An intracellular *Staphylococcus aureus* infection model using mature mouse osteocytes.

Amita Sekar[1,2], Marcos Lora[1], Devika Kannambadi[1], Birol Ay[3,4], Murat Bastepe[3,4], Orhun Muratoglu[1,2], Ebru Oral[1,2]
[1]Harris Orthopaedic Laboratory, Massachusetts General Hospital, Boston, MA
[2]Department of Orthopaedic Surgery, Harvard Medical School, Harvard University, Boston, MA
[3]Endocrine Unit Research Laboratories, Massachusetts General Hospital, Boston. MA
[4]Department of Medicine, Harvard Medical School, Harvard University, Boston, MA
Email: asekar2@mgh.harvard.edu

Disclosures: Amita Sekar (N), Marcos Lora (N), Devika Kannambadi (N), Birol Ay (N), Murat Bastepe (N) Orhun Muratoglu (Exactech, Stryker, Corin, Mako, Iconacy, Renovis, ConforMIS, Meril Healthcare, Kyocera, Arthrex, Ceramtec, Alchimist, Cambridge Polymer Group, Orthopedic Technology Group), Ebru Oral (1-Corin, Iconacy, Renovis, Arthrex, ConforMIS, Meril Healthcare, Exactech; 3B – WL Gore & Assoc; 7/8 – JBMR; 9 – SFB, ISTA)

INTRODUCTION: Osteomyelitis is a debilitating infection of the bone characterized by inflammation and bone loss with over 80% of the cases being post-operative (implant-associated) and post-traumatic [1]. *Staphylococcus aureus*, the predominant causative organism, is known to colonize the implant and peri-implant tissue, eventually resulting in deep bone infection [2]. During infection, *S. aureus* has been shown to penetrate deep into the bone and infiltrate bone cells, particularly terminally differentiated osteocytes, to persist as an intracellular infection [3]. Despite the knowledge of intracellular infection, there are currently no robust pre-clinical models to aid in designing effective strategies to eradicate a persisting intracellular pathogen. Here we aim to develop and characterize a stable *S. aureus* intracellular infection model using osteocytes as a tool to test the efficacy of local drug delivery applications.

METHODS: OCY454 cell line, derived from mouse long bones, was used in this study [4]. Proliferated OCY454 cells were differentiated in complete media (DMEM+10% FBS) at 37°C for 1-2 days until a dendritic morphology characteristic of mature osteocytes was observed. Total RNA was extracted from mature osteocytes and DMP-1 gene expression was determined by quantitative real-time PCR using specific primers to validate differentiated status. The mature OCY454 cells at 40-50% confluency, were differentiated in 6-well plates using complete media and were infected with *S. aureus* ATCC 12600 (MOI 1) for a period of 3 hours. The spent media was discarded, and the cells were washed with PBS to remove unbound bacteria. The infected cells were then subjected to a range of lysostaphin concentration treatments for 1 hour to optimize extracellular bacteria eradication. The absence of viable extracellular bacteria was determined by subjecting the cell supernatant to a real-time ATP-based luminescent assay. The cells were then processed for staining intracellular *S. aureus* using specific primary antibodies and Cy5-tagged secondary antibodies. Fluorescence microscopy together with Z-stack acquisition was performed to detect Cy5-stained *S. aureus* within DAPI and phalloidin-stained OCY454 cells. 3D rendering of the images was performed using the Nikon NIS Elements software package to observe the intracellular localization of the bacteria. The intracellular viability of the bacteria was determined by plating the cell lysate on tryptic soy agar plates. Both host cell and bacterial viability were further validated using real-time ATP-based viability assays.

RESULTS SECTION: Differentiated osteocytes showed increased expression of DMP1 on week 1, which validated osteocyte maturation. Lysostaphin extracellular bacteria killing assay was optimized and for all subsequent experiments, the cells were subjected to a concentration of 100µg/mL for 1 hour. Fluorescent microscopy revealed that in 3 hours, S. aureus was able to infiltrate the osteocytes, and intracellular localization of bacteria was observed within the cell cytoskeleton. The intracellular bacterial viability assay performed on the lysostaphin-treated cell lysate was able to recover approximately 10^3 CFU/mL when compared to ~5×10^5 CFU/mL in cell lysate obtained from infected cells treated with no lysostaphin.

DISCUSSION: We have shown the use of mouse osteocytes as a suitable model to simulate an *S. aureus* intracellular infection *in vitro*. The optimized enzymatic killing of extracellular bacteria enabled the focused study on intracellular *S. aureus*. The observations clearly indicate intracellular localization of the bacteria which was also determined to be viable within the cell. These preliminary findings aid in the understanding of intracellular infection dynamics and enable to development of a viable osteocyte-intracellular *S. aureus* co-culture model. The model will be used as a tool to test the efficacy of drug-eluting materials to eradicate bacteria which has infiltrated the bone cells.

SIGNIFICANCE/CLINICAL RELEVANCE: Deep bone infections warrant novel strategies to enable the effective eradication of resistant bacterial populations and intracellular persisters. A robust viable osteocyte intracellular infection model would aid in developing multimodal approaches to improve outcomes for osteomyelitis patients.

REFERENCES: *1. Lew DP and Waldvogel FA, Lancet 2004; 2. Walter G et al, Dtsch Arztebl Int. 2012; 3. de Mesy Bentley KL et al, J Bone Miner Res. 2017; 4. Xu LH et al, J Orthop Res. 2019*

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1.

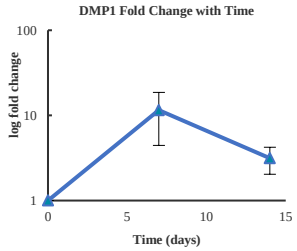

Fig 1: Relative DMP-1 gene expression of differentiated OCY454 cells plotted as log fold change over time. The error bars represent the standard deviation (n=3)

2.

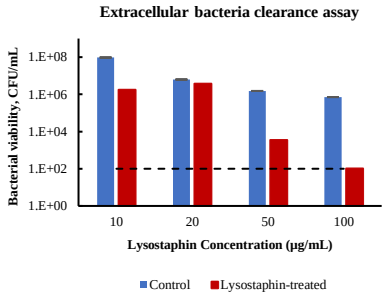

Fig 2: Extracellular bacteria exposed to indicated concentrations for a period of 1 hour. The bacterial CFU/mL corresponding to real-time luminescence units plotted for each concentration tested. The dotted line denotes the limit of detection. Extracellular bacteria with no lysostaphin treatment served as a control.

3.

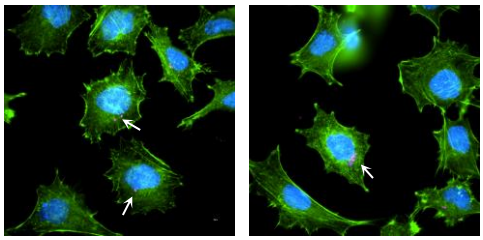

Fig 3: Fluorescent micrographs of *S. aureus* infected mouse osteocytes. White arrows indicate *S. aureus* localization (scale bar =10µm) DAPI stained cell nuclei; Phalloidin stained actin; Cy5-stained *S. aureus.*