

Time-Dependent Bactericidal Efficacy of Hydrogen Peroxide against Methicillin-Resistant *Staphylococcus Aureus* and *Acinetobacter Baumannii* in Second Degree Burn Wound

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ABSTRACT

Nosocomial infections are major health care problem and their developing antibiotics resistance have made it harder to treat. Hydrogen peroxide (H₂O₂) is widely used for contaminated wound sterilization, but its efficacy and time of sterilization against Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (*A. baumannii*) in burn wound has yet to be fully determined. In this study, we investigated the time-dependent bactericidal activity of 3% hydrogen peroxide. *In vitro* studies, MRSA and *A. baumannii* treated with hydrogen peroxide for 30 sec led to 100% bacterial kill. For *in vivo* studies, second degree burns was created on the back of mice followed by inoculation with 1 x 10⁸ CFU ml⁻¹ of MRSA and *A. baumannii*, isolated from clinical specimens of burn patients. Hydrogen peroxide application efficiently reduced bacterial burden 6.9 x 10⁵ CFU/g (control: 7.5 x 10¹⁰ CFU/g) for 30 sec to 3.2 x 10⁴ CFU/g (control: 9.2 x 10⁹ CFU/g) for 120 sec of application duration. The log₁₀ CFU bacterial load reduction with hydrogen peroxide application from 30 sec to 120 sec was found to be 0.96 (P<0.001). Furthermore, histological evaluation of the hydrogen peroxide (3%) treated and untreated burn wounds did not exhibit any significant tissue alterations. These results emphasize the potential use of 3% hydrogen peroxide as a possible treatment option for the decolonization of MRSA and *A. baumannii* bacteria in burn wounds without harming the tissue viability.

Introduction

Burns are common and major healthcare problem that requires care in specialized burns units [1]. The nature and extent of burn injury lead to immunosuppression in patients, resulting in increased susceptibility to various nosocomial pathogens [2]. MRSA accounts for 11.5% of the total *S. aureus* isolates from burn wound patients. Studies have shown that the prevalence of infections caused by MRSA has increased from 12% in 1992 to 80.03% in 2012 [3]. Many MRSA isolates are becoming multidrug-resistant and are susceptible to only tetracyclines and glycopeptides [4]. The commonly deployed antibiotics to treat MRSA mediated burn infections (tigecycline, vancomycin) are associated with several side effects. Also, *S. aureus*

strains resistant to these antibiotics have already emerged in hospital and community settings [5]. *A. baumannii* is a member of opportunistic bacteria and capable of developing drug resistance and biofilm formation [6]. The only available antibiotic treatments possesses additional damage to the patients [7]. Thus, a key challenge is to use simple, low-cost and effective treatments to develop non-antibiotic therapies to prevent MRSA and *A. baumannii* colonization in burn wounds.

One method for reducing wound bacterial load and colonization is the use of topical antiseptics agents. The advantage of antiseptics is that they are less selective in their action and bacteria are less

likely to develop resistance [8]. Hydrogen peroxide (3%) exhibits broad antimicrobial activity *in vitro* [9] and *in vivo* [10]. Studies have been conducted to observe the antimicrobial activity of hydrogen peroxide and its effect on wound healing. A randomized clinical trial of burn wounds showed that wounds treated with 2% hydrogen peroxide significantly improved skin graft take rates [11]. Another clinical trial demonstrated that hydro surgery with hydrogen peroxide was superior to controls without hydrogen peroxide for infected wounds, resulting in shorter hospital stay and enhanced graft viability [12]. The clinical use of hydrogen peroxide regarding the elimination of MRSA and *A. baumannii* is limited. Therefore, a better understanding of hydrogen peroxide in sterilization and its impact on the skin will enhance its potential use. In this paper, we studied the use of 3% hydrogen peroxide as one adjunct for reducing MRSA and *A. baumannii* burden in burn wound. We investigated time - dependent antimicrobial activity of hydrogen peroxide by creating the mice burn model infected with MRSA and *A. baumannii*. We also observed histological alterations in burn wound upon hydrogen peroxide application.

Materials and Methods

Materials

Tryptic soy broth (TSB), Luria-Bertani (LB) broth, Phosphate buffered saline (PBS), Mueller-Hinton (MH) agar and 3% hydrogen peroxide was purchased from Binafo Bioengineering Co. Ltd (Zhengzhou, China). MRSA and *A. baumannii* bacteria strains were obtained from the Department of Clinical Microbiology, Second Affiliated Hospital of Zhejiang University.

Experimental Animals

Pathogen-free BLAB/c mice (18-25 grams; 7-8 weeks of age) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The mice were allowed to acclimate in the animal facility for ten days with free access to food and water. Animals were divided into 8 groups (n = 6/ group) based on the hydrogen peroxide application duration. All procedures performed on mice were in accordance with the Guidelines for Animal Experimentation of Zhejiang University.

Antibacterial Activity by Disc Diffusion Method

The antimicrobial activity of hydrogen peroxide was performed using standard techniques. MRSA and *A. baumannii* separately cultured overnight was sub-cultured for 4 h. 50 µl aliquots of freshly prepared MRSA and *A. baumannii* suspension in PBS at optical density of OD 600 = 0.1, were seeded onto the MH agar plate. Sterile filter discs (5 mm) were impregnated with 3% hydrogen peroxide (PBS was used as a control) and incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of bacteria inhibitory zone.

MRSA and *A. Baumannii* Treatment with Hydrogen Peroxide

MRSA (1 x 10⁸ CFU ml⁻¹) and *A. baumannii* (1 x 10⁸ CFU ml⁻¹), suspensions in LB broth were centrifuged and rinsed twice with PBS. MRSA and *A. baumannii* pellets were then subjected to 100 µl of 3% hydrogen peroxide at predetermined time (30 sec, 60 sec, 90 sec and 120 sec). Bacterial suspension treated with hydrogen peroxide for various time periods was immediately introduced in 45 ml of PBS (reaching hydrogen peroxide final concentration to 0.001%) and centrifuged. Control group was treated the same, but with sterile PBS. After centrifugation, the supernatant was discarded, and bacteria were resuspended in 1 ml PBS. Aliquot of 50 µl were plated on MH agar and incubated at 37 °C for 24 h.

Creation of burn wound and MRSA/ *A. Baumannii* Infection

Mice dorsal surface was shaved and depilated with Veet (Reckitt Benckiser, China) 1 day before creation of burn wound. The following day, mice were anaesthetized and 1.5 cm diameter partial thickness burn (80°C for 6 sec) was created using burn creation machine (ZH-YLS-5Q, China) (Figure 1). This method was based on the protocol by Liu and colleagues [13]. Soon after, mice were resuscitated with 1 ml sterile saline injected intraperitoneally. 20 min after the creation of the burn (allowing the burn injury to cool down), elastic bandage tape was applied to burn wound and stripped from (1.5x1.5 cm) skin area, 8 times in one direction and 8 times in the opposite direction, until the skin became reddened with no apparent bleeding. This method is based on protocols by Tatiya et al [14]. This method allows bacteria to colonize in the wound. A 15 µl MRSA/ *A. baumannii* (1 x 10⁸ CFU ml⁻¹) suspension in PBS was smeared onto the burn wound with an inoculating loop. Burn wound was then covered with polyurethane film (Tegaderm, Shanghai, China) for 24 h to mimic a stable bacterial infection.



Figure 1: Mice burn model.

Treatment of Burn Infection and Microbiological Analysis

24 h after bacterial seeding onto the burn wound, it was treated with 2-5 ml of 3% hydrogen peroxide for duration of 30 sec, 60 sec, 90 sec and 120 sec, followed by a rinse with PBS. Mice in the control group were treated the same, but with sterile PBS. Mice were sacrificed, and wound tissue samples were excised using a punch skin biopsy (Skalr, USA). Samples were then homogenized in PBS and 10-folds serially diluted bacterial colonies were enumerated by plating on MH agar.

Hydrogen Peroxide Effect on The Burn Wound Histology

The effect of hydrogen peroxide on the burn wound was evaluated by hematoxylin and eosin (H & E) staining. Briefly, 24 h after the creation of burn wound, it was subjected to 3% hydrogen peroxide for 120 sec followed by a rinse with PBS. Control group was treated the same, but with PBS. Tissue samples (5×5 mm) were excised, fixed in buffered formalin (10%), and dehydrated with al-

cohol and finally embedded in paraffin wax into blocks. Sections of tissue samples were stained with H&E and examined under a light microscope (Nikon, Japan).

Statistical Analysis

Data were presented as means \pm standard deviation. Statistical analysis was executed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) P-values were determined using two-tailed Student's t-test. $P < 0.05$ were considered to be significant difference.

Results

In Vitro Antimicrobial Activity of Hydrogen Peroxide

Hydrogen peroxide application showed a promising antimicrobial activity. Zone of inhibition was calculated to be 30 mm (MRSA) and 28 mm (*A. baumannii*) compared with control group (Figure 2). Also, MRSA and *A. baumannii* treated with hydrogen peroxide for 30 sec led to 100 % bacterial kill in comparison with the control group (Figure 3).

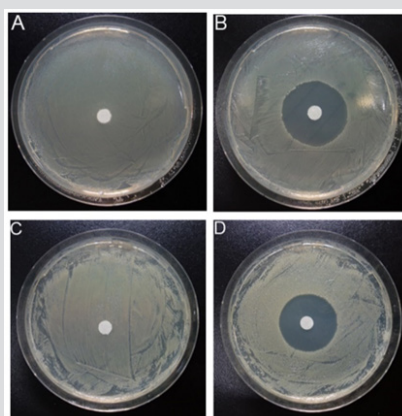


Figure 2: Antibacterial activity of 3% hydrogen peroxide. Top: halos representing zone of inhibition of MRSA (A) Control and (B) 3% hydrogen peroxide treated. bottom: halos representing *A. Baumannii* zone of inhibition, (C) Control (PBS) and (D) 3% hydrogen peroxide treated.

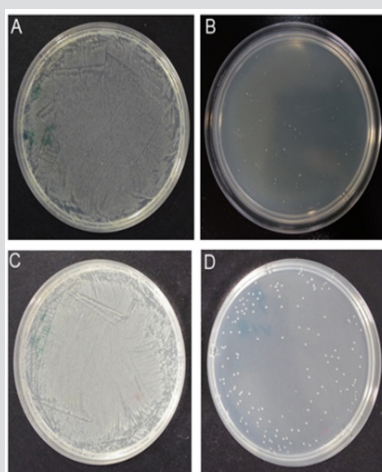


Figure 3: The antibacterial activity of 3% hydrogen peroxide. Top: (A) MRSA treated with PBS for 30 sec, (B) MRSA treated with hydrogen peroxide for 30 sec. bottom: (C) *A. baumannii* treated with PBS for 30 sec, (D) *A. baumannii* treated with hydrogen peroxide for 30 sec.

In Vivo Antimicrobial Activity of Hydrogen Peroxide

The antibacterial activity of 3% hydrogen peroxide against MRSA/*A. baumannii* was determined by measurements of bacterial load in burn wound. Application of hydrogen peroxide for specified duration (30 sec, 60 sec, 90 sec and 120 sec) suppressed MRSA/*A. baumannii* load in a time-dependent manner. Hydrogen peroxide application for 30 sec reduced 6.9×10^5 CFU/g bacterial load compared with control group (7.5×10^{10} CFU/g). The log₁₀ CFU bacterial reduction was found to be 5.07 ($P < 0.0001$). Furthermore,

increase in hydrogen peroxide application time further reduced the bacterial load, i.e. 60 sec (4.9×10^5 CFU/g vs. control 1.21×10^{10} CFU/g) log₁₀ CFU bacterial reduction of 4.39 ($P < 0.0001$), 90 sec (8.20×10^4 CFU/g vs. control 1.61×10^{10} CFU/g) log₁₀ CFU bacterial reduction of 5.30 ($P < 0.0001$), and 120 sec (3.2×10^4 CFU/g vs. control 9.2×10^9 CFU/g) log₁₀ CFU bacterial reduction of 6.03 ($P < 0.0001$). The total bacteria log₁₀ CFU reduction with hydrogen peroxide application from 30 sec to 120 sec was found to be 0.96 ($P < 0.001$) (Figure 4). These results show, increasing hydrogen peroxide application time can further reduce wound bacterial load.

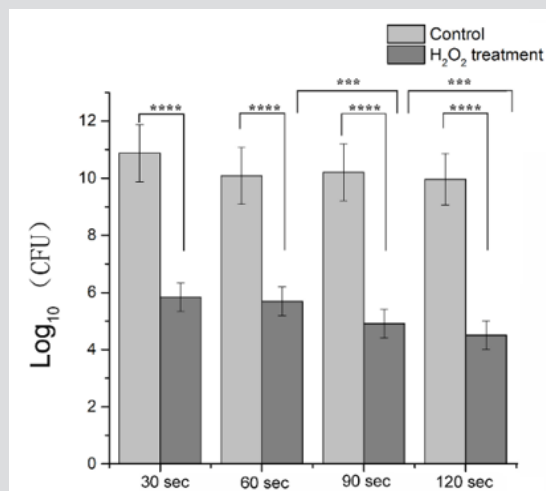


Figure 4: Antimicrobial effect of 3% hydrogen peroxide on MRSA and *A. baumannii* in burn wound. Bacterial counts were calculated by plating serially diluted homogenate onto agar plates and incubated at 37°C for 24 h. **** $P < 0.0001$, *** $P < 0.001$.

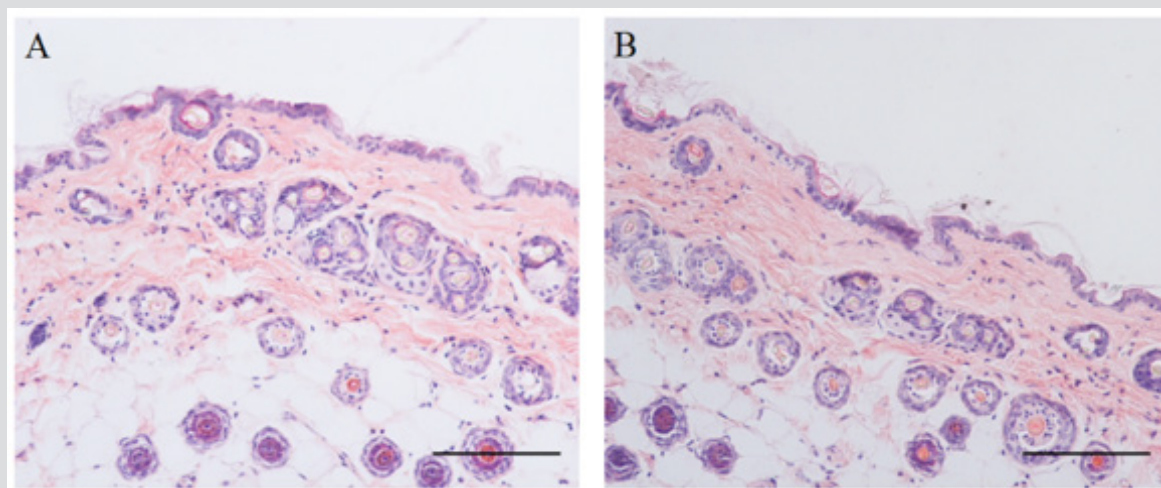


Figure 5: The histological analysis of mice skin treated with 3% hydrogen peroxide for 120 sec. Haematoxylin and eosin staining of (A) Control (PBS) and (B) hydrogen peroxide treated (magnification 100x; scale bar 100 μ m).

Hydrogen Peroxide Effect on Burn Wound

Histological studies of burn wound tissue treated with hydrogen peroxide for 120 sec revealed no significant difference compared to PBS treated control group. Congestion and edema were absent in both hydrogen peroxide and control treated groups (Figure 5).

Discussion

In this study, mice burn wounds infected with MRSA and *A. baumannii* were used to evaluate the antimicrobial effect of 3% hydrogen peroxide in time-dependent manner. MRSA and *A. baumannii* bacteria were smeared on burn wound, and 24 h later

a stable skin infection was created to mimic infected burn wound. Our results demonstrated reduction of bacterial load was based on the duration of hydrogen peroxide application and contact with bacteria. Histological evaluation of burn wound treated with hydrogen peroxide for 120 sec has no apparent changes when compared with the control group, showing that hydrogen peroxide did not alter the tissue viability.

Wound cleaning and sterilization are crucial for management of traumatic wounds to minimize the rate of infection [15]. Burn wounds especially the deep partial and full thickness burns consist of avascular necrotic tissue (eschar) providing a protein-rich environment for bacterial colonization and proliferation [1].

Antiseptics such as ethanol, triclocarban, chlorhexidine, chlorine, silver and iodine compounds destroy or inhibits the growth and development of bacteria and carries broader antimicrobial activity than antibiotics. Hydrogen peroxide is a well-known antiseptic, and 3% solution shows a broad spectrum of antimicrobial activity [9]. In our *in vitro* study, both gram-positive (MRSA) and gram-negative (*A. baumannii*) bacteria treated with 3% hydrogen peroxide for 30 sec resulted in 100% kill (Figure 3). This result shows that 3% hydrogen peroxide can be used for sterilization of burn and ICU units to effectively eliminating gram - positive and gram-negative bacteria, thus reducing the rate of nosocomial infections. The use of antiseptics increases the rate of wound healing by minimizing and preventing infection of open wounds [16]. Another argument about the use of antiseptics is to prevent bacterial resistance developed by the use of antibiotics [17]. In our *in vivo* study, 3% hydrogen peroxide efficiently reduced both MRSA and *A. baumannii*, which are difficult to treat with intravenous or topical antibiotics. Antiseptics reduce the use of antibiotics in burn patients. The presence of catalases in tissue can make the bactericidal activity of hydrogen peroxide less effective *in vivo* [18].

In our study, we concluded the hydrogen peroxide effect of reducing bacterial load is time dependent manner. Burn wound treated hydrogen peroxide for 120 sec significantly reduced bacterial load, thus reducing the risk of sepsis and biofilm formation (Figure 4). Also, we should keep in consideration that further increase in hydrogen peroxide application didn't reduce bacteria which might be associated with bacteria being penetrated deep into the wound and could not come in contact with hydrogen peroxide. Studies conducted on human and animals have shown that hydrogen peroxide has no negative impact on wound healing [19]. In a combined *in vitro* study and clinical trial, hydrogen peroxide accelerates wound reepithelialization [20]. In our study, the application of 3% hydrogen peroxide did not exhibit any alterations in tissue histology after a brief exposure of 120 sec (Figure 5). Hydrogen peroxide did not cause any immediate tissue damage, which might be associated with the hydrogen peroxide dilution in burn wound or short duration of application.

Conclusion

In Conclusion, 3% hydrogen peroxide is an effective antiseptic agent, which can reduce MRSA and *A. baumannii* related burn wound infection and sepsis. Hydrogen peroxide effectiveness increases with increase in application time without harming the tissue viability. However, clinical studies need to be conducted to evaluate its efficacy on infected burn wounds.

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