers in patients (n = 18, 11 were considered evaluable) with Stage III pressure ulcers and a wound volume of no more than 180 cm³. Patients received a total of six 4-minute, 40-kHz LFU treatments. After 2 weeks, bacterial colony counts in these wounds were reduced from 4 x 10⁶ CFU to 2 x 10⁵ CFU, a 50% reduction. However, a sham treatment group was not included in this study so whether the reduction in bacterial numbers was due to the mechanical effects of the saline mist or other factors cannot be determined. Additionally, no statistical analysis was performed due to the small sample size.
In recent years, other LFU medical devices that use frequencies between 20 and 35 kHz have been introduced and marketed for wound cleansing and debridement. These devices are believed to decrease wound bioburden, including both planktonic and biofilm bacteria. In vitro testing by Ensing et al. and others demonstrated that LFU administered concurrent with antibiotics enhances the effectiveness of the latter against planktonic forms of bacteria as well as bacteria housed in biofilms harvested from patients with prosthetic-related infections. Using the disk diffusion assay, Ensing et al. detected an increased antibiotic effectiveness in the presence of LFU administered at a time-average acoustic intensity of 167 mW/cm² and a frequency of 46.5 kHz. However, the 46.5-kHz LFU had no direct effect on planktonic or biofilm bacterial viability. This finding is in agreement with Qian et al. who, in vitro study using biofilms grown on polyethylene substrate also found that LFU enhanced the effectiveness of antibiotics against planktonic biofilm but did not produce any detrimental effects on bacterial viability alone. Qian et al. also noted that lower frequencies of ultrasound in the kilohertz range were more effective on biofilm viability than megahertz (MHz) frequencies in enhancing the effects of tested antibiotics.

Methods

Bacteria. MRSA cultures were obtained from a verified, methicillin-resistant clinical stock of *S. aureus* housed in the stock culture collection of the Clinical Laboratory Science Department of the School of Health Sciences, Winston-Salem State University (Winston-Salem, NC). The organism used in this study was originally isolated from a lower extremity wound and demonstrated a pattern of resistance to oral methicillin (oxacillin) on the antibiogram. Stock cultures were stored in a -20°C freezer before use in this study.

Media. MRSA cultures were thawed and then plated and grown on 5% sheep blood agar (SBA) medium overnight at 37°C before treatment stocks were prepared (106 colony forming units [CFU]/mL) in 0.9% isotonic saline for LFU treatment. Treated cultures then were replated to SBA and incubated as described. Normal saline in the same volume was added to untreated control stocks, which remained on ice for the duration of the experiment (treatment time of 30, 60, or 180 seconds) until all cultures were placed in the incubator. Ice was used to prevent bacterial proliferation.

**Antibiotic discs.** Antibiotic discs impregnated with 1 µg of oxacillin (BD Diagnostic Systems, Franklin Lakes, NJ) were inoculated aseptically to the culture at an inoculum of 37°C for 24 hours. The zone of inhibition (ZOI) then was measured according to the manufacturer’s standards (see Figure 1). The ZOI is the area on an agar plate where a micro-organism will not grow when it is susceptible to an antibiotic (contained in an impregnated disk) that has been placed on the plate. If this area is greater than the standard zone established for the antibiotic being tested, the organism is considered to be sensitive to the antibiotic.

**Equipment.** The low-frequency ultrasound generator (Arobella Medical LLC, Quoistic Wound Therapy System™, Minnetonka, MN) operates at a frequency of 35 kHz with an adjustable treatment intensity of up to approximately 2.0 W/cm². The treatment delivered in this experiment utilized 100% power output at an intensity of 2.0 W/cm². Intensity was calibrated using a proprietary laser interferometer that characterizes the displacement, frequency, and frequency waveform of the Quoistic Qurette™ applicator tip. In addition, acoustic output pressure, intensity, and power parameters were measured in an anechoic chamber and/or hydrophone tank to confirm desired output characterization (ie, intensity and power versus distance and acoustic field shape) was achieved. Saline jet irrigation was delivered through an orifice inside a metal curette capable of producing a distal displacement from 0 to 75 µm depending on the percent power setting. The low-frequency ultrasound energy was produced through the transduction of 60-cycle wall current into mechanical energy via a titanium alloy transducer using piezoelectric elements made of lead zirconate titanate (PZT), which produces energy in the kilohertz range and a frequency of 46.5 kHz. However, the 46.5-kHz LFU had no direct effect on planktonic or biofilm bacterial viability. This finding is in agreement with Qian et al. whose in vitro study using biofilms grown on polyethylene substrate also found that LFU enhanced the effectiveness of antibiotics against planktonic biofilm but did not produce any detrimental effects on bacterial viability alone. Qian et al. also noted that lower frequencies of ultrasound in the kilohertz range were more effective on biofilm viability than megahertz (MHz) frequencies in enhancing the effects of tested antibiotics.

**Observational culture changes.** All control (untreated) and LFU-treated cultures were visually inspected by a clinical specialist in microbiology. Colonial appearance including size, hemolytic pattern, and odor were recorded per clinical microbiology lab practice.

**Flow cytometry study.** Two samples each of 10² dilutions prepared from control (untreated MRSA) and 35-kHz, LFU-treated MRSA (treated for 60 seconds) were submitted to the flow cytometry facility for viability studies. These samples were initially stored at -4°C after treatment until transported on ice to the flow cytometry facility (-10 minutes). Cell suspensions were treated with a viability stain (fluorescein diacetate) at 1µg/mL and allowed to incubate for 30 minutes on ice. Viable cells were able to concentrate the fluorescence via the nonspecific esterase activity of the cell and to concentrate this fluorescence in the cell. Nonviable cells were not able to concentrate the fluorescence due to leakage of the cell wall. Using a BD FACs Canto instrument to detect fluorescence, 10,000 events were analyzed for green fluorescence per cells after gating on the cells using forward and side scatter. A known cancer cell, BT-474, was similarly treated and analyzed as a positive control to define viable and nonviable cut points. The distribution of fluorescent cells then was analyzed using FCS Express program and the mean and percent of viable and nonviable cells were determined.

**Oxacillin-resistance studies.** Three separate experiments with three replications of each experiment were conducted to determine if changes in the resistance pattern by the MRSA culture occurred. MRSA was plated at 10⁶ CFU/mL on SBA and allowed to air dry. An oxacillin (oral form of methicillin) disc was placed on the SBA plates previously inoculated with MRSA. Cultures then were incubated overnight at 37°C. After 24 hours, the ZOI then was calculated and the procedure was repeated for a second 24-hour period. Replated cultures did not receive additional 35-kHz treatments.
Data analysis. Descriptive statistics were calculated for the bacterial reduction and the ZOI study. A one-way analysis of variance (ANOVA) was utilized to assess differences between the experimental groups (control and three 35-kHz, LFU-treated groups; 30-, 60-, and 180-second exposure groups). A post-hoc comparison was performed using the Dunnett’s test, which is used specifically to compare doses to controls (in this case, exposure to LFU by length of treatment [time]).

Results

Bacterial reduction study. MRSA numbers dropped from 106/mL pre-treatment to 6 CFUs after 30 seconds of treatment, a >99.99% reduction (P <.001). A similar reduction was obtained for 60 seconds with bacterial numbers decreasing to 4 CFUs from 106/mL. After 180 seconds, no MRSA were detected on the SBA at 24 hours post 35-kHz LFU treatment. Results at each time point were similar whether treatment was conducted in or out of an ice bath.

Observational analysis revealed MRSA treated with ultrasound at all time points showed decreased colonial pigmentation and odor compared to control. LFU-treated MRSA also did not demonstrate hemolysis of the SBA as seen with controls (see Table 1).

Scanning electron microscopy. Scanning electron microscopy indicated that LFU delivered at 35 kHz almost completely eradicated the MRSA in the test samples. In only one individual were intact cocci located on any of the electron micrographs (see Figure 4a). In comparison, many individual cocci are intact in the control, untreated group shown on the micrographs in Figure 4b. Residue of MRSA destroyed during treatment was visible (see Figure 4a).

Flow cytometry study. LFU-treated MRSA emitted 61% less fluorescence than untreated MRSA, indicating a lower overall viability of the bacteria. The mean intensity of the LFU-treated MRSA decreased from 181 to 71 units (see Table 2) and MRSA viability in the treated group decreased 55.9%. Cells decreased from 1 million viable cells with a 7.5% decrease in control cell viability, compared to a 55.9% decrease for LFU-treated cells. Viability in the untreated control group was 92.5% compared to 44.1% for LFU-treated MRSA.

Bacterial resistance study. MRSA samples in the treatment group demonstrated decreased resistance to oxacillin (see Figure 5). ZOI all were >13 mL, the critical value for determining antibiotic resistance. No ZOI were apparent for the untreated controls. MRSA grew to the edge of the oxacillin table in the untreated control plates at 180 seconds (data not shown). The ZOI for the 30- and 60-second treatment groups were 14.3 mL and 16.7 mL, respectively, a 14% increase over the 30-second treatment. The 180-second treatment group exhibited the largest ZOI at 20.3 mL, increases of 30% and 18% respectively (P <.001) over the 30- and 60-second treatment groups. The dose-dependent decrease in bacterial colonies was visible and could be detected across the LFU-treated plates, even with a starting concentration of 10^6 CFUs/mL. After re-culturing susceptible MRSA previously exposed to the oxacillin test disk (24 hours earlier) and growing these cultures overnight for another 24 hours without any additional treatment, the same pattern of decreased resistance was observed (48 hours post initial exposure to the antibiotic).

However, the LFU treatment effect last only 72 hours post initial LFU treatment with no complete ZOI present. To confirm sensitivity to the oxacillin by 35-kHz LFU treated MRSA, an additional set of experiments was conducted using the same ZOI testing procedure with the substitution of the antibiotic cefoxitin. A similar pattern of sensitivity to this antibiotic disk also was detected post 35-kHz LFU treatment.

Discussion

Similar to findings of a scanning electron microscopy study by Kavros et al.25 reported in 2007 with the original 40-kHz LFU generator, MRSA exposed to the 35-kHz LFU exhibited cellular destruction with fracturing of MRSA cell walls, suggesting a common mechanism for bacterial destruction by LFU at both frequencies. In the present study, MRSA (10^6 cells/mL) was decimated by 60 seconds of exposure to 35-kHz LFU, which appears to physically rupture bacterial cells by shattering the cell walls because only one intact cocci could be located in the cellular debris. However, Kavros et al.9 did not provide data on actual in vitro reduction of bacterial numbers with the 40-kHz LFU. In the present study, 35-kHz LFU reduced MRSA numbers from 106/mL to 6 CFUs after 30 seconds of treatment. In contrast, recent findings reported by Serena et al.17 indicated that 40-kHz LFU delivered by a fine particle mist for 4 minutes of treatment time had little effect on S. aureus in vitro and produced a 1% increase in live MRSA numbers in vitro.

This suggests that 40-kHz LFU is less effective than 35-kHz LFU at reducing total numbers of bacterial cells in vitro. The methodology utilized in both the present study and by Serena was designed to maximize exposure of the bacteria to the acoustic energy field. Serena et al.26 used a Nucleopore filter to trap bacteria (to minimize any washout of bacteria as might occur with an agar dish) so LFU exposure was maximized. Likewise, the 35-kHz treatment was were treated in saline completely and the ultrasound probe completely submerged to prevent bacterial cell osmotic gradients. However, Kavros et al.9 did not observe any osmotic lysis even when the probe was submerged. Because both treatments were designed to maximize bacterial cell exposure, lack of concentrated energy delivery is not thought to have contributed to the difference in treatment effect between the two frequencies.

Additionally, the mechanical effects of both the 35- and 40-kHz spray are not thought to be the source of the differential effects of the two treatment devices because both use a gentle saline spray. The spray force from both units appears similar when applied to intact skin. However, formal pressure impact measurements of these LFU devices have not been reported in the literature. Further, the potential in vivo effects of the 35-kHz treatment device are not known. Unpublished case studies (Suzuki and Aronowick26) and anecdotal reports from clinicians suggest that clinical signs of infection decrease with treatment but no clinical studies have been conducted.

Both scanning electron microscopy and flow cytometry results indicate the mechanism of action for the 35-kHz LFU-mediated bacterial cell death is bacterial cell membrane, confirming physical disruption or damage to the cell membrane. The enhanced cell wall permeability of nonviable bacteria may have led to the loss of normal bacterial cell osmotic gradients. Resultant cellular swelling associated with the loss of an intact bacterial cell wall may have led to cell wall rupture and, ultimately, death. However, nonviable cell wall damage also may impair bacterial cell functioning or reduce bacterial virulence in surviving microbes.

The MRSA cultures treated with the 35-kHz LFU also demonstrated colonial characteristics different from the control (untreated organisms), including alterations in colony size, pigmentation, odor, hemolytic potential, and antibiotic resistance. These changes indicate gene expression may have been altered, resulting in a less virulent strain of MRSA.

The observed decrease in oxacillin resistance may be the most significant aspect of this study. This decreased resistance to oxacillin, the oral form of methicillin, was maintained for up to 48 hours after treatment. This is the first demonstration of the effectiveness of LFU and an antibiotic increased antibiotic effectiveness; the study current demonstrates that post treatment with LFU, only the antibiotic-resistant microbes are sensitive to oxacillin. Additionally, the high frequency of kHz ultrasound used did not produce a direct killing effect; it only changed the antibiotic's ability to affect the organism when LFU and an antibiotic are used together — ie, the 67-kHz LFU alone did not directly effect the bacteria and, when administered in combination with gentamicin, did not have any effect on S. aureus or S. epidermidis.27 Unlike the Pitt study results, 35-kHz LFU administered alone produced a significant killing effect on MRSA and enhanced the susceptibility of MRSA to oxacillin post-LFU exposure. Pitt’s findings also support a differential effectiveness of LFU frequencies on bacterial killing.

When examining ZOI studies, MRSA numbers appeared to decrease across all treatment groups in a dose-dependent manner. This was particularly evident in the 180-second treatment group. MRSA grew only sparsely in the upper treatment quadrants as opposed to the 30-second treatment plate, where growth was more robust. However, MRSA was not completely eliminated, as seen with the 10^6 CFU/mL concentration used in earlier experiments. MRSA survival may be related to the 10^6 CFU/mL concentration utilized in the ZOI experiments (a difference of 100 million compared to 1 million MRSA used in earlier experiments on the direct effects of 35 kHz on MRSA numbers). Confirmation testing with cefoxitin in additional ZOI studies confirmed that methicillin resistance had been reversed in MRSA and that a potential mechanism of this effect by LFU may be due to alteration of the mecA gene which confers resistance to methicillin in S. aureus. The detrimental effect
on bacteria was not detected on cultured human cells and application of 35-kHz LFU to human neuronal cultures for as long as 1 minute did not produce cell death (see Figure 6). Observations from this in vitro human cell study show an increase in neuron size and layering of cultured human neurons treated with 35-kHz ultrasound. Ongoing studies with neurons indicate no detectable cell wall damage occurs and the 35-kHz, LFU-treated cells do not exhibit an increased rate of cell death.

Taken together, these early findings indicate that LFU delivered at 35 kHz may have a different effect on mammalian than on simple prokaryotic cells. This effect may be related to the rigid structure of the bacterial cell wall. Bacterial cell walls may shatter once a resonant frequency is attained. In contrast, mammalian cells have no cell wall and are composed of a flexible and fluid phospholipid bilayer that may absorb and transmit the energy without fracturing. Furthermore, DNA in mammalian cells is sheltered in the nucleus and is not free in the cytosol, as seen in bacteria. The additional membranous barrier between the human DNA as compared to that of the bacteria also may convey some level of protection or moderate the level of energy exposure. It also is hypothesized that different acoustic energy frequencies may affect prokaryotic and eukaryotic cells differently.

**Limitations**

As with all in vitro studies, the observed results may or may not be supported with in vivo experiments. Likewise, it is important to confirm the effects of LFU on changing MRSA colonial characteristics, including antibiotic resistance patterns in other clinical isolates of MRSA and in living tissue. Research to ascertain the effects of LFU at different frequencies in reducing bacterial loads in living tissue as well as its potential effect on wound healing outcomes are needed.

**Conclusion**

The results of this study show that 35-kHz LFU reduces bacterial numbers in vitro through inducing direct damage to bacterial cell walls. The results also support an effect on the MRSA bacterial genome. Alteration of MRSA colonial characteristics, including hemolytic potential and antibiotic resistance, point to another mechanism by which 35-kHz LFU may modulate bacterial infectious potential. The genetic code of MRSA may be altered by 35-kHz LFU, making it less virulent (hemolytic potential, mecA gene modification,) and more susceptible to available chemotherapeutic agents (decreasing antibiotic resistance). If confirmed in vivo and in clinical studies, this modality may provide a mechanism for treating wound infections — especially those caused by antibiotic-resistant bacteria.

**References**


