Biomaterials 29 (2008) 4323–4332

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties

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ARTICLE INFO

Article history:
Received 23 June 2008
Accepted 21 July 2008
Available online 15 August 2008

Keywords:
Wound dressing
Chitosan
Hemostatic
Antimicrobial

ABSTRACT

Hemorrhage remains a leading cause of early death after trauma, and infectious complications in combat wounds continue to challenge caregivers. Although chitosan dressings have been developed to address these problems, they are not always effective in controlling bleeding or killing bacteria. We aimed to refine the chitosan dressing by incorporating a procoagulant (polyphosphate) and an antimicrobial (silver). Chitosan containing different amounts and types of polyphosphate polymers was fabricated, and their hemostatic efficacies evaluated *in vitro*. The optimal chitosan-polyphosphate formulation (ChiPP) accelerated blood clotting ($p = 0.011$), increased platelet adhesion ($p = 0.002$), generated thrombin faster ($p = 0.002$), and absorbed more blood than chitosan ($p < 0.001$). Silver-loaded ChiPP exhibited significantly greater bactericidal activity than ChiPP *in vitro*, achieving a complete kill of *Pseudomonas aeruginosa* and a >99.99% kill of *Staphylococcus aureus* consistently. The silver dressing also significantly reduced mortality from 90% to 14.3% in a *P. aeruginosa* wound infection model in mice. Although the dressing exerted severe cytotoxicity against cultured fibroblasts, wound healing was not inhibited. This study demonstrated for the first time, the application of polyphosphate as a hemostatic adjuvant, and developed a new chitosan-based composite with potent hemostatic and antimicrobial properties.

1. Introduction

Uncontrolled hemorrhage remains the leading cause of pre-hospital trauma deaths in both the combat and civilian settings [1,2]. A number of hemostatic agents have been developed that can arrest bleeding and stabilize the casualty before evacuation to definitive care. Of these, the HemCon chitosan dressing and the QuikClot zeolite powder are being used routinely in the battlefield, and case reports show that both were effective in reducing or stopping bleeding in >90% of applications [3,4]. However, the application of QuikClot generates heat that can cause burn injuries, and some studies have found no significant survival benefit of either QuikClot or HemCon over gauze in more extreme animal models of hemorrhage [5,6]. The challenge remains to develop more effective hemostatic dressings that can control bleeding that would otherwise lead to exsanguination.

Another challenge facing caregivers is the growing incidence of infection by antibiotic-resistant bacteria strains in combat trauma wounds [7,8]. Although the use of broad spectrum antibiotics has been implicated in the selection of these resistant pathogens, antibiotic prophylaxis remains the standard of care since early surgical debridement to reduce wound bacteria bioburden may not be possible under combat conditions [9,10]. To contain the problem of resistance, alternatives to antibiotics should be used to manage wound infection. Ionic silver is active against a wide range of pathogens including multi-drug resistant strains, and have a far lower propensity for resistance development [11,12]. Topical silver thus offers a useful first line intervention to stop the progress of infection that can lead to septicemia and death, while reducing the risk of inducing resistant strains that will be difficult to treat after evacuation.

In view of the dual challenges of bleeding and contamination in combat wounds, we conceived a chitosan-based dressing with improved hemostatic and antimicrobial properties. Chitosan is an attractive biomaterial for wound care because of its biocompatibility and intrinsic hemostatic and antimicrobial properties [13]. We hypothesized that the addition of polyphosphate (PP) polymers to chitosan can lead to a more potent hemostat. Polyphosphate, a linear polymer of inorganic phosphate, is found in high concentrations in platelet dense granules, and polymers with ≥45 phosphate units in their chain have been shown to accelerate blood coagulation and delay fibrinolysis [14]. Since polyphosphate dissolves in water to form a polyanion, it can ionically interact with chitosan to form polyelectrolyte complexes (PECs) [15].

A series of chitosan-polyphosphate dressings were fabricated and their hemostatic efficacies evaluated against chitosan and

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doi:10.1016/j.biomaterials.2008.07.034

gauze *in vitro*. After identifying the most effective hemostatic formulation (coded as ChiPP), silver nanoparticles were incorporated to enhance the dressing's antimicrobial activity, since chitosan's antimicrobial action is limited against certain species of bacteria and in non-acidic pH environments [16,17]. The silver dressing (coded as ChiPP-Ag) was then compared with ChiPP in time kill assays against two common wound pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, followed by *in vitro* characterization of silver elution and cytotoxicity, before evaluation in a murine model of wound infection.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree of 75–85% and MW ~190,000–310,000) was obtained from Sigma. Polyphosphate polymers with either 45 or 65 phosphate units per chain (abbreviated as PP_{45} and PP_{65}, respectively) were also obtained from Sigma. Silver nanoparticles were obtained from American BioTech Labs (UT, USA). Sterile gauze was obtained from Melintex Pharma Co (China), and cut into $0.5 \times 0.5\ cm^2$ for experiments. HemCon and Acticoat Absorbent dressings were obtained from HemCon Medical Technologies Inc (Or, USA) and Smith & Nephew (Singapore), respectively. All other materials were obtained from Sigma Chemical Co (Singapore) unless otherwise noted.

2.2. Fabrication of wound dressings

Chitosan powder (450 mg) was dispersed in 30 mL of 1.0% w/w of glacial acetic acid (Aldrich) and stirred vigorously overnight to achieve dissolution. A solution of PP was prepared by dissolving in water at 500 mg/mL, followed by standing at 4 °C until a homogenous solution was formed. Different amounts of PP were aliquoted into the chitosan solution to achieve a 6.7% w/w, 10% w/w or 15% w/w ratio in chitosan. This was immediately followed by vigorous shaking of the mixture and sonication for 20 min (minutes) to disperse the PP polymers and degas the resulting gel-like PEC. The solutions were frozen in a −80 °C freezer for 3 h (hours) followed by freeze-drying for at least 24 h. Each freeze-dried dressing was compressed before cutting into $0.5 \times 0.5\ cm^2$ (~40 mg each) for experiments, unless otherwise stated. The PECs were abbreviated as Chi-6.7%$PP_{45\ or\ 65}$, Chi-10%$PP_{45\ or\ 65}$, and Chi-15%$PP_{45\ or\ 65}$.

To fabricate the silver dressings, different amounts of silver nanoparticles (100–500 μg/cm^2) were mixed with the chitosan solution, followed by adding PP, freezing, and freeze-drying. Incorporation of silver into the ChiPP dressing did not affect its hemostatic properties.

2.3. Blood collection and platelet isolation

Swine blood was collected from a cannulated femoral artery under non-activation conditions, and anti-coagulated with acid citrate dextrose (20 mM citric acid, 110 mM sodium citrate, 5 mM D-glucose) at a v/v ratio of 9:1 or 1 U/mL heparin, as per protocol approved by the DSO Institutional Animal Care and Use Committee (IACUC). To isolate platelet solutions, whole blood was centrifuged at $180 \times g$ for 20 min, and platelet rich plasma was separated from the red blood cell fraction and further centrifuged at $1500 \times g$ for 15 min to pellet and concentrate the platelets. The platelet pellet was removed and resuspended in a buffer (140 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.1% glucose, pH 7.4, adjusted with 4% HEPES). The concentration of the platelet suspension was measured using the Cell-Dyn 500 hemostasis analyzer (Abbott Laboratories, IL, USA). Only suspensions with >100,000 platelets/mL were used in experiments.

2.4. Whole blood clotting

The blood clotting test was adapted from Shih et al. [18]. Dressings were placed into polypropylene tubes, and prewarmed to 37 °C. Citrated whole blood (0.2 mL) was then dispensed onto the dressings, and 20 μL of 0.2 M $CaCl_2$ solution added to start coagulation. The tubes were incubated at 37 °C and shaken at 30 rpm. After 10 min, red blood cells (RBCs) that are not trapped in the clot were hemolyzed with 25 mL of water, and the absorbance of the resulting hemoglobin solution was measured at 540 nm.

2.5. Platelet adhesion

The platelet adhesion assay was adapted from Vanickova et al. [19]. Before the start of the test, platelets were reconstituted to 2.5 mM $CaCl_2$ and 1.0 mM $MgCl_2$. A 1.5 mL of the platelet suspension was added per dressing. After incubation at 37 °C for 1 h, the dressings were removed and dip rinsed twice in phosphate-buffered saline (PBS) to remove the platelets that are not attached. The samples were then placed into PBS containing 0.9% Triton-X100 for 1 h at 37 °C to lyse adhered platelets. The lactate dehydrogenase (LDH) enzyme that was released was measured using a kit (Promega, USA) as per manufacturer's instructions. A platelet calibration curve was generated by serially diluting a known number of platelets, followed by lysis with 0.9% Triton-X100 and measuring of LDH. Samples were also fixed in 4% paraformaldehyde + 0.5% glutaraldehyde for electron microscopy studies.

2.6. Thrombin generation

Thrombin–antithrombin complex (TAT), a marker of thrombin neutralization, is an indicator of how much thrombin was formed over a period of time [20]. Dressings were incubated with 1 mL of heparinized whole blood for 30 min at 37 °C, before 20 μL of sodium citrate (0.633 M) was added to stop thrombin generation. TAT levels in blood samples were measured by an ELISA kit (Dade Behring, Germany) as per manufacturer's instructions.

2.7. Blood and simulated body fluid absorption

The absorption efficiency of dressings was determined in citrated whole blood and simulated body fluid (SBF), a solution with ion concentrations similar to that of human plasma. SBF consists of 142 mM Na^+, 5.0 mM K^+, 2.5 mM Ca^{2+}, 148 mM Cl^-, 4.2 mM HCO_3^-, 1.0 mM HPO_4^{2-} and 5.0 mM SO_4^{2-}, and was buffered at pH 7.4 with tris(hydroymethyl) aminomethane and 1 M hydrochloric acid at 37 °C. The pre-weighed dry dressings (W_{ini}) were placed into tissue culture plate wells containing 1.5 mL of SBF or citrated whole blood. Plates were sealed and incubated in a humidified chamber at 37 °C. At the end of 2 h, the dressings were removed, placed on absorbent paper towel for 3 s to remove surface water and freely draining liquid, followed by weighing to determine the wet weight (W_{wet}). Fluid uptake (g/g) was calculated as $(W_{wet} - W_{ini})/W_{ini}$.

2.8. Antimicrobial efficacy

Time kill assays adapted from Ip et al. [12] were used to evaluate the antimicrobial efficacy of ChiPP dressings containing different amounts of silver. Briefly, a suspension of *P. aeruginosa* (ATCC 27853) or *S. aureus* (ATCC 25923) was prepared from fresh colonies on tryptic soy agar (TSA) and the turbidity was adjusted to 0.5 McFarland standard. 7 μL of the bacteria suspension was added to each vial containing 2.5 mL of tryptic soy broth (TSB). Dressings were then added, followed by incubation at 37 °C, 220 rpm. Aliquots were taken at 0 h, 0.5 h, 2 h, 4 h, and 24 h, and plated directly or after serial dilution to count the number of surviving bacterial colonies. Only plates between 30 and 300 colonies were counted, and counts were used to determine the number of colony forming units per mL of TSB (cfu/mL). The broth was re-innoculated at 24 h and 48 h with the same type of bacteria, and plated 24 h after each repeat challenge. The log reduction was calculated as $\log_{10}$ [initial bacteria upon challenge (cfu/mL)] − $\log_{10}$ [surviving bacteria at timepoint after challenge (cfu/mL)]. Bactericidal activity was defined as a $\geq 3\ \log_{10}$ cfu/mL (≥99.9%) reduction in bacteria numbers.

2.9. Silver elution

The release of silver ions from a 1 cm^2 ChiPP-Ag dressing was evaluated in 16 mL of sterilized filtered distilled water. The tubes were incubated for 30 min, 2 h, 4 h, 24 h, 48 h, and 72 h at 37 °C. After incubation, the silver dressings were removed aseptically, allowed to drip into the tubes for approximately 10 s, and then discarded. The water was filtered through a 0.22 μm filter and stored at 4 °C. Silver concentrations were measured by Inductively Coupled Plasma (ICP). Water is generally viewed as the most appropriate release medium to assess steady state silver release into solution from the dressing, because organic and inorganic material in other media may reduce Ag^+ to inactive Ag^0, which cannot be distinguished by ICP [21].

2.10. Cytotoxicity

The cytotoxic effect of the HemCon, Acticoat, ChiPP, and ChiPP-Ag dressings was evaluated on fibroblast monolayer cultures. All dressings were cut into $1 \times 1\ cm^2$ under sterile conditions. Neonatal Human Dermal Fibroblasts (Cambrex Bio Science, MD, USA) were cultured in high glucose DMEM with L-glutamine and sodium pyruvate (Gibco), supplemented with 10% FBS (Invitrogen), 100 U/mL penicillin, 100 μL/mL streptomycin and 0.25 μg/μL amphotericin B. Fibroblasts at passages 8–10 were seeded into six-well plates at a density of 6.6×10^3 cells/cm^2, and incubated overnight. The culture medium was then removed and fresh medium without antibiotics added. Following that, dressings were added, and the cells incubated at 37 °C in 5% CO_2 for 24 h, 48 h, and 72 h. A negative control (no dressing) was included for each timepoint. At each timepoint, dressings were removed, the medium aspirated, and WST-1 (a cell proliferation reagent; Roche Diagnostics, Switzerland) in complete medium (with antibiotics) was added and used as per manufacturer's instructions. The results are expressed as cell viability relative to the control group at each respective timepoint.

2.11. P. aeruginosa *infection in a mouse full-thickness wound model*

Animal experiments were approved by the DSO IACUC. Male Balb/c mice (19–26 g) were anesthetized with an IP injection of ketamine (200 mg/kg) and xylazine (10 mg/kg). A 1×1 cm^2 full-thickness wounds down to the panniculus carnosus were generated in the center of the lower back after shaving and cleansing with alcohol. Wound perimeters were then traced onto sterile transparencies for subsequent square counting over graph paper to determine the initial wound area. Following wounding, mice received 35 μL of TSB containing $\sim 1 \times 10^{11}$ cfu/mL ($OD_{650} \sim 0.53$) of mid-log phase *P. aeruginosa* on the wound surface. Mice were dressed with either gauze or ChiPP-Ag dressings (2×2 cm^2, wetted with 200 μL of water) 30 min after infection. Both dressings were secured with Transpore™ surgical tape (3M, Singapore), although ChiPP-Ag dressings generally remain adhered to tissue for up to a week. There were 6–10 animals per treatment per timepoint (day 1, 2, 7, 14). Animals were monitored daily and euthanized when they became moribund. A pilot experiment was done to determine the inoculum required to reach desired mortality rates for the gauze (control) group.

2.12. *Evaluation of wound infection, healing, and mortality rates*

Dressings were removed at each timepoint, followed by weighing and collection of cardiac puncture blood for neutrophil counts using the Cell-Dyn 500 hemostasis analyzer. The overlying fibrinous clot was removed and the wound area measured as above, followed by surface decontamination with 70% ethanol before taking a 6 mm punch biopsy (Zuellig Pharma, Singapore) at the center of the wound. Biopsy samples were weighed and homogenized in 1 mL of sterile PBS-1% Tween-80 (to enhance bacteria dispersal) using a homogenizer (Heidolph Diax 900). The homogenizer was cleaned with large volume washes of ethanol and sterile PBS between samples. Undiluted samples and serially diluted samples of the tissue homogenate were plated on TSA for quantification of tissue bacteria load (cfu/g). Wound tissue samples were also fixed in formalin for histology. Sections were stained by routine Hematoxylin & Eosin (H&E), Masson Trichrome for collagen evaluation, and Gram–Twort stain for localization of bacteria. Mice that survived past 7 days were generally long term survivors, thus the day 14 groups were used to determine the mortality rates of respective treatments.

2.13. *Statistical analysis*

Data points are expressed as means ± standard deviations. Differences between means were analyzed for statistical significance using Student's *t*-test or one-way ANOVA with post hoc Scheffe test. Survival curves were compared by the Kaplan–Meier log-rank statistical method. *P* values <0.05 were considered significant.

3. Results

3.1. *Whole blood clotting*

In order to evaluate whether chitosan-polyphosphate PECs can increase the rate of blood clotting, whole blood was contacted with dressings for 10 min before hemolyzing RBCs that were not trapped in the clot that formed on the dressing surface. A higher absorbance value of the hemoglobin solution thus indicates a slower clotting rate. Chitosan with 6.7% w/w or 10% w/w PP_{65} or PP_{45} led to significantly lower absorbance values than chitosan (Fig. 1A). The clots formed on Chi-10%PP_{45} were also visibly larger than those on chitosan at 10 min (Fig. 1B). Since chitosan containing 15% w/w of either type of PP did not increase blood clotting rates significantly, these were not examined any further. Blood clotting rates on gauze were significantly slower than chitosan ($p < 0.05$), while the absorbance value of no-sample controls was significantly higher than that of chitosan-based materials ($p < 0.001$) but not gauze ($p = 0.362$). This indicated that gauze was not able to cause significant blood clotting within 10 min.

3.2. *Platelet adhesion*

Materials were contacted with platelet suspensions for 1 h, followed by measuring the LDH activity after lysis of adhered platelets. Significantly more platelets adhered onto the Chi-10%PP_{45} ($p < 0.01$) and Chi-10%PP_{65} ($p < 0.05$) than chitosan (Fig. 2A), while significantly fewer platelets were adhered on gauze than chitosan ($p < 0.001$). More platelets were also observed on Chi-10%PP_{45} than chitosan under SEM (Fig. 2C, F). In addition, larger platelet aggregates and more extensive platelet pseudopod formation can be observed on Chi-10%PP_{45} compared to chitosan (Fig. 2D, E, G, H).

3.3. *Thrombin generation*

TAT was measured in whole blood incubated with materials as an indicator of how much thrombin was formed over time. Only blood contacted with Chi-10%PP_{45} had significantly higher TAT levels than chitosan after 30 min (Fig. 2B). The level of TAT in blood contacted with Chi-10%PP_{45} was 100-fold higher than baseline no-sample controls. Significantly lower levels of TAT were measured in blood contacted with gauze ($p < 0.05$) than in blood contacted with chitosan-based materials.

3.4. *Blood and SBF absorption*

After immersion for 2 h in respective fluids, Chi-10%PP_{45} (coded as ChiPP) absorbed about 2 times as much blood as chitosan (16.3 ± 1.8 g/g compared to 8.2 ± 0.8 g/g, $p < 0.001$, one-way ANOVA with post hoc Scheffe test, $n = 5$). Both chitosan and ChiPP absorbed about the same amount of SBF (~ 17.5 g/g, not significant). Gauze absorbed about 5.2 g/g of both blood and SBF, which was significantly less than both chitosan-based materials ($p < 0.001$, $n = 5$).

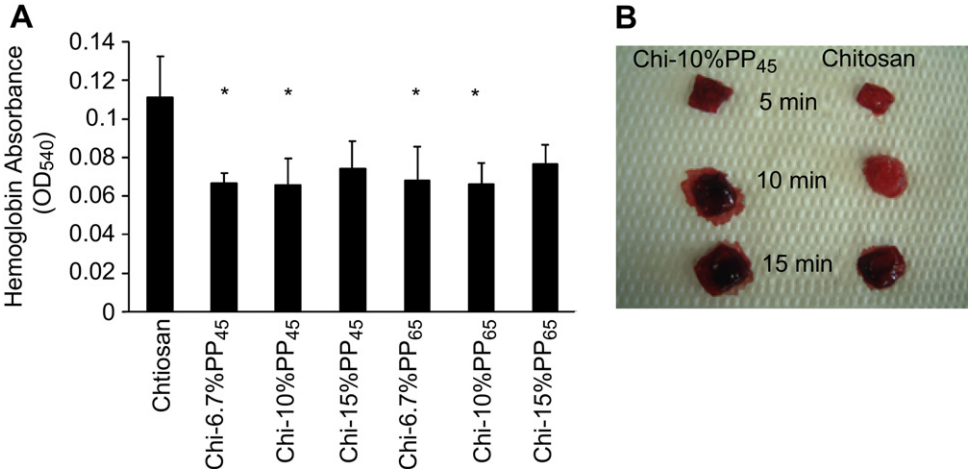

Fig 1. (A) Effect of the proportion and type of PP in chitosan on blood clotting rates, as measured by absorbance of hemoglobin from lyzed uncoagulated RBCs. *$p < 0.05$ compared to chitosan, analyzed by one-way ANOVA with post hoc Scheffe test, $n = 4$. (B) Photograph showing more rapid clot formation on Chi-10%PP_{45} compared to chitosan.

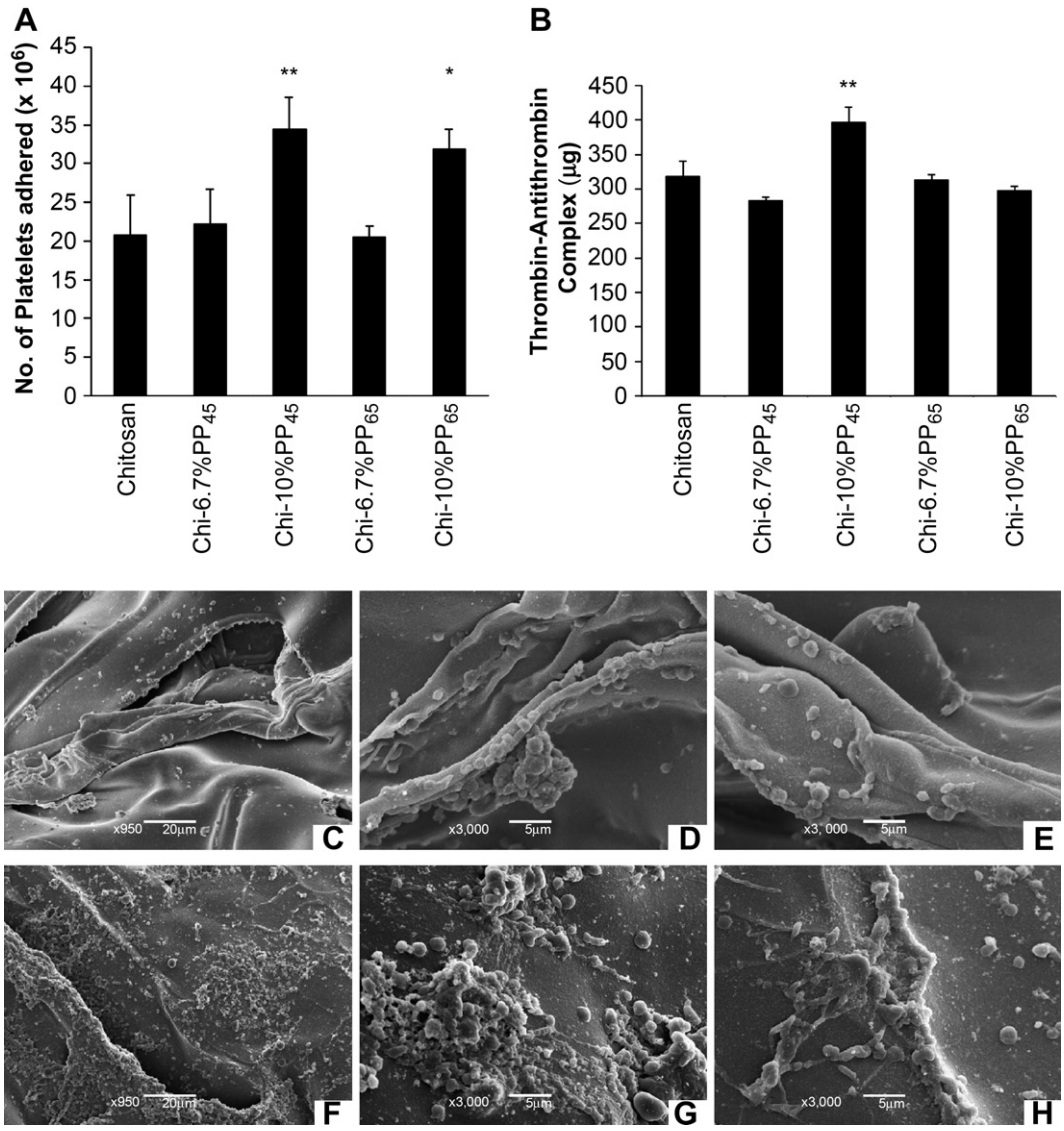

Fig 2. Effect of the proportion and type of PP in chitosan on (A) platelet adhesion and (B) thrombin generation over time, as measured by the levels of thrombin–antithrombin complex. $^{*}p < 0.05$ and $^{**}p < 0.005$ compared to chitosan, analyzed by one-way ANOVA with post hoc Scheffe test, $n = 4$. SEM micrographs show more platelets adhered onto Chi-10%PP_{45} (F) than chitosan (C); scale bar represents 20 μm. Platelets also formed more aggregates and pseudopods on Chi-10%PP_{45} (G, H) than chitosan (D, E); scale bar represents 5 μm.

3.5. Antimicrobial activity

Preliminary time kill assays found that dressings containing >320 μg/cm^2 silver did not demonstrate improved log reduction over time, thus dressings with 320 μg/cm^2 silver were used for further testing (coded as ChiPP-Ag). The antibacterial activity of ChiPP-Ag was compared with ChiPP against Gram-positive (Gm^+) *S. aureus* and Gram-negative (Gm^-) *P. aeruginosa* over 72 h, with repeat challenges at 24 h and 48 h. The log reduction values are collated in Table 1. ChiPP-Ag was highly effective against *P. aeruginosa*, reducing counts by 4.5 $\log_{10}$ within 4 h, and completely sterilizing the broth within 24 h (no colonies grew from undiluted aliquot samples). ChiPP-Ag remained highly effective and could achieve sterilization after each repeat challenge. ChiPP was bactericidal (>3 $\log_{10}$ kill) against *P. aeruginosa*, but kill rate was slower and less extensive than ChiPP-Ag. In the case of *S. aureus*, ChiPP-Ag achieved a >4.9 $\log_{10}$ kill at 24 h, 48 h, and 72 h; differences between the log reduction values at these timepoints were not significant. ChiPP achieved a 2.71 $\log_{10}$ kill against *S. aureus* within 24 h, but this killing was followed by reduced effectiveness at 48 h (0.07 $\log_{10}$ kill) and at 72 h (0.42 $\log_{10}$ growth).

3.6. Silver elution

The total available soluble silver was determined by Inductively Coupled Plasma (ICP) in water. Silver release per cm^2 dressing was 4.4 ± 0.6 μg at 2 h, increasing to 19.2 ± 2.5 μg at 24 h, to a maximum of 23.9 ± 2.1 μg of dissolved silver by 48 h ($n = 3$ at each timepoint).

3.7. Cytotoxicity

ChiPP and HemCon, as chitosan dressings without silver, exerted some cytotoxic effect on fibroblasts – cell viability relative to the no-dressing control was about 60% at 24 h for both dressings (Fig. 3A). Cells started to recover after 24 h, and the trend of decreasing viability in the ChiPP group from 48 h to 72 h was not significant. Light micrographs showed an almost confluent cell

Table 1
Log reduction of *P. aeruginosa* and *S. aureus* upon incubation with ChiPP and ChiPP-Ag dressings over a 3-day period, with repeat challenges at 24 h and 48 h ($n = 4$). All values were significantly different between ChiPP and ChiPP-Ag, except for the 30 min timepoint for *P. aeruginosa* ($p = 0.057$, Student's *t*-test)

Incubation time (h)	Log_{10} reduction[a]	
	ChiPP	ChiPP-Ag
Organism: *Pseudomonas aeruginosa*		
Inoculate 6.32 log_{10} cfu/mL at 0 h		
0.5	0.19 ± 0.35	0.71 ± 0.47
2	0.86 ± 0.36	2.29 ± 0.26
4	0.96 ± 0.37	3.86 ± 0.99
24	3.84 ± 0.21	6.32 ± 0
Re-inoculate 6.54 log_{10} cfu/mL at 24 h		
48	3.09 ± 0.64	6.54 ± 0
Re-inoculate 6.57 log_{10} cfu/mL at 72 h		
72	3.02 ± 0.95	6.57 ± 0
Organism: *Staphylococcus aureus*		
Inoculate 6.53 log_{10} cfu/mL at 0 h		
0.5	0.60 ± 0.12	0.90 ± 0.06
2	1.16 ± 0.40	2.54 ± 0.10
4	2.27 ± 0.19	3.43 ± 0.16
24	2.71 ± 0.44	5.23 ± 0.37
Re-inoculate 6.20 log_{10} cfu/mL at 24 h		
48	0.07 ± 0.39	5.50 ± 0.15
Re-inoculate 6.40 log_{10} cfu/mL at 48 h		
72	Growth	4.97 ± 0.52

[a] Log_{10} reduction values are calculated as log_{10} [initial bacteria upon challenge (cfu/mL)] − log_{10} [surviving bacteria at timepoint after challenge (cfu/mL)].

layer in wells containing ChiPP dressings at 72 h (Fig. 3B). This mild cytotoxicity is due to residual acetic acid in the dressings, and the different acetic acid contents of HemCon and ChiPP may have caused differences in cell viability at 48 h. The silver containing ChiPP-Ag dressing was severely cytotoxic to fibroblasts; relative cell viability was 14% at 24 h and dipped even lower to 4% at 48 h, before recovering to 26% at 72 h (Fig. 3C). Acticoat exerted a significantly more severe cytotoxic effect on fibroblasts, relative cell viability was 0.3%, 0.4% and 1.1% at 24 h, 48 h, and 72 h (Fig. 3D).

3.8. Evaluation of treatment using ChiPP-Ag or gauze on infected murine wounds

In both the gauze and ChiPP-Ag treatment groups, mice showed signs of bacterial infection (weight loss, lethargy). However, ChiPP-Ag dressing treatment significantly improved survival rates compared with gauze (85.7% *versus* 10%, $p = 0.001$, Kaplan–Meier log rank) (Fig. 4A). Cultures of blood and wound tissue from a moribund mouse treated by gauze on day 3 yielded high bacteria counts of 7.7×10^5 cfu/mL and 2.5×10^9 cfu/g, respectively, suggesting mortality due to bacteremia.

Wound and physiological parameters in treatment groups were only compared on day 1 and 2 because most mice in the gauze group died after day 2. The ChiPP-Ag dressing significantly reduced tissue bacteria counts and blood neutrophil percentages compared with gauze on both days (Table 2). Elevated blood neutrophil percentage is a predictor of bacteremia and sepsis [22]. Histology showed extensive inflammatory cell infiltrates in infected wounds, but infiltration appeared to be less in wounds treated with ChiPP-Ag than gauze (Fig. 5A, B). Tissue Gram stains revealed clusters of Gm^- (red) rods in wounds. No Gm^+ (blue) microorganisms were observed. For wounds dressed with ChiPP-Ag, bacteria were dispersed in the subcutaneous tissue but not on or near the wound surface (Fig. 5C). In contrast, bacteria were abundant on the surface of wounds dressed with gauze (Fig. 5D). In addition, bacteria penetration beyond the wound margins into adjacent uninjured skin was only observed in wounds dressed with gauze (Fig. 5E).

The lack of wound closure and continued low weight up to day 7 in the ChiPP-Ag treatment group suggested active wound infection. A quantitative assessment of tissue bacteria count showed that the initial inoculum of $\sim 3.5 \times 10^9$ cfu per wound led to 7.0 ± 0.6 log_{10} cfu/g at day 1, peaked to 8.2 ± 0.3 log_{10} cfu/g at day 2, and was not significantly reduced at day 7. By day 14, however, mice gained weight ($\sim 2.8\%$), their wounds closed significantly ($\sim 50\%$),

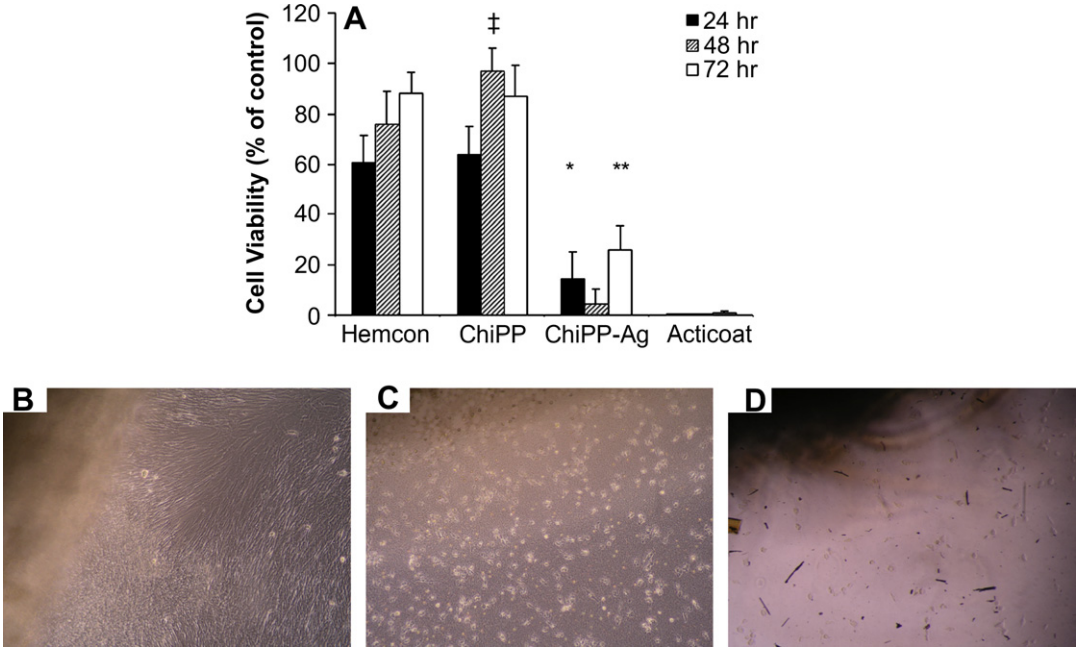

Fig 3. (A) Cytotoxic effect of chitosan-based dressings (ChiPP, HemCon) and silver-incorporated dressings (Acticoat, ChiPP-Ag) on fibroblast monolayers. ‡ indicates significant difference between HemCon and chitosan at each timepoint ($p < 0.05$, Student's *t*-test, $n = 5$). * and ** indicate significant difference between ChiPP-Ag and Acticoat at each timepoint ($p < 0.05$ and $p < 0.01$, respectively, Student's *t*-test, $n = 5$). Light micrographs of fibroblasts after 72 h incubation with dressings show a confluent monolayer with a few apoptotic cells near ChiPP (B), many round apoptotic cells near ChiPP-Ag (C), and almost no adherent cells near Acticoat (D).

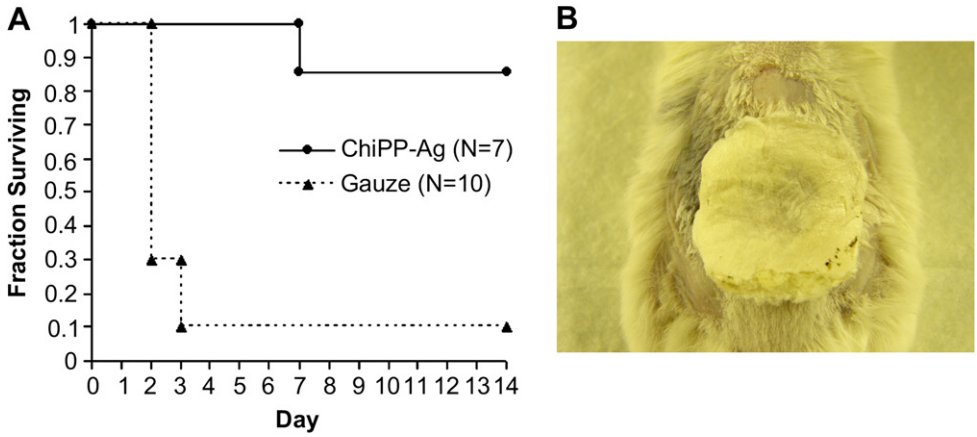

Fig 4. (A) Kaplan–Meier survival curves of mice with *P. aeruginosa* infection treated with ChiPP-Ag and gauze. (B) Photograph of mouse 24 h after application of ChiPP-Ag bandage.

and tissue bacteria counts were significantly lower (5.5 ± 0.6 $\log_{10}$ cfu/g) (Fig. 6). These results were corroborated by Gram stains showing clusters of Gm^- bacteria surrounded by leukocytes at day 7 that were no longer visible at day 14 (Fig. 7A, B). Neutrophil counts were not significantly different across all time-points (46–58%).

The effect of the ChiPP-Ag dressing on wound healing was examined by histology. At day 7, the regenerated epidermis near the wound edges has pushed up the slough layer and the underlying dermis contained disorganized, thin collagen fibrils (Fig. 7C). Nearer the center of the wound, however, the epidermis was still immature and the dermal layer had only a few short collagen fibrils (Fig. 7D). At day 14, the dermis showed thicker bands of organized collagen fibers (Fig. 7E), but collagen deposition at the wound site was still less dense than the adjacent undamaged dermis (Fig. 7F). H&E staining at day 14 showed abundant fibroblasts in the dermis (Fig. 7G) and a multilayered epidermis with good keratinocyte maturation at the center of the wound (Fig. 7H).

4. Discussion

The first part of this study evaluated the hemostatic activity of chitosan-polyphosphate dressings. Both components have been shown to activate coagulation by different mechanisms: Chitosan's protonated amine groups attracted negatively-charged residues on red blood cell membranes, causing strong hemagglutination [23–26]. Chitosan also adsorbed fibrinogen and plasma proteins, enhancing platelet aggregation [27,28]. On the other hand, polyphosphate specifically activated the contact pathway, which shortened both the time lag for initial thrombin generation as well as the time to peak thrombin generation [14]. Our hypothesis was that polyphosphate in the dressing would accelerate the production of sufficient amounts of thrombin to support earlier fibrin generation. At the same time, chitosan would recruit RBCs to enlarge and solidify the growing thrombus, leading to a stable clot that stops bleeding.

Our results demonstrated that the proportion of PP in chitosan was a critical parameter affecting hemostatic events. While chitosan containing 6.7% w/w and 10% w/w PP accelerated whole blood clotting compared to chitosan, the benefits of PP were negated at higher concentrations (15% w/w). A possible explanation is as follows: At lower PP levels, the accelerated thrombin generation and resultant fibrin formation could trap more RBCs that were aggregated on chitosan into the clot. However, at higher PP levels, free cationic amine groups of chitosan were reduced to an extent that significantly reduced its ability to electrostatically attract and bring RBCs in close proximity with fibrin. The dressing's ability to adhere RBCs was significant, not only because RBCs provided bulk to the clot, but more importantly because adhered RBCs have been shown to deform and expose procoagulant phospholipids (phosphatidylserine) on the membrane surface, similar to activated platelets [29]. These procoagulant sites allowed the assembly of prothrombinase complexes that catalyzes the conversion of prothrombin to thrombin [30].

In the case of platelet adhesion, only chitosan containing 10% w/w and not 6.7% w/w PP enhanced adhesion compared to chitosan. SEM micrographs also showed that some platelets adhered onto Chi-10%PP_{45} were activated, exhibiting a spread morphology with protruding pseudopods. These results suggest that PP may be required above a critical level to significantly shorten the lag time for initial thrombin generation and lead to faster platelet activation and adhesion [31]. Alternately, complexes containing different proportions of PP may have different surface charge distributions, which affected electrostatic interaction with plasma proteins and in turn affected platelet binding and subsequent activation [32].

The type of polyphosphate polymer in the complex may also affect hemostatic activity. Chi-10%PP_{45} induced significantly faster thrombin generation than chitosan, while Chi-10%PP_{65} dressings containing the same amount of polyphosphate did not. Since there were a greater number of polyphosphate chains (albeit 20 units shorter) in the Chi-10%PP_{45} complex compared with the Chi-10%PP_{65} complex, we tentatively postulate that the frequency of

Table 2
Comparison of wound and physiological parameters in ChiPP-Ag and gauze treatment groups at day 1 and 2

Parameters	Day 1		Day 2	
	ChiPP-Ag ($n=8$)	Gauze ($n=6$)	ChiPP-Ag ($n=8$)	Gauze ($n=5$, surviving)
Wound closure (%)	18.6 ± 10.7	14.0 ± 4.4	25.8 ± 19.2	16.6 ± 9.8
Weight loss (%)	6.45 ± 1.69^a	9.94 ± 2.73	10.16 ± 4.56	13.94 ± 3.26
Neutrophil (% WBC)	48.34 ± 10.53^a	66.95 ± 10.7	59.76 ± 12.83^b	76.57 ± 5.64
Tissue bacteria count [log (cfu/g)]	7.00 ± 0.60^b	8.81 ± 0.36	8.54 ± 0.66^a	9.12 ± 0.42

$^a p < 0.05$ and $^b p < 0.005$ compared to gauze, analyzed by Student's *t*-test.

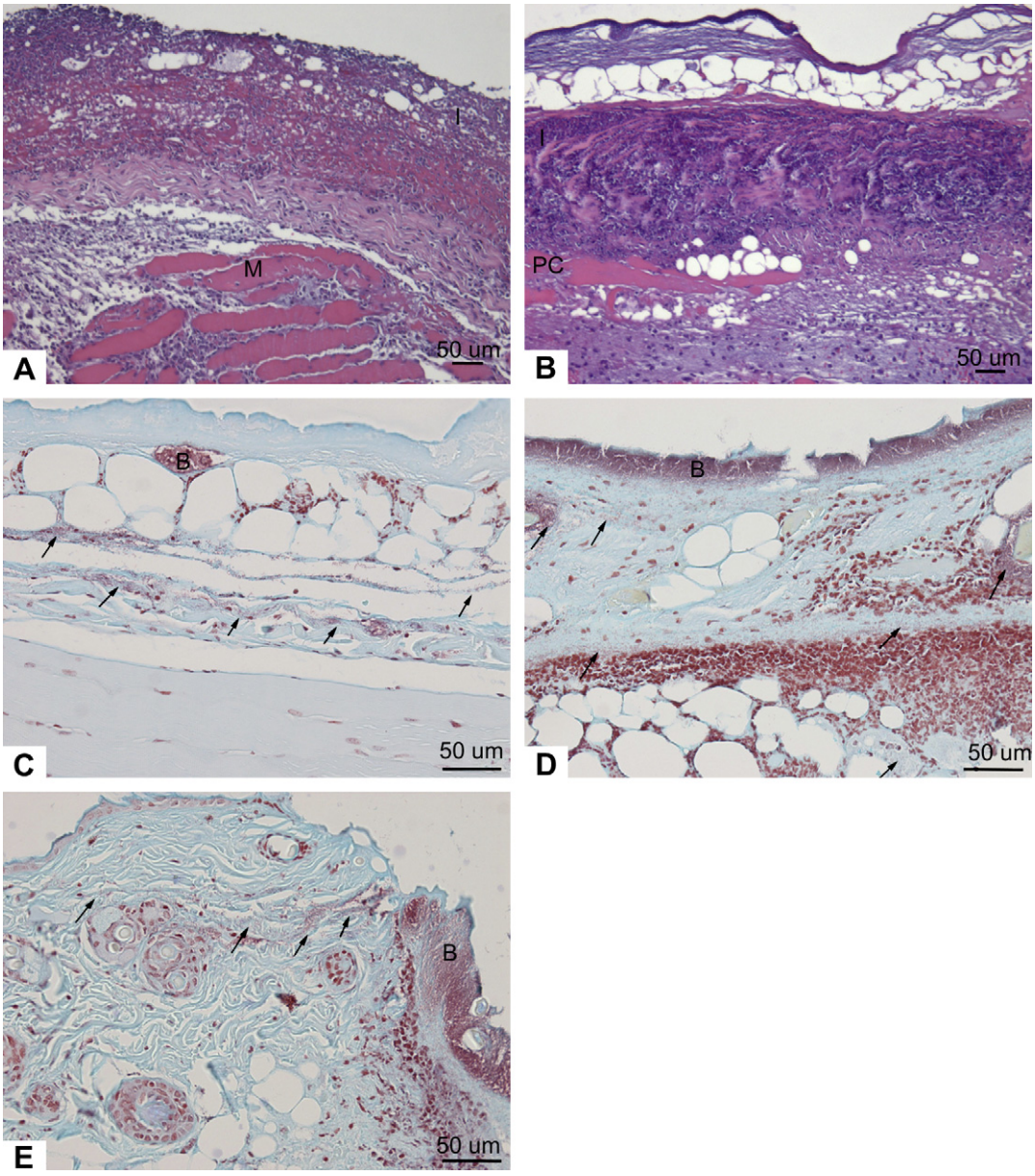

Fig 5. Histological examination of infected wounds at day 2. Large zones of inflammatory cell infiltrates were observed in both wounds dressed with ChiPP-Ag (A) and gauze (B), but infiltration appeared to be greater in wounds treated with gauze than ChiPP-Ag. Tissue Gram stains of wounds treated with ChiPP-Ag showed bacteria (arrows) in deeper tissue layers (C), while wounds treated with gauze had abundant bacteria growth on the wound surface (D). Bacteria were also observed in surrounding normal skin after gauze treatment (E). B: bacteria clusters, PC: panniculus carnosus, I: inflammatory infiltrate, M: muscle. Scale bar represents 50 μm.

complexed segments, rather than the size of complexed segments, may be more critical for increasing the number of procoagulant sites on the material, which accelerated the coagulation cascade turnover and led to faster thrombin formation.

Overall, the results of the hemostatic assays showed that the Chi-10%PP_{45} complex (coded as ChiPP) was most optimal for enhancing hemostasis – it led to faster blood clotting, more platelet adhesion, and faster thrombin generation. The ChiPP dressing also absorbed 2 times as much whole citrated blood as chitosan. Since the absorption of an electrolyte solution (SBF) by ChiPP was not significantly different from chitosan, the enhanced absorption should be due to specific attraction of blood proteins and other blood components. The ability of the ChiPP to absorb more blood should assist in stopping high flow hemorrhage. Most recently, PP containing 75 phosphate units was also found to increase fibrin fibril thickness and enhance fibrin clot strength [33]. Further investigation is required to determine if the shorter polyphosphate polymer (45 phosphate units) in our ChiPP dressing could also enhance fibrin clot structure and stability.

The second part of the study aimed to incorporate silver particles into the ChiPP dressing to improve its bactericidal properties (coded as ChiPP-Ag). Our data demonstrated that the ChiPP-Ag dressings had significantly faster and more potent bactericidal action than ChiPP against both pathogens that were tested; this rapid kill rate may reduce the opportunity for pathogens to develop resistance against antimicrobials [21]. Our results also showed that silver eluted from the dressing was more effective against *P. aeruginosa* than *S. aureus*. The lower bactericidal activity of silver against Gm^+ pathogens has been reported previously, and thought to be related to the thicker peptidoglycan cell wall which protects the cell from silver penetrating and subsequently denaturing proteins and inhibiting DNA replication [34]. ChiPP was not able to achieve a ≥99.9% kill rate against *S. aureus*. This may be because *S. aureus* had fewer negative charges on its cell walls, which reduced

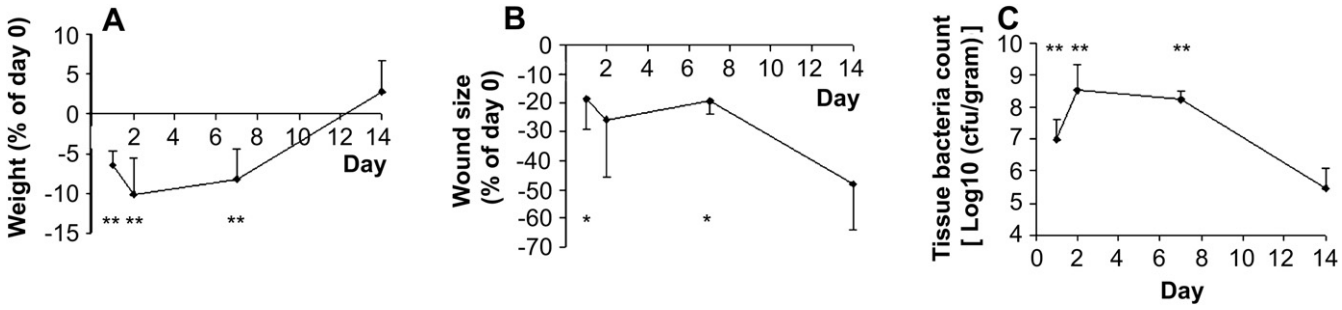

Fig 6. Graph showing (A) weight change, (B) wound size change, and (C) tissue bacteria count of mice treated with ChiPP-Ag over 14 days. $^{*}p < 0.05$ and $^{**}p < 0.005$ compared to day 14 value, analyzed by one-way ANOVA with post hoc Scheffe test, $n = 5–8$.

the degree of chitosan adsorption on its surface [16]. Adsorption was crucial for chitosan to exert its antimicrobial activity by destabilizing and disrupting cell membranes [35]. In addition, ChiPP had significantly reduced activity against *S. aureus* after 24 h, which may be attributed to the growth of small colony variants that were less susceptible to antimicrobials [35].

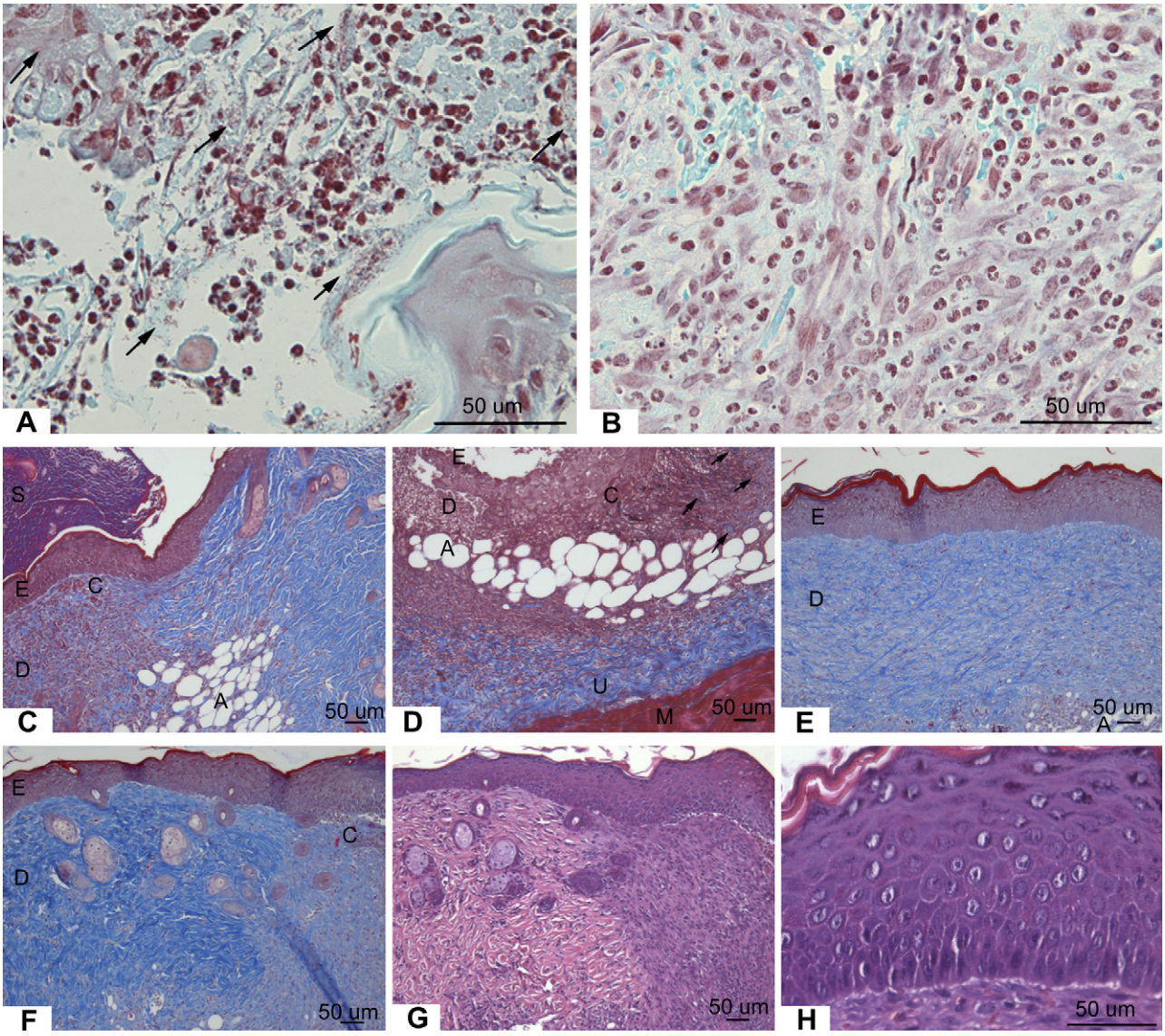

Fig 7. Gram stain of wounds treated with ChiPP-Ag showed bacteria (arrows) within inflammatory cell infiltrates at day 7 (A), which were no longer visible at day 14 (B). Masson Trichrome staining of wounds treated with ChiPP-Ag at day 7 showed collagen deposition (blue) at the wound edge (C), but only a few fine collagen fibrils (arrows) at the center of the wound (D). The blue layer above the muscle in (D) was undamaged subcutaneous tissue rather than regenerated dermis. At day 14, the dermis showed abundant collagen fibers that were thicker and more organized (E), but collagen deposition was still not as dense as the undamaged dermis (F). H&E staining of wounds treated with ChiPP-Ag at day 14 showed abundant fibroblasts in the dermis and good keratinocyte maturation in the epidermis (G, H). A: adipose tissue, C: blood capillaries, D: dermis, E: epidermis, M: muscle, S: slough, U: undamaged tissue. Scale bar represents 50 μm.

Silver elution from the dressing, at 24 μg/cm^2 over 48 h, was at the low end of the market range – between 17 μg/cm^2 for Aquacel and 3011 μg/cm^2 for Acticoat [36]. As expected, the lower silver release from our dressing induced a lower cytotoxic effect against fibroblast cells as compared with Acticoat, a nanocrystalline dressing that has had extensive clinical use. Acticoat's severe cytotoxicity has been reported in other *in vitro* studies [37,38], but *in vivo* use has found comparable healing and very rare cases of toxicity due to systemic absorption [39–42]. This apparent discrepancy may be explained by the rapid inactivation of silver ions in presence of physiologic concentrations of chloride and protein in the wound [43]. For the ChiPP and HemCon dressings, residual acetic acid in the dressing adversely affected cell viability in the first 24 h. It is unknown if neutralizing the dressings, which reduces the number of protonated amine groups, would affect the dressing's hemostatic properties.

A murine wound model contaminated with high levels of *P. aeruginosa* was used to study the effectiveness of the ChiPP-Ag dressings *in vivo*. The dressing was effective in reducing bacteria bioburden in full-thickness wounds compared to gauze, which helped significantly more mice survive the infection (85.7% *versus* 10%). However, quantitative tissue bacteria counts showed that bacteria numbers remained high up to day 7 in wounds dressed with ChiPP-Ag. This, together with tissue Gram stains showing localization of bacteria in deeper tissue layers but not near or on the surface, suggested that silver was not eluted at high enough concentrations to be effective in the presence of high levels of organic matter. Incorporating very high levels of silver, however, must take into consideration the risk of absorption by the systemic circulation and resultant toxicity. At current levels of silver, wound closure and the formation of a healthy granulating wound bed with abundant fibroblasts and collagen fibers were achieved by day 14.

5. Conclusions

In conclusion, a new ChiPP dressing has been developed that accelerated blood clotting, platelet adhesion, thrombin generation, and absorbed significantly more blood than chitosan. To our knowledge, these results provide the first evaluation and optimization of a chitosan-polyphosphate complex for hemostatic applications. Further investigation will more precisely delineate the mechanisms behind the improved hemostatic activity of ChiPP, and validate the findings *in vivo*. The ChiPP with incorporated silver was also effective in reducing mortality compared to standard gauze treatment in a full-thickness wound model contaminated with high levels of *P. aeruginosa*. Different forms of this hemostatic and antimicrobial material (*e.g.* granular, fibrillar) can be envisioned to accommodate different wound types and improve silver delivery to the wound. Further studies are also required to define the types of wounds that can be treated successfully by topical silver, so that systemic antibiotic administration can be minimized.

Acknowledgements

The authors would like to thank American BioTech Labs for donating the silver nanoparticles used in this study. We are also very grateful to Li-Li Tan (DSO National Laboratories, Singapore) for her assistance with histology.

References

[1] McManus JG, Eastridge BJ, Wade CE, Holcomb JB. Hemorrhage control research on today's battlefield: lessons applied. J Trauma 2007;62:S14.
[2] Kauvar DS, Lefering R, Wade CE. Impact of hemorrhage on trauma outcome: an overview of epidemiology, clinical presentations, and therapeutic considerations. J Trauma 2006;60:S3–11.
[3] Wedmore I, McManus JG, Pusateri AE, Holcomb JB. A special report on the chitosan-based hemostatic dressing: experience in current combat operations. J Trauma 2006;60:655–8.
[4] Rhee P, Brown C, Martin M, Salim A, Plurad D, Green D, et al. QuikClot use in trauma for hemorrhage control: case series of 103 documented uses. J Trauma 2008;64:1093–9.
[5] Ward KR, Tiba MH, Holbert WH, Blocher CR, Draucker GT, Proffitt EK, et al. Comparison of a new hemostatic agent to current combat hemostatic agents in a Swine model of lethal extremity arterial hemorrhage. J Trauma 2007;63:276–83.
[6] Ahuja N, Ostomel TA, Rhee P, Stucky GD, Conran R, Chen Z, et al. Testing of modified zeolite hemostatic dressings in a large animal model of lethal groin injury. J Trauma 2006;61:1312–20.
[7] Murray CK, Roop SA, Hospenthal DR, Dooley DP, Wenner K, Hammock J, et al. Bacteriology of war wounds at the time of injury. Mil Med 2006;171:826–9.
[8] Aronson NE, Sanders JW, Moran KA. In harm's way: infections in deployed American military forces. Clin Infect Dis 2006;43:1045–51.
[9] Murray CK, Hsu JR, Solomkin JS, Keeling JJ, Andersen RC, Ficke JR, et al. Prevention and management of infections associated with combat-related extremity injuries. J Trauma 2008;64:S239–51.
[10] Jackson DS. Sepsis in soft tissue limbs wounds in soldiers injured during the Falklands Campaign 1982. J R Army Med Corps 1984;130:97–9.
[11] Percival SL, Bowler PG, Russell D. Bacterial resistance to silver in wound care. J Hosp Infect 2005;60:1–7.
[12] Ip M, Lui SL, Poon VK, Lung I, Burd A. Antimicrobial activities of silver dressings: an in vitro comparison. J Med Microbiol 2006;55:59–63.
[13] Mi FL, Shyu SS, Wu YB, Lee ST, Shyong JY, Huang RN. Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing. Biomaterials 2001;22:165–73.
[14] Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A 2006;103:903–8.
[15] Mi FL, Shyu SS, Wong TB, Jang SF, Lee ST, Lu KT. Chitosan-polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. J Appl Polymer Sci 1999;74:1093–107.
[16] Chung YC, Su YP, Chen CC, Jia G, Wang HL, Wu JC, et al. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta Pharmacol Sin 2004;25:932–6.
[17] No HK, Park NY, Lee SH, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol 2002;74:65–72.
[18] Shih MF, Shau MD, Chang MY, Chiou SK, Chang JK, Cherng JY. Platelet adsorption and hemolytic properties of liquid crystal/composite polymers. Int J Pharm 2006;327:117–25.
[19] Vanickova M, Suttnar J, Dyr JE. The adhesion of blood platelets on fibrinogen surface: comparison of two biochemical microplate assays. Platelets 2006;17:470–6.
[20] Diquelou A, Lemozy S, Dupouy D, Boneu B, Sakariassen K, Cadroy Y. Effect of blood flow on thrombin generation is dependent on the nature of the thrombogenic surface. Blood 1994;84:2206–13.
[21] Brett DW. A discussion of silver as an antimicrobial agent: alleviating the confusion. Ostomy Wound Manage 2006;52:34–41.
[22] Shapiro NI, Wolfe RE, Wright SB, Moore R, Bates DW. Who needs a blood culture? a prospectively derived and validated prediction rule. J Emerg Med 2008 [prepublished online on May 15].
[23] Klokkevold PR, Fukayama H, Sung EC, Bertolami CN. The effect of chitosan (poly-*N*-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. J Oral Maxillofac Surg 1999;57:49–52.
[24] Okamoto Y, Yano R, Miyatake K, Tomohiro I, Shigemasa Y, Minami S. Effects of chitin and chitosan on blood coagulation. Carbohydr Polym 2003;53:337–42.
[25] Rao SB, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 1997;34:21–8.
[26] Yang J, Tian F, Wang Z, Wang Q, Zeng YJ, Chen SQ. Effect of chitosan molecular weight and deacetylation degree on hemostasis. J Biomed Mater Res B Appl Biomater 2008;84:31–7.
[27] Chou TC, Fu E, Wu CJ, Yeh JH. Chitosan enhances platelet adhesion and aggregation. Biochem Biophys Res Commun 2003;302:480–3.
[28] Benesch J, Tengvall P. Blood protein adsorption onto chitosan. Biomaterials 2002;23:2561–8.
[29] Fischer TH, Valeri CR, Smith CJ, Scull CM, Merricks EP, Nichols TC, et al. Non-classical processes in surface hemostasis: mechanisms for the poly-*N*-acetyl glucosamine-induced alteration of red blood cell morphology and surface prothrombogenicity. Biomed Mater 2008;3:15009.
[30] Peyrou V, Lormeau JC, Herault JP, Gaich C, Pfliegger AM, Herbert JM. Contribution of erythrocytes to thrombin generation in whole blood. Thromb Haemost 1999;81(3):400–6.
[31] Hong J, Larsson A, Ekdahl KN, Elgue G, Larsson R, Nilsson B. Contact between a polymer and whole blood: sequence of events leading to thrombin generation. J Lab Clin Med 2001;138:139–45.
[32] Yancheva E, Paneva D, Danchev D, Mespouille L, Dubois P, Manolova N, et al. Polyelectrolyte complexes based on (quaternized) poly[(2-dimethylamino)ethyl methacrylate]: behavior in contact with blood. Macromol Biosci 2007;7:940–54.
[33] Smith SA, Morrissey JH. Polyphosphate enhances fibrin clot structure. Blood 2008 [prepublished online on Jun 10].

[34] Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J Biomed Mater Res 2000;52:662–8.
[35] Raafat D, von Bargen K, Haas A, Sahl HG. Chitosan as an antibacterial compound: Insights into its mode of action. Appl Environ Microbiol 2008;74(12):3764–73.
[36] Parsons D, Bowler PG, Myles V, Jones S. Silver antimicrobial dressings in wound management: a comparison of antibacterial, physical and chemical characteristics. Wounds 2005;17:222–32.
[37] Burd A, Kwok CH, Hung SC, Chan HS, Gu H, Lam WK, et al. A comparative study of the cytotoxicity of silver-based dressings in monolayer cell, tissue explant, and animal models. Wound Repair Regen 2007;15:94–104.
[38] Lam PK, Chan ES, Ho WS, Liew CT. In vitro cytotoxicity testing of a nanocrystalline silver dressing (Acticoat) on cultured keratinocytes. Br J Biomed Sci 2004;61:125–7.
[39] Silver GM, Robertson SW, Halerz MM, Conrad P, Supple KG, Gamelli RL. A silver-coated antimicrobial barrier dressing used postoperatively on meshed autografts: a dressing comparison study. J Burn Care Res 2007;28:715–9.
[40] Vlachou E, Chipp E, Shale E, Wilson YT, Papini R, Moiemen NS. The safety of nanocrystalline silver dressings on burns: a study of systemic silver absorption. Burns 2007;33:979–85.
[41] Cuttle L, Naidu S, Mill J, Hoskins W, Das K, Kimble RM. A retrospective cohort study of Acticoat versus Silvazine in a paediatric population. Burns 2007;33:701–7.
[42] Trop M, Novak M, Rodl S, Hellbom B, Kroell W, Goessler W. Silver-coated dressing acticoat caused raised liver enzymes and argyria-like symptoms in burn patient. J Trauma 2006;60:648–52.
[43] Poon VK, Burd A. In vitro cytotoxity of silver: implication for clinical wound care. Burns 2004;30:140–7.