Effect of chitosan acetate bandage on wound healing in infected and noninfected wounds in mice

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Manuscript received: October 3, 2007
Accepted in final form: January 28, 2008

DOI:10.1111/j.1524-475X.2008.00382.x

ABSTRACT

HemCon® bandage is an engineered chitosan acetate preparation designed as a hemostatic dressing, and is under investigation as a topical antimicrobial dressing. We studied its effects on healing of excisional wounds that were or were not infected with *Staphylococcus aureus*, in normal mice or mice previously pretreated with cyclophosphamide (CY). CY significantly suppressed wound healing in both the early and later stages, while *S. aureus* alone significantly stimulated wound healing in the early stages by preventing the initial wound expansion. CY plus *S. aureus* showed an advantage in early stages by preventing expansion, but a significant slowing of wound healing in later stages. In order to study the conflicting clamping and stimulating effects of chitosan acetate bandage on normal wounds, we removed the bandage from wounds at times after application ranging from 1 hour to 9 days. Three days application gave the earliest wound closure, and all application times gave a faster healing slope after removal compared with control wounds. Chitosan acetate bandage reduced the number of inflammatory cells in the wound at days 2 and 4, and had an overall beneficial effect on wound healing especially during the early period where its antimicrobial effect is most important.

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 an increasing need for topical antimicrobial products that can be applied to potentially contaminated wounds. Many of the products that have been developed to meet this need are highly cytotoxic toward microbial cells, and the question is then raised as to what extent are they also toxic toward mammalian cells,[1] and does this mammalian cytotoxicity then interfere with the normal coordinated process of tissue wound healing?

Products as diverse as antimicrobial peptides,[2] silver,[3–5] povidone-iodine,[6,7] and natural product extracts[8] have been examined in the context of their effectiveness as topical antimicrobial agents, and their possible adverse or beneficial effect on wound healing. The consensus opinion appears to be that a balance needs to be drawn between killing microbial cells and damaging host tissue, thereby adversely affecting wound healing, and that the precise position of this balance may depend on the virulence and pathogenicity of the infecting microbes.

Chitin or poly-*N*-acetyl glucosamine is widespread in nature as a structural material particularly in marine arthropod shells. Chitin is generally an insoluble material but can be deacetylated to form the more soluble polymer chitosan. HemCon® bandage is a compressed chitosan acetate dressing that was developed as a hemostatic agent and its polycationic nature of chitosan is such that the substance possesses natural antimicrobial properties.[9] In a previous publication[10] we reported on the use of chitosan acetate bandage as an antimicrobial dressing in our model of excisional wounds in mice heavily contaminated with bacteria. We used both Gram-negative and Gram-positive species that had been genetically engineered to be bioluminescent to allow the progress of the infection to be noninvasively monitored by bioluminescence imaging. When the Gram-negative species *Pseudomonas aeruginosa* and *Proteus mirabilis* were used as causative agents in the wound infection without treatment, mice developed sepsis and died, but application of the chitosan acetate bandage to the contaminated wound killed bacteria rapidly and saved the mice from death. In the case of Gram-positive species, *Staphylococcus aureus,* it was necessary to use temporary immunosuppression with cyclophosphamide (CY) to allow the bacteria to establish a long-lasting but not fatal localized wound infection. Application of the chitosan acetate bandage reduced the intensity and duration of infection as judged by bioluminescence imaging. Because all these mice survived, it was possible to measure the effects of these treatments on the healing of the excisional dorsal wounds. The three individual treatments, CY injection, *S. aureus* infection, and application of chitosan acetate bandage, all had significant effects on wound healing alone, and they were also used in various combinations.

In this report we present the effects of these three treatments singly and in all possible combinations on rates of wound healing in mouse dorsal excisional wounds and

Table 1. Experimental groups (n=8 mice/group) in experiment 1

No treatment	CY alone	*Staphylococcus aureus* alone	CY+*Staphylococcus aureus*
CAB alone	CY+CAB	*Staphylococcus aureus*+CAB	CY+*Staphylococcus aureus*+CAB

CY, cyclophosphamide, CAB, chitosan acetate bandage.

investigate in more detail the effects of chitosan acetate bandage alone on this model of wound healing in normal mice.

MATERIALS AND METHODS

Bacteria and culture conditions

We used the Gram-positive species *S. aureus* (strain 8325-4) carrying the entire bacterial lux operon integrated in the chromosomes (termed Xen 8.1, Xenogen Inc., Alameda, CA). Cells were cultured in brain–heart infusion (BHI) broth with aeration at 37 °C and used in mid-log growth phase (OD at 600-nm=0.6–0.8; 10^8 cells/mL). For inoculation of wounds, cell suspensions were concentrated by centrifugation and resuspension in phosphate-buffered saline solution (PBS). It should be noted that although bioluminescence imaging was used in our previous experiments with *S. aureus* bacterial species (and other bacterial species)[10] in the present studies no imaging was done, but the strain of *S. aureus* was kept the same for comparative purposes.

Preparation of chitosan acetate bandages

HemCon® Dressing (10 mm×10 mm×5.5 mm) was prepared by HemCon, Medical Technologies Inc. (Portland, OR) by methods fully described in McCarthy et al.[11]

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 (NIH Publication No. 86-23, Revised 1985). 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 and infection. The operative area of skin was cleaned with alcohol, then surgical scissors and forceps were used in combination with a template to make a square, full-thickness excisional wound down to but not through the panniculus carnosus measuring 5 mm×5 mm. There was no visible bleeding within the wounds. Some groups of mice received two doses of CY 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 did not suffer any systemic infection as judged by loss of weight and adverse health effects were minimal.

Wound healing studies

Thirty minutes after anesthetized mice received single square wounds measuring 25 mm^2, they received inocula of mid-log phase *S. aureus*=25×10^7 cells suspended in 50-μL PBS and applied with a 200-μL pipette tip into the wound bed as previously described.[12,13] The moistened bandages were applied to the normal wounds 60 minutes after wounding and to infected wounds 30 minutes after infection. Eight experimental groups (8 mice/group) are described in Table 1. Chitosan acetate bandage test pieces 1 cm×1 cm were first wetted with sodium acetate buffer (100 mM, pH 4.5) before application.[10]

Follow up

Records were kept of which day the mice lost their bandage. After the bandage was lost (or throughout the whole course of the experiment in the groups with no bandage) the dimensions of the wounds (length and width) were measured daily using vernier calipers and the areas were calculated.

Bandage removal study

Thirteen groups of five mice (including a no bandage control group) were wounded as described above and chitosan acetate bandages applied 1 hour after wounding. Bandages were removed from all mice (five in a group, which occupied a time period of roughly 15 minutes per group) at the median times after application shown in Table 2. At early time points (1 hour to 3 days) bandages were very adherent and required copious irrigation with warm (40 °C) water to soften the bandage enough to allow it to be removed with forceps. At later time points the bandage became successively easier to remove. Some mice spontaneously lost bandages between days 7 and 9. Wound dimensions were recorded daily after bandage removal.

Statistics

Data points are given as mean ± standard deviation or in the case of median wound healing time ± 95% confidence interval. The wound areas of individual mice at each time point were used for curve generation using Microcal Origin 6.1 program.[14] Areas under the curve for each mouse were calculated using the calculus integration function. The integrals were used for comparing the effects of different treatment regimens. Differences in the mean areas under the curve (MAUC) between control and treatment groups and between different treatment groups were compared for statistical significance using one-way

Table 2. Experimental groups showing delay before removing bandage (n=5 mice per group) in experiment 2

None	1 hour	6 hours	20 hours	32 hours	48 hours	3 days	4 days	5 days	6 days	7 days	8 days	9 days

ANOVA Microsoft Excel. The value of $p < 0.05$ was considered significant.

RESULTS

Healing of excisional wounds on the mouse back in groups 1–4

The time courses of excisional wound healing (25 mm^2 area) in mouse groups 1–4 are shown in Figure 1. Normal mice demonstrated an initial increase in wound size with a reduction from the original size not occurring until day 6, while normal mice that had *S. aureus* introduced into the wound showed no initial expansion and wounds started to contract as early as the day after wounding. Mice treated with CY alone showed an expansion two and a half times bigger than normal mice and no contraction until after day 10. Wounds in mice that had been treated with CY and then infected with *S. aureus* showed the size remained constant until after day 5, when wound contraction occurred at the same rate as that observed in control mice and eventual healing at the same time. Statistical comparisons of the areas under the curve showed that CY decreased the healing process compared with controls, while S. *aureus* increased the healing process whether or not CY was also present (Figure 1B).

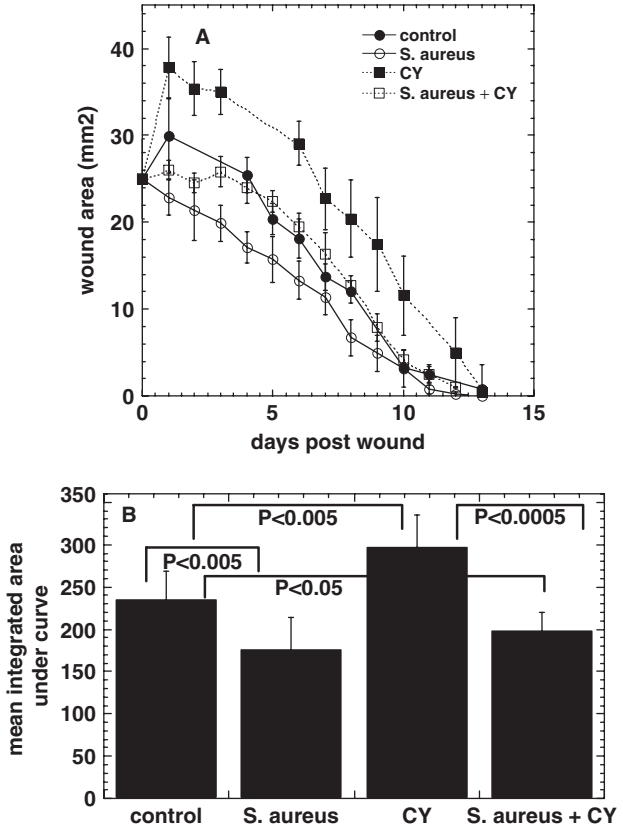

Figure 1. (A) Mean wound areas (error bars are SD) with time in groups of mice (n=8, groups 1–4) as follows: control excisional wounds 5 mm×5 mm; wounds inoculated 30 minutes after wounding with 250 million *Staphylococcus aureus;* wounds in mice that received two doses of cyclophosphamide (first of 150 mg/kg given 4 days before wounding; second of 100 mg/kg given 1 day before wounding); wounds in mice that received both cyclophosphamide and *S. aureus*. (B) Mean values (error bars are SD) of wound areas integrated over time (day 0–13) for individual mice (n=8 per group 1–4) as above.

Wound healing in mice with chitosan acetate bandage in groups 5–8

The healing curves of mouse groups 5–8 with chitosan bandage are shown in Figure 2. The adherence of the bandage to the wound meant that we could not measure

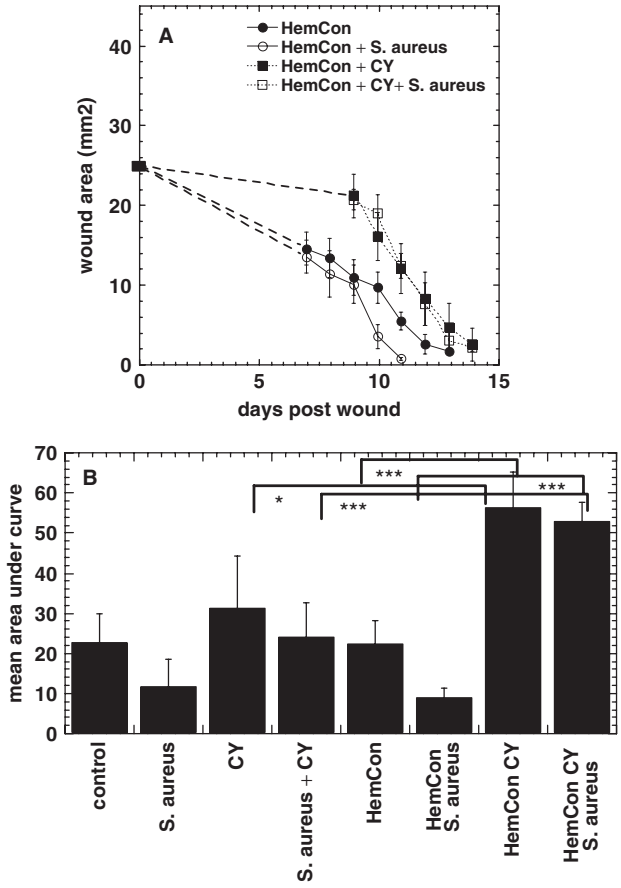

Figure 2. (A) Mean wound areas (error bars are SD) with time in groups of mice (n=8, groups 5–8) as follows: excisional wounds with HemCon applied 60 minutes after wounding; wounds inoculated 30 minutes after wounding with 250 million *Staphylococcus aureus* followed by HemCon 30 minutes later; wounds in mice that received two doses of cyclophosphamide and HemCon 60 minutes after wounding; wounds in mice that received both cyclophosphamide and *S. aureus* followed by HemCon. Wound areas were measured starting from the time when the bandage detached naturally (B). Mean values (error bars are SD) of wound areas integrated over time (starting at day 9–15) for individual mice (n=8 per group) in groups 1–8. Statistical significance ($p < 0.05$, *; $p < 0.1$; $p < 0.01$, ***).

wound size during the time the bandage was present (at least not without some sophisticated imaging technique that could see through the bandage) and we have inserted a dotted line to cover the portion of the healing curve that could not be measured. Normal wounds or *S. aureus* infected normal wounds had substantially healed when the bandage was lost; however, once the bandage was removed, wounds that had received *S. aureus* healed faster than the uninfected wounds. By contrast when the bandage was lost from CY-treated mice whether or not the wound was also infected with *S. aureus,* the mean area was only marginally less than the original area. These two groups, however, healed at the same rate after bandage loss. We could only integrate the individual healing curves from day 9 to the time at which eventual healing occurred, and we also calculated these MAUC values from day 9 onward for the healing curves from the mouse groups 1–4 to allow more comparisons to be made (Figure 2B). The bandage decreased the rates of wound healing in CY-treated mice both in the presence and absence of *S. aureus*.

Effect of chitosan acetate bandage on healing of normal wounds

We attempted to isolate the effects of chitosan acetate bandage on excisional wound healing in normal noninfected mice, by removing the chitosan acetate bandage at a number of time points after its application. We finally used 13 different time points as described in Table 2. In the last four groups (6–9 days) some mice had already lost their bandage naturally, and their wound sizes were measured from the time of bandage loss. For the sake of convenience and visibility we have plotted the mean wound healing curves of these 13 groups of mice in two groups: control plus 1 hour to 2 days (Figure 3A); control plus 3 days to 9 days (Figure 3B).

When the bandage was removed from mice between 1 and 20 hours after wounding (when it had already dried) the wounds had a greater (but decreasing with time) expansion than that seen in nonbandaged wounds (Figure 3A); however, subsequent healing was rapid and the wounds eventually healed somewhat quicker than normal wounds. The enforced delay in wound healing caused by the bandage adhering strongly to the tissue started to negatively impact wound healing from day 5 onward.

We calculated the median time to complete healing of all these groups of mice and the data are displayed in Figure 4A. It can be seen that there is an increase in healing time compared with controls for short periods 1 and 6 hours, a reduction in healing time compared with controls for periods of 32 hours to 4 days, and an increase in healing time that became progressively larger for times from 6 to 9 days. The median time for healing of the 3-day bandage removal wound was significantly shorter than the control, and than that of bandages removed at 1 hours, 6 hours, and all times between 6 and 9 days ($p < 0.05$).

The other factor that appeared to vary in these groups of mice was the rate of healing once the bandage had been removed. We calculated the mean slopes of the wound healing curves of these groups of mice after bandage loss and the data are displayed in Figure 4B. Every group of mice had a faster rate of wound closure after chitosan acetate bandage removal (range 3.7–5.3, mean 4.5 ± 0.5) compared with control wounded mice (3 ± 0.52) and the difference was highly significant ($p < 0.0001$). There was a trend towards increased healing rate with increasing time of chitosan acetate bandage application from 1 hour until 7 days when the healing rate slowed down rapidly at 8 and 9 days.

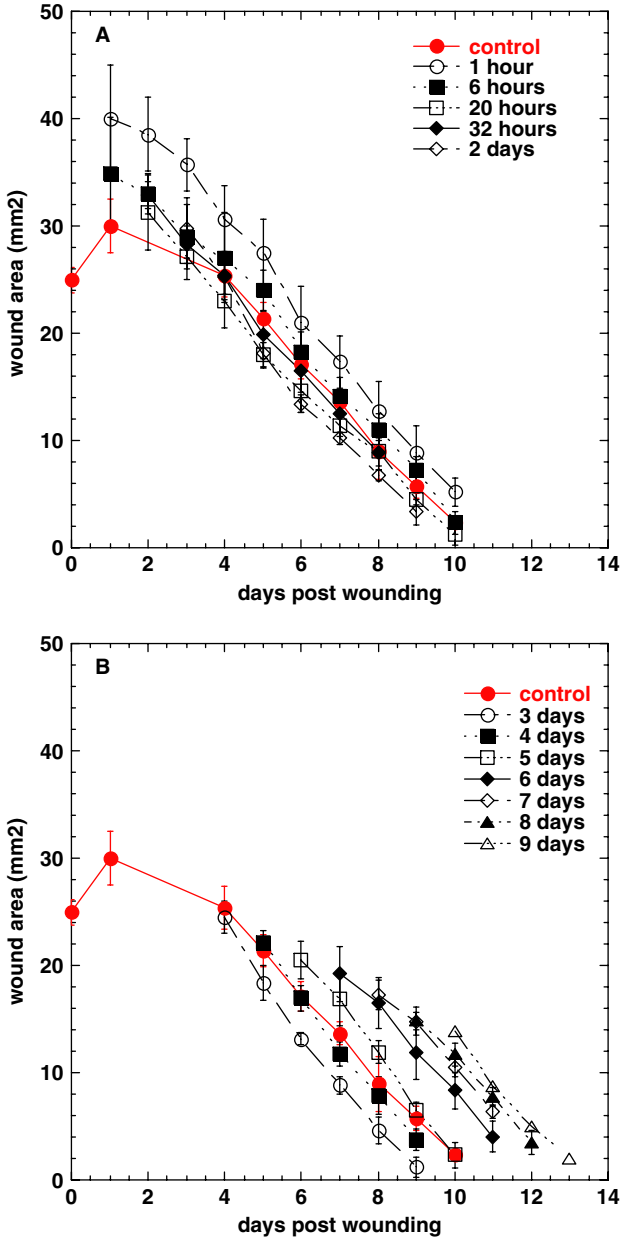

Figure 3. (A) Mean wound areas (error bars are SD) with time in groups of mice (n=5) as follows with excisional wounds without HemCon, or with HemCon applied 60 minutes after wounding and removed by soaking at different times after application from 1 hour to 2 days. (B) Same as (A) but showing mice without HemCon, compared with groups where HemCon was removed from 3 to 9 days after application.

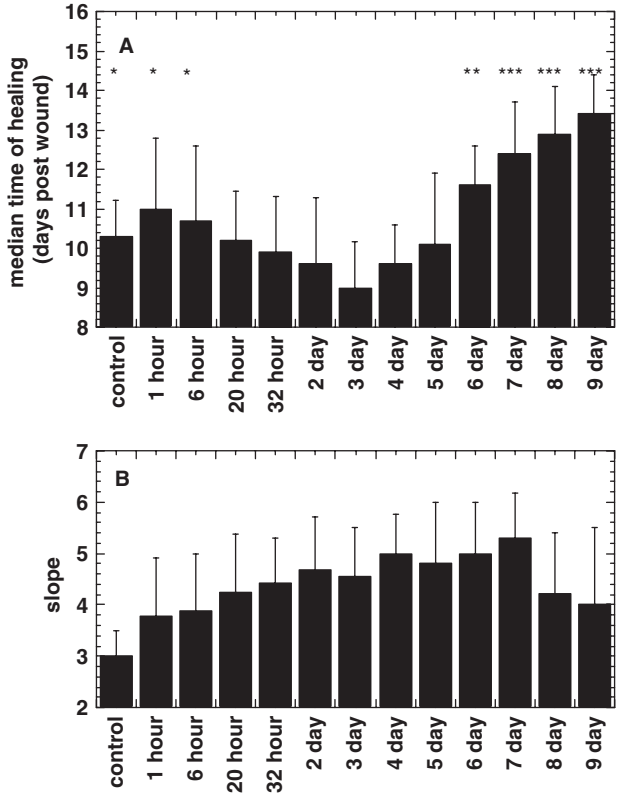

Figure 4. (A) Median time (error bars are 95% confidence interval) of complete wound closure of mice groups shown in Figure 3A and B. Statistical significance ($p < 0.05$, *; $p < 0.1$, **; $p < 0.01$, ***) vs. healing time for 3-day removal. (B) Mean slopes (error bars are SD) of the linear part of the wound healing curves of the mice groups ($n=5$) shown in Figure 3A and B.

Histopathology of chitosan acetate bandage-treated wounds

It was possible to remove the wounds with the chitosan acetate bandage still adherent to the tissue and after fixation in formalin and paraffin embedding and sectioning many slides still had visible chitosan acetate bandage attached to the tissues. Standard hematoxylin and eosin staining of wounds removed from control mice and chitosan acetate bandage-treated mice at 1, 2, and 4 days postwounding are shown in Figure 5A–L. At 1 day postwounding little cell or tissue response is seen either to the control wound (Figure 5A) or to the chitosan acetate bandage-treated wound (Figure 5G). At 2 days post wounding in control wounds (Figure 5B and C) inflammatory cells can be seen especially at the wound edge (Figure 5B). By contrast in the chitosan acetate bandage-treated wounds there are fewer inflammatory cells visible in the wound edge (Figure 5H), and virtually none in the wound bed (Figure 5I). At day four the difference between control wounds and chitosan acetate bandage-treated wounds became even more marked, with major inflammation visible in control wounds (Figure 5D–F). In chitosan acetate bandage-treated wounds at 4 days (Figure 5J–L) inflammation is markedly reduced and in addition there is an accumulation of fibrinous material visible on top of the wound immediately below the chitosan.

DISCUSSION

It is well known in medicine that infected wounds heal more slowly and with worse overall results than noninfected wounds[15,16] so the stimulation of wound healing in mice that had their wounds infected with *S. aureus* was somewhat surprising. In fact a large part of the wound care market is concerned with the antibacterial effects of various topical preparations and wound dressings.[17,18] *S. aureus* is less virulent and invasive than Gram-negative species such as *P. aeruginosa,* and in our experience does not invade the mouse systemically even when relatively large infective doses are introduced into mouse wounds, or the mice are immunosuppressed with CY. We had previously shown that the formation of *S. aureus* subcutaneous infections[19] or excisional wound infections[10] in mice was not efficient unless the mice were rendered temporarily neutropenic by systemic CY treatment.

However, there are several reports in the literature that *S. aureus* in particular (or possibly uniquely) can stimulate rather than suppress wound healing in small animal models. This was first shown in 1983[20] when live *S. aureus* inoculated into scalpel wounds in rats dramatically accelerated healing as measured by wound strength. Seven strains of *S. aureus* demonstrated the accelerated wound-healing effect, while *S. epidermidis* (three strains), *S. hominis* (one strain), and *P. aeruginosa* (two strains), did not. Subsequent studies found that that nonviable *S. aureus*[21] and purified *S. aureus* peptidoglycan[22] could also accelerate wound healing, and could overcome corticosteroid-induced suppression[23] and also CY-induced suppression[24] of incisional wound healing in rats. The only significant differences observed in experiment one after chitosan application was a reduction of the wound healing process in the presence of CY. This is not surprising because the effect of CY is to dramatically increase the initial wound expansion and chitosan then clamps it in place.

We found there are two effects of chitosan acetate bandage application on normal excisional wounds. Firstly, there is a clamping effect that may be either good or bad depending on whether the wound is expected to expand or not. In our model relative benefit mainly depended on the time of application, with times from 32 hours to 5 days being beneficial and the maximum time of benefit being 3 days. Secondly, there is a stimulation effect on the rate of wound healing when the bandage is finally removed. The slope of the healing curve was significantly steeper in all wounds that had had chitosan acetate bandage applied, with the greatest effect for application times between 4 and 7 days.

One effect to be considered is the action of an applied bandage in preventing excessive loss of water from an open excisional wound. This phenomenon has been previously reported[25] when application of an asymmetric chitosan bandage reduced the evaporation rate of water from the wound.

There are many reports in the literature on various preparations of chitosan on processes related to wound healing, and by and large chitosan is thought to stimulate wound healing. These reports, however, are difficult to

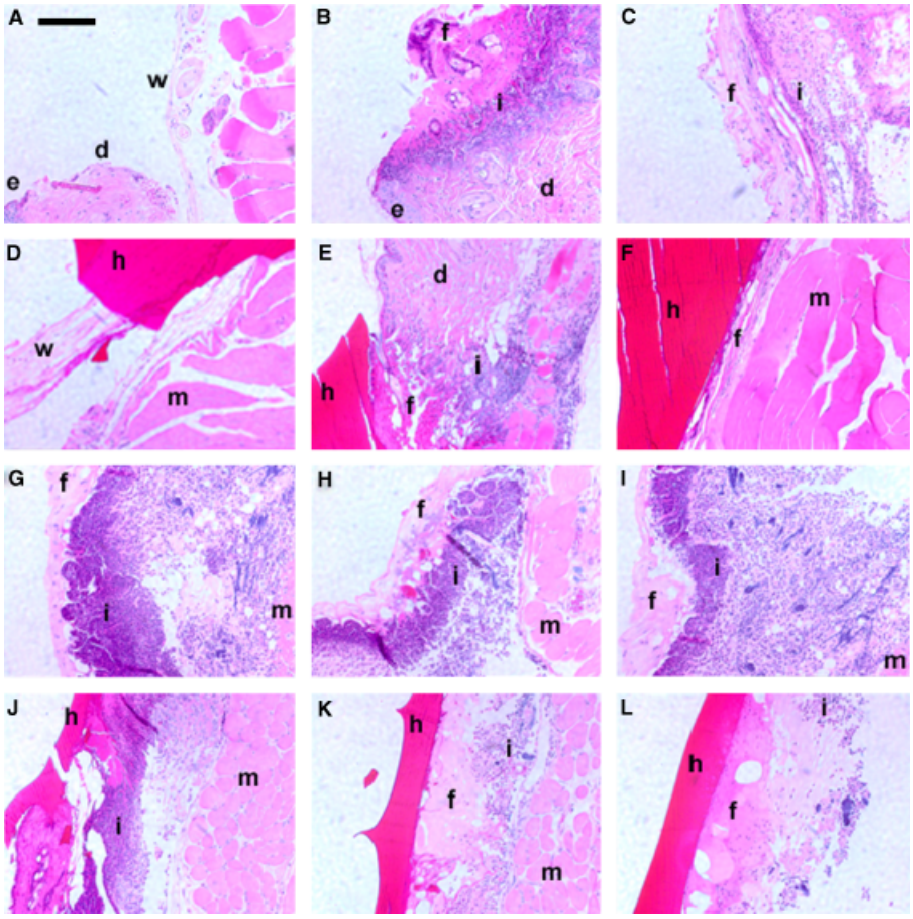

Figure 5. H&E histology of wound tissue samples removed from control wounded mice (A–C; G–I) and HemCon treated wounded mice (D–F; J–L) at 1 day (A, D), 2 days (B, C, E, F) and 4 days (G, H, I, J, K, L) after wounding. Scale bar is 200 μm and abbreviation letters are as follows; h, HemCon; w, wound bed; d, dermis, e, epidermis; i, inflammatory cells; f, fibrin; m, muscle.

compare as some authors have used derivatives or copolymers of chitosan, and various others have used diverse chitosan preparations to deliver growth factors to stimulate wound healing. These have included epidermal growth factor,[26] and basic fibroblast growth factor.[27] The reports on the effects of chitosan alone on wound healing have included both in vitro and in vivo studies. Hamilton et al.[28] found increases in fibroblast attachment and proliferation when the cells were in contact with chitosan films depending on degree of deacetylation and chemical composition. Mori et al.[29] found that chitosan activated macrophages in particular by increasing expression of major histocompatibility complex class I, class II, Fc receptors, transferrin receptor, mannose receptor, Fas, and macrophage inflammatory protein 2. Ueno et al.[30] applied "cotton fibre-type" chitosan to x-irradiated wounds in dogs and found increased neovascularization and mRNA for vascular endothelial growth factor. Kojima et al.[31] used a polyester nonwoven fabric impregnated with chitosan and found increased collagen synthesis and prolyl hydroxylase activity in rat wounds. Azad et al.[32] showed that a chitosan mesh membrane with a 75% degree of deacetylation and a thickness of 10 μm used as a dressing in skin-graft donor sites in patients, gave better healing, and a positive effect on the reepithelialization and the regeneration of the granular layer compared with the alternative Bactigras dressed area.

In conclusion, we have shown that a topically applied chitosan bandage can have beneficial effects on wound healing in addition to its antibacterial properties. The bandage holds the wound edges immobile during the time it is adherent and reduces inflammatory cell infiltrate at days 2–4. The clamping effect may be beneficial in avoiding initial wound expansion in the first days after wounding, or detrimental in preventing wound healing after adherence for longer times. After removal of the bandage, wounds at all time-points heal faster than wounds without bandage.

ACKNOWLEDGMENTS

This work was supported by HemCon Medical Technologies Inc., and by the US National Institutes of Health (grant R01 AI050875 to M.R.H.). Ana P. Castano was supported by Department of Defense CDMRP Breast Cancer Research Grant (W81XWH-04-1-0676). Tatiana N. Demidova-Rice was supported by a Wellman Center Graduate Student Fellowship. We thank Xenogen Corp. and Dr. Kevin P. Francis for the generous gift of stable bioluminescent *S. aureus*. We thank HemCon Medical Technologies Inc. for supplying samples of HemCon® Bandage. We are grateful to Simon J. McCarthy and William P. Wiesmann for helpful discussions, advice, and support.

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