Determination of the effects of a novel antimicrobial agent used in conjunction with Gentamicin on *Staphylococcus aureus* using a porcine model: preliminary evaluations

Jose Valdes, Joel Gil, Andrew Herr^, Andrew Harding and Stephen C Davis*

^University of Cincinnati, Dept. of Molecular Genetics, Biochemistry & Microbiology / University of Miami, Miller School of Medicine, Dept. of Dermatology and Cutaneous Surgery, Miami, FL

Abstract:

The incidence of wound infections, especially associated with *Staphylococcus aureus*, is a major concern for healthcare providers.[1] *S. aureus* is one of the more common pathogens found in chronic wounds. [2,3] Furthermore, *S. aureus* readily forms biofilms, which are surface-adherent bacterial communities that render the bacteria resistant to antibiotics or host immune responses, and greatly increase healthcare treatment costs.[4,5] Previous work indicated that zinc is required for the initial formation of staphylococcal biofilms, and that DTPA, a zinc chelator, could potentially inhibit biofilm formation by *S. aureus* and *S. epidermidis*.[6,7] The goal of this study was to examine the effects of a novel DTPA formulation used in conjunction with Gentamicin on wounds infected with *S. aureus*. Forty-four deep, partial-thickness wounds were created on the paravertebral area on two pigs. Four wounds each were randomly assigned to eleven treatment groups and inoculated with *S. aureus* ATCC 6538. Wounds were treated with approximately 200 mg of each treatment. On day 2, wounds were assessed using a flush and scrub technique to recover planktonic and biofilm-associated bacterial count, respectively.[8,9,10] All treatment groups at differing concentrations of DTPA alone reduced bacterial counts (planktonic and biofilm) as compared to untreated control wounds. The highest concentration of DTPA (30000 µM) alone showed the largest reduction of both planktonic and biofilm bacterial counts as compared to other DTPA concentrations. DTPA at 5000, 10000 and 30000 µM concentrations when combined with Gentamicin showed a 99.99% reduction of *S. aureus* planktonic bacterial count as compared to untreated wounds. The highest concentration of DTPA (30000 µM) combined with Gentamicin also showed the highest percentage of reduction (99.99%) in *S. aureus* biofilm bacterial counts compared to untreated wounds. These studies suggest that DTPA, at concentrations of 2500 to 30000 µM, in combination with Gentamicin showed a reduction in both planktonic and biofilm-associated *S. aureus* as compared to Gentamicin alone, suggesting a strong synergistic effect of this compound with this antibiotic. The use of novel therapies that can reduce the bacterial load of wounds would have significant clinical implications.

Introduction:

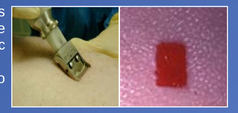
A leading factor in the pathogenesis of chronic wounds is bacterial infection. Bacteria colonizing wounds can evoke a persistent inflammatory response which is deleterious to the healing process. Cells like neutrophils and macrophages upregulate pro-inflammatory cytokines like IL-1 and TNF-α, which in turn lead to elevated levels of matrix metalloproteinases (MMPs), decreased growth factor expression, and ultimate aberration of the healing process.[11,12,13] One of the most common species of bacteria cultured from chronic wounds is *Staphylococcus aureus*.[1,2,3] *S. aureus*, as well as many other species of bacteria, have the ability to encase itself in an extracellular polysaccharide matrix (EPS) called a glycocalyx. Once a population adopts this sessile phenotype, it is substantially more resistant to host defense mechanisms as well as exogenous antimicrobials.[4] Novel methods to overcome the protective characteristics of biofilms are therefore very desirable. One viable treatment option is a zinc chelator named DTPA. The theoretical efficacy of DTPA against biofilms bases itself on *S. aureus* requirement of zinc to create the intercellular adhesion quality of a biofilm. By introducing DTPA into the environment of *S. aureus*, the chelator can bind zinc, sequestering it from the bacteria and thereby inhibit biofilm formation.[6] *S. aureus* preserved in the planktonic state can then be more easily targeted and eradicated by traditional, topical antimicrobials.[9] The goal of our study was to test the ability of DTPA to inhibit *S. aureus* biofilm formation and determine the concentration in which it is most effective using a standardized porcine wound model. Furthermore, we assessed its effects when used in combination with a common topical antibiotic, Gentamicin.

Materials & Methods:

Swine (2 animals) were used as our experimental animal due to the morphological, physiological, and biochemical similarities between swine skin and human skin

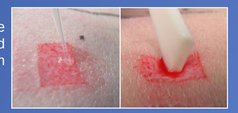
Animal Wounding:

- 44 deep partial thickness wounds measuring 10mm x 7mm x 0.5mm were made in the peravertebral and thoracic areas of each animal
- 4 wounds were randomly assigned to each of the 11 treatment groups

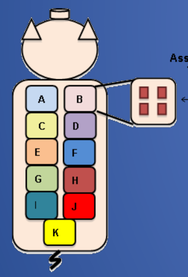


Inoculation:

- 25 µl of inoculum of 10^6 CFU/ml were inoculated into each wound and scrubbed lightly using a sterile Teflon spatula.



Experimental Design:



Pig 1: Treatment Groups

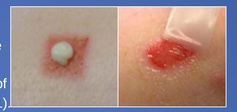
A. 30 µM DTPA
B. 100 µM DTPA
C. 300 µM DTPA
D. 1000 µM DTPA
E. 30 µM DTPA + Gentamicin 0.1%
F. 100 µM DTPA + Gentamicin 0.1%
G. 300 µM D TPA + Gentamicin 0.1%
H. 1000 µM DTPA + Gentamicin 0.1%
I. Vehicle
J. Gentamicin 0.1%
K. Untreated

Pig 2: Treatment Groups

A. 2500 µM DTPA
B. 5000 µM DTPA
C. 10000 µM DTPA
D. 30000 µM DTPA
E. 2500 µM DTPA + Gentamicin 0.1%
F. 5000 µM DTPA + Gentamicin 0.1%
G. 10000 µM D TPA + Gentamicin 0.1%
H. 30000 µM DTPA + Gentamicin 0.1%
I. Vehicle
J. Gentamicin 0.1%
K. Untreated

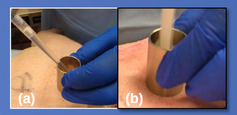
Treatment Application:

- Within 20 minutes of inoculation each wound was treated with the appropriate treatment.
- Each wound was treated with ≅200mg of the appropriate treatment (days 0 and 1).
- All wounds were covered with polyurethane film dressings.



Wound Recovery:

- A sterile surgical steel cylinder (22 mm inside diameter) was placed over the wound area and 1 ml of all purpose neutralizer solution was pipetted inside.
- The site was gently flushed by pipetting in and out three times to remove the loosely attached bacteria(a), this aliquot represents the planktonic bacteria.
- The same wound was then encircled using another sterile cylinder and 1 ml of all purpose neutralizer solution was again pipetted into the cylinder and this time scrubbed with a sterile Teflon spatula for 30 seconds to remove the firmly attached bacteria (b), this aliquot was aspirated and represents biofilm bacteria.

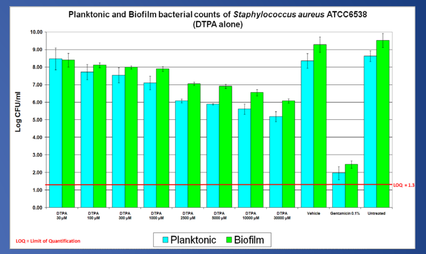


Bacterial Quantification:

- Serial dilutions (c) were made and quantified using the Spiral Plater System (d) (which deposits a defined amount (50µl) of suspension over the surface of a rotating agar plate).
- *Staphylococcus aureus* was grown on differential and selective medium Mannitol Salt Agar (e) at 37±2°C for 24 hours.

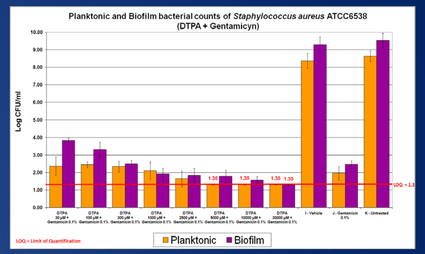
Results :

Wounds Treated with DTPA alone:



- The wounds treated with 30000 µM DTPA contained the lowest counts of *S. aureus* compared to all other concentrations of DTPA.
- Treatment with this concentration resulted in a 99.96% reduction compared to the untreated group.
- Increases in DTPA concentration were directly proportional to the increases in the percentage of both planktonic and biofilm bacterial reduction.
- All wounds, with the exception of those treated with 30 µM DTPA, contained more *S. aureus* in the biofilm phenotype than in a planktonic state.
- The 0.1% Gentamicin positive control produced substantially more reduction in wound bioburden than any of the DTPA concentrations alone.

Wounds Treated with DTPA/Gentamicin Combination:



- The lowest planktonic *S. aureus* counts were seen in wounds treated with 5000, 10000, and 30000 µM DTPA + Gentamicin 0.1% Compared to the untreated wounds, all three concentrations reduced bioburden by 99.99% to levels below the limit of quantification.
- The 30000 µM DTPA + Gentamicin 0.1% resulted in the lowest amount of bacteria in the biofilm phenotype, reducing counts to below the limit of quantification.
- Reductions of planktonic and biofilm bacteria were directly proportional to the concentrations of DTPA used.
- All wounds, with the exception of those treated with 1000 µM DTPA + Gentamicin 0.1%, contained more *S. aureus* in the biofilm phenotype than in the planktonic state.
- The 2500 µM, 5000 µM, 10000 µM, and 30000 µM DTPA + Gentamicin 0.1% treatments all resulted in both planktonic and biofilm counts lower than in the wounds treated with only Gentamicin 0.1%.

Conclusions:

- The increased reduction of *S. aureus* within wounds was directly proportional to the increased concentration of DTPA (with and without Gentamicin 0.1%).
- The 30000 µM concentration of DTPA used alone produced a planktonic bacterial count of 5.19±0.29 Log CFU/ml and a biofilm count of 6.09±012 Log CFU/ml, whereas 30000 µM DTPA used in conjunction with Gentamicin 0.1% resulted in wounds containing planktonic and biofilm bacterial loads below the level of quantification (1.30 Log CFU/ml). These were over a 3.89±0.29 and 4.79±01.2 Log differences in colony forming units per milliliter, respectively.
- The 2500 µM, 5000 µM, 10000 µM, and 30000 µM DTPA + Gentamicin 0.1% treatments produced larger reductions in planktonic and biofilm bacterial counts of *S. aureus* than the Gentamicin 0.1% alone.
- These data suggest that the zinc chelator, DTPA, has an antimicrobial effect that is synergistic when used in combination with Gentamicin.
- Additional studies on the effectiveness of DTPA with other antimicrobials to reduce infections are warranted.

Study Sponsor:
3G BioTech, LLC
Boston, MA

Contact Information:
Stephen C Davis
Professor
University of Miami, Miller School of Medicine
Dept of Dermatology and Cutaneous Surgery
Sdavis@medmiamiedu
Ph: 305-243-4897

References:

1. Sahm DF Marsilio MK, Piazza G Antimicrobial resistance in key bloodstream bacterial isolates: electronic surveillance with the Surveillance network database – USA Clin Infect Dis 1999, 29:259263
2. Fazli M, Bjarnsholt T, KirketerpMøller K, Jørgensen B, Schou Andersen A, Krogfelt K, Givskov M, and TolkerNielsen T Nonrandomized distribution of Pseudomonas aeruginosa and Staphylococcus aureus in chronic wounds *Journal of Clinical Microbiology* 2009, Epub
3. KirketerpMøller K, Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, TolkerNielsen T, Høiby N, Givskov M, Bjarnsholt T Distribution, organization, and ecology of bacteria in chronic wounds *J Clin Microbiol* 2008 Aug; 46 (8):271722 Epub 2008 May 28
4. Costerton JW, Stewart PS and Greenberg EP Bacterial biofilms: A common cause of persistent infections *Science* 1999, 284:13181322
5. Otto M Staphylococcal biofilms *Curr Top Microbiol Immunol* 2008, 322:207228
6. Conrady D, Brescia C, Horii K, Weiss A, Hassett D and Herr A: A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms *PNAS* 2008, 105(49):1945661
7. Geoghegan JA, Corrigan RM, Gruszka DT, Speziale P O'Gara JP, Potts JR, and Foster TJ Role of surface protein SasG in biofilm formation by Staphylococcus aureus *J Bacteriol* 2010, 192(21):566373
8. Serralta VW, HarrisonBalestra C, Cazzaniga AL, Davis SC, and Mertz PM Lifestyles of bacteria in wounds: presence of biofilms? *Wounds* 2001, 13(1):2934
9. Davis SC, Ricotti C, Cazzaniga AL, Welch E, and Mertz PM Microscopic and physiological evidence for Biofilm Associated Wound Colonization *Invivo Wound Repair and Regen*: 2008, 16(1):239
10. Martineau L and Davis SC Controlling Methicillin Resistant Staphylococcus aureus and Pseudomonas aeruginosa wound infections with a novel biomaterial *J Invest Surgery* 2007, 20; 217227
11. Konturek PC, BZozowski T, Konturek SJ, Kwiecien S, Dembinski A, Hahn EG Influence of bacterial lipopolysaccharide on healing of chronic experimental ulcer in rat *Scan J Gasteroenterol* 2001, 36(12):123947
12. Ladwig GP, Robson MC, Liu R, Kuhn MA, Muir DF, Schultz GS Ratios of activated matrix metalloproteinase9 and tissue inhibitor of matrix metalloproteinase1 in wound fluids are inversely correlated with healing of pressure ulcers *Wound Repair Regen* 2002, 10(1):2637
13. Power C, Wang JH, Sookhai S, Street JT, Redmond HP Bacterial wall products induce down regulation of vascular endothelial growth factor receptors on endothelial cells via a CD14dependent mechanism: implications for surgical wound healing *J Surg Res* 2001, 101(2):13845