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Use of chitosan bandage to prevent fatal infections developing from highly contaminated wounds in mice

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Abstract

HemCon® bandage is an engineered chitosan acetate preparation used as a hemostatic control dressing, and its chemical structure suggests that it should also be antimicrobial. We tested its ability to rapidly kill bacteria in vitro and in mouse models of infected wounds. We used the Gram-negative species *Pseudomonas aeruginosa* and *Proteus mirabilis* and the Gram-positive *Staphylococcus aureus* that had all been stably transduced with the entire bacterial lux operon to allow in vivo bioluminescence imaging. An excisional wound in Balb/c mice was inoculated with 50–250 million cells followed after 30 min by application of HemCon bandage, alginate sponge bandage, silver sulfadiazine cream or no treatment. HemCon was more adhesive to the wound and conformed well to the injury compared to alginate. Animal survival was followed over 15 days with observations of bioluminescence emission and animal activity daily. Chitosan acetate treated mice infected with *P. aeruginosa* and *P. mirabilis* all survived while those receiving no treatment, alginate and silver sulfadiazine demonstrated 25–100% mortality. Chitosan acetate was much more effective than other treatments in rapidly reducing bioluminescence in the wound consistent with its rapid bactericidal activity in vitro as well as its light-scattering properties. *S. aureus* formed only non-lethal localized infections after temporary immunosuppression of the mice but HemCon was again more effective in reducing bioluminescence. The data suggest that chitosan acetate rapidly kills bacteria in the wound before systemic invasion can take place, and is superior to alginate bandage and silver sulfadiazine that may both encourage bacterial growth in the short term.
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1. Introduction

Infections that develop in traumatic and surgical wounds remain a major problem despite decades of advances in antibiotics and antiseptics. The relentless worldwide increase in multidrug resistance in pathogenic bacteria has led to the present time being described as "the end of the antibiotic era" [1]. There is therefore an increasing need for topical antimicrobial products that can be applied to potentially contaminated wounds.

Chitin is a biopolymer consisting of poly *N*-acetyl glucosamine and is widespread in nature as a structural material particularly in marine arthropod shells. Chitin is generally an insoluble material but can be deacetylated by treatment with hot sodium hydroxide to form the soluble polymer chitosan. Chitosan preparations of various molecular weights, degrees of deacetylation and with further molecular derivatization patterns have attracted much attention because of their potentially beneficial

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biological properties [2,3]. These properties include hemostasis [4], antimicrobial activity [5] stimulation of healing [6], tissue-engineering scaffolds [7], and drug delivery [8]. The broad spectrum antimicrobial activity of *N*-carboxybutyl chitosan suggested it could be used as a wound dressing [9]. HemCon bandage is a compressed chitosan acetate dressing that was developed as a hemostatic agent [10]. It is used to stem blood flow, especially flow from severely bleeding wounds [11]. Previously the application of continuous pressure with gauze bandage was the preferred primary intervention technique used to stem severe bleeding from wounds. Alternative hemostatic bandages such as collagen wound dressings or dry fibrin thrombin wound dressings are not sufficiently resistant to dissolution in high blood flow. Action reports by combat medics during freedom operations in Afghanistan and Iraq have shown success in treating bullet wounds, shrapnel, land mine and other injuries, when conventional wound dressings have failed [12,13].

The nature of combat injuries is such that bacterial contamination is frequently present in traumatic wounds. One of the natural purposes of free and unimpeded bleeding from wounds is to flush out potentially contaminating microorganisms that may have gained entry to the wound from the environment. The question then arises if a hemostatic bandage is successfully used to control potentially life-threatening bleeding, will it increase the chances of infection developing in the wound? The polycationic nature of chitosan is such that the substance possesses natural antimicrobial properties [5]. Therefore it is possible that HemCon bandage could simultaneously have two highly desirable properties in a wound dressing: hemostasis and microbicidal activity. The present study was designed to test the antibacterial properties of HemCon in a mouse model of highly contaminated wounds. We used bacterial strains that were genetically engineered to stably express the lux operon that encodes bacterial luciferase and the enzymes necessary to synthesize the luciferase substrate so the bacteria ‘‘glow in the dark’’ [14]. This allows the use of a low-light imaging camera to follow the progress of the infection in real time [15]. The strains that were studied in the present investigation include two Gram-negative bacterial species that are invasive and can lead to development of sepsis (*Pseudomonas aeruginosa* and *Proteus mirabilis*) and a Gram-positive species (*Staphylococcus aureus*) that leads to a more chronic localized infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

We used the Gram negative species *P. mirabilis* (ATCC 51393) and *P. aeruginosa* (ATCC19660) and the Gram-positive *S. aureus* (strain 8325-4) carrying the entire bacterial lux operon integrated in their chromosomes for stable expression that allowed them to be used for bioluminescent imaging (strains Xen 44, Xen 5 and Xen 8.1, respectively, Xenogen Inc, Alameda, CA). Cells were cultured in brain–heart infusion (BHI) broth with aeration at 37 °C. Cells were used for in vitro studies in mid-log growth phase (OD at 600-nm = 0.6–0.8; 10^8 cells per mL). For inoculation of wounds, cell suspensions were concentrated by centrifugation and resuspension in PBS.

2.2. Preparation of chitosan and alginate bandages

HemCon® dressing (100 mm × 100 mm × 5.5 mm) was prepared by HemCon, Inc (Portland, OR) by methods fully described in [16]. Briefly chitosan acetate sponges (74% chitosan, 9% moisture, 17% acetic acid by weight) were prepared by freeze drying dilute aqueous acetic acid solutions of chitosan with a fractional degree of deacetylation of $81\pm2\%$ and number average molecular weight (GPC with polyethylene oxide standards) 75 ± 10 kDa and polydispersity 5 ± 1 (Primex, Iceland) in Teflon™ coated aluminum mold (20 mm deep × 110 mm width × 110 mm length) at room temperature. The resultant sponges were compressed, annealed and γ-irradiated to yield sterile, dissolution resistant and adhesive dressings. Chitosan dressing test pieces (10 mm × 10 mm) were cut from the standard dressing and the bottom 2.0 mm thickness was excised to yield 10 mm × 10 mm × 2.0 mm thickness test dressings with chitosan sponge density close to 0.2 g/cm^3.

Alginic acid sodium salt (Aldrich, Milwaukee, WI; 16.0 g) was dissolved in deionized water (800 g) at room temperature. The solution was poured to a depth of 15 mm into coated aluminum mold described above, which were placed on cooled stainless steel plate shelves at −30 °C in a freeze dryer (Model 25EL, Virtis, Gardiner, NY) and the solutions were allowed to freeze. The freezing induced formation of a phase separated plaque of interspersed regions of ice (20–100 μm thick) and alginic acid sodium salt (5–10 μm thick). Lyophilization of the frozen plaque resulted in removal of the ice to leave a sponge of alginic acid sodium salt (15 mm thick, 95 mm wide, 95 mm long, density 0.025 ± 5 g/cm^3). The alginic acid sponge was subsequently pressed to 5.5 mm thickness by compression between parallel platens for 2 min at 80 °C. Alginic acid sodium salt sponge test samples (10 mm wide × 10 mm long × 1.5 mm thick) were prepared by die cutting of the original sponges and excising the first 1.5 mm thickness of sponge base using a safety razor blade.

2.3. In vitro experiments

HemCon bandage was prepared as a 1% (w/v) solution in distilled and sterilized water to yield a solution pH = 4.5. One mL aliquots of bacterial suspensions (initial concentration 10^8 cells mL^{-1}) were incubated with 4 mL of HemCon bandage solution and aliquots withdrawn at time points and placed in wells of 96 well micro titer plates (Fisher Scientific) for serial dilution. The aliquots were serially diluted 10-fold in PBS to give dilutions of 10^{-1}–10^{-6} times the original concentrations and were streaked horizontally on square BHI agar plates as described by Jett et al. [17]. This allowed a maximum of seven logs of killing to be measured. Plates were incubated at 37 °C overnight.

2.4. Animal experiments

All animal experiments were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital and were in accordance with NIH guidelines. Male Balb/c mice weighing 20–25 g were shaved on the back and depilated with Nair (Carter-Wallace Inc, New York, NY) the day before the experiment. Mice were anesthetized with an IP injection of ketamine/xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine) for surgery, infection and imaging. The operative area of skin was cleaned with alcohol and surgical scissors and forceps were used to construct a full-thickness excisional wound down to but not through the panniculus carnosus measuring 5 × 5 mm. There was no visible bleeding within the wounds. For studies involving *S. aureus* wound infection mice received two doses of cyclophosphamide injected IP in sterile PBS (first of 150 mg/kg given 4 days before wounding; second of 100 mg/kg given 1 day

before wounding). Mice were euthanized according to protocol when their condition was assessed to be moribund.

2.5. Bioluminescence imaging

The low light-imaging system (Hamamatsu Photonics KK Bridgewater, NJ) has been previously described in detail [18]. It consisted of an intensified charge-coupled device-camera mounted in a light-tight specimen chamber fitted with a light-emitting diode allowing a background gray-scale image of the entire mouse to be captured. In the photon-counting mode, an image of the emitted light was captured using an integration time of 2 min at a maximum setting on the image intensifier control module. Using ARGUS software the luminescence image was presented as a false-color image superimposed on top of the grayscale reference image. The image-processing component of the software gave mean pixel values from the luminescence images on defined areas within each wound on a 256-grayscale. The analysis area was defined on each image to encompass all luminescence within the wound or the surrounding area. For comparisons of bioluminescence images in Fig. 4 the same bit-range was used for all the images.

2.6. In vivo infection studies

Thirty minutes after anesthetized mice received single wounds measuring 25 mm^2, they received inocula of mid-log phase bacteria suspended in 50-μL PBS and applied with a 200-μL pipette tip into the wound bed as previously described [18,19]. The doses were as follows; *P. aeruginosa* $= 5 \times 10^7$ cells; *P. mirabilis* $= 25 \times 10^7$ cells; *S. aureus* $= 25 \times 10^7$ cells. Mice were imaged in the luminescence camera immediately after infection and then daily until sacrifice or until the luminescence signal disappeared. Bandages or antimicrobial cream were applied to the infected wounds 30 min after infection.

Four experimental groups (6–20 mice per group) consisting of: (1) no treatment control, (2) HemCon bandage, (3) Na alginate bandage, and (4) silver sulfadiazine (AgSD) cream (Par Pharmaceutical, Woodcliff Lake, NJ) were used for the Gram-negative experiments, and three groups (no antimicrobial cream) for *S. aureus.* Chitosan acetate test pieces 1 cm × 1 cm were first wetted with sodium acetate buffer (100 mM, pH 4.5) before application, while sodium alginate bandages were wetted with PBS. We checked that alginate bandages had no antibacterial properties if they were wetted with sodium acetate buffer (100 mM, pH 4.5) instead of PBS. Fifty mg AgSD cream was weighed out and applied to the infected wound with a gloved fingertip.

2.7. Follow up

Mice were subjected to bioluminescence imaging twice on first day (before and after application of bandage or cream) and thereafter daily. Records were kept of which day the mice lost their bandage. Some mice showed symptoms of bacterial infection (weight loss, ruffled fur, and inactivity). Mice were euthanized when their condition was assessed to be moribund.

2.8. Statistics

Data points are given as mean ± standard error of the mean (SEM). Differences between means were analyzed for statistical significance by the unpaired 2-tailed Student's *t*-test assuming equal or unequal differences in the standard deviation as appropriate. Survival curves were compared by the Kaplan-Meier log-rank statistical method. *P* values < 0.05 were considered significant.

3. Results

3.1. In vitro experiments

We tested the in vitro antimicrobial effects of dissolved HemCon bandage in water. Because a 1% water solution of HemCon has a pH of 4.5 due to the acetic acid content of the formulation, we used a buffer solution of sodium acetate/acetic acid at pH 4.5 as a control. Fig. 1 depicts the killing curves with time. *P. aeruginosa* was the most sensitive organism to HemCon solution, giving six logs of killing within 1.5 h, while *P. mirabilis* was slightly less sensitive giving almost as much killing in 2 h. *S. aureus* was the least sensitive with 3.5 logs of killing in 2.5 h. The pH 4.5 buffer gave no measurable killing and even allowed some bacterial growth.

3.2. Infected wound models

When the suspensions of bacteria were introduced into the wound the bacteria attached to the tissue in the wound bed and the 50 μL of PBS evaporated over the course of 30 min. The majority of mice that received excisional wounds infected 30 min after wounding with 50 million CFU of *P. aeruginosa* or 250 million CFU of *P. mirabilis* developed systemic infections and died. Eighty percent of *P. aeruginosa* infected mice were dead within 7 days (Fig. 8), while *P. mirabilis* infection developed even quicker with 75% fatality within 2 days (Fig. 6). By contrast when we contaminated an excisional wound with 250 million *S. aureus,* the bacterial luminescence disappeared in a few

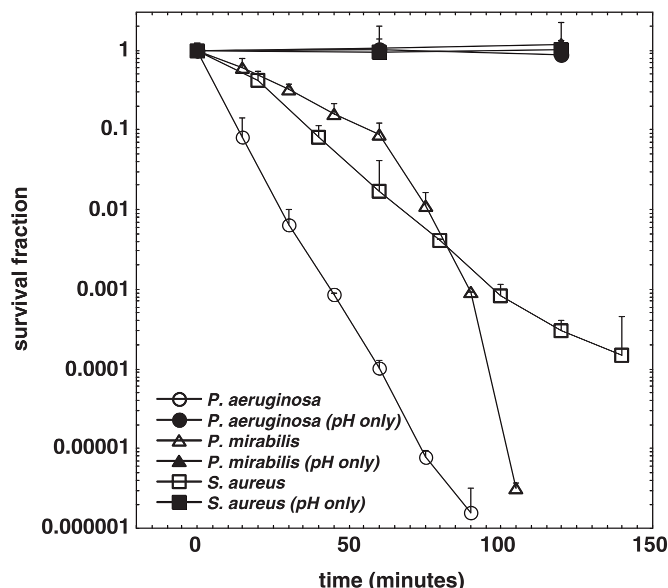

Fig. 1. In vitro antimicrobial effects of dissolved HemCon bandage. Bacteria (10^8/mL) were incubated with a 1% aqueous solution of HemCon bandage (pH = 4.5) or with a control acetate buffer (pH = 4.5) and aliquots withdrawn at the indicated times for determination of CFU. Points are means of three independent determinations and bars are SEM.

hours (data not shown). This finding is similar to results we found previously [20] using an intramuscular injection of bioluminescent *S. aureus* to form a subcutaneous soft-tissue infection. In that study it was necessary to pre-treat the mice with two injections of cyclophosphamide to render the animals temporarily (8–10 days) neutropenic before adding the bacteria. It appears that once the bacteria have established an infection in the absence of neutrophils, they will resist destruction by the returned neutrophils for some time (2–3 weeks). In the present excisional wound model a similar process took place after 250 million *S. aureus* were added to a wound on a neutropenic mouse. None of the mice died but infections were established that lasted for up to 9 days (Fig. 9).

3.3. Bandage attachment

When we applied the HemCon bandage to the wound we chose to moisten it with 100 mM sodium acetate buffer pH = 4.5, which is the same pH that the bandage solution in water possessed. This was done because the buffering capacity of the mouse wound is unknown, and using water to moisten the bandage may have allowed the pH to rise above the value where chitosan is antimicrobial. The sodium alginate bandage was moistened with water as it does not possess any antimicrobial activity and was used as a control for material quenching (scattering) of bioluminescence light emitted during imaging. When we placed a moistened HemCon bandage on the wound approximately 30 min after adding the bacteria, it adhered well to the tissue (Fig. 2). Alginate bandages also initially adhered to the wound but did not remain attached for as many days as HemCon. Fig. 3 compares the adhesion times of all the HemCon and aliginate bandages used regardless of infectious organism present. The median HemCon adhesion time is 6 days and the median adhesion time of alginate is only 1 day ($P < 0.002$).

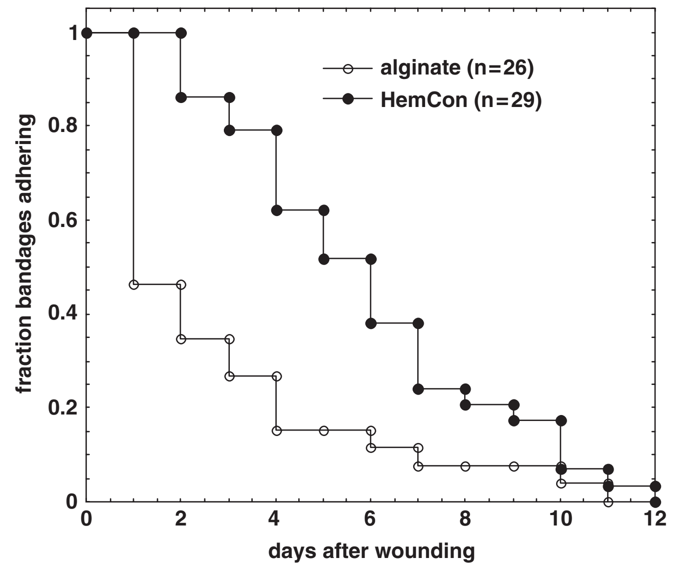

Fig. 3. Comparison of adhesion times of HemCon and alginate bandages to infected mouse wounds.

3.4. P. mirabilis wound infections

Fig. 4 shows some representative overlaid images from the bioluminescence imaging system. It can be seen that at 5 min after application of HemCom (panel B) the luminescence is significantly reduced but still visible under the center of the bandage, while at the same time point after application of alginate to the infected wound (panel F) there was a small band of bioluminescence visible at the edge of the bandage. This illustrates the rapid antimicrobial effect of HemCon that can destroy the bacteria that would otherwise leak from the edge of the bandage. The next day HemCon has only a trace of luminescence visible (panel C), while the luminescence has grown dramatically in the tissue surrounding the alginate bandage (panel G). The no treatment wound at 24 h (panel K) has as strong a luminescence as alginate, but without the central dark area due to the bandage. At 48 h post-infection HemCon shows only minimal luminescence (panel D), while alginate (panel H) and no treatment (panel L) have further increased. Panel M illustrates the overnight culture of blood from a moribund mouse with an alginate bandage streaked on an agar plate and demonstrating that mice died from sepsis due to bacteremia.

The time course of the mean bioluminescence signal from the various groups of mice is shown in Fig. 5. The luminescence from the HemCon wounds steadily dropped throughout the course of the study, but

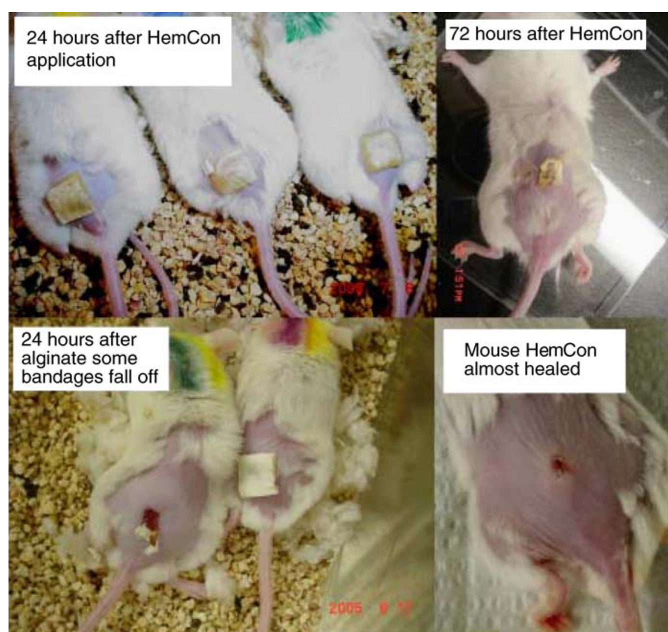

Fig. 2. Photographs of representative mice with HemCon or alginate bandages.

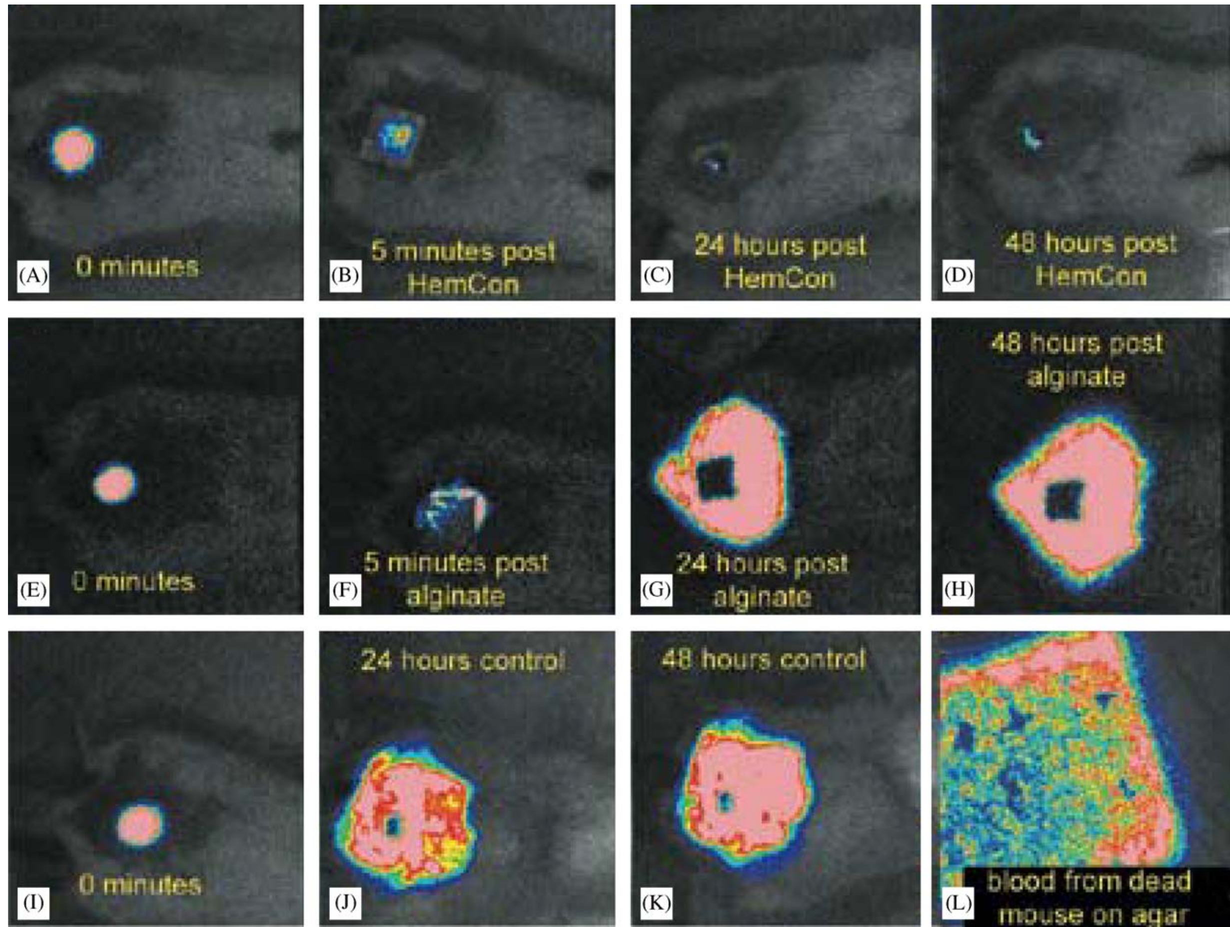

Fig. 4. Representative bioluminescence images at same bit range of mice with *P. mirabilis*-infected wounds with treated HemCon (A–D), alginate (E–H), or no treatment control (J–L). Panel (M) shows overnight incubation of blood sample from moribund mouse demonstrating bioluminescent bacteremia.

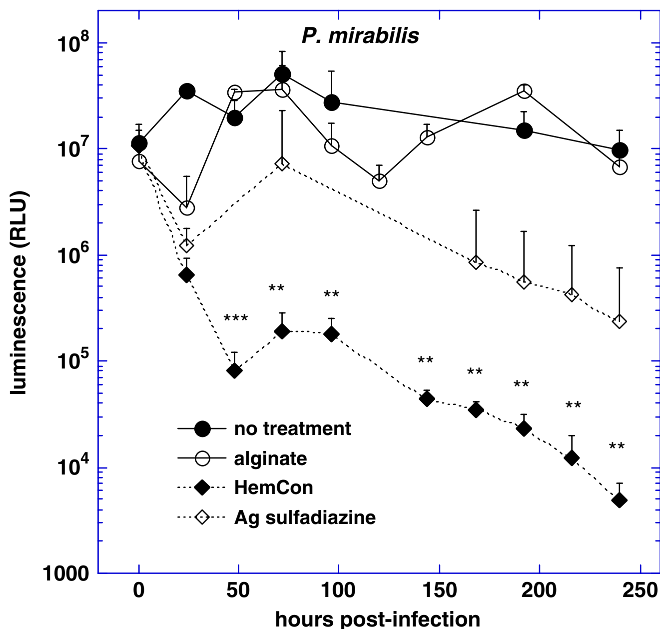

Fig. 5. Mean bioluminescence values (bars = SEM) of mice with *P. mirabilis* infection treated with HemCon, alginate, AgSD, or no treatment. ***$P<0.001$; **$P<0.01$ vs. all other treatments.

a small amount remained until day 15 embedded in the granulation tissue. The luminescence in the alginate wound dropped after 24 h (probably due to the light-scattering properties of the bandage, but recurred strongly as the bacteria grew in the tissue surrounding the wound. The mean luminescence in control wounds increased from day 1 and remained at a high level throughout, although the number of mice in this group and the alginate group was reduced due to death. The luminescence in the wounds treated with AgSD cream initially dropped but recurred strongly after 3 days, although it remained at a lower level compared to no treatment and alginate, but at a higher level than HemCon.

The Kaplan-Meier survival curves are shown in Fig. 6. Mice treated with HemCon demonstrated 100% survival. Mice treated with alginate demonstrated 65% survival, while mice treated with AgSD cream showed a 50% survival, and those receiving no treatment had a 25% survival. The P values for the survival curves are as follows: HemCon vs. alginate = 0.048, HemCon vs. AgSD = 0.023, Hemcon vs. no treatment = 0.0045, alginate vs. AgSD = 0.32 NS, alginate vs. no treatment = 0.036, AgSD vs. control = 0.39 NS.

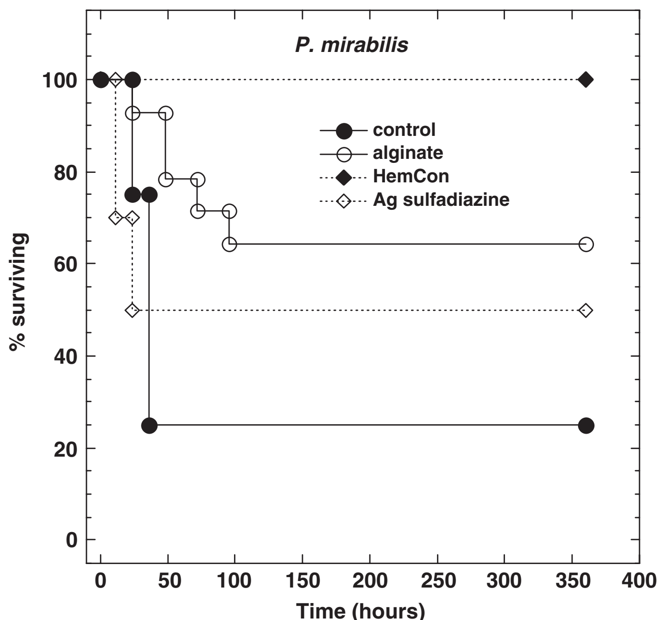

Fig. 6. Survival curves of mice with *P. mirabilis* infection treated with HemCon ($n = 8$), alginate ($n = 12$), AgSD ($n = 10$), or no treatment ($n = 10$).

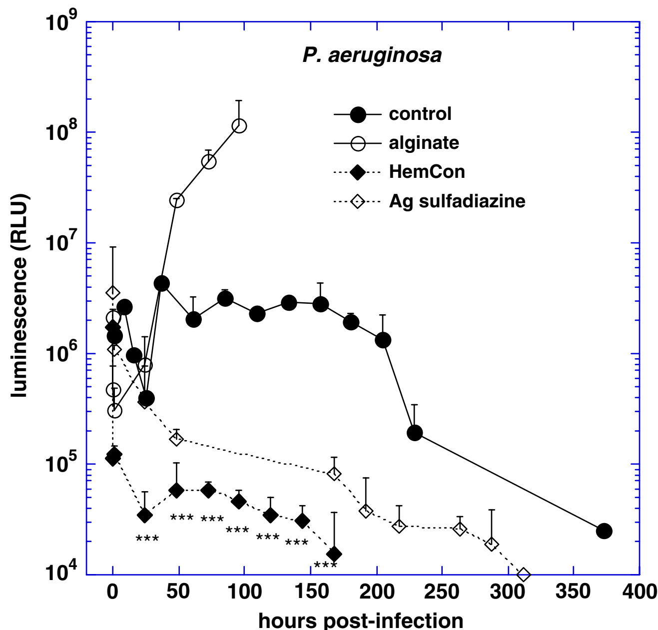

Fig. 7. Mean bioluminescence values (bars = SEM) of mice with *P. aeruginosa* infection treated with HemCon, alginate, AgSD, or no treatment. ***, $P<0.001$ vs. no treatment and alginate. HemCon was only significantly different from AgSD ($P<0.01$) at 24 and 36 h.

3.5. *P. aeruginosa* wound infections

The time course of the mean bioluminescence signal from the various groups of mice infected with *P. aeruginosa* is shown in Fig. 7. Both the HemCon and alginate mice showed an initial sharp drop on day 1 as the bandage quenched the bioluminescence signal. However the signal in the HemCon mice continued to drop and remained at a relatively low level until it disappeared at day 7–8. By contrast the bioluminescence signal in alginate mice increased dramatically as the bacteria grew both beneath the bandage and around the edges of the bandage, until all the mice were dead within 3 days. The mean bioluminescence in the AgSD treated mice showed an initial modest drop, followed by a large increase. In both the no treatment and AgSD groups, the mice that exhibited the largest increase in bioluminescence signal between 24 and 48 h post-infection were the most likely to die.

The Kaplan-Meier survival curves are shown in Fig. 8. Mice treated with HemCon demonstrated 100% survival. Mice treated with AgSD demonstrated 75% survival, while mice receiving no treatment showed a 20% survival and mice with alginate bandages all died. The P values for the survival curves are as follows: HemCon vs. AgSD = 0.134, NS, HemCon vs. no treatment = 0.0002, Hemcon vs. alginate <0.0001, AgSD vs. no treatment = 0.0002, AgSD vs. alginate = <0.0001, no treatment vs. alginate = 0.0035.

3.6. *S. aureus* wound infections

The bioluminescence emitted by the Gram-positive *S. aureus* is dramatically lower on a per cell basis than

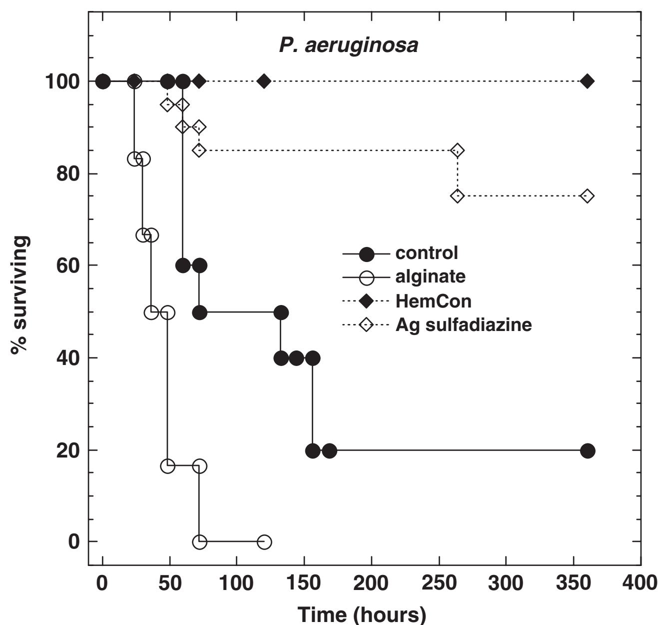

Fig. 8. Survival curves of mice with *P. aeruginosa* infection treated with HemCon ($n = 8$), alginate ($n = 6$), AgSD ($n = 20$), or no treatment ($n = 8$).

the bioluminescence emitted by both the Gram-negative species. This fact has been previously reported [21] and is due to the genetic construction used in introducing the lux operon. Nevertheless bioluminescence can still be used to follow the course of the infection in mouse wounds as shown in Fig. 9. Mice that received HemCon bandages showed a rapid decline in bioluminescence signal that

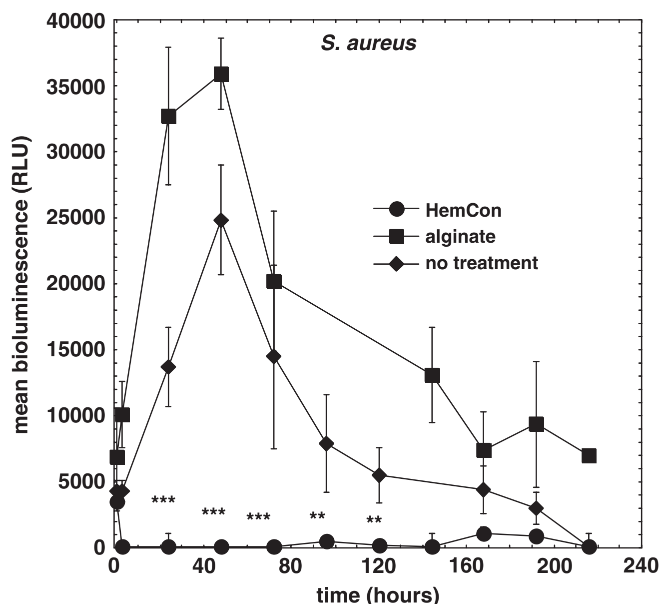

Fig. 9. Mean bioluminescence values (bars = SEM) of cyclophosphamide-treated mice with *S. aureus* infection treated with HemCon, alginate, or no treatment. ***, $P<0.001$; **, $P<0.01$ vs. all other treatments.

remained at almost undetectable levels until the bandages were lost when a small amount of bioluminescence signal was still seen embedded in the granulation tissue in the wound. No treatment wounds showed a steady increase in bioluminescence signal until day 3 when it then started a steady decline until it disappeared at day 9. Mice treated with alginate bandages showed an even higher mean increase in bioluminescence than no treatment mice that peaked 1 day later on day 4 and also declined at a slower rate.

4. Discussion

This study has shown that small pieces of HemCon bandage applied to recently contaminated mouse wounds strongly adhere to the tissue, and are highly active in killing bacteria. This fast bactericidal action prevents the bacteria from proliferating in the wound and subsequently invading the mouse tissue, gaining access to the bloodstream and causing death from sepsis.

We used three bacterial strains that are virulent in mice, two Gram-negatives; *P. aeruginosa* and *P. mirabilis* and the Gram-positive *S. aureus.* We had previously shown that *P. aeruginosa* gives rise to potentially fatal infections when introduced into excisional wounds on the mouse back that can be successfully treated with photodynamic therapy [19]. *P. mirabilis* is more frequently known as a urinary tract pathogen than as a cause of wound and burn infections [22]. Nevertheless McManus et al. showed [23] that *P. mirabilis* was highly virulent in a rat model of a contaminated surface scald and led to local bacterial proliferation in the wound occurring followed by the development of bacteremia. *S. aureus* is less virulent and invasive than the Gram-negative species discussed above. We had previously shown that the formation of *S. aureus* subcutaneous infections in mice was not efficient unless the mice were rendered temporarily neutropenic by systemic cyclophosphamide treatment [20]. In a similar fashion to the previous study *S. aureus* did not form an established infection in an excisional wound unless the mice were first rendered neutropenic.

The severity of an experimental wound infection is a function of bacterial virulence, infective dose and the presence of conditions that encourage initial bacterial proliferation after introduction into the wound. We originally chose to use the non-antimicrobial alginate bandage as a control for the optical shielding of the bioluminescence emitted from the bacteria when an opaque light-scattering substance was placed directly over the contaminated wound. However the results showed that the alginate bandage increased the number of mice dying rapidly of *P. aeruginosa* sepsis, compared to no treatment controls. We believe that the effect of the alginate bandage was to keep the bacterial inoculum moist in the wound but without exerting an antibacterial effect. This dramatic potentiation of infection did not occur when alginate was used on *P. mirabilis* infected wounds.

We decided to use only a single application of silver sulfadiazine cream against the Gram-negative infections in order to avoid possible confusion caused by repeatedly rubbing cream on the infected wound. However, it is the case that in clinical practice repeated applications of AgSD would be used in patients. This single application was much less effective than the HemCon bandage in the Gram-negative wound infections. We suppose that there were two opposite effects occurring simultaneously. The in vitro antibacterial properties of silver sulfadiazine cream have been well established [24] and it is the treatment of choice for topical antimicrobial application to many wounds and burns [25]. However it is also a cream and could have acted as a protectant to the bacteria in the wound in a similar fashion to the alginate bandage, preventing them from drying out whilst they attached to the tissue. The active preparation must leach out of the cream vehicle before it can effectively come into contact with the bacteria.

The mechanism of the antimicrobial effects of chitosan acetate has been fairly well established [5]. The large numbers of positive charges on the primary amino groups of the polyglucosamine backbone at acidic pH values interacts with the outer membrane of Gram-negative bacteria, destabilizes the lipopolysaccharide and permeabilizes both the outer and inner membranes [26] leading to leakage of cellular contents [27]. The particular features of the HemCon bandage that make it a particularly effective hemostatic agent consist of "micro-channels" that allow blood to rapidly penetrate into the sponge where the polycationic charges on the chitosan interact with the anionic charges on erythrocytes and "cross-link" them into a clot independent of platelets or plasma clotting factors

[28]. In a similar fashion the chitosan sponge may allow bacteria to penetrate into these micro-channels and consequently the bacteria would come into close contact with the cationic charges. Another possible advantage of HemCon bandage for infected wounds is that it is likely to efficiently bind bacterial lipopolysaccharide (endotoxin) released from the Gram-negative bacteria, which can be another exacerbating factor in wound infections [29].

Acknowledgments

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