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● *Technical Note*

HIGH INTENSITY FOCUSED ULTRASOUND (HIFU) HEATING IMPROVES PERFUSION AND ANTIMICROBIAL EFFICACY IN MOUSE *STAPHYLOCOCCUS* ABSCESS

Rachel Wardlow, Kaustuv Sahoo, Danielle Dugat, Jerry Malayer, and Ashish Ranjan
Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma, USA

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Abstract—Chronic wounds typically require long-duration treatment with a combination of antibiotics administered systemically. This incurs adverse side effects and can require aversive surgical treatments and limb amputations. To improve non-invasive antimicrobial therapy, the objective of this study was to investigate antimicrobial chemotherapy combined with high-intensity focused ultrasound (HIFU) heating (HT). A *Staphylococcus aureus* abscess (80 ± 30 mm^3) was generated in the mouse flank region. Once the average temperature (~42 °C–46 °C) in the abscess was reached with HIFU-HT, a broad-spectrum antimicrobial (ciprofloxacin, 10 mg/kg) and perfusion marker (Evans blue dye, 40 mg/kg wt) were administered intravenously *via* the tail vein. Four hours later, mean abscess perfusion and colony-forming units (CFUs) per gram of abscess were determined. HIFU-HT increased abscess perfusion by ~2.5-fold (4 ± 0.6 μg/mL Evans blue) compared with control (1.5 ± 0.7 μg/mL), and improved antimicrobial efficacy to decrease percentage average survival of *S. aureus* by ~20% (46 ± 7 CFUs/g of abscess) *versus* that seen with ciprofloxacin alone (61 ± 4 CFU/g). Our *in vivo* data suggest that HIFU-HT can improve antimicrobial treatment responses against deep-seated bacteria in abscess wounds *via* enhanced perfusion. (E-mail: ashish.ranjan@okstate.edu)

Key Words: **High-intensity focused ultrasound, Heating, *Staphylococcus aureus*, Abscess, Perfusion, Antibiotic.**

INTRODUCTION

Acute and chronic wound infections caused typically by *Staphylococcus aureus* are a serious condition in children and patients with diabetes mellitus and peripheral vascular disease (Carstens et al. 2016). Despite significant progress, successful therapeutic outcomes in wound infections remain a challenge because of the poor ability of local or systemically administered antimicrobial agents to reach infection sites (Wardlow et al. 2016). Non-healing wounds infested with biofilms also have reduced vascular flow and perfusion, and can have antibiotic resistance caused by efflux pumps, modifying enzymes and receptor mutations that allow bacteria to survive (Fraimow 2009). To overcome these barriers, long-duration treatment (generally >6 wk) with combinations of antimicrobials, resection of tissues and, in some extreme cases, amputation is required in infected patients (Henry et al. 1993). Therefore, there is an important need to enhance therapy of biofilm-infested bacterial wounds.

We hypothesize that localized mild heating (~42 °C–46 °C) of a wound will permit enhancement of perfusion rates of antibiotics and killing of *Staphylococcus aureus* biofilm bacteria. Sturtevant et al. (2015) observed upregulation of *S. aureus* stress response genes (hsp60, which contributes to the misfolded protein response, and murAB/Z, the first step required in staphylococcal cell wall synthesis and cell wall repair) at elevated temperature (>40 °C). We and others have reported that high-intensity focused ultrasound (HIFU) under magnetic resonance (MR) and ultrasound (US) guidance delivers focused US waves, providing a flexible and powerful tool for clinical control of anatomically specified, well-tolerated mild local tissue heating (HT) and drug delivery in solid tumors (Bing et al. 2015; Maples et al. 2015; Negussie et al. 2010; Partanen et al. 2012; Ranjan et al. 2012). HIFU-HT (~42 °C) can also decrease resistance within vascular beds to elevate local perfusion (de Smet et al. 2013; Frazier et al. 2016; Lasithiotakis et al. 2010; Lefor et al. 1985; Manzoor et al. 2012; Ranjan et al. 2012; Song et al. 2001). Because

Address correspondence to: Ashish Ranjan, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74074, USA. E-mail: ashish.ranjan@okstate.edu

S. aureus biofilms are typically characterized by dense, highly hydrated clusters of bacterial cells encased in a polysaccharide matrix (Bing et al. 2015), we propose that HIFU-HT can improve perfusion in the abscess capsular regions and decrease bacterial stress to improve antibiotic therapy outcomes (Locke et al. 2005; Wardlow et al. 2016). To test this idea, in this study we combined antibiotic therapy with HIFU-HT in mouse *S. aureus* abscess for investigation of changes in perfusion and antimicrobial efficacy. Data suggest that the proposed HIFU-HT antibiotic delivery methodology can improve bacterial killing rates in abscess wounds.

METHODS

Chemicals and bacterial strain

Ciprofloxacin was obtained from Alfa Aesar (Ward Hill, MA, USA). Tryptic soy agar (TSA) and tryptic soy broth (TSB) were obtained from Becton Dickinson (Sparks, MD, USA). Female BALB/c mice were obtained from Charles River (Wilmington, MA, USA). Evans blue dye and trichloroacetic acid (TCA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Two-millimeter zirconia beads and 2- and 7-mL polypropylene screw-cap microvials were obtained from Biospec Products, Inc. (Bartlesville, OK, USA). A human clinical isolate of *S. aureus* (mecA negative by polymerase chain reaction) sensitive to amoxicillin/clavulanic acid, ampicillin, ciprofloxacin, gentamicin and vancomycin was obtained from a local medical diagnostic laboratory.

Scanning electron microscopy of heated and unheated S. aureus *biofilms*

To test the impact of biofilm on biofilm architecture, *S. aureus* biofilms were established in an MBEC 96-well plate device system that allows for high-throughput screening of therapeutic agents (Parker et al. 2014). Briefly, MBEC wells were filled with 150 μL of *S. aureus* culture (1.0×10^7 colony-forming units [CFUs]/mL) and then incubated for 5 d in a humidified gyrorotary shaker rotating at 150 rpm at 37 °C to establish a biofilm on the pegs located on the lid of the device. Then, biofilm was heated to ~42 °C–46 °C for 30 min in a water bath. Heated and unheated pegs were collected separately, fixed with 10% formaldehyde, washed with sodium cacodylate buffer, incubated for 1 h in 1% osmium tetraoxide in cacodylate buffer, serially dehydrated in increasing concentrations of ethanol (50%, 70%, 90%, 95% and 100%) and dried in hexamethyldisilazane. Individual samples (biofilm alone, heated biofilms) were mounted on microscopy stubs with tape and then coated with gold–palladium and viewed under the scanning electron microscope (magnification: 30,000×).

Abscess treatment and perfusion study design

All laboratory and animal-related procedures were approved and carried out under the guidelines of the Oklahoma State University (OSU) Institutional Biosafety Committee and Animal Care and Use Committee, respectively. Eighteen C57BL/6 mice (3 mice/group, 6–8 wk old) with *S. aureus* abscesses were assigned to one of four groups for efficacy—(1) untreated control, (2) ciprofloxacin, (3) HIFU-HT and (4) ciprofloxacin + HIFU-HT)—and perfusion assessment (Evans blue dye ± HIFU-HT). Treatments were performed at a mean abscess volume of 80 ± 30 mm^3.

Establishment of mouse S. aureus *abscess model*

Staphylococcus aureus was grown overnight in TSB, centrifuged, washed in phosphate-buffered saline and serially diluted to achieve a final suspension containing 3.0×10^8 CFU/mL. Mice (n = 3/group) were injected subcutaneously with 50 μL of inoculum (3.0×10^8 CFU/mL) in the flank regions. Subsequently, the mice were observed for 3 to 5 d for development of clinical symptoms of abscess formation.

Mouse abscess HIFU-HT protocol

An integrated ultrasound–HIFU Alpinion system (VIFU2000, Bothell, WA, USA) was used for abscess identification, sonication and treatment characterization. The HIFU transducer has a 1.5-MHz central frequency, 45-mm radius and 64-mm aperture diameter with a central opening 40 mm in diameter. Mice were anesthetized with 2%–5% isoflurane and restrained in custom-built mouse holders attached to a 3-D positioning stage. The abscess in the flank region was dipped in degassed water maintained at 37 °C for coupling with the HIFU transducer. Through real-time ultrasound guidance, the abscess was positioned so that the target was in the center of the focal zone of the transducer. VIFU-2000 software was used to define the target boundary and slice distance in the x, y and z directions for automatic rastering of the transducer (Fig. 1a). Before actual studies, we calibrated the instrument to heat to a temperature of ~42 °C–46 °C in abscess-bearing mice by optimizing the HIFU parameters (duty cycle, pulse repetitive frequency, total acoustic power and time), as previously described (Dromi et al. 2007; Yuh et al. 2005). Temperature in the capsular region was measured by placing a fiber optic temperature sensor (Neoptix, Canada), close to the abscess wall. HIFU treatment parameters used were as follows: 5-Hz pulse repetition frequency (PRF), 50% duty cycle, and 5.9-W acoustic power. A 3×3-raster pattern of focal points along the x-axis was used to generate a heat gradient of ~42 °C–46 °C. The subsequent focal point needed to be 2 mm away to effectively take advantage of the heat gradient created at a single focal point. Each focal point with dimensions of $1 \times 1 \times 10$ mm in the x, y and z axes, respectively, was