Effects of Myeloperoxidase on Methicillin-Resistant *Staphylococcus aureus*-Colonized Burn Wounds in Rats

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Objective: To achieve better therapeutic results in burn wound infections and to examine alternatives to antibiotics, we designed this study to elaborate the role of myeloperoxidase (MPO) on infected burn wounds in rats.

Approach: We compared chemical properties as well as bacteriostatic ability of MPO in different concentrations with NeutroPhase. Subsequently, we applied MPO (MPO group), NeutroPhase (NeutroPhase group), NaCl+H₂O₂ (NaCl+H₂O₂ group), or NaCl (control group) on rat dorsal burn wounds inoculated with methicillin-resistant *Staphylococcus aureus* (MRSA). Their effects on MRSA-colonized wounds were evaluated by microscopy, histologic section, and Western blot.

Results: MPO produced more H⁺ and HClO⁻, leading to a more acidic environment. Moreover, MPO inhibited the growth of MRSA more intensely after 6 h of inoculation *ex vivo*. *In vivo* the open wound rate in the MPO group was significantly lower, while the contraction rate and epithelialization rate of MPO group were higher than that of the control group, NaCl+H₂O₂ group, and NeutroPhase group on day 20. The hematoxylin and eosin staining of MPO group showed better wound healing than other groups. More vascular endothelial growth factor (VEGF) was expressed in wound tissue of MPO group by Western blot.

Innovation: This is the first study to use MPO for MRSA-colonized burn wound therapy.

Conclusion: MPO displayed more effective bacteriostatic ability, possibly beneficial for MRSA-colonized wound healing.

Keywords: wound healing, MRSA, burn wound, MPO, bacteriostatic ability

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INTRODUCTION

WOUND REPAIR is a complex process, including inflammatory reactions, granulation tissue formation, and matrix remodeling.¹ Infection is a common problem following a burn wound, which could hinder the healing process.² The openness of burn wounds, body surface temperature,

and exudation of plasma components create ideal conditions for bacterial growth and reproduction, making the wounds become the gateway to pathogenic microorganism invasion.³ Together with the presence of localized necrotic tissue and compromised systemic immune function, it can easily lead to invasive infection complicated

by sepsis.^{4–6} *Staphylococcus aureus* is a leading cause of severe infections in burns throughout the world.⁷ Antibiotic resistance is one of the major issues in the management of *S. aureus* infections.⁸ Methicillin-resistant *S. aureus* (MRSA) is one of the most common pathogens in community and hospital.⁹ It has been identified as the leading cause of nosocomial infections in the burn sector, especially in developing countries.^{9,10} The morbidity, mortality, length of hospital stays, and costs of patients with MRSA burns are increasing in recent years.¹¹ In 2016, Parhizgari *et al.* found that the *mecA* gene showed 86.4% of *S. aureus* isolated from burn patients in a central burn hospital in the southwest of Iran were MRSA. The highest rate of resistance was observed for penicillin (97.5%) and erythromycin (77.8%). None of the isolates was resistant to vancomycin.¹² Due to the drug resistance of bacteria, the choice of antimicrobials now is more difficult.¹³ For this reason, nonantibiotic medication may be a good option.

Studies have shown that hypochlorous acid (HOCl) can disrupt the lipids of cell wall components, destroy the bacterial cell membrane, and block ATP production, thus disintegrating the biofilm and eliminating bacteria.¹⁴ Hypochlorous acid is the most powerful bactericidal oxidant of the innate immune system produced by neutrophils.¹⁵ Because of its oxidative bactericidal principle, it is not easy for bacteria to develop resistance to HOCl. NeutroPhase, a commercial liquid dressing with hypochlorous acid as the main active ingredient, has an obvious curative effect in treating infected burn wounds.¹⁴ However, the chemical nature of hypochlorous acid is not stable, so it is not easy to ensure and maintain the effective concentration of hypochlorous acid when used. To achieve better therapeutic results, alternatives to unstable HOCl products are sought.

Myeloperoxidase (MPO) can catalyze the production of hypochlorous acid. It is a heme-containing enzyme that consists of two dimers linked by disulfide bonds. Each dimer contains one beta-heavy chain subunit (59 kDa) and one alpha-subunit (14 kDa).¹⁶ MPO is stored in neutral granules of neutrophils and is not released until the neutrophil is activated or degranulated to achieve its corresponding physiological function, which oxidizes chlorine in the presence of hydrogen peroxide (H_2O_2) to catalyze the production of hypochlorous acid, that is, the main active ingredient of NeutroPhase.¹⁷ Wang *et al.* found that the ability of MPO to produce hypochlorous acid is dependent on its enzymatic activity, which is strongly influenced by glycosylation, and that glycan bound to rigid heme is likely to

alter its structure and increase MPO enzyme and antibacterial activity in the wound microenvironment.¹⁸ At present, MPO is mainly used in auxiliary detection of cardiovascular disease, as elevated MPO activity is associated with many diseases, including atherosclerosis, myocardial infarction, atrial fibrillation, multiple sclerosis, Alzheimer's, and transplant rejection.^{19–23} However, its effects on infected wound repair remain to be elucidated.

In summary, this study is intended to explore the role of MPO in the healing process of MRSA-colonized burn wounds. It may provide new ideas for the development of new antimicrobial agents for the treatment of traumatic infections.

CLINICAL PROBLEM ADDRESSED

MRSA is one of the most common pathogens in community and environments. It has been identified as the leading cause of nosocomial infections in the burn sector, especially in developing countries. The morbidity, mortality, length of hospital stays, and costs of patients with MRSA-colonized burns are increasing in recent years. In 2016, Parhizgari *et al.* found that the *mecA* gene showed 86.4% of the *S. aureus* isolated from burn patients in a central burn hospital in the southwest of Iran were MRSA.¹² The highest rate of resistance was observed for penicillin (97.5%) and erythromycin (77.8%). To achieve better therapeutic results of burn wound infections, it is urgent to look for new alternatives to antibiotics.

MATERIALS AND METHODS

Preparation of MPO and NeutroPhase Solution and measurement of ion concentration

MPO from human leukocytes was purchased from Sigma-Aldrich (St. Louis, MO). Ten nanomolars, 50 and 100 nM solutions were obtained by dissolving MPO in 0.9% NaCl and 3% H_2O_2 solution. NeutroPhase was purchased from NovaBay Pharmaceuticals (Emeryville, CA), the effective constituent is 0.01% HOCl. All solutions were immersed in a water bath. Subsequently, pH and H^+ ion concentrations were measured at 1, 2, 3, 4, 5, 6, 7, and 8 h.

MRSA activation

The MRSA standard strain (ATCC 43300), obtained from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), was inoculated on an agar plate and incubated at 37°C overnight. A single colony was then picked from an agar plate and diluted in 2 mL of tryptone broth, mixed well, and then placed at 37°C over-

night at 180 r/min in a shaking incubator. The next day, the bacterial culture was diluted 1:20. The TSB solution was shaken on a constant temperature of 180 r/min at 37°C for about 6 h to amplify the strain to a logarithmic phase, and the turbidimetric method was used to adjust the concentration of the bacterial solution to about 1.0×10^9 colony-forming unit (CFU)/mL.

Bacteriostasis experiment

The colonies of MRSA preserved at -80°C were inoculated on the inoculation loop and inoculated on the flat surface of sterile MH agar medium and cultured in a 37°C incubator for 24 h. After activation, the MRSA colonies were diluted with 0.9% sterile physiological saline. After shaking for 15 s, the bacterial suspension was adjusted to a concentration of 2.0×10^9 CFU/mL. The bacterial suspension was added to tubes with solutions containing 3% H_2O_2 + 0.9% NaCl, and then NeutroPhase, 10 nM MPO, 50 nM MPO, or 100 nM MPO, respectively. Culture medium was mixed to the bacterial suspension to obtain a final bacterial suspension concentration of 1.0×10^9 CFU/mL. Tubes were placed in a 37°C water bath. Drawing culture medium at 0, 6, 12, and 18 h, respectively, in 0.9% sterile saline to make a series of dilutions. Bacterial suspensions, 0.1 mL, from each tube were inoculated on sterile MH agar medium plates, placed in a 35°C incubator for 24 h, and then colonies were counted. Parallel tests were taken three times, and average results were recorded and used to generate $\log_{10}$ CFU/mL curve for the time (*i.e.*, time/sterilization curve).

Animal experiments

Male Sprague Dawley rats (8 weeks old) were purchased from the Laboratory Animal Center of Third Xiangya Hospital, Central South University, and housed at a specific pathogen-free environment under standard conditions. The animals were treated humanely. All animal experiments were conducted according to the approved animal protocols of the Third Xiangya Hospital of Central South University, in accordance with Animal Care and Use Committee protocols of Central South University. Four rats were used for each treatment group. All animals were individually housed and given 1 week for acclimatizing to laboratory conditions. Twenty-four hours before surgery, the dorsal hair was clipped and depilated (Nair; Church & Dwight Co., Princeton, NJ). On the day of surgery, rats were first anesthetized with an injection of 1% sodium pentobarbital (6 mL/kg) in the abdominal cavity. To create second-degree burns wounds, a cylindrical glass container with a

basal diameter of 1 cm was filled with hot water (94°C) and then placed on the dorsal skin of rats for 15 s.²⁴ Each wound was inoculated with 5×10^8 CFU of MRSA.

NaCl, NaCl+ H_2O_2 , MPO+NaCl+ H_2O_2 (MPO, 50 nM), and NeutroPhase treatments were prepared in gelatin hydrogel as a vehicle. Each wound was then covered with polyurethane dressings (Tegaderm; 3M, St. Paul, MN). Wound treatments and Tegaderm dressing changing were conducted three times per week. Rats were anesthetized with isoflurane inhalation (Isoflurane, USP; Novaplus, Lake Forest, IL) and photographed from a standard height. On day 7, half of the animals from each group were euthanized and the remaining animals were not euthanized until the wound healed. Entire wounds were harvested for further investigation, including a surrounding skin area of 0.5 cm.

Wound closure analysis

Two independent, blinded observers measured the initial areas of wound size on day 0, areas of wound contraction, reepithelialization, and the open wound using planimetric methods (ImageJ; National Institutes of Health, Bethesda, MD) over a time period of 14 days, two times per week. Wound kinetics was quantified as follows: open wound rate = open wound/initial area of wound size, contraction rate = contraction/initial area of wound size, and epithelialization rate = reepithelialization/(initial area of wound size – contraction).²¹

Figure 1. Morphology of MRSA colonies in each group at 6, 12, and 18 h (scale bar, 5 mm). MRSA, methicillin-resistant *Staphylococcus aureus*.

Histological analysis

The samples of central wound cross sections underwent routine histological processing at day 20 after operation (hematoxylin and eosin [H&E] stain; BioGenex). Skin biopsy was fixed with 4% phosphate-buffered formaldehyde, embedded in paraffin, sectioned, and stained with H&E for histopathological evaluation. All slides were coded and assessed in a blinded manner by a pathologist.

Western blot

Wound tissues were harvested when the open wound rate of the control group reached 50% on day 7. Lysates were prepared using the protein extraction reagent RIPA lysis buffer (Beyotime, Shanghai, China). Protein samples were loaded on

10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gels following the manufacturer's protocols (Bio-Rad, Hercules, CA) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and then probed with rabbit primary antibodies against vascular endothelial growth factor (VEGF; Abcam, Cambridge, MA) overnight at 4°C, and the blots were incubated with IgG-horseradish peroxidase (HRP)-labeled secondary antibodies (Beyotime Biotechnology, Jiangsu, China) for 1 h at room temperature. Immunoreactivity was detected with enhanced chemiluminescence (ECL), and densitometry was

Figure 2. (A) The total bacterial count of each group at 6, 12, and 18 h ($n=5$). (B) The time- $\log_{10}$ CFU/mL curve of each group at 6, 12, and 18 h ($n=5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CFU, colony-forming unit.

Figure 3. (A) pH of NeutroPhase and MPO groups ($n=5$, $*p<0.05$). Concentrations of H^+ (B) and HOCl (C) were calculated according to the pH and the formation of $2O_2^- + 2H^+ + Cl^- \rightarrow O_2 + HOCl + OH^-$ ($n=5$) $*p<0.05$, $**p<0.01$. HOCl, hypochlorous acid; MPO, myeloperoxidase.

performed using the Quantity One software (Bio-Rad Laboratories, Inc., Munich, Germany).

Statistical analysis

Values are expressed as mean $\pm$ standard deviation in the text and figures unless otherwise noted. Statistical significance was determined by Student's *t*-test or analysis of variance using SPSS 17.0 (SPSS, Inc., Chicago, IL). A value of $p < 0.05$ was considered significant.

RESULTS

Antibacterial test

In this section, the test solutions that contained MPO had the most effective inhibition against the growth of MRSA at 6 and 12 h than the NeutroPhase and NaCl+H₂O₂ (Fig. 1). At 18 h, the total bacterial count in the NaCl+H₂O₂ group was higher, while the other groups had similar antibacterial results (Fig. 2A). In this section, effective inhibition against the growth of MRSA emerged at 6 h after drawing culture medium in the bacterial suspension, the total bacterial count of the NeutroPhase and NaCl+H₂O₂ groups was about 12- and 18-fold than that of the 10 nM MPO+NaCl+H₂O₂ group, and even more than that of the 50 and 100 nM MPO+NaCl+H₂O₂ group. At 18 h, the antibacterial action still existed, but the gap between the NeutroPhase group and MPO group was narrowing. The same trend was also observed in the time-log₁₀ CFU/mL curve (Fig. 2B).

Ionic measurement

Except the initial time, the pH in the solution that had different concentrations of MPO was lower

than the NeutroPhase group (Fig. 3A), by reason of time needed for the reaction between the MPO and H₂O₂ to release H⁺ from the beginning. By calculating through chemical equation, results revealed that the solution containing MPO showed significantly higher concentrations of H⁺ from 1 h than the NeutroPhase group and became stable at 8 and 9 h (Fig. 3B). Compared with the NeutroPhase group, the concentrations of HClO in the group containing MPO were higher at 1 and 2 h (Fig. 3C).

Acceleration of wound closure in the presence of MPO

Four groups of scalded rats revealed different healing rates of MRSA-colonized burn wound in macroscopic view (Fig. 4). The open wound in MPO+NaCl+H₂O₂ group was significantly smaller than the other groups on day 16 (Fig. 5A). With time prolonged, the open wound ratio of the MPO+NaCl+H₂O₂ group was $\sim 5\%$, even smaller than that of the NeutroPhase, NaCl+H₂O₂, and control groups, which attained 12%, 18%, and 22% individually on day 28. Unsurprisingly, the contraction and epithelialization ratios of the MPO+NaCl+H₂O₂ group were 73% and 79% on day 28 (Fig. 5B, C), significantly higher than the other groups during the healing process.

Histomorphological examination

To explore the mechanism how MPO accelerated wound healing, histological analysis was performed. In general, MPO+NaCl+H₂O₂ treatment led to less skin damage compared with other groups (Fig. 6A). Significantly narrower skin lesions were found in MPO+NaCl+H₂O₂-treated wounds, whereas the depth of the lesions was similar across all groups.

Figure 4. The effects of wound healing in each group *in vivo* were evaluated via MRSA-colonized burn wound models. Standardized photographs of wounds were taken from day 0 to 28 after wounding (scale bar, 20 mm).

Figure 5. Wound closure analysis. (A) Time course of open wound closure of each group. (B) Wound contraction rate of each group over time. (C) Wound reepithelialization rate of each group over time ($n=5$) * $p<0.05$, ** $p<0.01$.

Figure 6. (A) H&E staining of epidermal leading edge in each group on day 16 (scale bar, 200 μ m). (B) Western blot of VEGF from wounds in each group on day 16 ($n=3$). H&E, hematoxylin and eosin; VEGF, vascular endothelial growth factor.

Importantly, epithelial tissue was more common in the MPO+NaCl+H₂O₂ group.

Western blot

The day the open wound ratio of control group reached almost 50%, we took all the wound skins of the four groups to examine the expression of VEGF. The result showed that the MPO+NaCl+H₂O₂ group expressed almost twofold VEGF than that of the other groups (Fig. 6B).

DISCUSSION

MRSA is now one of the leading organisms causing invasive infections in burns. Although vancomycin is an independent treatment for MRSA infection, resistant strains such as the vancomycin intermediate *S. aureus* have been isolated in recent years.⁷ Securing the more effective methods to treat the wounds infected by MRSA is a difficult task. Nonpharmacological treatments have emerged in the decade. Thakur *et al.* assessed the antimicrobial properties of the cationic-bilayered nanoemulsion of fusidic acid as an antimicrobial agent in an *in vivo* burn model infected with MRSA.²⁵ Kouhkheil *et al.* combined pulsed wave low-level laser therapy and mesenchymal stem cell-conditioned medium on the healing of an infected wound with MRSA in diabetic rats and showed that this can reduce infection and promote healing.²⁶

The heme-enzyme MPO, the most abundantly expressed protein in polymorphonuclear neutrophils (PMNs), catalyzes PMNs to generate H₂O₂ and form potent reactive oxygen species, such as HOCl. MPO plays a key role in the killing of ingested bacteria. Biochemical data suggest that MPO is by far the most deadly antibacterial toxin that neutrophils can utilize, and that the enzyme is active in phago-

somes, where it produces an oxidant that reacts with the ingested bacteria.^{27–29} However, Slater *et al.* found that the activated MPO delivered by neutrophil microparticles inhibited epithelial wound healing of the intestinal mucosa.³⁰ In addition, at low levels of exposure to pathogens, MPO may not be required to prevent infections, as other antimicrobial agents can eliminate invasion. The vital role of MPO in innate host defense will manifest only when exposure to pathogens overwhelms the capacity of other host defense mechanisms.²⁹

Thus, in the present study, we investigated the effects of MPO on healing of burn wounds colonized by MRSA. *In vitro*, we found that MPO significantly decreases the amount of MRSA colonies in the MH agar medium plates compared with H₂O₂ and NeutroPhase. Previous studies showed that the bacterial killing speed of MPO-deficient neutrophils was slower than normal neutrophils. For example, incubating MPO-deficient neutrophils with *Serratia marcescens* and *S. aureus* required 3–4 h to kill the bacteria, while normal cells need only 30–45 min to achieve the same.³¹ Much evidence suggests that H₂O₂ alone is not a particularly effective microbicide in the context of phagocytosis and is more likely to work through MPO. It has been determined that the reagent HOCl, or HOCl produced by the MPO/H₂O₂/chloride system, is very effective in killing a variety of microorganisms.²⁹ Furthermore, in our study, the MPO+NaCl+H₂O₂ group yielded more HOCl.

Aratani *et al.* first reported that MPO knockout (KO) mice are considerably more susceptible than wild-type mice to intraperitoneally injected *Klebsiella pneumoniae* infection,^{32,33} to the development of pulmonary infection following the intranasal instillation of certain fungi or bacteria,^{33,34} and to

intra-abdominal infection by endogenous organisms in the cecal ligation and puncture model.³⁵ In the present study, MPO not only caused the MRSA-colonized wounds to contract faster but also showed a significant increase in the degree of epithelialization compared with other groups.

By Western blot, we found that more VEGF was expressed in the wound of the MPO group. Many studies have also reported that VEGF potently induces human endothelial cell proliferation, angiogenesis, and wound healing.^{36,37} Therefore, MPO may be a major contributor to host resistance to infection, and beneficial for wound healing.

In conclusion, this study provided a new modality for wound healing by using MPO. The *in vitro* studies revealed that MPO released more HClO to further inhibit the growth of MRSA. *In vivo*, significantly accelerated MRSA-colonized burn wound healing was observed in the animal model experiments. MPO can serve as a potential clinical therapeutic agent, which can be applied for the treatment of wound healing in the future.

INNOVATION

This is the first study to use MPO for MRSA-colonized burn wound therapy. *S. aureus* is a leading cause of severe infections in burn wounds. With antibiotic resistance, MRSA is one of the most common pathogens in community and hospital. By elucidating the role of MPO on burn wounds with MRSA infection, we provide a new strategy for the development of a new antimicrobial agent for the treatment of traumatic infections.

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KEY FINDINGS

- MPO inhibited the growth of MRSA more effectively than a hypochlorous acid wound cleanser.
- MPO catalyzed the production of H⁺ and HClO⁻, leading to an acidic environment.
- MRSA-colonized burn wounds treated with MPO showed enhanced wound healing compared with wounds treated with NaCl, NaCl+H₂O₂, or NeutroPhase.
- Abundant VEGF was expressed in MPO-treated MRSA-colonized burn wounds.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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ABBREVIATIONS AND ACRONYMS

CFU = colony-forming units
H&E = hematoxylin and eosin
MPO = myeloperoxidase
MRSA = methicillin-resistant *Staphylococcus aureus*
PMNs = polymorphonuclear neutrophils
VEGF = vascular endothelial growth factor