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Antibiofilm effect of ultrasound combined with microbubbles against *Staphylococcus epidermidis* biofilm

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ABSTRACT

Biofilms are difficult to eradicate due to their resistance to antibiotics and host immune cells. Ultrasound microbubbles have emerged as a new treatment modality with the underlying mechanisms largely unknown. In this study, we exposed 24-h-old *Staphylococcus epidermidis* biofilms established in OptiCell™ chambers to ultrasound in combination with microbubbles, and investigated the activities of vancomycin and neutrophils against *S. epidermidis* biofilms after treatment. The antibiofilm mechanims of ultrasound microbubbles were explored in terms of bacterial permeability and biofilm-associated gene expression. After treatment of ultrasound (1 MHz, 0.5 W/cm^2, 50% duty cycle) combined with microbubbles in the concentration of 1% and 4% (v/v) for 5 min, bacterial permeability to extracellular fluorescent dyes was enhanced and the expression of *icaA* was down-regulated while that of *agrB* and RNAIII up-regulated. Post-treatment biofilms were more sensitive to vancomycin by demonstrating reduced biomass than those exposed to vancomycin alone ($P < 0.05$). The phagocytosis, oxidative burst activity as well as chemotaxis of neutrophils in response to biofilms were also significantly increased. The bioeffect of ultrasound combined with microbubbles was generally more significant than that of ultrasound alone, and dependent on microbubble concentration. This study demonstrated that ultrasound combined with microbubbles could enhance the activities of antibiotics and neutrophils against biofilms possibly via mechanical and biochemical mechanisms, and may provide an efficient and non-invasive antibiofilm alternative apart from chemical and biological approaches.

1. Introduction

Biofilms are responsible for more than 80% of bacterial infections in humans (Römling and Balsalobre, 2012), and *Staphylococcus epidermidis* has emerged as the most prominent pathogen for biofim-associated infections, especially among patients with compromised immunity and indwelling medical devices (Otto, 2009; Schoenfelder et al., 2010; Dong and Speer, 2014). Due to its structural integrity and the barrier function of extracellular matrix, *S. epidermidis* biofilms are recalcitrant to antibiotics and human immune cells, causing persistent infections and even failure of medical devices (Otto, 2014Schoefelder et al., 2010). The major component of *S. epidermidis* biofilms extracellular matrix is polysaccharide intercellular adhesion (PIA), a homopolymer of β-1,6-linked N-acetylglucosamine (GlcNAc) residues, encoded by the *icaADBC* operon, with *icaA* encoding the rate-limiting enzyme N-acetylglucosaminyl-transferase (Otto, 2012, 2014). On the other hand, biofilm structural integrity lies in cell-to-cell communication system, namely quorum sensing (QS) (Le and Otto, 2015). The well-characterized QS system in *S. epidermidis* biofilm is accessory gene regulator (*agr*) with two transcription units, RNAII and RNAIII (Le et al., 2015). RNAII locus contains four genes, *agrB*, *agrD*, *agrC* and *agrA*, with *agrB* encoding transmembrane endopeptidase for the production of quorum signal molecule (Le and Otto, 2015). RNAIII encodes detergent-like peptides such as phenol-soluble modulins (PSMs) which contribute to the dispersion and detachment of biofilms (Le and Otto, 2015). Current data indicated that *agr* is differentially expressed during the course of infection, and a low activity of *agr* is generally associated with biofilm formation and persistence (Yao et al., 2005; Le and Otto, 2015).

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The limited capacity and potential safety concerns of chemical and biological approaches to eradicate biofilms raise the prospect that mechanical strategies may produce better outcomes (Gomes et al., 2014; Erriu et al., 2014). A favored approach during the past two decades has been the use of low-intensity ultrasound (US) (Erriu et al., 2014). Our previous investigation (Dong et al., 2013) and other similar studies (Qian et al., 1999; Rediske et al., 2000; Carmen et al., 2004; Carmen et al., 2005; Ensing et al., 2005; He et al., 2011), *in vitro* and *in vivo* alike, demonstrated that US could act synergistically with antimicrobial substances against bacterial biofilms and increase the antibacterial effect up to several hundred fold, a phenomenon also termed bioacoustic effect (Qian et al., 1999). Additionally, no obvious harm was caused to host tissues as shown in in vivo experiments (Rediske et al., 2000; Carmen et al., 2004; Ensing et al., 2005). Very recent data also indicated that microbubbles (MBs), 1–8 μm-sized particles consisting of a gas core and a stabilizing shell, could further enhance the bioacoustic effect of US (He et al., 2011; Dong et al., 2013). So far, the use of ultrasound microbubbles in the field of anti-infectious treatment is still in its infancy, and the mechanisms of bioacoustic effect remains far from clear (Erriu et al., 2014).

Our present study is a continuum of the previous work with a focus on the mechanisms underlying antibiofilm effect of US combined with MBs. In order to provide a relatively comprehensive understanding in this matter, we investigated: (1) mechanical effect of US combined with MBs on *S. epidermidis* bacterial permeability; (2) biochemical effect of US combined with MBs on biofilm-associated gene expression; (3) the response of antibiotics as well as neutrophils to biofilms treated by US combined with MBs.

2. Materials and methods

2.1. Strain and microbubbles (MBs)

A single colony of *S. epidermidis* ATCC 35984 (RP62A) was inoculated into 5 mL of tryptic soy broth (TSB) (Oxoid) and allowed to grow overnight at 37 °C with agitation (180 rpm). Bacteria were harvested and resuspended in TSB to a turbidity of 0.5 McFarland. Microbubbles (MBs) were prepared following established protocols (Zheng et al., 2012). Briefly, five milligram of 1,2-distearoyl-sn-glycerophosphatidylcholine (DSPC) (Sigma-Aldrich, St Louis, USA) and 2 mg of 1,2-dipalmi- toyl-sn-glycerol-3-phosphor-ethanolamine (DPPE) (Sigma-Aldrich, St Louis, USA) were mixed with 0.5 mL of 10% glycerol in a 1.5 mL vial and incubated at 45 °C for 30 min. After filled with perfluoropropane gas, vials were vigorously shaken for 60 s using a dental amalgamator (YJT, Shanghai Medical Apparatuses and Instruments, China). The MBs solution was then sterilized by ^{60}Co irradiation. The diameters of prepared MBs were 4–6 mm, with a density of approximately 1.2×10^9/mL. The MBs solution was further diluted to 1% (vol/vol) and 4% (vol/vol) for experimental use as previously (Dong et al., 2013).

2.2. Biofilm development and ultrasound exposure setup

S. epidermidis biofilm was developed in an OptiCell™ chamber (Nunc, Rochester, NY) as previously described (Dong et al., 2013). Ten milliliter of bacteria suspension at 0.5 McFarland was injected into the OptiCell™ chamber and incubated at 37 °C for 24 h. Bacteria adhered to the bottom membrane and formed biofilm. A specific experimental setup was used to facilitate ultrasound exposure as previously demonstrated (Dong et al., 2013). Briefly, the medium inside the OptiCell™ chamber was replaced with 10 mL TSB with or without MBs. Then, the chamber was gently reversed and placed horizontally in a water tank with the biofilm and MBs on the underside of the uppermost membrane. The ultrasound transducer with a surface area of approximately 3.72 cm^2 was fixed below the chamber. Membranes of the OptiCell™ chamber were acoustically transparent (Meijering et al., 2007). The gene transfer machine produced by Institute of Ultrasound Imaging, Chongqing Medical University was used in experiments. Biofilms and MBs were exposed to 5 min of 1 MHz pulsed ultrasonic waves at an acoustic intensity of 0.5W/cm^2, with a 50% duty cycle. Then, the OptiCell™ chambers were incubated at 37 °C for another 24 h. After treatment under four different conditions (except for crystal violet assay), namely the control (not treated), US only, US + 1% MBs, and US + 4% MBs, three squares of 1 cm^2 of biofilms were cut from the OptiCell™ membrane in the exposed region and subjected to further assays.

2.3. Bacterial permeability by confocal laser scanning microscopy (CLSM)

Changes in cell membrane permeability by ultrasound were assessed as described elsewhere (Fan et al., 2010). Propidium iodide (PI) from LIVE/DEAD BacLight kit (Invitrogen Molecular Probes, USA) was diluted and injected into OptiCell™ chamber with preformed biofilms to reach a final concentration of 120 μM. The uptake of extracellular macromolecules can be used to reflect the permeability of bacterial membrane, and bacteria with damaged membranes would be stained fluorescent red. Thirty minutes after treatment under the four experimental conditions, squares of biofilms were rinsed with phosphate buffer saline (PBS) to remove the extracellular dyes and observed under Nikon A1R laser confocal microscope (Nikon, Tokyo, Japan). Signals were obtained using the red (excitation 568 nm, emission 600/50 nm) channel.

2.4. Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) was used to quantitatively analyze the expression of *ica*A, *agr*B and RNAIII, all are key genes in biofilm development. After treatment under four different conditions, biofilms were stripped from the square surface and dispersed in TSB by sonication at 20 kHz for 5 s (Tomy UD-201, Tokyo, Japan). Our preliminary experiment showed that this procedure did not affect bacterial viability. Bacterial RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using a PrimeScript™ RT-PCR kit (TaKaRa, Ohtsu, Shiga, Japan). RT-PCR was performed in the thermal cycler system (CFX-96, Biorad, USA) by using cDNA templates, specific forward and reverse primers (Table 1), SsoFast EvaGreen Supermix (Biorad, USA) and deionized H_2O. The reaction was performed using a 2-step protocol with 40 cycles of 95 °C for 2 s and 60 °C for 4 s following initial denaturing of DNA at 95 °C for 30 s. A melt curve analysis was performed at the end of every run to verify single PCR products. The expression levels of *icaA*, *agrB* and RNAIII were normalized to the internal standard gene 16S rRNA as previously described (Chini et al., 2007). All RT-PCR experiments were performed in triplicate.

Table 1
Primer sequences used in real-time polymerase chain reaction.

Primer	Direction	Sequence (5′–3′)
16S rRNA	Forward	CGTGGAGGGTCATTGGAAAC
	Reverse	CCACTGGTGTTCCTCCATATCTC
agrB	Forward	TTCGTTTAGGGATGCAGGTA
	Reverse	TACCGTGTGCATGTCTCCTA
RNAIII	Forward	TGAGTTGTTGAGCCATCCA
	Reverse	ACCTAACACTGAGTCCAAGGAAACTA
icaA	Forward	GGAAGTTCTGATAATACTGCTG
	Reverse	GATGCTTGTTTGATTCCCTC