Antibacterial Properties of an Iron-based Hemostatic Agent In Vitro and in a Rat Wound Model

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Abstract

Objectives: Topical hemostatic agents are currently employed on the battlefield for control of major hemorrhage and have potential for use in civilian settings. Some of these compounds may also be antibacterial. Given the behavior of these compounds, the purpose of this study was to assess the potential antibacterial properties of an iron oxyacid–based topical hemostatic agent against three problematic species of wound-contaminating microorganisms: *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus epidermidis*.

Methods: Bacteria were treated in vitro with the test powder for 30 minutes and then assessed for viability. Long-term (8-hour) inhibition of bacterial growth was also examined. In vivo, a rat full-thickness 1-cm^2 skin wound was studied. Wounds were contaminated, treated, and then quantitatively cultured 24 hours later.

Results: The lethal dose for 99% of the organisms (LD_{99}) for the compound against each organism ranged from 0.89 (±0.28) to 4.77 (±0.66) mg/mL ($p < 0.05$). The compound produced sustained inhibition over 8 hours at both 1 and 5 mg/mL ($p < 0.05$ for each), for *P. aeruginosa, S. epidermidis,* and *S. aureus*. In vivo, activity was noted against only *P. aeruginosa*, with the largest magnitude reduction being on the order of 3-log colony-forming units (CFU; $p < 0.01$).

Conclusions: The iron-based agent studied possesses significant in vitro and lesser in vivo antibacterial effects. Further optimization of the delivery, dosing, and evaluation of this agent in a larger animal model with more humanlike skin structures may reveal important wound effects beyond control of bleeding.

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Injuries associated with major blood loss are important sources of death and disability among civilian and military trauma victims. Approximately 20% of combat deaths due to exsanguination occur as a result of a potentially accessible or compressible injury, and large vascular injuries of the extremities are a leading cause of preventable death.[1] While not as common, preventable death from isolated extremity injuries also occurs following civilian trauma.[2] Hence, the development of an easily applied hemostatic agent has great potential to reduce morbidity and mortality. Additionally, such an agent might help to preserve a blood supply that is already limited both in the battlefield and in civilian settings.

One class of topical procoagulants is the iron oxyacids. Based on FeO^{42-} salts, the agents are water soluble and are powerful oxidants that degrade quickly upon hydration, according to the following reaction: $4FeO_4^{2-} + 10H \rightarrow 4Fe(OH)_3 + 3O_2 + 8OH^{-3}$

These compounds have been successfully tested in applications such as water disinfection, degradation of organic and inorganic pollutants, and sewage treatment.[4,5] In these applications, advantages of iron oxyacids over other disinfectants and oxidants include nontoxic by-products and direct antimicrobial proper-

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ties, raising the possibility that they might be employed in wound care, as well.[5–7]

Given the behavior of these compounds, the purpose of this study was to assess the potential antibacterial properties of an iron oxyacid–based topical hemostatic agent against three problematic species of wound-contaminating microorganisms: *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus epidermidis*. In particular, we addressed the following hypotheses. First, given that one proposed mechanism of action of the compound is the release of oxidative species during hydration, we proposed that 30 minutes of exposure, in vitro, of bacteria to material during hydration (as would occur when the material was placed in a contaminated wound) would reduce the number of viable organisms. Second, given that iron compounds may produce antimicrobial effects independent of the oxyacid hydration chemistry outlined above, we hypothesized that addition of fully hydrated test material to actively growing liquid cultures would curtail bacterial growth over time. Last, we argued that the material, when applied to a contaminated wound, would reduce the number of viable bacteria present in that wound 24 hours after treatment. An additional goal throughout these studies was to generate an initial estimate of the magnitude of effect in different in vitro and in vivo models to allow informed study design (e.g., sample size calculations) in future work.

METHODS

Study Design

This was a laboratory study utilizing both in vitro and in vivo models to assess the antibacterial properties of a hemostatic agent. All animal procedures were approved by our institutional committee on the use and care of animals as being in compliance with local and federal regulations.

Animal Subjects

Young female specific pathogen-free Sprague-Dawley rats (250–300 g) were kept in standard housing with food and water ad libitum and 12-hour light/dark cycles.

Study Protocol

In Vitro Studies

Bacterial Strains. *P. aeruginosa* PA07,[8] *S. epidermidis* (ATCC No. 700565), and *S. aureus* (ATCC No. 43300) were used. Both staphylococcal strains are methicillin-resistant. Liquid and solid cultures were grown in Luria-Bertani (LB) broth and agar, respectively.

Test Compound. The test compound was a proprietary topical hemostatic agent provided by the manufacturer (Biolife LLC, Sarasota, FL). In vitro doses were based on empiric preliminary experience (data not shown) of what concentrations of agents would produce a measurable dose response.

Hydration-associated In Vitro Antibacterial Activity. Liquid cultures were washed twice then resuspended in sterile water to optical density (OD_{600nm}) = 0.1, corresponding to approximately 10^8 colony-forming units (CFU)/mL for each strain. These were added to tubes containing dry compound such that the final concentration of compound would equal 0, 0.1, 0.5, 1.0, 5.0, 10, and 50 mg/mL, respectively. Preliminary experience indicated very good intersample agreement in this range of doses, and each experimental condition was repeated in triplicate for a total of 21 measurements per species. The samples were vortexed and allowed to stand for 30 minutes at room temperature. Each sample was then revortexed, and quantitative culture on LB agar was performed, with a lower limit of detection of 100 CFU/mL.

Persistent In Vitro Bacteriostatic Activity. The test compound was added to 10 mL of LB medium and allowed to hydrate for 30 minutes with frequent mixing. Tubes were then inoculated and incubated at 37°C and 200 rpm for 8 hours. Experiments were also performed in triplicate. Bacterial proliferation was measured as sample turbidity at 600 nm, during incubation, using a visible-wavelength spectrophotometer (Ultrospec 2100 pro, Amersham Pharmacia, GE Healthcare, Piscataway, NJ).[8,9] Results were reported as normalized turbidity: the OD_{600} of the treated sample divided by the OD_{600} of a simultaneously studied untreated control culture.

In Vivo Study

Rat Wound Contamination Model. The animal model used for the experiments has previously been described.[9,10] Although important differences in dermal anatomy exist between rodents and humans, the aim of this work was to provide a living dermal system that could be easily replicated in sufficient numbers to allow quantitative preliminary analysis of compound effectiveness. We also draw a distinction here between wound contamination and wound infection. In the treatment timeline described below, the material was applied to wounds that were contaminated (i.e., contained pathogenic organisms) but not yet infected (i.e., contaminated and displaying an inflammatory host response). While by these definitions the material harvested at 24 hours for culture was technically infected, we use the operational term contaminated throughout to describe the wound model used in our study.

Young female specific pathogen-free Sprague-Dawley rats (250–300 g) were anesthetized with oxygen with 1% isoflurane, and hair from the skin overlying the dorsal cervical spine and interscapular region was removed with an electric razor. Next, a 1-cm-diameter circular full-thickness piece of skin was excised. A bacterial inoculum (n = 5 rats per bacterial strain) of 2.5×10^8 CFU suspended in 250 μL of sterile saline was injected into the loose subcutaneous connective tissue.

The dry test compound was administered as it would be in the field, by generously applying the powder to the entire surface of the wound. Moisture from the subcutaneous tissue led to a brittle but adherent shell on the wound surface within a few minutes of application. Accordingly, the primary means of standardizing treatment was by carefully controlling the size of the

wound (and thus the moist surface area available for compound contact) rather than by controlling the amount of agent itself. A sterile piece of Telfa nonadherent dressing (Tyco Healthcare/Kendall, Mansfield, MA) was affixed to the wound with four sutures. Animals were recovered from anesthesia and returned to standard housing.

After 24 hours, animals were euthanized under anesthesia and the sterile dressing was removed from the wound. The entirety of the contaminated subcutaneous tissue, including any adherent test compound, was debrided down to but not including the interscapular paraspinous muscles. The excised material was homogenized and used for quantitative culture as described above. In other animals (n = 3 per condition), the contaminated wound and the surrounding uncontaminated skin and muscle were excised en bloc and preserved in formalin for hematoxylin and eosin staining.

Data Analysis

In vitro bacterial killing assays were analyzed and reported as the lethal dose for 99% of the organisms (LD_{99}) concentration[11] of the test compound for each organism (the concentration which would be expected to kill 99% of the organisms present). As we measured a range of agent concentrations spanning 0 to 50 mg/mL, the LD_{99} was determined by linear interpolation between the compound concentrations killing just less and just more than 99% of the bacteria present in an untreated control sample. For sustained killing assays, normalized turbidity was recorded as the OD_{600} of a treated sample divided by the OD_{600} of an untreated control bacterial inoculum. Normalized turbidities in which the mean value was more than two standard deviations (SD) below 1 were considered statistically significant. Comparisons of wound bacterial burden between untreated animals and animals treated with the test compound were performed on log-transformed data using Student's t-tests.

RESULTS

Immediate In Vitro Antibacterial Activity

The test compound demonstrated bactericidal activity when combined with organisms in liquid culture. No organisms of any species were recovered by quantitative culture at exposure levels of 5 mg/mL or more at 30 minutes duration. We report the estimated dose required to produce 99% killing (Figure 1A).

Persistent In Vitro Bacteriostatic Activity

The agent also demonstrated sustained bacteriostatic effects. When bacterial colonies were inoculated into liquid medium containing fully hydrated material, inhibition of bacterial growth, as measured by liquid culture turbidity, was seen at both 1 and 5 mg/mL concentrations of the compound for 8 hours (Figure 1B).

In Vivo Performance in a Rat Wound Model

The compound was also used to treat wounds contaminated with each of the three bacterial pathogens (Figure 1C). There was an approximately 1,000-fold

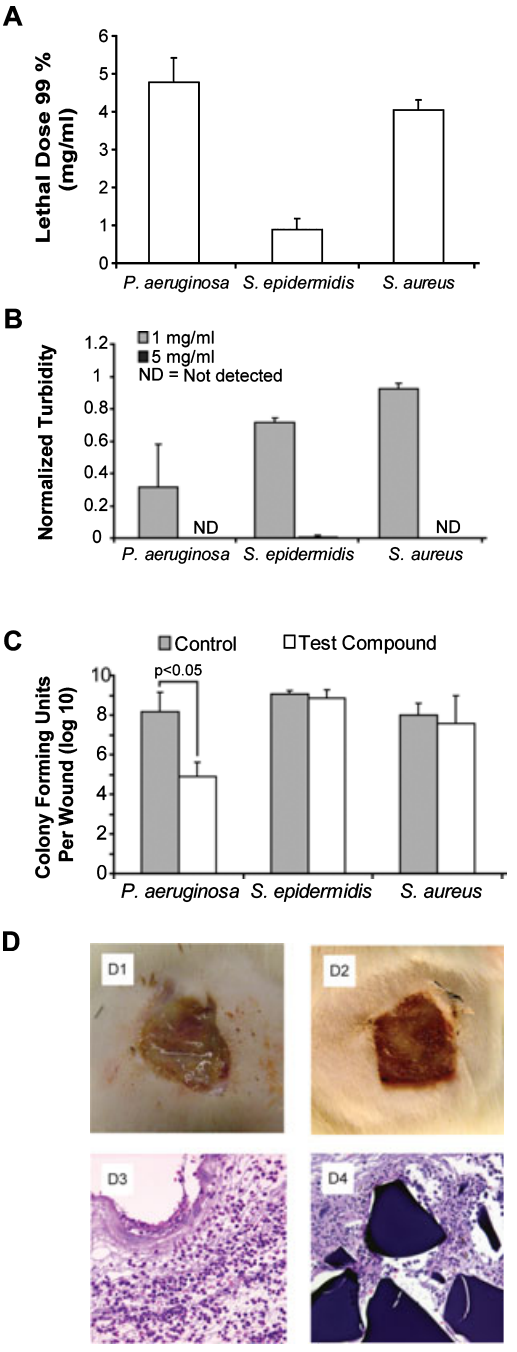

Figure 1. (A) Lethal dose for 99% of the organisms (LD_{99}) concentrations (mg/mL) of the test compound against three bacterial strains. n = 3 replicates per condition. Bars represent SD. (B) Inhibition of bacterial growth following exposure to prehydrated material. Eight-hour sample turbidity was measured and normalized for turbidity of control samples containing test compounds but no bacteria. In each case, the mean value was more than 2 SDs below 1.0, i.e., the value of no effect. (C) In vivo bacterial killing results. n = 5 in each case. (D) D1, appearance of untreated wound 24 hours after inoculation with 10^8 CFU of *P. aeruginosa.* D2, identically contaminated wound treated with the test agent. A dry shell is seen covering the denuded tissue. D3, 40× hematoxylin and eosin stain of section through untreated wound showing marked infiltration with inflammatory cells. D4, similarly prepared section through a treated wound showing incorporation of particles of the test compound into the inflammatory debris.

reduction in wound colonies of *P. aeruginosa (*$p < 0.05$), but only very modest (and statistically insignificant) reductions with *S. epidermidis* and *S. aureus*. In a subset of animals, the entirety of the wound was excised for histology. Representative images are shown in Figure 1D. The material harvested was essentially a neutrophil-rich exudate into the subcutaneous space, with no other cell types being present to any significant extent. For that reason, no formal semiquantitative scoring of the material was performed.

DISCUSSION

We examined the antimicrobial effects of a topical hemostatic agent in three assay systems including an in vivo wound model. Against three important wound pathogens, *P. aeruginosa* and methicillin-resistant strains of *S. epidermidis* and *S. aureus,* we noted significant in vitro activity of these materials. In vivo effects were seen only against *P. aeruginosa*. Nevertheless, the current work represents a further step toward application of this material as a wound disinfectant in the setting of wound-associated blood loss. Among the issues remaining to be explored is to what extent these findings can be translated into human experience. Wounds treated with these compounds would presumably contain large amounts of clotted blood and other debris that would alter the nature of the contact between compound and contaminating pathogen.

Previous reports have noted the bactericidal and viricidal activity of iron oxyacids under various conditions.[7,12–14] The nontoxic nature of their hydration products has led to their use in water treatment and flocculation.[4–6,15] Our experiments in liquid culture support these observations. Although the necessary dose of hemostatic agent varied between pathogens, bacteria present at the moment of compound hydration were effectively killed. This effect extended beyond immediate chemistry associated with hydration; fully hydrated test material demonstrated persistent suppression of bacterial proliferation for 8 hours.

The ability of the test agent to reduce bacterial burden in wounds contaminated with the Gram-negative organism *P. aeruginosa* was substantial. We witnessed an approximately 3-log reduction in CFU of *P. aeruginosa*. This effect size is 10-fold larger than what the U.S. Food and Drug Administration considers a clinically relevant effect for nonspecific skin decontaminants such as surgical prep solutions.[16] This magnitude of improvement was not seen against the two Gram-positive organisms tested. Further work is needed to determine if this is due to a difference in bacterial susceptibility to these compounds within wounds as a function of cell wall composition.

The unique nature of the application of this material raises considerable methodologic challenges. Unlike liquids or creams, the agent tested is a dry powder that hardens as a shell over the treated area. Accordingly, methods used to standardize the delivery of these other treatments are not fully meaningful when applied to the test agent in this report. Measuring bacterial burden in its traditional sense (CFU/tissue mass) is also problematic as the adherent iron-laden material represents the majority of the mass of any sample taken. For this reason, we report our results as CFU per wound (where the wound size and inoculum size have been standardized) rather than in the more conventional CFU/g that is often reported. We are unaware of an alternative approach in the literature and acknowledge this as an inherent limitation of the study of this material in vivo.

LIMITATIONS

These results suggest a potentially useful antibacterial effect against common and problematic organisms in a clinically important setting. Its pilot nature was intended to establish feasibility, and several experimental limitations remain that may warrant future study. First, we studied only three bacterial species, one Gram-negative and two Gram-positive. While we are unaware of any unique susceptibility of *Pseudomonas* or *Staphylococcus* to iron compounds, the effectiveness of the tested material against unexamined species cannot be commented on. Second, we studied a wound contamination model wherein noncommensal bacteria were present in wounds that had not yet had time to mount an inflammatory response. This situation is the one likely to be encountered at the time of initial treatment of significant soft tissue injury. Our results should not be extended to use of the material against wounds in which infection (e.g., contamination and a coexisting inflammatory response) is already present.

The primary application of iron oxyacids in this context is as fast-acting hemostatic agents to be used in the setting of difficult-to-control external hemorrhage. Our wound model is simpler than what would be required to fully reproduce a life-threatening wound with hemorrhage and contamination. We are not able to comment on what effect a very large volume of blood in the wound would have on antibacterial effects.

The rat wound model used, while practical for initial studies, is not as structurally similar to human clinical conditions as other larger, more expensive animal models such as porcine wound systems.[17] Unlike porcine models where pretherapeutic biopsies can be performed to assure equal bacterial inoculation between groups, this is not structurally feasible in the rat cutis. Standardization of our inoculum sizes in vivo could only be ensured by careful introduction of a fixed volume of bacteria-containing media, a method employed with success by others when dealing with exotic topically applied materials in the rat cutaneous contamination model.[18] Furthermore, the nature of the test compound is as a dry powder applied to a damp wound. The effective dose is therefore a function of wound surface area, moisture content, and the capacity of the material to remain adherent to the site during treatment. We were able to specifically control for the first feature, but have no reason to suspect significant differences in moisture content between rats at the time of application. While we were able to produce a standardized wound area, the interscapular area was the only location where a wound could be developed ''out of reach'' of the animal during its 24-hour treatment. As a result, animals were unable to serve as their own controls by the use of mul-

tiple wounds in individual test subjects, as is often done in larger animal models.

CONCLUSIONS

We found significant antibacterial properties of an iron-based hemostatic agent against three important clinical pathogens in an in vitro setting. In vivo effectiveness was limited to the one Gram-negative organism studied (*P. aeruginosa)*. These data serve as a useful launching point for further examination of the potential value of this compound.

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