Effect of low intensity pulsed ultrasound therapy on *Staphylococcus aureus* biofilms

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Introduction

"Antimicrobial resistance (tolerance) poses a catastrophic threat...and routine operations like hip replacements or organ transplants could be deadly because of the risk of infection."

Professor Dame Sally Davies, Chief Medical Officer UK

- *Staphylococci* are one of the leading causes of hospital acquired infections
  - ~70% infections of surgical implants and prostheses in orthopaedic surgery[1]
    - ~40% *Staphylococcus aureus*-related
  - Annual treatment cost ~£300million[2] (£20,000-£100,000 per patient)

Implant-related procedures are complicated by infection in ~1-2% cases. Current treatment strategies have varying levels of success. In general, the more effective treatment strategies involve greater patient morbidity and inferior patient outcomes[3, 4].

One strategy to overcome this problem would be to look beyond traditional antimicrobial drug therapies and investigate other treatment modalities. Biophysical modalities, such as ultrasound, have shown potential, particularly in the food industry, but are poorly explored in a clinical context[5,6].

Low intensity pulsed ultrasound (LIPUS, 30 mW/cm^2, 1.5 MHz, 200µs burst width) has regulatory approval as a treatment for fracture non-unions.

Aims

This study aimed to:

1. Evaluate the eradication efficacy of LIPUS in an *in vitro S. aureus* biofilm
2. Determine whether there was a antimicrobial potentiation effect with LIPUS
3. Explore the mechanism of action associated with any antibiofilm effect associated with LIPUS

Materials and Methods

- *MSSA* reference strain (ATCC #29213, *MSSA*-N)
- Antimicrobials
  - Gentamicin

The Dissolvable bead assay[7] was used, with biofilms grown for 24 hours.

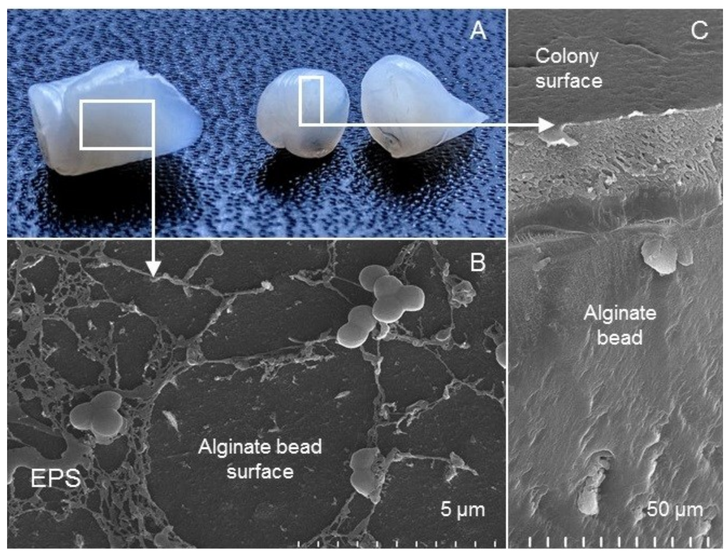

Figure 1. Alginate bead and Cryo-scanning electron microscopy (Cryo-SEM) images*. Fig. 1A. Photograph of an intact alginate bead (left) and a fractured alginate bead (right); Fig. 1B. Cryo-SEM image taken of the bead surface showing the extracellular polymeric substance (EPS), secreted by and encasing the colonies of S. aureus. The undamaged alginate bead substratum is seen in the background; Fig. 1C Cryo-SEM image taken of a fractured frozen bead. No organisms are seen within the alginate bead core below the bead surface.*

Cryo-SEM images that the biofilms are located exclusively at the surface of the beads (*Fig. 1*). Ensuring a homogenous exposure to the treatment. Biofilms were washed to remove non-adherent bacteria, treated (*Fig. 2*), and then dissolved in pH-neutralised citric acid to allow enumeration.

Ultrasound Frequency **f**: 1.5 ± 5% MHz
Modulating Burst Width $\mathbf{t_p}$: 200 ± 10% µs
Repetition Rate **REF**: 1.0 ± 10% KHz
Acoustic Power $\mathbf{P_1}$: 116mW
Spatial Average - Temporal Average (SATA) $\mathbf{I_e}$: 30 ± 30% mW/cm^2

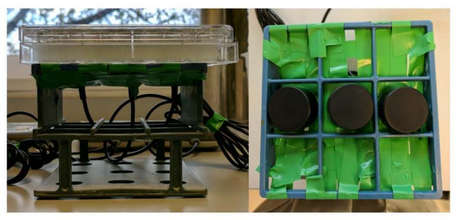

Figure 2. Acoustic parameters (left) and experimental set-up for sonication of MSSA-'N' biofilms (right). *The sonicating probes were in direct contact with the 24-well polystyrene plate using ultrasound gel. Each well housed three alginate beads with nutrient broth +/- gentamicin during treatment.*

Results

- LIPUS alone was not found to have a direct bactericidal effect against *S. aureus* biofilms
- There was a 4-8 fold reduction in the effective eradication concentration of gentamicin with the addition of ultrasound (*Fig. 3*)
- Application of LIPUS was associated with a 25% increase in cellular respiration (*Fig. 4*)

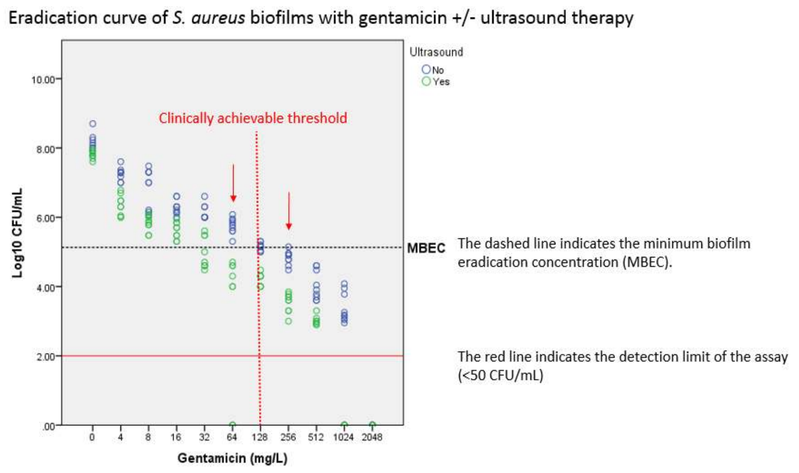

Figure 3. *Eradication curve of biofilm-associated S. aureus (CFU/mL) detected following a 3-hour exposure to gentamicin +/- ultrasound therapy. Dashed line represents a 99.9% reduction. Solid line represents detection limit of assay.*

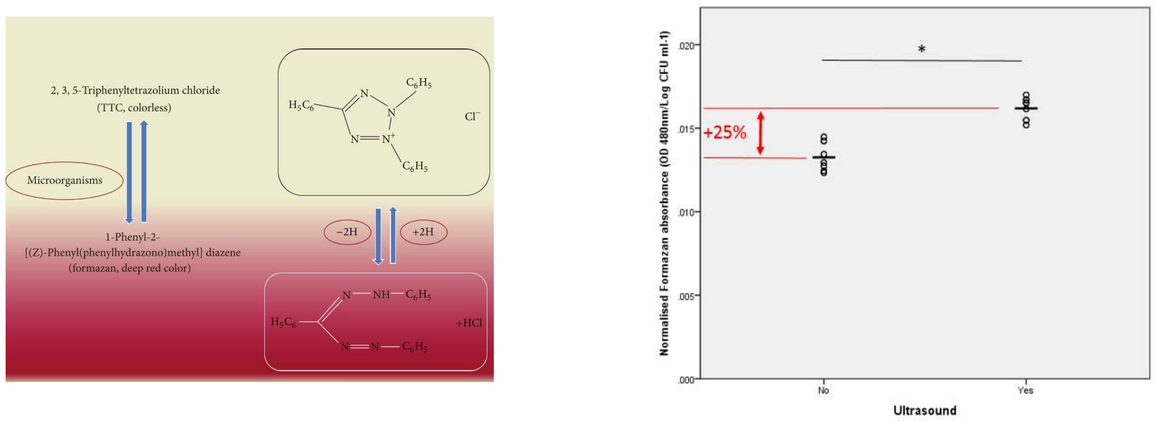

Figure 4. The tetrazolium-formazan biotransformation system *(left) TTC is a redox dye that is reduced by bacterial respiration to form formazan which appears red in solution. The amount of formazan produced can be quantified using optical density reading[10].* ***Normalised formazan absorbance (OD / Log CFU ml^{-1}) of S. aureus biofilms +/- ultrasound treatment.*** ** denotes statistical significance ($p<0.0001$)*

The effective eradication concentration of gentamicin for biofilm-associated *S. aureus* was 256mg/L (*Fig. 3*), which is 50-100 times greater than the concentration to effectively eradicate *S. aureus* in its planktonic form. This is important clinically as this concentration of gentamicin cannot be achieved *in vivo* even with the use of local antibiotic depots (Table. 1)

Table 1. Summary of recommended peak serum concentrations used clinically and the median elution concentration from antibiotic-loaded cements of commonly used antimicrobials

	Gent	Vanc	Dapto	Clinda	Cipro	Rif	Linez
Recommended C_{Max}*	5-12	15-20	4-16	10-14	2-4	4	12.5
Typically achieved local concentrations from antibiotic-loaded bone cement‡	128	128	-	128	128	-	128

*Recommended peak serum concentration levels (mg/L) in adults[8]

‡ Reflects the midrange between the high initial interfacial gap levels and the human *in vivo* levels (mg/L) recorded from sites more distant from the cement such as seroma[9]

Conclusion

- **LIPUS alone did not have a bactericidal effect**
  - Due to the thickness of its cell wall[1], sphericity, and size[11]
- **Gentamicin potentiation effect with low intensity pulsed ultrasound**
  - 4-8 fold reduction in the effective biofilm eradication concentration to below clinically achievable level
    - Due to etching of the glycocalyx

      → improved delivery of nutrients and oxygen to the bacteria.

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