Arthroplasty Today 18 (2022) 130–137

Contents lists available at ScienceDirect

Arthroplasty Today

journal homepage: http://www.arthroplastytoday.org/

Original Research

Common Wound Irrigation Solutions Produce Different Responses in Infected vs Sterile Host Tissue: Murine Air Pouch Infection Model

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ARTICLE INFO

Article history:
Received 12 April 2022
Received in revised form
8 August 2022
Accepted 28 August 2022
Available online xxx

Keywords:
Arthroplasty Infection
Basic science
Irrigation solution
Cytotoxicity

ABSTRACT

Background: Despite desirable microbicidal actions of irrigation solutions in surgical site infection treatment, several studies demonstrate potential cytotoxic effects. This study investigated tissue damage caused by irrigation solutions in the presence or absence of infection.
Methods: Air pouches were created in 60 mice and evenly divided into 2 groups as infected with *Staphylococcus aureus* and control. Groups were then subdivided both by type of solution and by timing after irrigation. Solutions included control (0.9% saline), bacitracin (33 IU/ml), 0.2% sodium oxychlorosene, 0.05% chlorhexidine gluconate, and 0.013% benzalkonium chloride.
Results: Inflammation decreased in infected pouches compared to the sterile ones for all solutions except bacitracin on day 0 and for all on day 7. On day 0, infected pouches had increased necrosis with bacitracin ($P = .006$), chlorhexidine gluconate ($P = .18$), and benzalkonium chloride ($P = .07$); on day 7, there was decreased necrosis in infected pouches for all solutions ($P < .05$) except for sodium oxychlorosene ($P = .18$). Edema decreased in infected pouches on day 0 for all solutions. On day 7, infected pouches had decreased edema with 0.9% saline, bacitracin, and benzalkonium chloride ($P < .05$) and increased edema with chlorhexidine gluconate ($P < .05$) and sodium oxychlorosene ($P = .069$). Bacitracin allowed for more bacteria growth than sodium oxychlorosene ($P = .024$), chlorhexidine gluconate ($P = .025$), and benzalkonium chloride ($P = .025$).
Conclusions: The presence of bacteria led to less immediate tissue inflammation and edema, while tissue necrosis varied over time. The current study may guide surgeons on which solution to use and whether to irrigate a possibly sterile wound or joint.

Introduction

Surgical site infections are often treated via irrigation and debridement to decrease bacterial load [1–3]. Normal saline is an effective, standard irrigation solution for most wounds. Antibiotics are frequently used as irrigation additives but can cause bacterial resistance. Moreover, some antibiotics are bacteriostatic and others bactericidal; the susceptibilities of the underlying organism(s) are frequently unclear. Bacitracin is a peptide antibiotic with gram-positive coverage that disrupts bacterial cell wall synthesis and enzymes involved in cell membrane function [4]. Antiseptics and surfactant-containing irrigants are gaining popularity since they actively kill bacteria in the wound and may disrupt biofilms [5,6]. The antiseptic chlorhexidine gluconate has been used intraoperatively with success in reducing bacterial infection [7]. However, some studies show that it produces a negative cytotoxic effect and is not superior to normal saline [8,9]. The antiseptic Clorpactin (0.2% sodium oxychlorosene; United-Guardian Inc., Hauppauge, NY), a hypochlorous acid derivative, is highly bactericidal to nearly all microorganisms [10]. Clorpactin has been studied in general, plastic, and otolaryngology surgeries and demonstrated good

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https://doi.org/10.1016/j.artd.2022.08.019

antimicrobiosis effects with minimal host-tissue damage via oxidation and hypochlorination [11,12]. The irrigant Bactisure (ethanol, acetic acid, sodium acetate, benzalkonium chloride, water; Zimmer-Biomet, Warsaw, IN) has shown success in reducing bacterial loads in vitro and in vivo. Its bactericidal effect is due to the surfactant properties, chelating agents, and salts that disrupt the extracellular matrix, making bacteria more susceptible to the body's immune system as well as preventing biofilm formation [13]. Unfortunately, drawbacks do exist with these irrigants [7,14–16]. They may cause severe damage to primary cells including keratinocytes, leukocytes, fibroblasts, and osteocytes [17,18]; inhibit cellular growth and attachment [19]; and create direct tissue damage [20].

Our previous in vitro work demonstrated that some irrigation solutions had toxic effects on osteoblasts, fibroblasts, and tenocytes, and cells could either recover over time or had severe, permanent damage [21]. This in vivo study, approved by the Ascension Animal Committee, applied a validated murine air pouch model for 2 primary goals: to investigate the effectiveness of irrigation solutions used in practice to minimize bacterial infection and to evaluate the extent of tissue damage caused by the solutions in the presence or absence of infection [22].

Material and methods

Materials

Female BALB/C strain mice were obtained from Jackson Laboratory (Bar Harbor, ME), and *Staphylococcus aureus* (lux) from Caliper Life Science (Xenogen 29; Hopkinton, MA). Irrigation solutions included normal saline, Bacitracin (Sigma Aldrich Corp., St. Louis, MO), Clorpactin (United-Guardian Inc., Hauppauge, NY), Irrisept (Irrimax Corporation, Innovation Technologies, Inc., Lawrenceville, GA), and Bactisure (Zimmer-Biomet, Warsaw, IN).

Experimental design

The validated air pouch model study was approved by the Institutional Animal Care and Use Committee (IACUC #:104-17-Amendment 05/2019) [23,24]. Air pouches were created in 60 mice by injecting 1.5 ml of air subcutaneously on the back of the mice twice in 3 days. Animals were randomized into 2 groups—infected (n = 30) and noninfected (control, n = 30)—and then subdivided by type of irrigation solution, with saline as control. There were 3 animals in each subgroup at each time point (Fig. 1). Half the animals were sacrificed immediately after the pouch irrigation, and the other half after 1 week. All pouches were analyzed for bacterial growth as well as host-tissue damage. Tissue damage was observed on both a macroscopic level as well as by histology by specifically examining inflammation, necrosis, and edema as markers of tissue irritation, damage, or need for regeneration.

Irrigation solutions' preparation

Solutions were prepared as used in practice as well as at the senior author's institution. Bacitracin (33 IU/ml) and Clorpactin (sodium oxychlorosene, 0.2% dilution) were freshly prepared as aqueous solutions. The Bacitracin concentration was based on the available solution at our institution and its use in our previous in vitro study on human osteoblasts [21], while the Clorpactin concentration was based on the recommendation by original solution development [10]. Irrisept (chlorhexidine gluconate 0.05% in sterile water) and Bactisure (ethanol 1%, acetic acid 0.6%, sodium acetate 0.2%, benzalkonium chloride 0.013%, and water) were from manufactures.

Bacterial inoculation

Pouches in infected groups were inoculated by injecting 10^6 colony-forming units (CFU) *S. aureus* in 0.5 mL of broth/pouch. After inoculation, all 30 mice in the infected group were returned to their cages for 7 days prior to the treatment (irrigation). Fifteen out of 30 infected mice were immediately sacrificed after irrigation, while the remaining 15 mice were returned to their cages for 7 additional days and then sacrificed. The 30 noninfected mice did not require 7 days of inoculation prior to the treatment of irrigation. Of the 30 noninfected mice, 15 were sacrificed immediately after irrigation,

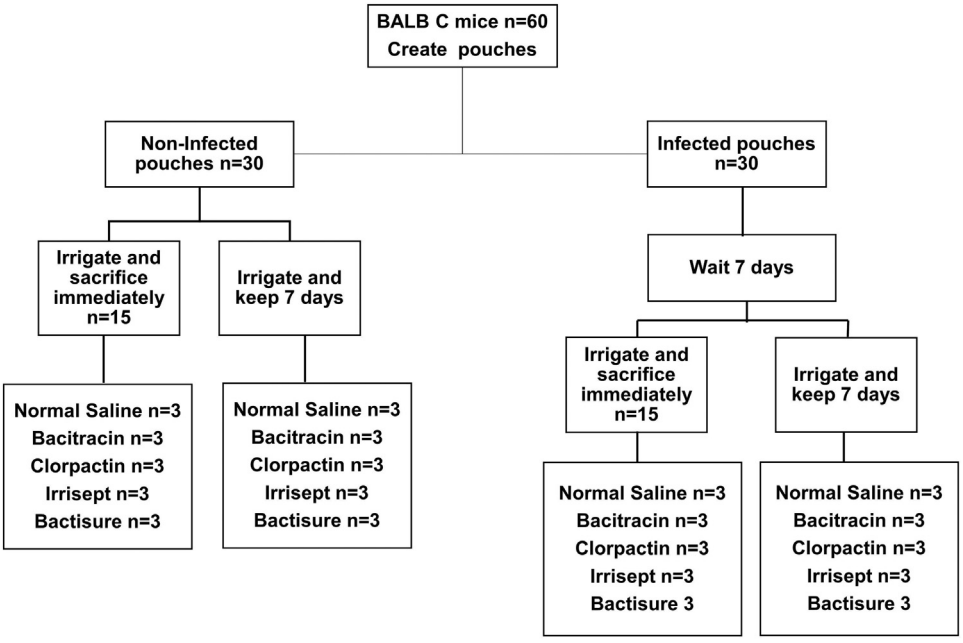

Figure 1. A diagram of the experimental design including treatment groups and animal numbers (BALB/C strain mice) for each condition.

while 15 of the noninfected mice were sacrificed after 7 days (Fig. 1).

Air pouch irrigation

The mice were anesthetized using ketamine (120 mg/kg) and xylazine (10 mg/kg), shaved, and prepped, and a 5-mm incision was created on the dorsal area of the established pouch. Pouches were held open and soaked in 3 mL of the designated irrigant. After soaking, each pouch was washed with normal saline. Irrigation fluids were immediately collected (wash fluid) for analysis. After treatment, half of the animals (infected and noninfected) were immediately sacrificed, and the pouch tissue was harvested and fixed in 10% neutral-buffered formalin. The other mice had their skin incision closed, were kept for 7 days, and then euthanized, and the pouch tissue was harvested and fixed in neutral-buffered formalin.

Bacterial analysis by optical density measurement

The collected wash fluids from the infected group were quantitatively analyzed for bacterial growth after irrigation. Bacterial analysis by optical density (OD) is a previous validated method of measuring bacterial concentration in suspension [25,26]. Wash samples of 100 μl were added to 1.9 ml of Luria-Bertini broth and stored at 4°C overnight. The next day, tubes were placed in a shaking water bath for 24 hours at 37°C and 225 rpm. A 20-μl sample was taken from the culture at 0, 6, and 24 hours, and OD600 was measured using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA). Five OD600 readings were taken for each sample.

Histology

The fixed pouches were paraffin-embedded and processed for histology. Five-micron hematoxylin and eosin sections were evaluated for signs of healing and host cell status. All slides were reviewed by a trained, blinded pathologist and scored for comparisons based on both his expertise and previous validated applications of inflammation and cytotoxicity in air pouch models [24,27,28].

The inflammation score was based on the percent observations of polymorphonuclear leukocytes per high-power field (HPF), 1 for 1%-10%/HPF, 2 for 10%-20%/HPF, 3 for 30%-60%/HPF, and 4 for >60%/HPF. Necrosis was scored based on the number of necrotic foci per HPF. Edema was scored based on the number of edematous areas per HPF represented by areas of fluid leading to spacing between cells. Necrosis was each measured as a number per HPF and labeled as 1 for 1 foci of necrosis per HPF, 2 for 2 foci of necrosis per HPF, and so on. Areas of edema were labeled in the same manner as necrosis. Ten HPFs for each pouch were measured for inflammation, necrosis, and edema and then averaged.

Statistical analysis

A power analysis was performed using a priori F analysis using analysis of variance for multiple conditions to attain 85% confidence. The statistical comparison was based on histological findings as determined by the blinded pathologist. This involved determining average and standard deviation for the quantitative presence of inflammation, foci of necrosis, and edematous areas. Single-factor analysis of variance was used to analyze bacterial growth. A standard 2-tailed t-test was used to determine differences among sterile vs infected pouches for each solution. The standard 2-tailed t-test was also used to determine differences between each solution for both sterile and infected pouches.

Results

Bacterial growth

The 24-hour cultures showed that Clorpactin, Irrisept, and Bactisure controlled infection, whereas saline and Bacitracin washes allowed bacteria growth, with washouts having up to 3.9×10^7 CFU/ml and 6.7×10^7 CFU/ml, respectively. Bacitracin allowed for significantly more bacteria growth than Clorpactin ($P = .024$), Irrisept ($P = .025$), and Bactisure ($P = .025$). Standard deviations for saline and Bacitracin groups were quite high. Three of the 6 washouts from the saline control group showed a high-grade infection (~10^8 CFU/ml), and the other 3 showed low-grade infections (~10^4 CFU/ml to 10^5 CFU/ml). In the Bacitracin washouts, 4 of 6 had high-grade infection (~10^8 CFU/ml), 1 middle-grade infection (~10^6 CFU/ml), and 1 completely cleared infection (~0 CFU/ml) (Table 1 and Fig. 2).

Histological analysis

A gross histological examination showed varying degrees of host-tissue damage in reaction to the irrigants initially and after 7 days in the sterile (Fig. 3) and infected pouches (Fig. 4). A detailed quantitative histological analysis at time 0 (immediately after irrigation) and 7 days after irrigation provided information on inflammation, necrosis, and edema.

Inflammation

At time 0, infected pouches had significantly less inflammation than sterile pouches with all solutions except Bacitracin ($P < .05$) (Fig. 5a). At 7 days, inflammation significantly decreased in infected pouches for all solutions compared to sterile pouches, except for Bacitracin ($P < .005$) (Fig. 5b). When comparing day 0 to day 7, in sterile conditions, there was a significant increase in inflammation for Bactisure ($P < .0005$) and a significant decrease for Bacitracin-treated groups (Fig. 5c). In contrast, with infected conditions, there was a significant decrease in inflammation for all irrigants ($P < .005$) except for Bactisure which had increased inflammation ($P < .0005$) and no change with saline (Fig. 5d).

Table 1
Bacterial count (CFU) obtained from the culture of wash fluid collected during irrigation of the infected pouches after 0, 6, and 24 h of incubation at 37°C.

Irrigation solution	Colony-forming units (CFU)		
	0 h	6 h	24 h
Control	7.6×10^5 ($\pm 5.9 \times 10^5$)	3.0×10^6 ($\pm 5.4 \times 10^6$)	3.9×10^7 ($\pm 6.0 \times 10^7$)
Bacitracin	9.9×10^5 ($\pm 1.5 \times 10^6$)	1.2×10^7 ($\pm 2.0 \times 10^7$)	6.7×10^7 ($\pm 5.4 \times 10^7$)
Clorpactin	1.0×10^5 ($\pm 1.2 \times 10^5$)	3.5×10^5 ($\pm 4.9 \times 10^5$)	7.9×10^5 ($\pm 6.6 \times 10^5$)
Irrisept	8.2×10^4 ($\pm 1.4 \times 10^5$)	5.8×10^5 ($\pm 5.1 \times 10^5$)	1.2×10^6 ($\pm 8.7 \times 10^5$)
Bactisure	1.7×10^6 ($\pm 3.7 \times 10^6$)	1.6×10^6 ($\pm 2.5 \times 10^6$)	1.3×10^6 ($\pm 1.4 \times 10^6$)

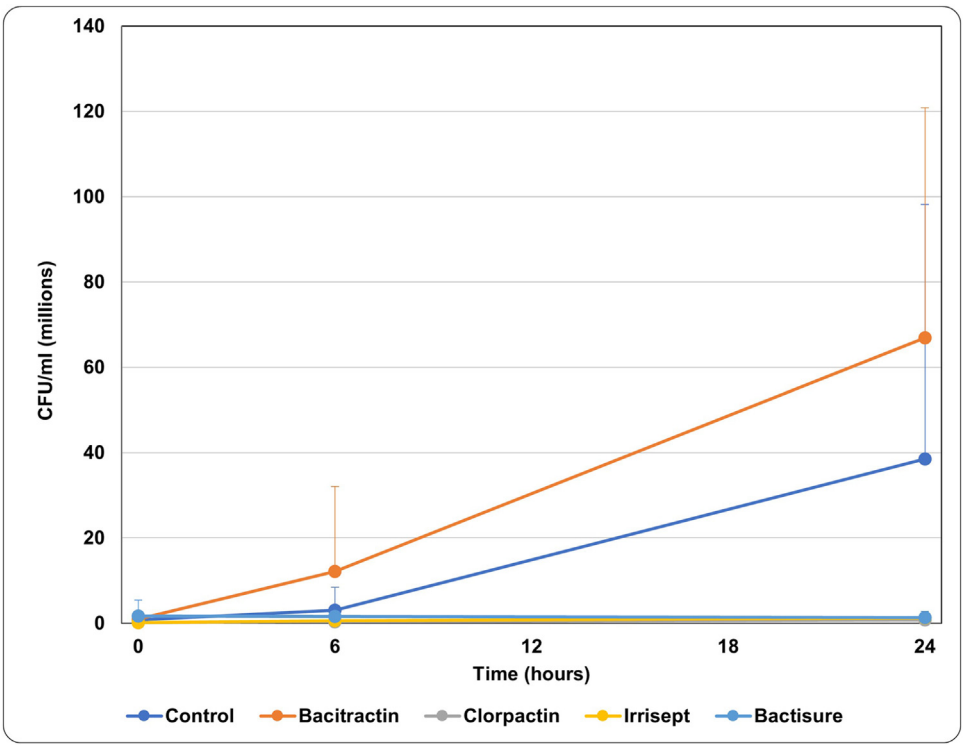

Figure 2. Visual representation of the wash fluid collected during irrigation of the infected pouches after 0, 6, and 24 hours of incubation at 37°C. Significant differences noted at 24 hours between Bacitracin vs Clorpactin ($P = .024$), Bacitracin vs Irrisept ($P = .025$), and Bacitracin vs Bactisure ($P = .025$).

Necrosis

At time 0, there was a decrease in necrosis in infected pouches compared to sterile pouches for saline ($P = 1.1E\text{-}8$) and Clorpactin ($P = .027$) and an increase for Bacitracin ($P = .006$), Irrisept ($P = .18$), and Bactisure ($P = .07$) (Fig. 6a). At 7 days, there was a significant decrease in necrosis in infected pouches compared to sterile pouches for all solutions ($P < .05$) except Clorpactin ($P = .18$) (Fig. 6b). When comparing day 0 to day 7, in sterile conditions,

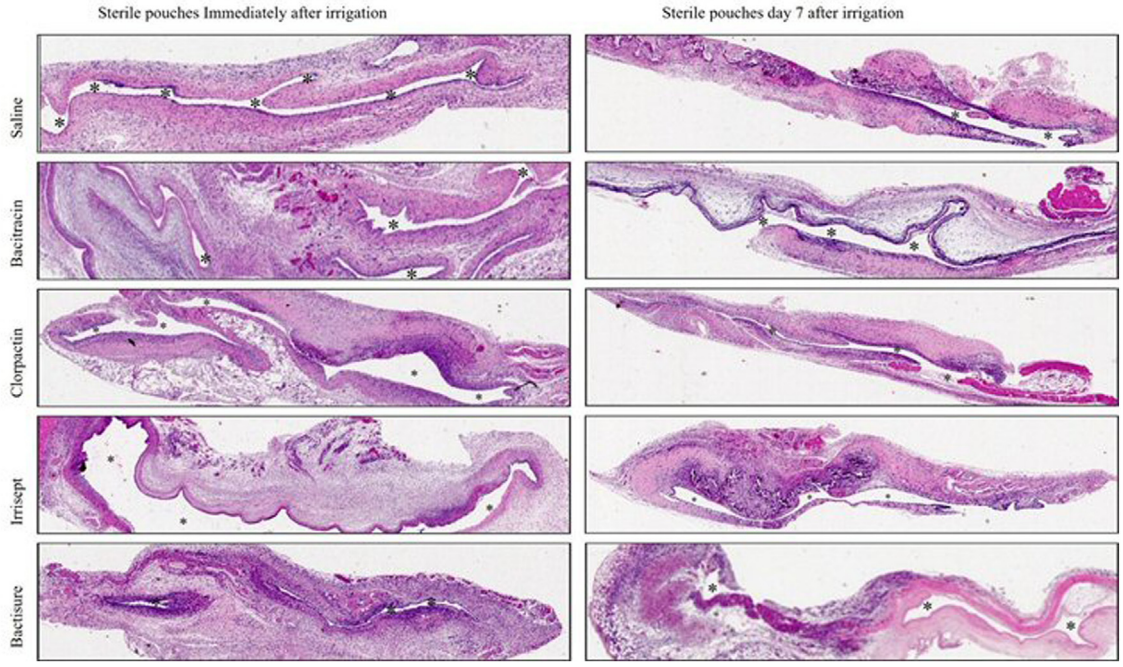

Figure 3. Representative images of hematoxylin and eosin-stained sterile pouches for all tested irrigation solutions on day 0 (immediately after irrigation) and day 7 after irrigation. Images taken at 4× magnification using PathScan Enabler (Meyer Instruments, Inc. Houston, TX). Black stars in the sections indicate the pouch cavity.

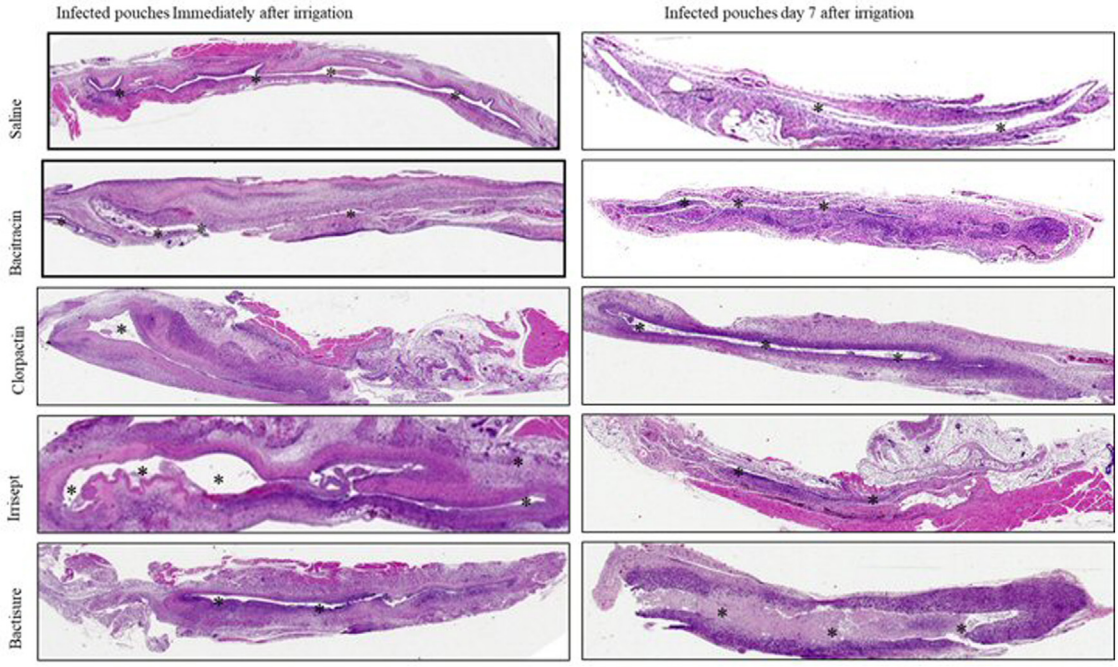

Figure 4. Representative images of hematoxylin and eosin-stained infected pouches (with Xen 29-10^6 CFU/ml) for all tested irrigation solutions on day 0 (immediately after irrigation) and day 7 after irrigation. Images taken at 40× magnification using the PathScan Enabler. Black stars in the sections indicate the pouch cavity.

there was a significant increase in necrosis over time for Bacitracin ($P = .004$), Irrisept ($P = .002$), and Bactisure ($P < 2.68E-09$) but not for saline or Clorpactin (Fig. 6c). Under infected conditions, there was a significant increase in necrosis for saline ($P = 6.98E-06$), Clorpactin ($P = .026$), and Bactisure ($P < .0005$) and a decrease with Bacitracin ($P = .228$) and Irrisept ($P < .009$) (Fig. 6d)

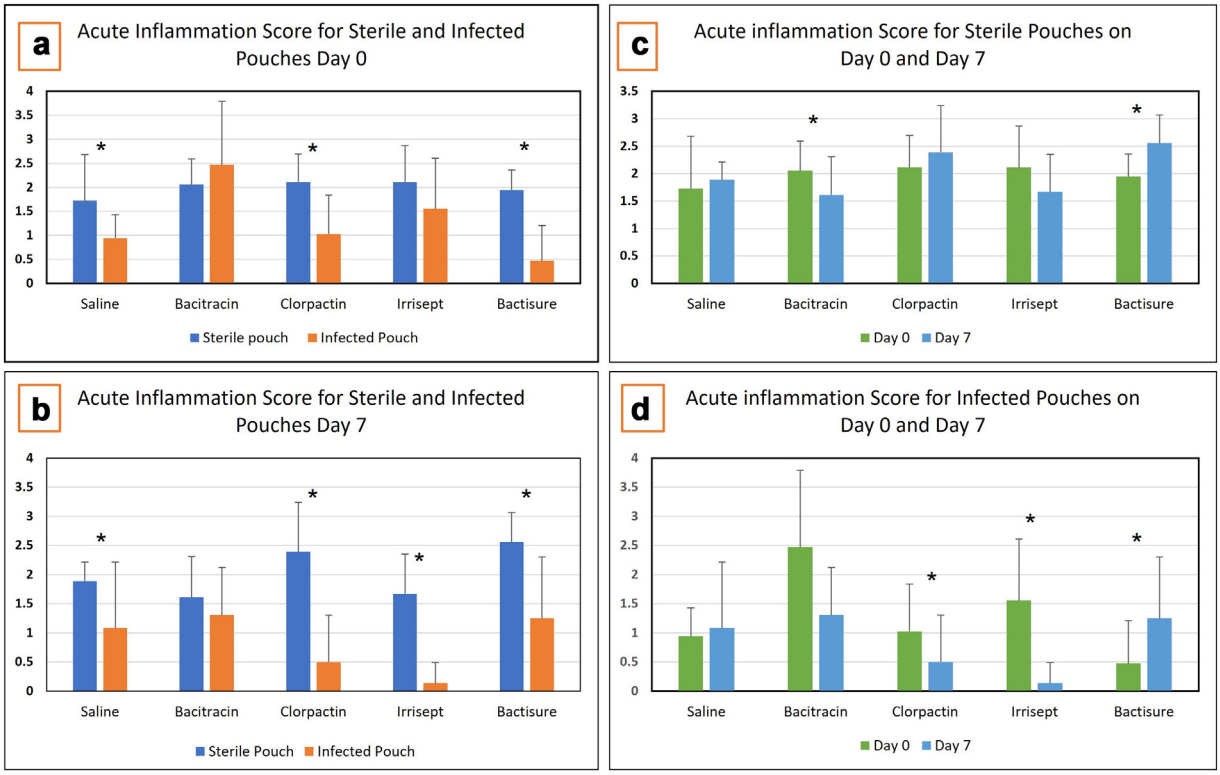

Figure 5. Acute inflammatory scores of the pouch tissue subjected to various irrigation solutions under sterile and infected conditions of the pouch immediately and at 7 days after irrigation. The asterisk symbols indicate statistical significance. (a) demonstrates acute inflammation score for sterile and infected pouches on day 0, (b) demonstrates acute inflammation score for sterile and infected pouches on day 7, (c) demonstrates acute inflammation score for sterile pouches only on day 0 and day 7, (d) demonstrates acute inflammation score for infected pouches only on day 0 and day 7.

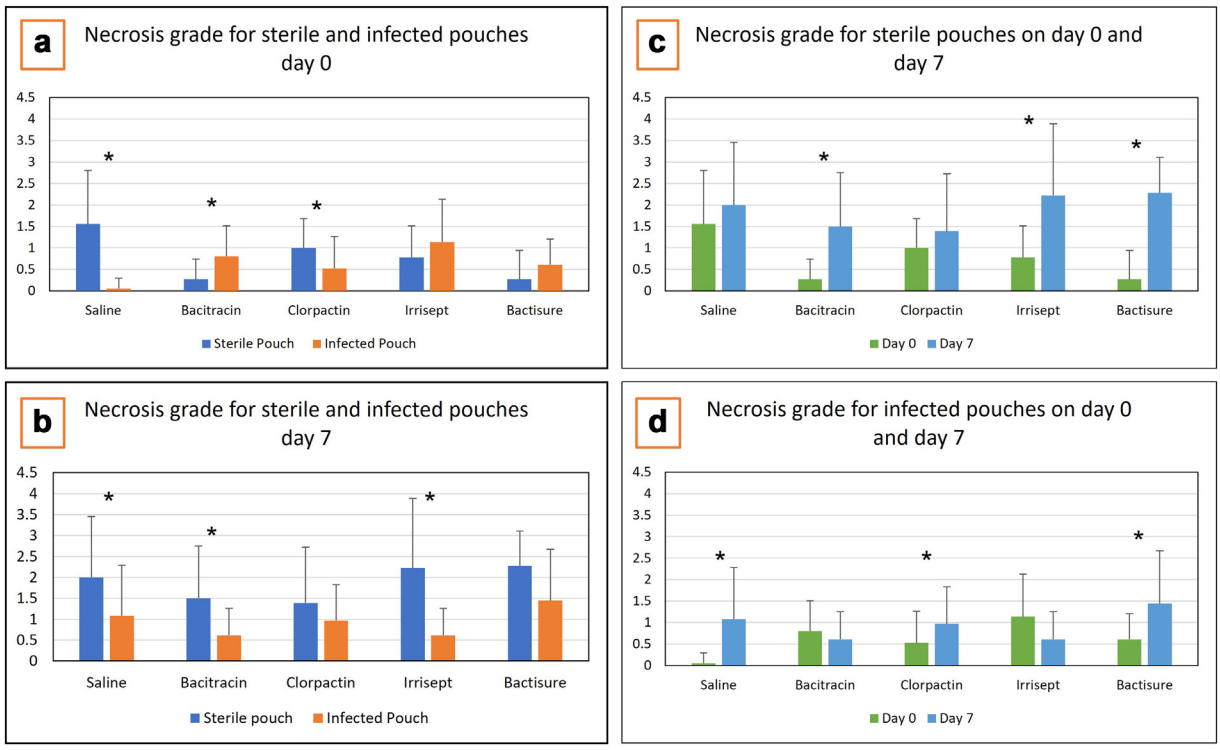

Figure 6. Necrosis scores for the pouch tissue subjected to various irrigation solutions under sterile and infected conditions of the pouch immediately and at 7 days after irrigation. The asterisk symbols indicate statistical significance. (a) demonstrates necrosis grade for sterile and infected pouches on day 0, (b) demonstrates necrosis grade for sterile and infected pouches on day 7, (c) demonstrates necrosis grade for sterile pouches only on day 0 and day 7, (d) demonstrates necrosis grade for infected pouches only on day 0 and day 7.

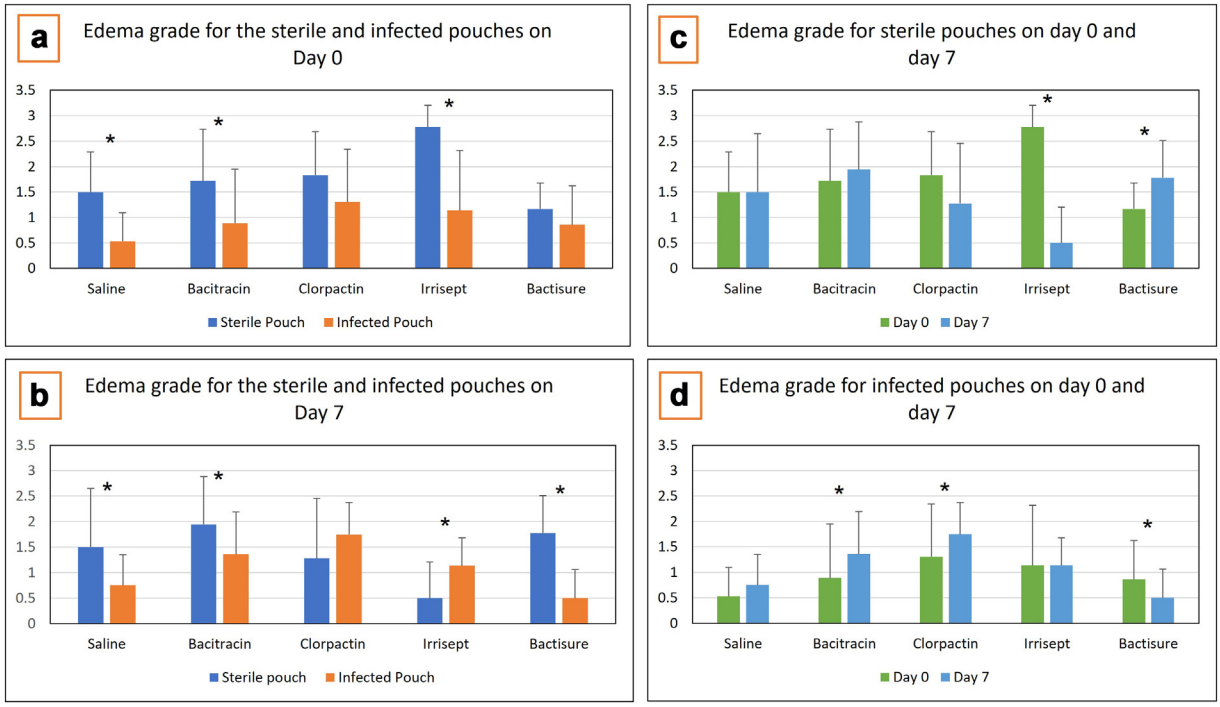

Figure 7. Edema scores for the pouch tissue subjected to various irrigation solutions under sterile and infected conditions of the pouch immediately and at 7 days after irrigation. The asterisk symbols indicate statistical significance. (a) demonstrates edema grade for sterile and infected pouches on day 0, (b) demonstrates edema grade for sterile and infected pouches on day 7, (c) demonstrates edema grade for sterile pouches only on day 0 and day 7, (d) demonstrates edema grade for infected pouches only on day 0 and day 7.

Edema

At time 0, there was a significant decrease in edema in infected pouches compared to sterile pouches in all conditions with statistical significance ($P < .01$) for saline-, Bacitracin-, and Irrisept-washed pouches (Fig. 7a). At 7 days, less edema was seen in infected pouches than in sterile pouches for saline ($P = .003$), Bacitracin ($P = .024$), and Bactisure ($P = 3.2E-9$), while significantly increased edema was observed for Irrisept ($P = .0006$) (Fig. 7b). When comparing day 0 to day 7, under sterile conditions, there was a significant increase in tissue edema with Bactisure ($P = .006$) and decrease with Irrisept ($P = 1.8E-13$) (Fig. 7c). The same comparison for infected pouches showed significant increases in edema for Bacitracin ($P = .039$) and Clorpactin ($P = .038$), and significantly less edema was found only for Bactisure ($P < .0249$) (Fig. 7d).

Discussion

While antiseptic and surfactant agents are used to minimize and/or eliminate infection, the risks of tissue necrosis, edema, inflammation, or other negative effects on native tissue needs consideration. This study demonstrated that the negative impact of the irrigation solutions on native tissue was dependent on the presence or absence of infection and exposure time. In the presence and absence of infection with *S. aureus*, there were clear differences among the tested solutions. When analyzing bacterial growth and histologic tissue inflammation, sterile pouches had increased inflammation compared to infected pouches in all solutions except Bacitracin when observed immediately after irrigation and in all solutions including Bacitracin at 7 days. For sterile conditions, all solutions created tissue necrosis that increased or persisted over 7 days. In contrast, when infected, less necrosis occurred in all groups. The effect decreased over time for Bacitracin and Irrisept and increased for saline, Clorpactin, and Bactisure. Edema, a measure for tissue reaction, had less direct patterns over time. But edema was less prominent in infected vs sterile samples for most irrigation solutions. It appeared that the antimicrobial action of the irrigants was tissue-friendly when bacteria were present but was somewhat injurious to native tissue when uninfected.

Toxic effects of irrigants have been studied due to the concern for tissue damage by authors such as Heling et al., Kaysinger et al., and Muller and Kramer [17,18,29]. We examined tissue damage indicators including necrosis, inflammation, and edema in infected and sterile conditions. The irrigation solutions tested herein demonstrated efficiency in treating *S. aureus* infection [17,18]. We confirmed irrigant efficacy and decreased bacterial loads in collected washout fluid. Results were statistically significant for Clorpactin, Irrisept, and Bacitracin. We previously showed similar effects on human osteoblasts' viability and recovery after an in vitro exposure. Irrisept, Clorpactin, and Bacitracin all demonstrated some toxic effects on the cells; the damage was to some extent reversible with solutions other than Irrisept [21]. Difference in testing method (in vitro vs in vivo) should be taken into consideration when comparing studies. It will be helpful to establish a uniform definition on cytotoxicity parameters across investigations to evaluate the effects of specific solutions. While we did not specifically examine cell recovery, there was analysis of tissue damage on macroscopic and cellular levels by evaluating edema, necrosis, and inflammation.

The present study showed that Bacitracin, Clorpactin, Irrisept, and Bactisure had more significant effects on edema and tissue inflammation under sterile conditions than in *S. aureus*-infected tissues. Tissue necrosis did not follow a clear pattern but increased over time in the sterile pouches. For inflammation and edema, the study findings suggested that exposing sterile tissues to antiseptic agents may be more damaging than if they are used in the presence of infection. There are several possible explanations for this finding. Local infections are much more susceptible to bactericidal solutions than systemic infections or those involving deeper tissues [30]. It is possible that the mechanisms of antiseptic solutions allow for prioritization of direct bactericidal effects prior to secondary host-tissue damage. Additionally, the full extent of tissue damage may take longer than the time investigated in the presence of infection in tissues as compared to that in sterile tissue. The nature of measuring permanent tissue damage would likely require a specific method to measure the tissue's ability to regenerate after a set time as well as a method of quantifying the extent of damage in various tissues. The natural immunological habitat can be affected by the presence of acute infection, foreign bodies, by pH, temperature, and other factors, and this may explain different patterns of host-tissue cytotoxicity in the presence vs absence of *S. aureus* [30]. When weighing risk vs benefit, it may be valuable to ask whether the area to be irrigated (tissue or joint) is infected or not, understanding that the irrigation solution may create significant native tissue damage if the area is not infected.

There were limitations to the study. While the murine pouch has been validated as a model for tissue analysis, it is unclear if this can be extrapolated from soft tissue to infections involving joints or in proximity of healing fractures. For example, the force required and applied for irrigation in a clinical setting cannot be replicated directly in the murine pouch model due to size difference. Also, it is difficult to standardize the accurate solution retention in the pouch as well as in clinical settings which leads to high standard deviation. There were multiple HPFs examined for cytotoxicity in each pouch; however, this study still has some limitations in interobserver reliability since 1 trained pathologist examined each section. While the antiseptic solutions used here were commonly used reagents, they represent a limited sample of commercially available irrigants, and we used only 1 antibiotic solution. Additionally, epithelial tissue damage is a concern with some of the antiseptic solutions; this study focused mostly on the deep tissue infection but did not analyze the potential for more superficial tissue damage.

While it was not our intention to investigate the microbicidal effects of the specific irrigants, the data herein combined with the findings from Kaysinger et al. and Muller and Kramer may imply that lower concentrations of antiseptics in infected tissues would be useful [17,18]. It will be important for surgeons to correlate a patient's full clinical presentation prior to deciding on whether to irrigate and which solution to use. Ultimately, it may be valuable to ask the question of whether the tissue or joint involved looks infected, knowing that there may be a higher likelihood of significant native tissue damage if it is not infected.

Conclusion

Bacitracin, Clorpactin, Irrisept, and Bactisure irrigation had negative soft-tissue effects expressed as edema and tissue inflammation under sterile conditions. These effects were minimized in the presence of *S. aureus* infection. Soft-tissue effects expressed as necrosis did not follow a clear pattern. The irrigants Clorpactin, Irrisept, and Bactisure also effectively controlled *S. aureus* infections. Although these irrigants are clearly effective, one should consider not using these irrigants when infection is not suspected.

Conflicts of interest

David C. Markel receives royalties from Stryker; is in the speakers' bureau of or gave paid presentations for Smith & Nephew; is a paid employee of The CORE Institute/HopCo; is a paid consultant for Stryker and Smith & Nephew; has stock or stock

options in HopCo and Arboretum Ventures; receives research support from Stryker, OREF, and Ascension Providence; and is a board member in the Michigan Orthopedic Society and Michigan Arthroplasty Registry Collaborative Quality Initiative. The other authors declare no potential conflicts of interest.

For full disclosure statements refer to https://doi.org/10.1016/j.artd.2022.08.019.

Acknowledgments

The authors would like to acknowledge Dr. Les Clifford (PhD, DVM) for his contributions toward the histopathology reading and analysis in this study.

This study was approved by the Ascension Animal Committee. The previously validated murine air pouch model was approved by the Institutional Animal Care and Use Committee (IACUC #:104-17-Amendment 05/2019).

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