

Cold Plasma Treatment of Infected Periprosthetic Tissue Enhances Neutrophil Effector Activities
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INTRODUCTION: Infection by biofilm-forming *Staphylococcus aureus* (*S. aureus*) presents one of the most detrimental outcomes to orthopedic surgery. Biofilm formation complicates treatment options by preventing infiltration by immune cells and enhancing secretion of virulence factors, which further target the immune response and dampen its effect¹. Neutrophil recruitment in the host defense against *S. aureus* infection is required for bacterial clearance both in humans and rodent models. Neutropenic patients and those with NADPH oxidase defects are particularly susceptible to *S. aureus* infection². Further, neutrophil-depleted mice develop nonhealing skin lesions and are unable to clear the bacteria³. The need to combat *S. aureus* immune evasion coupled with the evolving crisis of antibiotic resistance highlight the increasing need for non-antibiotic strategies to combat bacterial infections. Cold plasma represents just such a strategy. Cold plasma is an energized gas which generates a cocktail of reactive oxygen and nitrogen species (ROS/RNS) and has been shown in a large body of *in vitro* research to be anti-microbial. In addition to inhibiting bacterial pathogenesis through direct killing and modulation of virulence factor production⁴, cold plasma has also been shown to enhance innate immune effector activities including migration, phagocytosis, degranulation, and NETosis⁵. However, these findings have yet to be validated *in vivo*. The goal of this study was to investigate the effect of cold plasma on the innate immune response and neutrophils in an *in vivo* rat model of orthopedic infection and surgical revision.

METHODS: Animals and Surgical Procedure: This study was IACUC approved. At the index surgery (Day -7) male Sprague-Dawley rats were anesthetized and a craniolateral incision between the vastus lateralis and the biceps femoris was created to access the femur. An Accupen (RISystem) was used to place 1.7 mm titanium screws at the proximal and distal ends, and to create a mid-diaphyseal empty drill hole to expose the medullary cavity. A collagen sponge impregnated with 250 μ L of *S. aureus* ATCC 25923 at 1×10^6 CFU (infected) or saline (uninfected) was positioned over the screws and the empty hole. Seven days later, a revision surgery (Day 0) was performed through the previous incision. Both screws were removed for microbial analysis and sterile saline was used to lavage the surgical site before treatment with 0.3% povidone iodine solution for 2 minutes. An 8-hole RatFix (RISystems) plate was secured using 1.7 mm titanium screws with 2 proximal and 2 distal to the open mid-diaphyseal hole. A final saline lavage was performed prior to closing the incision in 2 layers. The rats were treated with perioperative analgesics (extended-release buprenorphine 0.65 mg/kg for 72 hours SC, and meloxicam 2 mg/kg for 24 hours SC). Experimental Groups: At either 6-hours, 4- or 14-days post-revision surgery (n=10/timepoint, 5 infected, 5 control) the rats were humanely euthanized and the hardware and tissue surrounding the incision were aseptically collected for microbial analysis, RNA, and protein extraction. Microbial Analysis: The tissue was vortexed for 40 seconds in 5 mL sterile phosphate buffered saline (PBS) which was used for colony counts. The hardware was washed in 1 mL PBS three times and then placed in 0.3% Tween20 for 5 minutes of sonication. Serial dilutions of the fluid from the muscle or hardware were transferred to petrifilms, incubated at 37 C for 24 hours, and counted to determine log CFU/mL. RNA Analysis: RNA sequencing and gene expression analysis (NovoGene) was performed on periprosthetic soft tissue from 3 rats/group (Uninfected, Infection + Betadine, Infection + Plasma). RNA was isolated (NucleoSpin RNA kit, Macherey-Nagel), cDNA was generated (EcoDry Premix with Oligo dT, Takara), and qPCR (SYBR Green Master Mix, Thermo Fisher) was used to confirm the results. Samples were run in triplicate.

RESULTS: In order to investigate cold plasma's anti-microbial properties *in vivo*, plasma was used to treat infected periprosthetic tissue at the time of surgical revision. While plasma treatment of infected tissue at the time of revision did not significantly reduce bacterial load (log CFU) at 6-hours, 4-days, or 14-days post-treatment, it was observed that plasma treatment increased circulating leukocytes 4-days post-treatment. RNA sequencing data revealed enhanced gene expression of neutrophils at the 4-day timepoint, and GSEA analysis of RNA sequencing data revealed significant enrichment of genes involved in neutrophil activation and chemotaxis when compared to untreated infected tissue. These findings indicate that plasma treatment correlates with a heightened early immune response which is more capable of targeting invading pathogens than the immune response in the uninfected groups. Cold plasma treatment also significantly upregulated IL-1 β gene expression, a major pro-inflammatory cytokine produced by neutrophils that serves as a recruitment molecule for additional immune cells, propagating the pro-inflammatory response. Interestingly, when bacterial load was correlated with IL-1 β expression in infected untreated animals, it was negatively correlated, but after cold plasma treatment it became positively correlated, highlighting a shift in the host-pathogen relationship in plasma-treated groups. Further, gene expression of *S. aureus* virulence factors associated with immune evasion techniques were found to be significantly downregulated in plasma-treated groups. Finally, it was observed that at 14-days post plasma treatment, draining lymph node size returned to that of uninfected controls whereas untreated animals retained significantly inflamed lymph nodes. These findings suggest that plasma treatment plays a role in modulating the host-pathogen relationship, resulting in a renewal of the immune response.

DISCUSSION: The properties of cold plasma make it an excellent non-antibiotic candidate for treating bacterial infections^{4,5}. While we did not observe changes in bacterial load when we applied plasma treatment *in vivo*, our RNA sequencing results suggest a renewal of the innate immune response by neutrophils. The observed change in the innate immune response was correlated with the independent observation that plasma treatment reduced the expression of bacterial virulence factors in our *in vivo* model, making the bacteria less pathogenic. Taken together these data suggest that plasma treatment alters the host-pathogen relationship during an infection. Additional studies will be critical in determining if cold plasma treatment in combination with standard clinical antibiotic regimens will improve bacterial clearance following revision surgery.

SIGNIFICANCE: Cold plasma represents a viable non-antibiotic treatment option for orthopedic infections through its combined antibacterial and immunostimulatory properties which may be used additively with the current standard of care.

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