**ABSTRACT**

Summary: Bacterial biofilms are a possible mechanism in chronic rhino-sinusitis. As an alternative to costly and invasive treatments, high frequency ultrasound could be used to reduce biofilms and incidence of recurrent chronic rhino-sinusitis. In this study we evaluate the effect of ultrasound on bacterial colony counts and specifically their effect on biofilm formation.

Methods: Testing was performed using clinical isolates of Staphylococcus aureus, including a methicillin sensitive strain (MSSA) from a chronic rhino-sinusitis patient and a methicillin-resistant strain (MRSA) from a chronic wound. The test samples were grown using a CDC reactor and a modified ASTM protocol for growing a repeatable biofilm. The MSSA samples were treated with high frequency ultrasound, and the MRSA samples were treated with ampicillin (100 µg/ml) and a combination of ultrasound and ampicillin. These samples were then evaluated using both plate count analysis and confocal scanning laser microscopy (CSLM).

Results: The plate count analysis of the MSSA samples treated with ultrasound only showed a 1.08±0.13 Mean Log Reduction (MLR) when compared to the control. The MLR for combined treatment of ultrasound and ampicillin were significantly greater than treatment with ampicillin alone (p<0.0003). Based on Live/Dead-stained microscopy, the primary mode of action of ultrasound treatment is biofilm removal.

Conclusion: The reduction of bacterial density shows the use of ultrasound may be a viable adjunct treatment for eradication of biofilm infections. Although early in vitro results are promising, further analysis is necessary for optimal dosage and frequency of ultrasound for complete biofilm eradication.

**INTRODUCTION**

Bacterial biofilms have been implicated as a possible causative factor in chronic sinusitis. The use of high frequency ultrasound could be a viable drug free alternative option to current pharmaceutical and invasive surgical treatments. In this study we evaluate the effects of ultrasound on bacterial colony counts and specifically their effect on biofilm formation.

Testing was performed using clinical isolates of Staphylococcus aureus, including a methicillin sensitive strain (MSSA) and a methicillin-resistant strain (MRSA). Biofilm was grown and tested at the Center of Biofilm Engineering (Montana State University, Bozeman MT).

Experiments were performed to investigate the effects of high frequency ultrasound on MSSA biofilm grown on polycarbonate coupons and effects of high frequency ultrasound combined with ampicillin on MRSA biofilm grown on polycarbonate coupons.

**METHODS AND MATERIALS**

**Biofilm Growth**

Both MSSA and MRSA biofilms were grown on polycarbonate coupons in a CDC Biofilm Reactor (model CBR 90, Biosurface Technologies Corporation, Bozeman, MT) using a modified ASTM standard for growing repeatable biofilms on polycarbonate surfaces (Designation E 2562-07).

**Plate Count Analysis**

To evaluate the effectiveness of high frequency ultrasound, the treated coupons were assessed relative to untreated controls using viable plate count methods. Samples not treated with antibiotics were placed in tubes containing 10 ml of phosphate-buffered saline (PBS), while samples treated with antibiotics were placed in 10 ml of Dey-Engly (DE) neutralizing broth. A bacterial suspension was produced by removing the bacteria from the coupons with a sequence of a vortex, sonication, and vortex. This bacterial solution was serially-diluted in PBS, plated on Tryptic Soy Agar (TSA), incubated for 24-28 hours at 37°C, and then the number of colony forming units (CFU) were counted. The CFU per unit area (CFU/cm²) was then calculated based on the dilution and coupon dimensions. The log density was determined by the logarithmically transformed CFU/cm² counts and the mean log density (MLD) were calculated from repeated coupons.

**Ultrasound**

Ultrasound was applied to the treated samples (AxioSonic Sinus System prototype). This system (Figure 1) has an external transducer optimized for the transdermal application of ultrasound to the sinuses. The system delivers 300 J/cm² to the treatment site. The coupons were placed in a fixture (Figure 2) and covered with plastic wrap, acoustic gel was applied, and then samples were treated with ultrasound.

**RESULTS**

**Experiment 1**

Evaluation of Ultrasound on Methicillin Sensitive Staphylococcus aureus (MSSA):

To evaluate the effect of high frequency ultrasound on MSSA, samples were removed from the CDC Biofilm Reactor and treated with high frequency ultrasound. The control samples received no ultrasound treatment.

**Experiment 2**

Evaluation of Ultrasound and antibiotics on Methicillin Resistant Staphylococcus aureus (MRSA):

This experiment evaluated if high frequency ultrasound treatment could improve the efficacy of ampicillin against MRSA. Samples were removed from the biofilm reactor and pre-treated with ampicillin (100 µg/ml) for 1 hour before being treated with high frequency ultrasound. After treatment, the samples were exposed to ampicillin (100 µg/ml) for an additional 24 hours. Antibiotic only samples were exposed to ampicillin (100 µg/ml) for 25 hours and received no ultrasound. The control samples received no ultrasound or antibiotic treatment.

**DISCUSSION**

In past studies the results of ultrasound on bacterial biofilms have been inconclusive. We believe this is due to inconsistencies with the application of ultrasound and variation in the acoustic beam throughout the treatment area. Uniform coverage of the treatment area is critical for the optimal reduction of biofilms and requires a transducer head designed for the indication.

To determine if the reduction of biofilm was due to biofilm removal or killing bacteria, qualitative analysis of the coupon surfaces were performed on both MSSA and MRSA samples using a confocal scanning laser microscopy (CSLM). Both treated and untreated samples were treated with the LIVE/DEAD™ Viability Kit (Life Technologies, Carlsbad, CA) and the images were then imaged (Figure 3, Figure 4) with a Leica SP2 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany).

Images were collected from 5-6 random microscopic fields for each coupon and analyzed by multiple scientists with CSLM expertise, including one who was not involved with the project and blinded to the identity of the images. All scientists indicated that the treated coupons had less biofilm than the control coupons and there was no obvious difference between ratios of red to green cells on the samples. These results indicate that treatment was removing biofilm.

**CONCLUSIONS**

Both the MSSA and MRSA bacterial biofilms treated with 300 J/cm² of ultrasound were reduced by over 90%. This reduction of bacterial density shows the use of ultrasound may be a viable adjunct treatment for eradication of biofilm infections. Although early in vitro results are promising, further analysis is necessary for optimal dosage and frequency of ultrasound for optimal biofilm eradication.