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Mix populations of 2 strains for *Pseudomonas aeruginosa* combined with MRSA after nitric oxide effect in porcine deep partial thickness wound model

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Abstract:

Wounds are colonized frequently by heterogeneous microflora.[1,2] Pseudomonas aeruginosa (PA) and Staphylococcus aureus (SA) are two of the most isolated bacterial species from wounds, and both typically form highly organized biofilms.[3] Nitric oxide (NO) is a short-lived, diatomic, lipophilic gas with antimicrobial activity.[4] Recently, NO and its derivatives have been shown to exhibit broad-spectrum antimicrobial activity against bacteria, viruses, and parasites.[5,6] Using Pseudomonas aeruginosa (a military isolate PA09-010 and a ATCC 27312 strain) were combined with Methicillin Resistant Staphylococcus aureus MRSA USA300 to demonstrate the efficacy of the NO to reduce polymicrobial infected wounds. Fifty-nine (59) deep partial-thickness wounds (10mm x 7mm x 0.5mm) were made with a specialized electrokeratome. Wounds were inoculated with MRSA USA300 in one animal combined with PA09-010 and with PA27312 in the other, then wounds were covered with polyurethane film dressings to allow for biofilm formation. After 48hours three wounds were recovered for baseline enumeration. Wounds were treated with several NO formations with various release properties. Wounds were recovered for bacterial bioburden on day 7. All treatments reduced MRSA bacterial counts compared to baseline counts, however the bacterial counts either for PA09-010 and PA27312 were lowest compared to baseline counts. The largest difference in efficacy against two strains of bacteria was the NO Formulation: Low/Fast. This treatment reduced the MRSA USA300 bacteria down to 4.47±0.28 Log CFU/ml but only reduced the PA 09-010 bacteria down to 8.87±0.56 Log CFU/ml. Results showed that the same formulation NO Low/Fast, reduced less MRSA USA300 when was combined with PA27312. These studies demonstrate that NO formulations reduce the mixed microbial burden of multiple microorganisms. Other studies have been demonstrated that NO formulations have better efficacy against PA than MRSA.[7] We have previously shown MRSA was significantly reduced when was inoculated together with PA09-10.[8] Simultaneous presence of mixed populations of bacteria in wounds may favor one species survival over the other.[2] A better understanding of mechanisms of host-bacteria interactions, in single or mixed species biofilms, may lead to development of novel therapeutic approaches to treat wound infections.

Introduction:

Infections in wounds can delay healing and lead to systemic complications. Biofilm produced by pathogens protects these organisms from immune response and antibiotics. Additionally, wounds infections are often diverse polymicrobial communities, with interactions that may increase virulence and resistance to treatment.[9] Other investigators has sown the antimicrobial efficacy of Nitric Oxide against common pathogens in wounds, inoculated with *S. aureus* and *P. aeruginosa*.[10] This study investigated the antimicrobial efficacy of various topical Nitric Oxide releasing formulations against wound infections by mixed populations of *P. aeruginosa* and methicillin-resistant *S. aureus* with mature biofilm.

References

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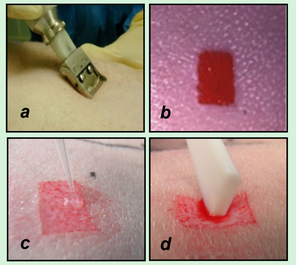
Materials and Methods:

1. Experimental Animals:

Swine (2) were used as our experimental animal due to the morphological, physiological, and biochemical similarities between porcine skin and human skin.[11]

2. Wounding Technique:

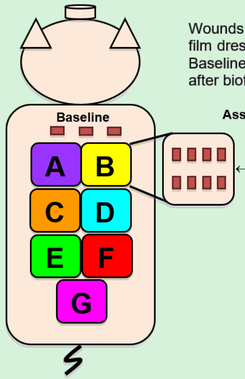
- An electrokeratome (photo a) was used to create 59 deep partial-thickness wounds measuring 10 mm x 7 mm x 0.5 mm (photo b) on the paravertebral and thoracic area of each animal.



3. Inoculation:

- Suspensions were prepared by combining 10^6 CFU/mL equal volumes of MRSA USA300 and PA 09-010 for one animal, and MRSA and PA 27312 for the other animal.
- After wound creation, 25 µl of the mixed inoculum suspension (photo c) was used to inoculate each wound by scrubbing the mixed inoculum by 30 seconds into each wound with a teflon spatula (photo d). Wounds were covered with a polyurethane film dressing to allow for biofilm formation.[2]

4. Experimental Design:



Wounds were covered with a polyurethane film dressing (Tegaderm, 3M, St. Paul, MN). Baseline wounds were recovered 48 hours after biofilm formation.

Assessment Time

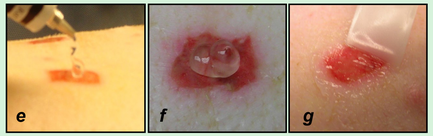
← Day 7

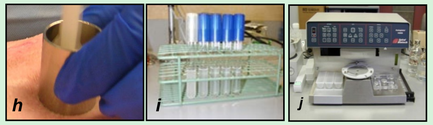
Treatment Groups

A. Negative Control: **Vehicle**
B. NO Formulation**: High**
C. NO Formulation**: Mid/Fast**
D. NO Formulation**: Mid/Slow**
E. NO Formulation**: Low/Fast**
F. NO Formulation**: Low/Slow**
G. Positive Control (**Mupirocin**)

5. Treatment Application:

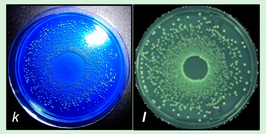
- Test formulations were applied over the surface of the infected wounds daily after biofilm formation. (photo e)
- Each formulation (≅ 200 mg) was applied to cover the wounded area and surrounding unwounded skin.
- Formulations were sprod out gently using a sterile Teflon spatula (photo g) and covered with a polyurethane film dressing (Tegaderm, 3M).





6. Wound Recovery:

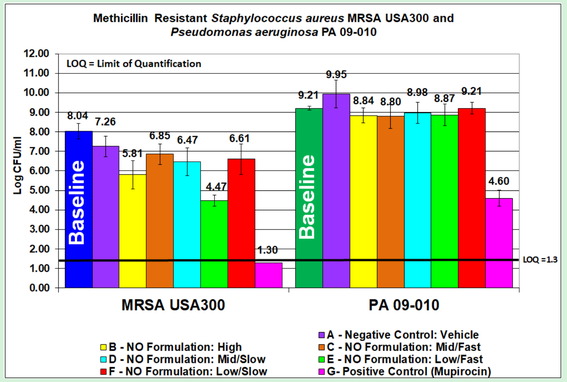
- Baseline wounds were recovered 48 hours after inoculation prior to treatment. On day 7 after inoculation, eight (8) wounds per treatment group group were recovered from each animal.
- One (1) mL of neutralizer solution was pipetted into a sterile steel cylinder at the center of each wound and scrubbed with a sterile Teflon spatula for 30 seconds (photo h).
- Serial dilutions were made (photo i) and quantified using the Spiral Plater System (which deposits a defined amount (50µL) of suspension over the surface of a rotating agar plate (photo j)
- MRSA USA300 was isolated on ORSAB (Oxacillin Resistance Screening Agar Base photo k) and *P. aeruginosa* strains were isolated on Pseudomonas Agar with CN supplement (photo l). Plates were incubated at 37±2°C for 36-48 hours.
- The colony forming units per mL (CFU/mL) were calculated and comparison of the means was analyzed.



Results:

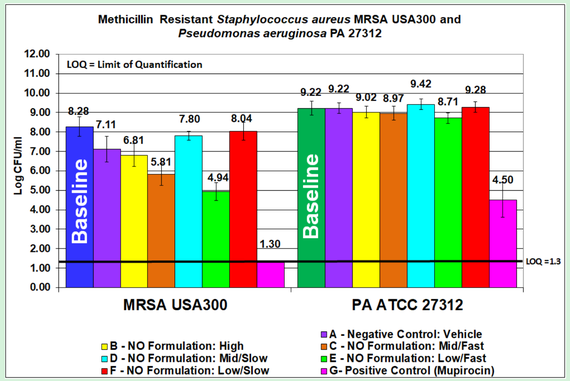
MRSA USA300 + PA 09-010

- 48 hours after inoculation, the baseline for MRSA USA300 + PA 09-010 was 8.04±0.40 Log CFU/mL for MRSA USA 300 and 9.21±0.09 Log CFU/mL for PA 09-010.
- On day 7, Mupirocin reduced MRSA compared to baseline (99.99%).
- Among NO treatments against MRSA USA300, the Low/Fast, High and Mid/Slow showed the highest bacterial reduction compared to baseline (3.57±0.12, 2.24±0.3 and 1.57±0.31 Log CFU/mL, respectively).
- These values represent a 99.97%, a 99.42% and a 97.31% of bacteria reduction, respectively.
- All NO formulations had lower MRSA burden than vehicle control.
- All NO formulations reduced PA 09-010 counts compared to vehicle control at day 7. The greatest reduction among NO formulations was observed for the Mid/Fast formulation, followed by High, and Low/Fast formulations, respectively.



MRSA USA300 + PA 27312

- 48 hours after inoculation, the baseline for MRSA USA300 + PA 27312 was 8.28±0.51 Log CFU/mL for MRSA USA 300 and 9.22±0.36 Log CFU/mL for PA 09-010.
- On day 7, Mupirocin reduced MRSA compared to baseline (99.99%).
- Among NO treatments against MRSA USA300, the Low/Fast, Mid/Fast and High formulations showed the highest bacterial reduction compared to baseline (3.34±0.06, 2.47±0.05 and 1.47±0.07 Log CFU/ml, respectively]
- These values represent a 99.95%, a 99.66% and a 96.58% of bacteria reduction, respectively.
- Low/Fast, Mid/Fast and High formulations had lower MRSA burden than vehicle control.
- NO formulations (Low/Fast, Mid/Fast and High formulations) reduced PA 27312 counts compared to vehicle control at day 7.



Conclusions

- Overall, the amount of MRSA USA300 bacteria recovered from the wounds was much less, in every treatment group, than either of the *Pseudomonas aeruginosa* strains (PA 09-010 and PA 27312).
- The most drastic difference in efficacy against two strains of bacteria was the NO Formulation: Low/Fast. This treatment reduced the MRSA USA300 bacteria down to 4.47±0.28 Log CFU/ml but only reduced the PA 09-010 bacteria down to 8.87±0.56 Log CFU/ml.
- These results demonstrate that the Low/Fast formulation contains properties that differentiate between the metabolism of MRSA USA300 and PA 09-010 / PA 27312.
- Studies on the effects of the Nitric Oxide against other pathogenic mixed microorganisms and its effect on healing is warranted.

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