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Basic Research

Is NS-EDTA Effective in Clearing Bacteria From Infected Wounds in a Rat Model?

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Abstract

Background Irrigation is one of the key procedures in open fracture management to eliminate pathogens and prevent infection. Metal ion deprivation could inhibit bacterial adhesins and weaken adhesion to the host tissue. EDTA in solution can competitively bind to a metal ion and thus might be able to inhibit bacterial adhesins.

Questions/purposes (1) Is normal saline-EDTA toxic to fibroblasts and endothelial cells? (2) In a contaminated wound rat model, does irrigation with normal saline-EDTA solution decrease the risk of positive bacterial cultures and infection when compared with normal saline and soap solutions? (3) In an infected wound rat model, are fewer surgical débridements and irrigations with normal saline-EDTA solution required to obtain culture-free wounds when compared with normal saline and soap controls?

Methods Normal saline-EDTA solution refers to 1 mmol/L EDTA dissolved in normal saline (pH adjusted to 7.4). Normal saline and soap solutions acted as controls. The toxicity of these solutions to fibroblasts and endothelial cells was assessed in vitro by Annexin V/propidium iodide staining and flow cytometer counting (a well-established method to quantitatively measure the number of dead cells). We established contaminated and infected wound models (bone-exposed or not) with either *Staphylococcus aureus* or *Escherichia coli* in rats to investigate the efficacy of normal saline-EDTA solution (n = 30 for the contaminated model and n = 50 for the infected model). For contaminated wounds, the proportion of positive bacterial cultures and infections was compared after irrigation and débridement among the three groups. For infected wounds, we performed irrigation and débridement every 48 hours until the cultures were negative and compared the number of débridements required to achieve a negative culture with survival analysis.

Results Normal saline-EDTA showed no additional toxicity to fibroblasts and endothelial cells when compared with normal saline (normal saline [97%] versus EDTA [98%] on fibroblasts, p = 0.654; normal saline [97%] versus EDTA [98%] on endothelial cells, p = 0.711). When bone was exposed in the contaminated models, EDTA irrigation resulted in fewer positive bacterial cultures with *S aureus* (EDTA: 23%, normal saline: 67%, soap: 40%, p = 0.003) and with *E coli* (EDTA: 27%, normal saline: 57%, soap: 30%, p = 0.032); however, infection risk was only lower with EDTA irrigation (*S aureus* with EDTA: 10%, normal saline: 33%, soap: 37%, p = 0.039; *E coli* with EDTA: 3%, normal saline: 27%, soap: 23%, p = 0.038). In the infected wound model, EDTA irrigation resulted in earlier culture-negative wounds (fewer surgical sessions) compared with

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normal saline and soap solutions (nonbone-exposed wounds infected by *S aureus*: p = 0.003, infected by *E coli*: p = 0.001; bone-exposed wounds infected by *S aure*us: p = 0.012, infected by *E coli*: p = 0.022).

Conclusions After in vitro assessment of toxicity and in vivo evaluation of efficacy, we concluded that normal saline-EDTA is superior to normal saline and soap solution in our laboratory models.

Clinical Relevance The use of normal-saline EDTA as an irrigation solution may reduce the infection rate of wounds. Future studies in large animals and humans might prove our observation in rat models that normal saline-EDTA has an advantage over normal saline as an irrigation solution.

Introduction

Adhesins are a group of components on the surface of pathogenic bacteria that interact with host tissues and allow establishment of adhesion [6]. Most of the bacterial adhesins are cell-surface proteins with multiple ion-binding sites [22, 23, 35]. The presence of ions, including calcium, zinc, and magnesium, is required for proper adhesion function. Mutation of the ion-binding motif or deprivation of specific ions could lead to decreased adhesion of bacteria-to-host tissues [10, 24-26, 33]. EDTA is a widely used chelating agent for the treatment of heavy metal poisoning in humans. It competitively chelates ions, including calcium, zinc, and magnesium, to form a complex [7, 29].

Open fracture management requires thorough irrigation and débridement to prevent infection and promote healing [16, 18, 32]. Controversy exists regarding the choice of irrigation solution and additives [2, 3, 8, 13, 31]. Killing the bacteria seems to be the most straightforward strategy. However, antiseptic additives including povidone-iodine were found to be toxic to host tissues at a working concentration [13, 27]. Brennen and Leaper showed that all antiseptics have a negative effect on microvascular flow and endothelial integrity in a rabbit model [5]. As a result, antiseptic additives did not decrease the infection rate in the management of open fractures according to many previous studies [13, 14].

In addition, enhancing bacterial removal is another feasible option to decrease the infection rate. From this point of view, there is a strong biologic rationale for using surfactants (compounds that lower the surface tension and are widely adopted as detergents) as an irrigation solution additive. Surfactants enhance bacteria removal through irrigation by interfering with the adhesion of pathogens to host tissues [17]. Studies have shown that surfactants improve bacteria removal compared with normal saline [1, 15, 19, 28]. However, recent studies have recommended normal saline irrigation without surfactants, citing concerns about toxicity and adverse healing effects [4, 12].

In general, normal saline is still the preferred choice for an irrigation solution by most surgeons in the management of open fractures [31]. This highlights the need for an irrigation solution with potent bacteria-removing/killing capacity and low toxicity. As we described previously, EDTA is nontoxic to host tissues and is possibly able to interfere with bacterial adhesion effectively.

Therefore, we asked: (1) Is normal saline-EDTA toxic to fibroblasts and endothelial cells? (2) In the contaminated wound model, does irrigation with normal saline-EDTA solution decrease the risk of positive bacterial cultures and infection when compared with normal saline and soap solutions? (3) To obtain culture-free wounds in the infected wound model, are fewer surgical irrigations with normal saline-EDTA solution required compared with normal saline and soap controls?

Materials and Methods

This study consisted of an in vitro toxicity experiment followed by several animal models to assess the efficacy of normal saline-EDTA. The in vitro toxicity experiment compared normal saline-EDTA with normal saline using a cell viability test (Annexin V/PI staining). We then established multiple models of different wounds (bone-exposed or not) and contaminated or infected the wounds with either *Staphylococcus aureus* or *Escherichia coli* in rats to investigate the efficacy of normal saline-EDTA irrigation. The use of human fibroblasts and endothelial cells in this study was approved by the ethics committee of Shanghai Jiaotong University Affiliated Sixth People's Hospital and informed consent was obtained from all donors in accordance with the Declaration of Helsinki. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of our hospital.

Cell Viability Analysis

Fibroblasts and endothelial cells were widely adopted to test the toxicity of irrigation solution in vitro because the granulation tissues were mainly deposited by these cells [5, 13, 20, 27]. In this study, we tested and compared the toxicity of normal saline-EDTA with normal saline in human umbilical vein endothelial cells and fibroblasts. Both cell lines were cultured in α-MEM (Corning, New York, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Dublin, Ireland). Culture media were replaced with fresh media every 2 days. Cells were passaged in a 1:3 ratio once they reached full confluence. The viability test was conducted with cells in passage 5. For viability testing, the

cells were stimulated with normal saline or normal saline-EDTA for 15 minutes. We removed the irrigation solutions and added fresh media. After 6 hours, cell viability analysis was conducted with an Annexin V/propidium iodide (PI) apoptotic analysis kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. The cells were counted by Guava easyCyte Flow Cytometers (Merck KGaA, Darmstadt, Germany). Viable cells were negative for the Annexin V and PI; late apoptotic cells were positive for both, whereas early apoptotic cells were positive for Annexin V only. The viable, early apoptotic, and late apoptotic cells were located in left-lower, right-lower, and right-upper quadrants of the flow cytometry gram, respectively. The viable rates were calculated automatically by the software provided by the manufacturer of flow cytometry. The experiments were conducted in three replicates three independent times. The mean rates of viable cells were compared and statistically analyzed.

Animal Models

We designed four models (Group I: contaminated wound without exposed bone; Group II: infected wound without exposed bone; Group III: contaminated wound with exposed bone; Group IV: infected wound with exposed bone) according to different clinical scenarios to compare the efficacy of normal saline-EDTA solution with normal saline and soap solution. The wounds with bone exposure are clearly different from the wounds affecting soft tissue only. Therefore, we created two types of wounds (bone-exposed or not) in this study and then infected or contaminated the wounds. A representative Gram-positive bacterium, *Staphylococcus aureus* (ATCC 29213), or a Gram-negative one, *Escherichia coli* (ATCC 25922), acted as the pathogen. After each model was established, rats underwent randomization in a 1:1:1 ratio and were assigned to one of three treatment groups: normal saline, soap, or normal saline-EDTA (Fig. 1).

Bacterial Inoculum Preparation

We maintained a stock culture of the *E coli* and *S aureus* on tryptic soy agar with 5% sheep blood (BD™ Trypticase™ Soy Agar II with 5% Sheep Blood; BD, Heidelberg, Germany) and prepared fresh culture 24 hours before surgery. We prepared the inoculum by collecting the organisms on a cotton swab, washing the cells three times in normal saline, and adjusting the cells to a concentration of 1×10^8 colony-forming units (CFUs)/mL according to a standard curve of optical density. Each rat would receive a bacterial inoculum of 1×10^7 CFU in a volume of 100 μL.

Irrigation Solution Preparation

The normal saline-EDTA solution was prepared by dissolving EDTA at a concentration of 1 mmol/L in normal saline; the pH was adjusted to 7.4. The soap solution was prepared with 0.45% of castile soap in normal saline. All irrigation solutions underwent autoclaving before application.

Irrigation and Débridement Procedures

All rats were intraperitoneally injected with 400 mg/kg chloral hydrate for anesthesia before surgical procedures. Those rats deemed stable for general anesthesia received thorough irrigation and débridement with a standardized volume (300 mL) of respective solutions using a 50-mL syringe. The principle for débridement was to establish margins of viable and perfused tissues. After these procedures, all rats in the three groups received additional irrigation with 100 mL of normal saline to remove residual additives or to act as a control.

Contaminated Model Groups

To create a model of contaminated wound without exposed bone (Group I), a 2-cm incision was created at the dorsal

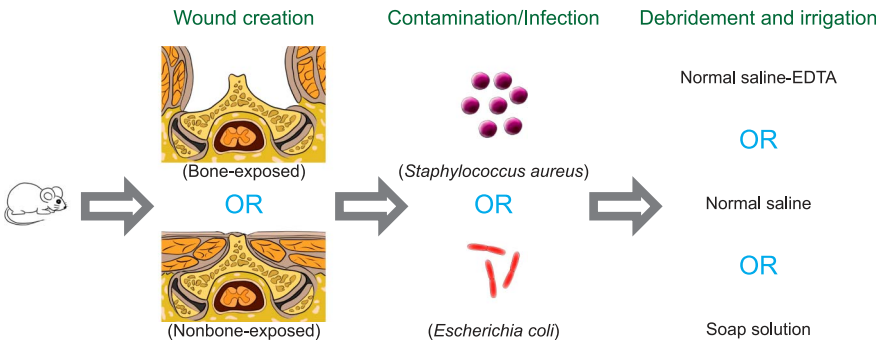

Fig. 1 A schematic illustration of the study design is shown.

skin in rats and inoculated with *E coli* or *S aureus*. To create models of contaminated wound with exposed bone (Group III), we adopted a modified protocol described in a previous study [19]. In brief, we surgically exposed a posterior spinous process in a rat through a 1-cm incision, punctured the bone with a needle, and then inoculated it with *E coli* or *S aureus*. After 6 hours, irrigation and débridement was conducted in both groups, then a bacterial culture sample was obtained with a cotton swab, and the wound was sutured. The wound was subsequently reopened after 48 hours to look for purulence (confirmed by a smear test and Gram), which was the definition of infection in this study. The sign of bacterial phagocytosis by leukocytes was defined as positive results (infection existed). All macroscopic judgments in this study were consistent with the confirmation of a smear test.

Infected Model Groups

To create a model of an infected wound without exposed bone (Group II), a standardized full-thickness skin defect (18 mm in diameter) was created and inoculated with *E coli* or *S aureus*. For the model of infected wound with exposed bone (Group IV), a 18-mm skin defect was created, bone was exposed as we described for Group III, and then inoculated with *E coli* or *S aureus*. After 48 hours, irrigation and débridement was conducted, and the wound was covered with sterile dressings. Then, a bacterial culture sample was obtained with a cotton swab. Repeat irrigation and débridement of the open wound was conducted every 48 hours until the cultures obtained after the last irrigation and débridement were negative after 48 hours.

Blinding

For Annexin/PI staining and cytometer counting, the group information was blinded to the researchers during solution treatment and subsequent assessment (Annexin V/ PI staining and cytometer counting). Notably, the soap solution could be identified easily by observation. Thus, the blinding method was only effective for normal saline and EDTA groups.

The group information was blinded to the researchers when they performed the surgical débridement. Although the group information was also blinded in the irrigation procedures, the blinding method was only effective for normal saline and EDTA groups because the soap solution could be identified easily by observation.

Statistical Analysis

Differences in dichotomous variables between groups were compared using Pearson's chi-square statistic or Fisher's exact test. Kaplan-Meier curves were computed and compared using the log-rank statistic. The Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) was used for statistical analysis.

Results

Normal saline-EDTA showed no additional toxicity to fibroblasts and endothelial cells when compared with normal saline (Fig. 2). The mean percent viability of fibroblasts was 97% with normal saline and 98% with normal saline-EDTA ($p = 0.654$); the mean percent viability of endothelial cells was 97% with normal saline and 98% with normal saline-EDTA ($p = 0.711$). In contrast, soap solution resulted in the death of 67% fibroblasts and 72% endothelial cells ($p < 0.001$ versus normal saline or normal saline-EDTA). We also stimulated the cells with 10 mmol/L EDTA (10-fold of working concentration) and this resulted in no significant cell death (the mean viability of fibroblasts: 95%; endothelial cells: 96%).

In the contaminated model with bone exposure (Table 1), EDTA irrigation resulted in fewer positive bacterial cultures with *S aureus* (normal saline-EDTA: seven of 30 [23%], normal saline: 20 of 30 [67%], soap: 12 of 30 [40%]; $p = 0.003$) and with *E coli* (normal saline-EDTA: eight of 30 [27%], normal saline: 17 of 30 [57%], soap: nine of 30 [30%], $p = 0.032$); however, infection risk was only lower with EDTA irrigation (*S aureus* with EDTA: three of 30 [10%], normal saline: 10 of 30 [33%], soap: 11 of 30 [37%], $p = 0.039$; *E coli* with EDTA: one of 30 [3%], normal saline: eight of 30 [27%], soap: seven of 30 [23%], $p = 0.038$). When bone was not exposed (Table 2), the incidence of positive cultures (*S aureus* with normal saline-EDTA: seven of 30 [23%], normal saline: six of 30 [20%], soap: five of 30 [17%], $p = 0.812$; *E coli* with normal saline-EDTA: six of 30 [20%], normal saline: six of 30 [20%], soap: five of 30 [17%], $p = 0.930$) and infection (*S aureus* with normal saline-EDTA: one of 30 [3%], normal saline: two of 30 [7%], soap: one of 30 [3%], $p = 0.770$; *E coli* with normal saline-EDTA: zero of 30 [0%], normal saline: one of 30 [3%], soap: zero of 30 [0%], $p = 0.364$) was not statistically different.

In the infected model without exposed bone, the mean number of irrigation and débridement procedures was decreased after EDTA irrigation (*S aureus*: 1.42 ± 0.64; *E coli*: 1.34 ± 0.56) compared with normal saline (*S aureus*: 1.74 ± 0.80, mean difference 0.32 [95% confidence interval {CI}, 0.03-0.61]; *E coli*: 1.66 ± 0.77, mean difference 0.32 [95% CI, 0.03-0.61]) and soap (*S aureus*: 1.94 ± 0.77, mean difference 0.52 [95% CI, 0.23-0.81]; *E coli*: 1.90 ± 0.81, mean difference 0.56 [95% CI, 0.27-0.85]). When bone was exposed, EDTA irrigation

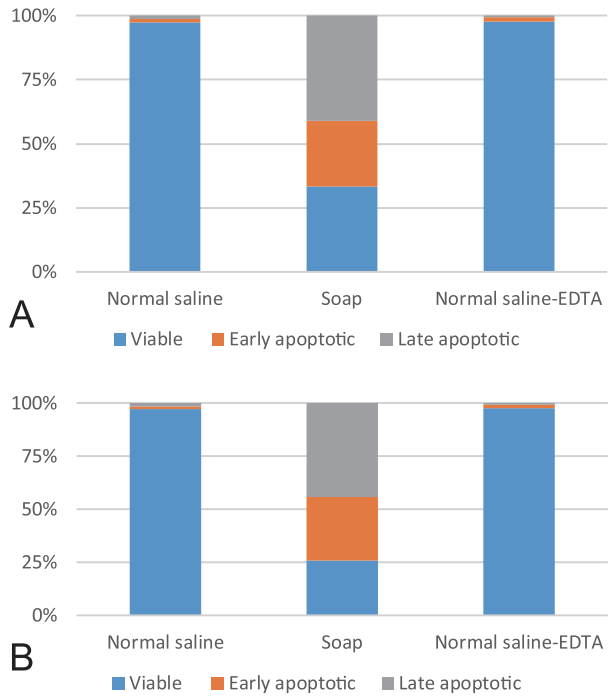

Fig. 2A-B Annexin V/PI staining of fibroblasts and endothelial cells was performed after stimulation of normal saline, soap, or normal saline-EDTA. (**A**) Fibroblasts were stimulated with normal saline (97.2% viability), soap (33.4% viability), or normal saline-EDTA (97.6% viability) for 15 minutes. (**B**) Likewise, endothelial cells were stimulated with normal saline (97.3% viability), soap (27.6% viability), or normal saline-EDTA (97.7% viability) for 15 minutes. Annexin V/PI staining was conducted and detected by flow cytometry after 6 hours.

resulted in a fewer number of irrigation and débridement procedures (*S aureus*: 1.90 ± 0.84; *E coli*: 1.74 ± 0.69) compared with normal saline (*S aureus*: 2.32 ± 1.11, mean difference 0.42 [95% CI, 0.02-0.82]; *E coli*: 2.14 ± 1.14, mean difference 0.40 [95% CI, 0.03-0.77]) and soap (*S aureus*: 2.48 ± 1.03, mean difference 0.58 [95% CI, 0.18-0.98]; *E coli*: 2.22 ± 0.95, mean difference 0.48 [95% CI, 0.11-0.85]). A survival analysis revealed that normal saline-EDTA irrigation resulted in culture-negative wounds after fewer irrigation and débridement procedures compared with normal saline and soap solutions (Fig. 3; nonbone-exposed wounds infected by *S aureus*: p = 0.003, infected by *E coli*: p = 0.001; bone-exposed wounds infected by *S aureus*: p = 0.012, infected by *E coli*: p = 0.022).

Discussion

The cornerstone of wound management is appropriate débridement to remove necrotic tissues and establish margins of viable and perfused tissues. The main purpose of irrigation is to eliminate adhered bacteria and prevent/diminish infection. One strategy to better achieve this goal is interfering with bacterial adhesion by inhibiting the function of bacterial adhesins [37]. Current methods including inhibition of adhesins and their host receptors, vaccination with adhesins, and interfering with receptor-adhesin interactions are highly valuable but only against specific infections of one or several types of bacteria [11], highlighting the need for a universally effective method. In this study, we report on a novel irrigation solution and assess its toxicity in vitro and efficacy in a rat model. With EDTA, irrigation can eliminate multiple ions, including calcium, zinc, and magnesium, which are required for the proper functioning of bacterial adhesions. As a result, normal saline-EDTA solution may enhance bacterial removal and decrease infection rates.

The current work had several limitations. First, we used a single strain of *S aureus* and *E coli*, classic representatives of Gram-positive and Gram-negative pathogens, like many previous studies [21, 36]. Because the proper functioning of most bacterial adhesions requires metal ions, it is highly possible that EDTA would also be effective against other pathogens. Second, this study was conducted in rats, which

Table 1. Proportion of positive cultures and infection of acute-phase and bone-exposed wounds (Group III) after irrigation and débridement

Irrigation solution	Normal saline	Soap	Normal saline-EDTA	p value
Staphylococcus aureus				
Positive culture	20/30 (67%)	12/30 (40%)*	7 (23%)*	0.003
Infection	10/30 (33%)	11/30 (37%)	3/30 (10%)*	0.039
Escherichia coli				
Positive culture	17/30 (57%)	9/30 (30%)*	8/30 (27%)*	0.032
Infection	8/30 (27%)	7/30 (23%)	1/30 (3%)*	0.038

Figures are numbers.
*p < 0.05 versus normal saline; bacterial culture was obtained immediately after irrigation and débridement; infection was defined as the presence of pus in the wound on reopening.

Table 2. Proportion of positive cultures and infection of acute-phase and nonbone-exposed wounds (Group I) after irrigation and débridement

Irrigation solution	Normal saline	Soap	Normal saline-EDTA	p value
Staphylococcus aureus				
Positive culture	7 /30 (23%)	6/30 (20%)	5/30 (17%)	0.812
Infection	1/30 (3%)	2/30 (7%)	1/30 (3%)	0.770
Escherichia coli				
Positive culture	6/30 (20%)	6/30 (20%)	5/30 (17%)	0.930
Infection	0/30 (0%)	1/30 (3%)	0/30 (0%)	0.364

Figures are numbers; *p < 0.05 versus normal saline; bacterial culture was obtained immediately after irrigation and débridement; infection was defined as the presence of pus in the wound on reopening.

clearly have a different response to microbial infection compared with humans. Compared with other irrigation additives, EDTA greatly reduced the infection risk (all models no more than 10%) and the efficacy was remarkable [9, 30]. Despite these limitations, our results justify the study of normal saline-EDTA in wound irrigation research using larger animals and, if those results are promising, then eventually in humans. Third, current techniques do not allow us to measure the ions in the tissues or the margin of wound. The mechanism remains undetermined and future studies are still needed. Clearly, the main conclusion of this study that normal saline-EDTA is effective and safe is not affected.

EDTA is widely used in treatment of heavy metal poisoning. The safety and nontoxicity of EDTA has been well established, which could accelerate the translation of our study into clinical practice. The enhanced removal/killing of pathogens could also be achieved by many additives.

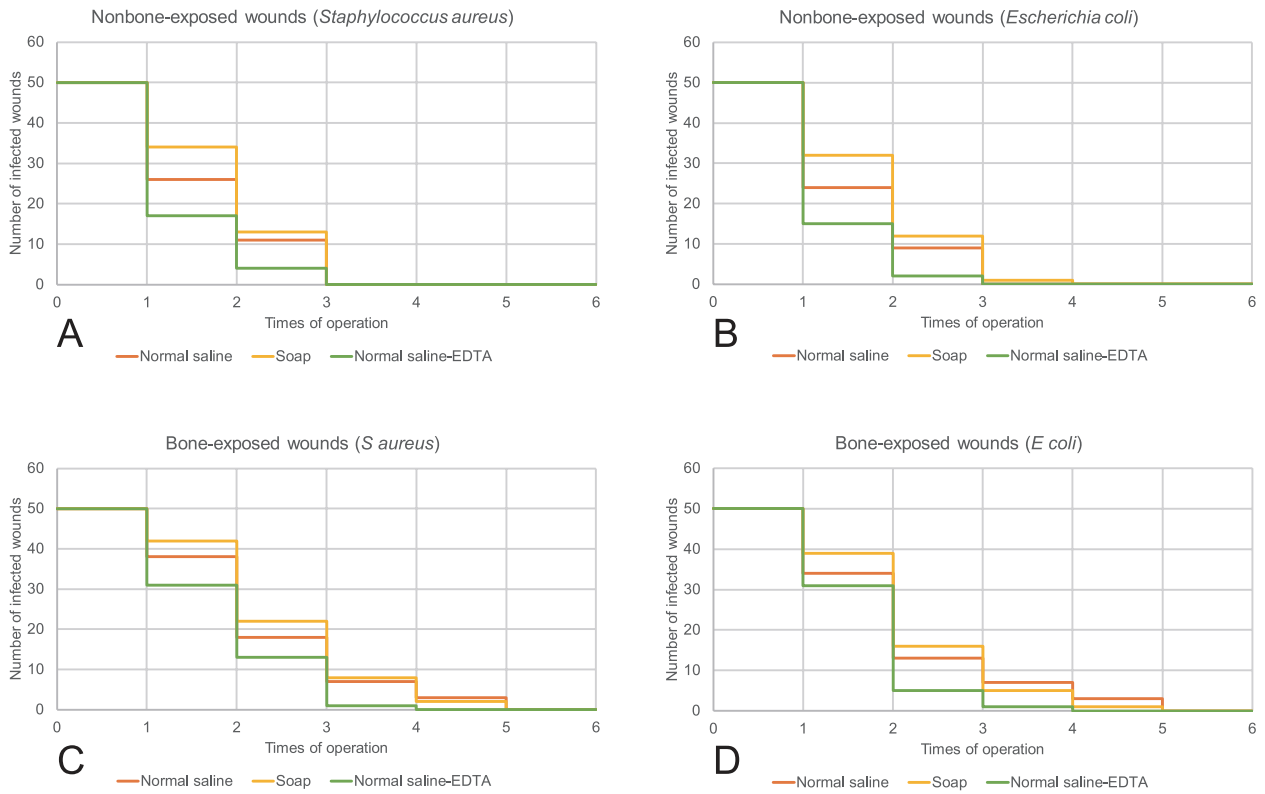

Fig. 3A-D Survival curves of culture-positive wounds of *S aureus* or *E coli* are shown. (**A**) This is the survival curve for nonbone-exposed wounds with positive culture of *S aureus* (p = 0.003). (**B**) This is the survival curve for nonbone-exposed wounds with positive culture of *E coli* (p = 0.001). (**C**) This is the survival curve for bone-exposed wounds with positive culture of *S aureus* (p = 0.012). (**D**) This is the survival curve for bone-exposed wounds with positive culture of *E coli* (p = 0.022).

However, the reduced bacterial load in wounds did not result in decreased infection risk because the therapeutic effects were offset by the toxicity to host tissues [9, 30], highlighting the advantage of EDTA irrigation.

In our model, EDTA and soap solution decreased bacterial load, but it did not necessarily reduce the development of infection. Residual wound pathogens did not necessarily lead to an infection because the host's immunity eliminated a certain quantity of pathogens. On the other hand, necrotic tissues can facilitate bacterial proliferation. In these two aspects, the ideal irrigation solution should be able to remove as many pathogens as possible with minimal host tissue toxicity.

Irrigating with EDTA resulted in earlier bacteria clearance from infected bone.

Although the soap solution, like many other additives, was more potent in removing pathogens, the higher toxicity restrained the infection rate decrease [18, 34].

In summary, we found that in an in vitro toxicity model and two rat models (one on contamination and one on wound infection), normal saline-EDTA irrigation solution was no more toxic than simple normal saline and was more effective than normal saline or soap solution in reducing contamination and clearing bacterial infection. This is potentially important because infection is a major challenge in the management of open fractures. However, such contentions must be considered preliminary and will need to be tested in larger animal models and, if those prove similarly promising, perhaps in clinical studies.

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