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INFECTION

Optimal concentration of ethylenediaminetetraacetic acid as an irrigation solution additive to reduce infection rates in rat models of contaminated wound

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Aims

In wound irrigation, 1 mM ethylenediaminetetraacetic acid (EDTA) is more efficacious than normal saline (NS) in removing bacteria from a contaminated wound. However, the optimal EDTA concentration remains unknown for different animal wound models.

Methods

The cell toxicity of different concentrations of EDTA dissolved in NS (EDTA-NS) was assessed by Cell Counting Kit-8 (CCK-8). Various concentrations of EDTA-NS irrigation solution were compared in three female Sprague-Dawley rat models: 1) a skin defect; 2) a bone exposed; and 3) a wound with an intra-articular implant. All three models were contaminated with *Staphylococcus aureus* or *Escherichia coli*. EDTA was dissolved at a concentration of 0 (as control), 0.1, 0.5, 1, 2, 5, 10, 50, and 100 mM in sterile NS. Samples were collected from the wounds and cultured. The bacterial culture-positive rate (colony formation) and infection rate (pus formation) of each treatment group were compared after irrigation and debridement.

Results

Cell viability intervened below 10 mM concentrations of EDTA-NS showed no cytotoxicity. Concentrations of 1, 2, and 5 mM EDTA-NS had lower rates of infection and positive cultures for *S. aureus* and *E. coli* compared with other concentrations in the skin defect model. For the bone exposed model, 0.5, 1, and 2 mM EDTA-NS had lower rates of infection and positive cultures. For intra-articular implant models 10 and 50 mM, EDTA-NS had the lowest rates of infection and positive cultures.

Conclusion

The concentrations of EDTA-NS below 10 mM are safe for irrigation. The optimal concentration of EDTA-NS varies by type of wound after experimental inoculation of three types of wound.

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Keywords: Antibacterial agents, Therapeutic irrigation, Surgical wound infection

Article focus

- Are different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) safe for irrigating the wounds during debridement?
- What are the optimal EDTA-NS concentrations for wound irrigation in three animal models?

Key messages

- EDTA-NS concentration lower than 10 mM is a safe concentration in wound irrigation solution.
- Three different animal model wounds have different optimal concentrations of EDTA-NS in wound irrigation.

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Strengths and limitations

- Our studies performed a "head-to-head" comparison of different EDTA-NS concentrations in multiple animal models to determine the optimal concentration of EDTA-NS solution for wound irrigation. Our studies were adequately powered (up to 30 rats in each concentration).
- Although our findings in rats should have a good indication for translation to humans that the optimal concentration might vary by type of wound, the optimal concentrations of EDTA-NS in large animals and humans is still unknown. Thus, the optimal concentration of EDTA-NS in large animal experiments and the clinical applications needs further investigation.

Introduction

For several decades, surfactants, bactericides, and antiseptics have been used during surgery as additives in irrigation solutions to prevent surgical site infections (SSIs).[1] Still, the benefit of surfactants, bactericides, and antiseptics for managing wound infections is controversial,[2] even though overwhelming evidence from preclinical studies demonstrates that the practice of using these agents as additives for irrigation solutions is a feasible method for reducing the quantity of adhered pathogens in wound.[3,4 5] Clinical studies have recently determined that povidone-iodine irrigation does not significantly reduce the risk of intraoperative infections compared to normal saline (NS) irrigation.[6,7] Findings from the fluid lavage of open wounds (FLOW) investigator's research recommend employing NS irrigation over surfactant-additive irrigation.[8] The cytotoxicity of surfactants, bactericides, and antiseptics is usually considered to be responsible for their ineffectiveness in reducing infection rates.[6–10]

Bacteria adhere to host tissue by bacterial adhesins, specialized cell-surface proteins that enable bacteria to adhere to host cells.[11–13] Positive metal ions, including calcium (Ca^{2+}), zinc (Zn^{2+}), and magnesium (Mg^{2+}), are required for the normal functioning of bacterial adhesins.[14–17] Malfunctioning of adhesins by mutating their ion-binding sites, or depriving of specific ions as are necessary for adhesion, leads to reduced bacterial adhesion to tissue.[18–20] Following this adhesion-interfering philosophy for wound infection prevention, in a bone exposed rat model, investigators demonstrated that ethylenediaminetetraacetic acid (EDTA), an agent that strongly binds and then forms complexes with $Ca2^{+}$, $Zn2^{+}$, and $Mg2^{+}$,[20–22] could effectively deactivate the protein of adhesins of bacteria that cause the decrease of bacterial adhesion with low additional tissue toxicity compared to NS irrigation alone.[23] Several recent studies have demonstrated that EDTA is effective as a wound irrigation solution in both open fracture models and intra-articular animal models. In these studies, EDTA shows the powerful ability to remove bacterial and reduce the incidence of wound infection.[23–28] EDTA is widely used in biomedical applications for chelation and thus has a well-established safety profile, and is known for low cytotoxicity, highlighting its potential for translation.[29] Further evidence supporting its low cytotoxicity is that, as most researchers know, EDTA is commonly used along with trypsin for cell passage.

These findings have been extrapolated to more different models including implant-exposed wounds contaminated with more various pathogenic bacterial species (e.g. *Pseudomonas aeruginosa, Enterococcus*, *Acinetobacter baumannii*, *Enterobacter,* and *Proteus mirabilis*).[22–25] For those modelling arthroplasties with an intra-articular implant, wound irrigation with EDTA-reduced infection caused no adverse effects, and was superior to povidone-iodine, benzalkonium chloride, and NS. In the above studies, 1 mM was the concentration of EDTA used in irrigation solutions.[23,24 25] However, it is unknown whether 1 mM EDTA in irrigation solutions is an optimal concentration for reducing or preventing bacterial SSIs. In the present study, we evaluated the cell toxicity of different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) and the efficacy of different concentrations of EDTA as an additive to NS irrigation solutions in preventing infection in three different rat models, with the goal of identifying an optimal concentration.

Methods

Cell viability analysis. The cellular component of granulation tissue consists primarily of fibroblasts and endothelial cells, which have been used in toxicity assays of commonly used irrigation solutions.[30] We compared the different concentrations of EDTA-NS with normal saline in fibroblasts (L929), endothelial cells (human umbilical vein endothelial cells (HUVECs)), rat chondrocytes, and osteoblast cells (MC3T3). All cell lines were obtained from the Cell Resource Center, Peking Union Medical College (National Infrastructure of Cell Line resource, NSTI). Briefly, cells (passage 5) were plated (5,000 cells/well in 96-well plates) in different media and cultured at 37°C, with 5% CO_2, until 80% confluence had been achieved (fibroblasts and endothelial cells were cultured in alpha-minimal essential medium (α-MEM) (Gibco, Dublin, Ireland); chondrocytes were cultured in DM/F12 (Gibco); osteoblasts were cultured in DMEM-high glucose (Gibco). All culture media included 10% fetal bovine serum (Gibco) and 1% penicillin (Gibco). They were then treated with different concentrations of EDTA-NS and normal NS, respectively. The irrigation solution was removed after 15 minutes intervention, and the fresh media were added into the wells at cell incubator (37°C, 5% CO_2). Following co-incubation, the Cell Counting Kit-8 (CCK8) (Dojindo, Kumamoto, Japan) assay was performed to evaluate cell viability, and the absorbance was measured with a multifunction plate reader (Varioskan LUX; Thermo Fisher Scientific, Waltham, Massachusetts, USA) by using

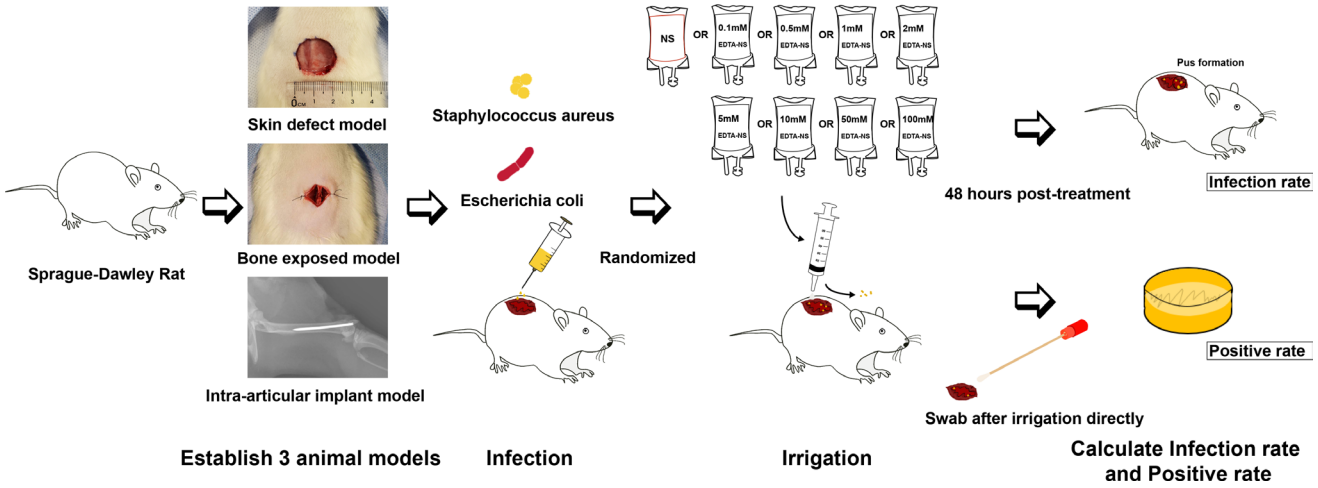

Fig. 1

A schematic diagram for the three postoperative animal models.

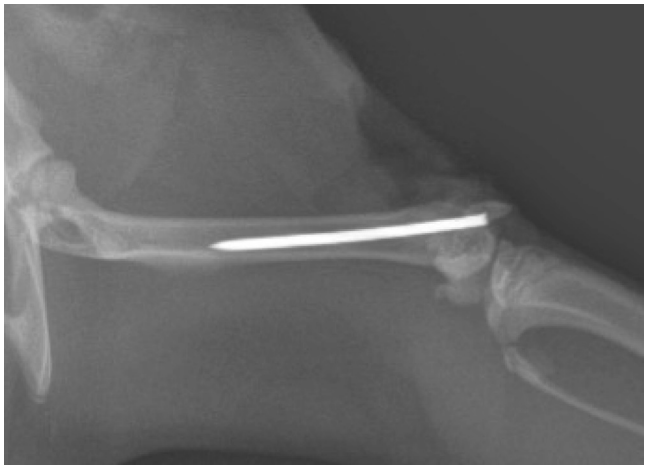

Fig. 2

Representative lateral radiograph after intra-articular implant. A unilateral suprapatellar arthrotomy was performed. A sterile operation-grade Kirschner wire (diameter, 0.088 mm; length, 20 mm) was inserted into the canal of the rat's femoral bone, and the protruding part of the wire was positioned in the knee joint. Then, the joint and skin incision was closed with 4 to 0 sutures after irrigation by ethylenediaminetetraacetic acid dissolved in normal saline.

a 450 nm filter, the room temperature kept at between 18°C to 25°C, and humidity no less than 60%.

Experimental animals and ethical approval. Female Sprague-Dawley rats (six to eight weeks old; bought from Laboratory Animal Center of Shanghai Jiao Tong University) were used for this study. Rats were housed in a stable temperature and humidity environment (20°C to 23°C, 50%) and maintained under a 12-hour light/dark cycle. Free food and water were provided. All the rats were allowed to acclimate (at least one week) to the laboratory conditions before testing. All operations were performed under sterile conditions. Rats were randomly assigned to treatment conditions using a randomization table, and the process was blinded to reduce the bias of this research.

The study and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of our hospital. All study methods were in accordance with China's regulations on experimental animal usage, which were consistent with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.[31]

Animal models and surgery. In total 1,620 animals were randomized into one of three infection model groups (540 rats/animal model). These models simulated different clinical situations (soft-tissue wound, bone exposed, and arthroplasty) in which SSI occurred following orthopaedic procedures, and we utilized these three infection models to directly compare the efficacies of various concentrations of EDTA-NS irrigation solution. Representative Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria (270 rats/strain bacteria in each animal model group) were used to contaminate the wounds in these model animals. We chose these two strains because they were commonly used in experimental rodent models of infection,[32] and because these two species were among the most common bacterial species that cause clinical infections.[33] Infection was confirmed clinically, 48 hours post-treatment, with visual inspection of wound (pus formation) prior to euthanasia of animals and collection of microbiology samples (Figure 1). Sterile procedures were used throughout the experiments (inoculum preparation, irrigation solution preparation, and surgical procedures). For all surgical procedures, rats were first anaesthetized with isoflurane, and the area around the surgical field was shaved and disinfected with povidone-iodine solution (Lionser; Hangzhou, China).

In the skin defect model, a standard full-thickness skin wound (18 mm in diameter) was made on the rat's back. The wound was then inoculated with either *S. aureus* or *E. coli*, respectively. Similar to the clinical scenarios, six hours after surgery, the rat's wound was debrided and irrigated with EDTA-NS or NS with 0 mM EDTA (see below). After that, the wounds were assessed by swabbing the

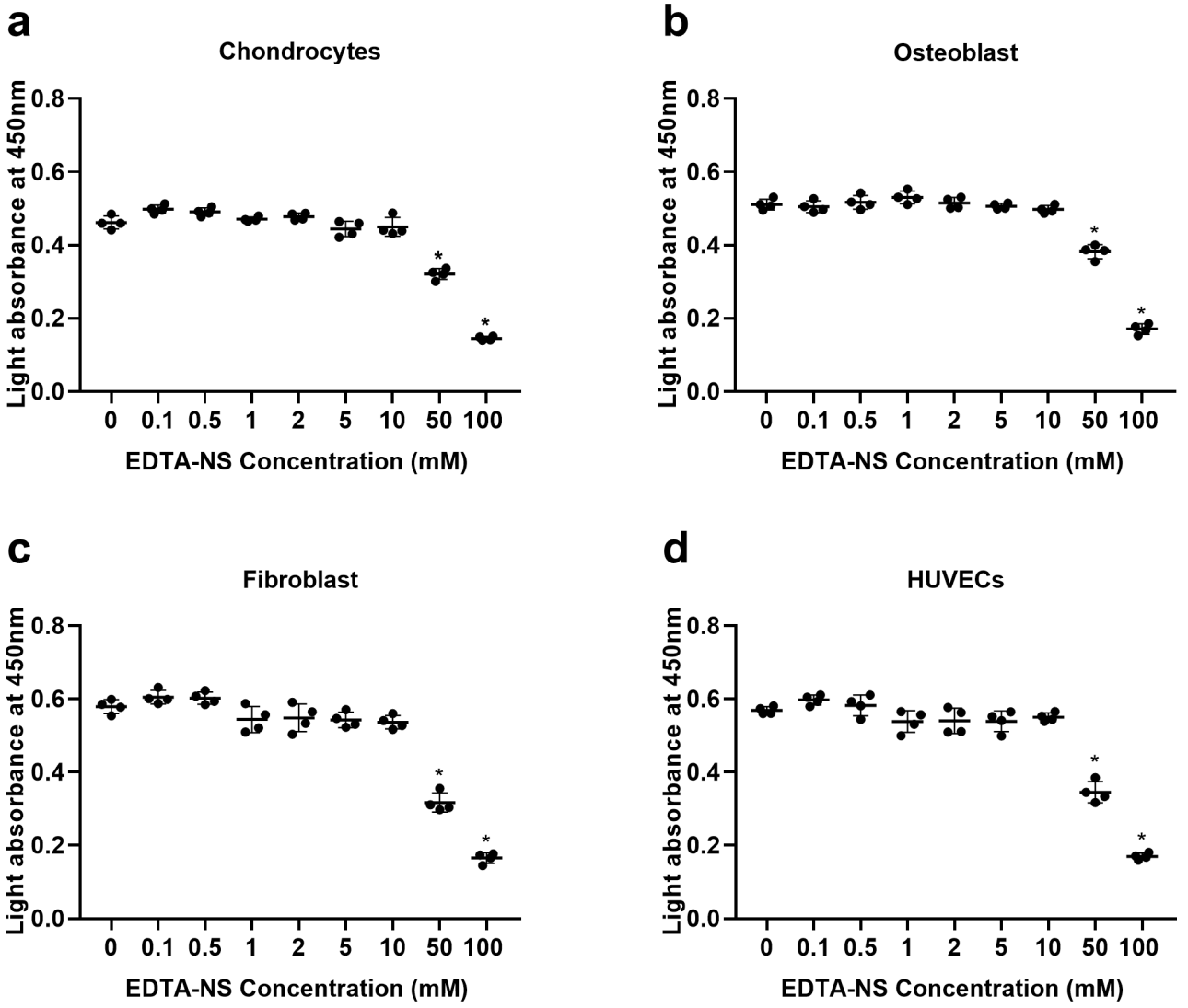

Fig. 3

Cell Counting Kit-8 (CCK8) tests were used to evaluate the safety of different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) in influencing four kinds of cell proliferation: a) chondrocyte, b) osteoblast, c) fibroblast, and d) human umbilical vein endothelial cells (HUVECs). Data are presented as means and SDs: dots represent raw data, middle lines represent each group's mean, and upper and lower lines represent the SD. The concentrations of EDTA-NS within 10 mM did not affect the proliferation of different cells (0 vs 0.1, 1, 2, 5, 10 mM; $p > 0.05$, one-way analysis of variance (ANOVA)). The concentration of EDTA-NS in 50 mM and 100 mM showed higher cell toxicity in vitro compared with NS-only group (0 vs 50 and 100 mM; *$p < 0.001$, one-way ANOVA).

wounds with a cotton swab guide by Levine method[34] and culturing, then calculating the positive rate (see below).

In the bone exposed model, we prepared the rats as described above with the additional use of the protocol of Huyette et al.[35] Briefly, a 1 cm-wide incision was made over the rat's spine. Next, we exposed the spinal process and injected the bone with a needle connected to a syringe containing *S. aureus* or *E. coli*. Six hours after surgery, the wound was debrided and irrigated, cultured for bacterial contamination to calculate positive rate with Levine swab method, and closed surgically.

In the intra-articular implant model, we sought to replicate what occurs in a contaminated intra-articular implant. First, we performed unilateral suprapatellar arthrotomy (3 cm longitudinal skin incision over the knee) and inserted a sterile operation-grade Kirschner wire (diameter, 0.088 mm; length, 20 mm) into the canal of the rat's femoral bone and positioned the protruding part of the wire in the knee joint (Figure 2).[36] We closed the incision temporarily and then injected either *S. aureus* or *E. coli* into the arthrotomy sites. The incision remained closed for one hour, allowing the bacteria to adhere to the implant and adjacent tissue. Next, we reopened the incision and irrigated the implant and adjoining tissue with different concentrations of EDTA-NS or NS with 0 mM EDTA (see below). Finally, the implant and exposed tissue (soft tissue, bone, and joint capsule) were swabbed with

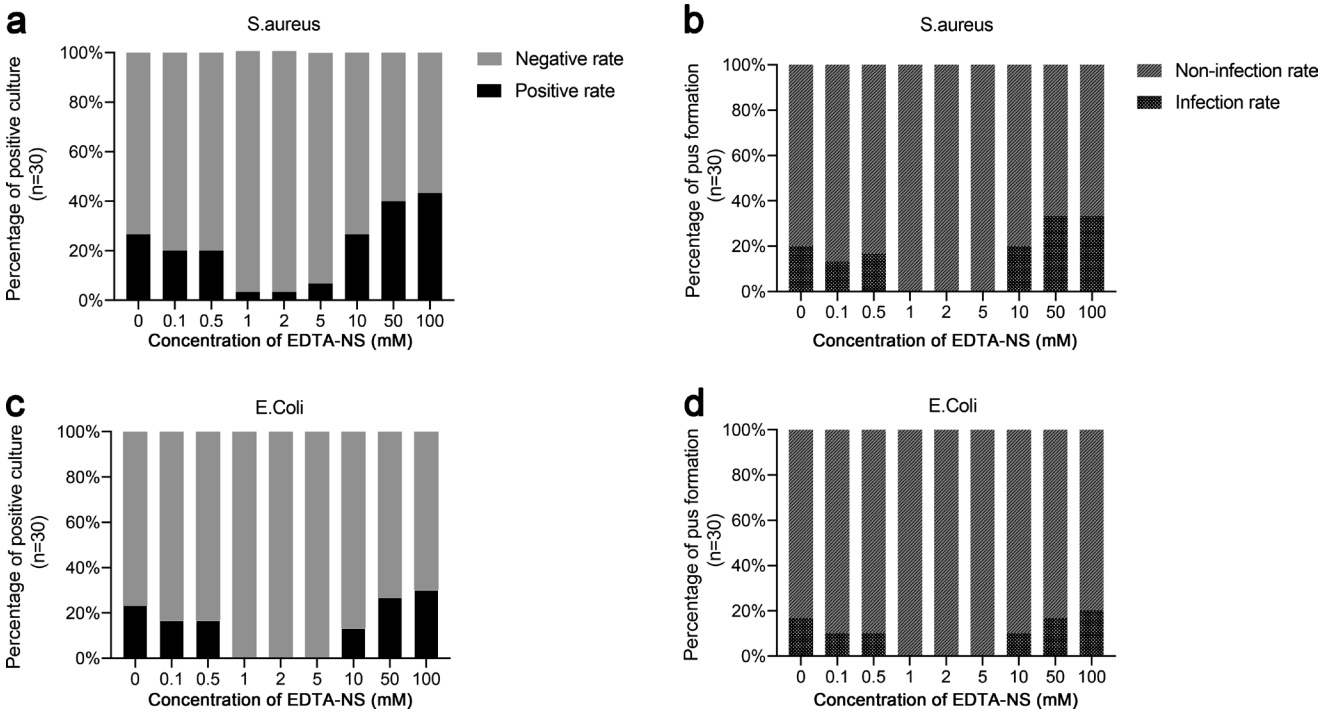

Fig. 4

Efficacy of different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) irrigation solutions in preventing infections in the skin defect model experimentally contaminated immediately with a) and b) *Staphylococcus aureus* or c) and d) *Escherichia coli*. Filled circles in both graphs are mean percentages of cultures with at least one *S. aureus* colony, or one *E. coli* colony assessed 24 hours after plating wound samples. Filled squares represent the percentage of rats with pus formation in the contaminated wound (n = 30 culture plates or animals for each concentration).

a cotton swab guide by Levine method, cultured, and calculated positive rate, and then the incision was closed.

Preparation of bacterial inoculum. Aseptic plated media were used for culturing and maintaining a stock of *S. aureus* (American Type Culture Collection, ATCC 29213; Manassas, Virginia, USA) and *E. coli* (ATCC 25922) using standard laboratory culturing techniques. We used tryptic soy agar (TSA) with 5% sheep blood serum (TSA II, Cat. No. 254,053; Becton-Dickinson, Heidelberg, Germany). New cultures were freshly prepared 24 hours before surgery and wound inoculation. To make the two inocula, we collected a sample of the bacteria on a sterile cotton swab, washed the bacteria three times with NS into a sterile collection tube, and then adjusted the cell concentration to 1×10^8 colony-forming units per millilitre (CFU/ml) by a standard curve of optical density in 600 nm detected by a multifunction plate reader (Varioskan LUX; Thermo Fisher Scientific).[37] Each rat received approximately 2.5×10^6 CFUs of bacterial inoculum in a volume of 25 µl.

Preparation of irrigation solution. To prepare different concentrations of sterile EDTA-NS solution, EDTA (Sinopharm Chemical Reagent, Shanghai, China) was dissolved at a concentration of 0.1, 0.5, 1, 2, 5, 10, 50, and 100 mM in NS (Sinopharm Chemical Reagent), and then titrated with 0.1 M sodium hydroxide to achieve a pH 7.4. NS with 0 mM EDTA was used as a control. All the EDTA-NS solutions were prepared one hour before the experiment and stored at 37°C to maintain optimum temperature. Before use, all solutions were filtered through 0.22 µm filters (Sigma-Aldrich, St. Louis, Missouri, USA, Cat. No. GSWP04700) to make sure the solutions were sterile.

Debridement and irrigation. We followed a standard debridement and irrigation procedure, as previously described.[23] The wounds of anaesthetized rats underwent debridement and irrigation with 300 ml of different concentrations of EDTA-NS (EDTA group) or NS alone (control group). Solutions were delivered to the wound by a 50 ml syringe with maximal manual pressure. The irrigation pressure was low (defined as the irrigation pressure between 5 and 15 lb/square inches).[38] The wound irrigation procedure was performed by the same researcher (JL) in all groups of animals. Finally, the wounds were irrigated with 100 ml of NS to remove any residual EDTA.

Bacterial wound cultures and analysis. All of the wounds were assessed for bacterial contamination by first swabbing the wounds or implant with a sterile cotton swab, and then streaking a petri dish containing TSA agar with 5% sheep blood serum (Becton-Dickinson) with the swab. The petri dishes were incubated at 37°C for 24 hours to allow the bacteria to grow.[39] The bacterial identification was confirmed with 16S ribosomal DNA sequencing by using the MicroSeq 500 microbial identification system (Thermo Fisher Scientific).[40] A culture was considered to be positive if at least one bacterial colony appeared after the incubation period. A wound was considered to be infected if the pus formed after surgery.

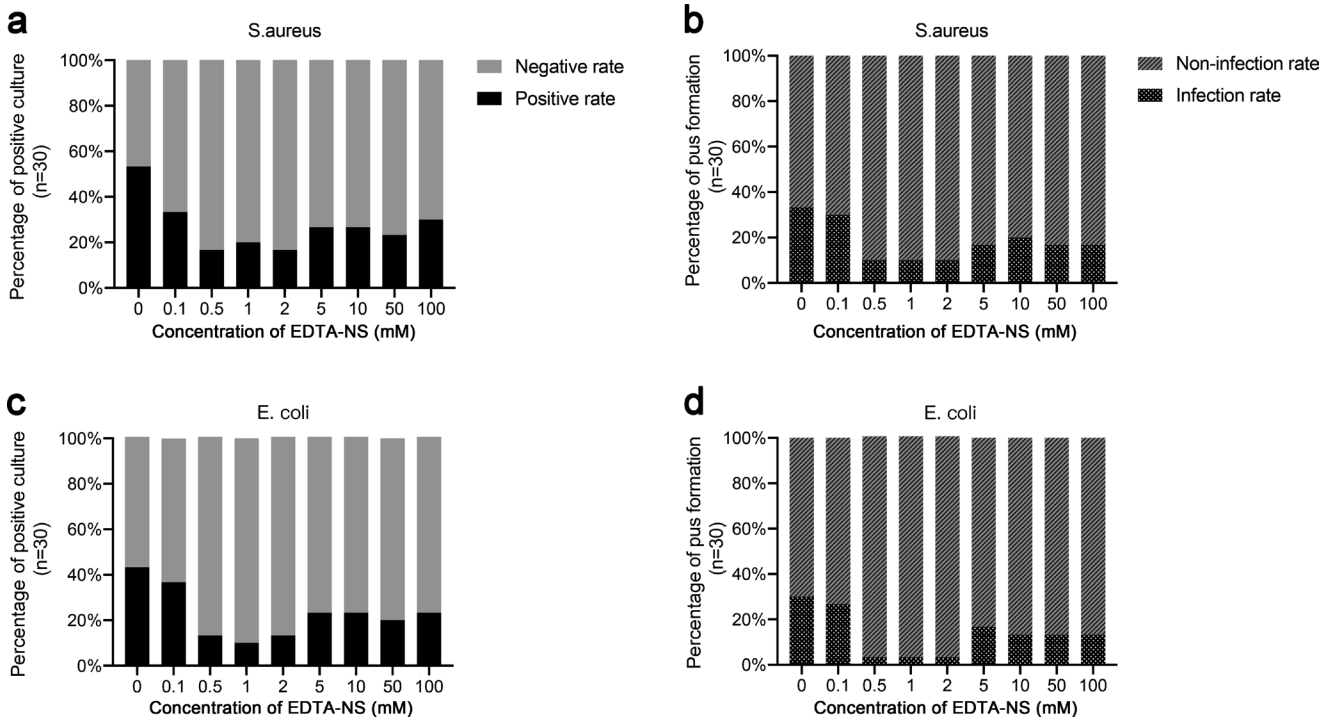

Fig. 5

Efficacy of different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) in irrigation solutions in preventing infection in the bone-exposed model contaminated immediately with a) and b) *Staphylococcus aureus* or c) and d) *Escherichia coli*. Filled circles in both graphs are mean percentages of cultures with at least one *S. aureus* colony, or one *E. coli* colony assessed 24 hours after plating wound samples. Filled squares represent the percentage of rats with pus formation in the contaminated wound (n = 30 culture plates or animals for each concentration).

Sample size calculation and blinding. We set 0.05 as the value of type I error (α) and 80% as the power (1-β) to calculate the sample size. The superiority margin was set at 0.1. According to previously published data, in order to detect a 33% difference between EDTA-NS irrigation (37%) and NS (70%) we required 28 rats per group, applying the sample size calculation method from Charan and Kantharia.[24] Given that the possible loss of rats, we finally set the sample size to be 30 rats per group. For the CCK8 assay, the group information during irrigation solution intervention and subsequent assessment was blinded to the researcher (TG). For surgical debridement, irrigation, and wound cultures, the group information of different concentrations of EDTA-NS was also blinded to the researchers (JL, HW, HZ).

Statistical analysis. Pearson's chi-squared statistic or Fisher's exact test was used to evaluate differences in dichotomous variables between the different concentrations of EDTA-NS in each group. One-way analysis of variance (ANOVA), followed by Tukey hypothesis testing to correct for multiple comparisons, which were used to evaluate differences in continuous variables between the different concentrations of EDTA-NS in each group. GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, California, USA) was used for statistical analysis. All statistical significance was defined as $p < 0.05$.

Results

The toxicity of various concentrations of EDTA-NS was measured by CCK-8. Four types of cells treated below 10 mM EDTA-NS showed normal cell proliferation compared to NS-treated cells, which indicated that the viability of four kinds of cells showed no significant differences below 10 mM EDTA-NS. In contrast, cytotoxicity was observed in four types of cells exposed to concentrations of 50 mM and 100 mM EDTA-NS (Figure 3).

Three different rat models were used to evaluate the efficacies of different concentrations of EDTA-NS irrigation solutions for preventing infections in experimental wounds: 1) skin defect model, 2) bone exposed model, and 3) intra-articular implant model. The primary outcomes were presence/absence of infection (defined as pus formation) and positive bacterial colonies formed from cultures of wound specimens (defined as at least one *S. aureus* colony or *E. coli* colony).

For the skin defect model, irrigation with 1, 2, and 5 mM EDTA-NS produced significantly lower prevalence of infection and lower positive culture rates compared with other concentrations (Figure 4). When we irrigated the wound with higher concentrations of EDTA-NS (10, 50, and 100 mM), the capacity to disinfect the wound quickly declined. Visual inspection of the graphs in Figure 4 also suggests that for moderate concentrations of EDTA-NS (1, 2, and 5 mM), disinfection efficacy may be better towards *E. coli* than towards *S. aureus*. For

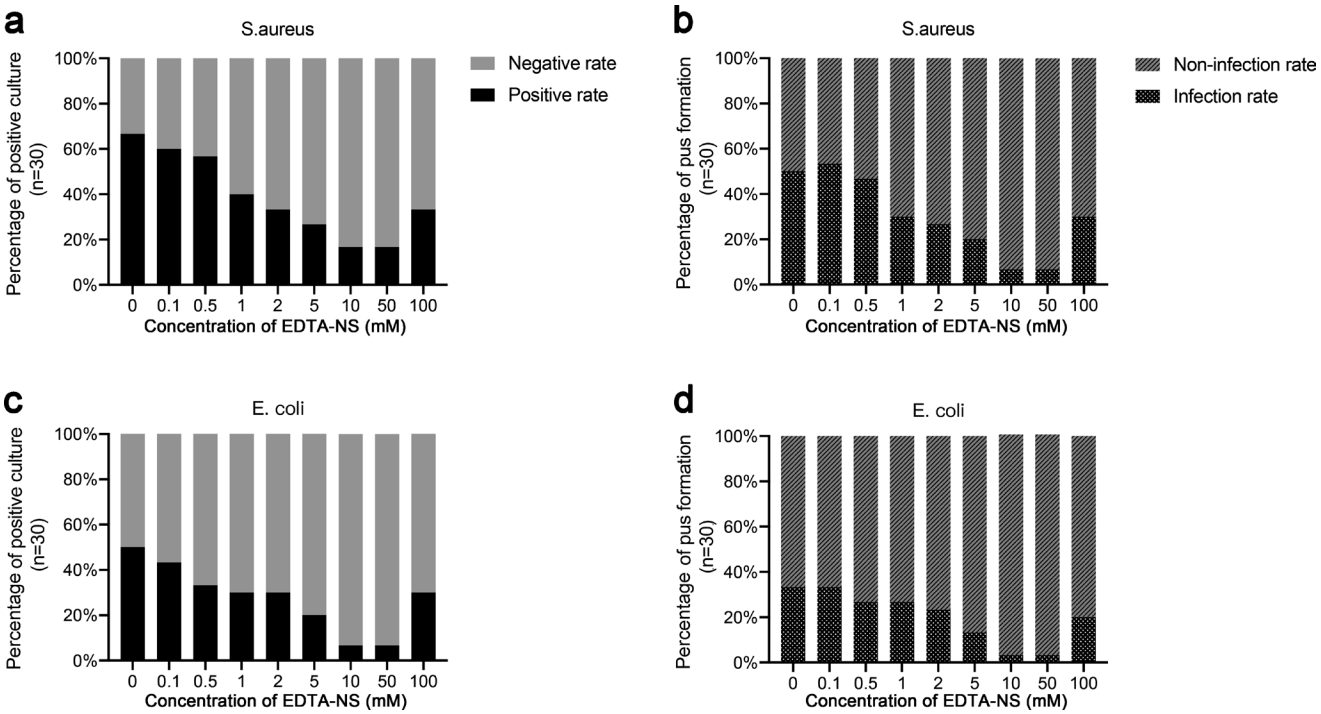

Fig. 6

Efficacy of different concentrations of ethylenediaminetetraacetic acid dissolved in normal saline (EDTA-NS) in irrigation solutions in preventing infection in the intra-articular implant model contaminated with a) and b) *Staphylococcus aureus* or c) and d) *Escherichia coli*. Filled circles in both graphs are mean percentages of cultures with at least one *S. aureus* colony, or one *E. coli* colony assessed 24 hours after plating wound samples. Filled squares represent the percentage of rats with pus formation in the contaminated wound (n = 30 culture plates or animals for each concentration).

the bone-exposed animal model, irrigation with 0.5, 1, and 2 mM EDTA-NS produced lower infection and positive culture rates compared to the other concentrations (Figure 5). In contrast to the results for the skin defect model, irrigation with higher concentrations of EDTA-NS produced better wound disinfection than NS. For the infected intra-articular implant model, we found that the lowest positive culture and infection rates occurred when irrigating the wound with 10 and 50 mM EDTA-NS irrigation (Figure 6).

Discussion

Although the efficacy of EDTA-NS solution has been well established by previous studies,[22–25] to the best of our knowledge it was unknown if one concentration of EDTA-NS is superior to others for reducing SSIs. In the present study, we evaluated a range of EDTA-NS concentrations to determine if there was an optimal concentration that was effective and safe in reducing infections in three rat models: 1) soft-skin defect, 2) bone exposed, and 3) intra-articular implant. Before conducting any prospective human trials, it was essential to first assess different concentrations of EDTA-NS in animal models.

We experimentally infected rats with *S. aureus* or *E. coli,* two bacterial species that are often related to orthopaedic infections.[33] Our results indicate that infections in different kinds of wounds respond differently to varying concentrations of EDTA irrigation solutions. For example, in soft-skin wounds, 1, 2, and 5 mM EDTA-NS solutions were more effective in reducing infections; in bone-exposed wounds, 0.5, 1, and 2 mM EDTA-NS were more effective; and in infections of intra-articular implants, 10 and 50 mM were more effective. The reasons why different suitable concentrations of EDTA-NS are used in various animal models are still unknown, and we infer that the explanation is related to the ability of bacteria to adhere to different kinds of tissue and cell.[41,42] Also, we observed that increasing the concentration of EDTA (> 10 mM EDTA in NS) failed to improve the irrigation solution's efficacy to remove bacteria from the wounds modelled. This may be due to increased osmotic pressure at higher concentrations of EDTA-NS, or to tissue toxicity.[23]

Arthroplasty is a sterile surgical procedure done in humans and, as such, it is considered to have a low risk for bacterial infections. In theory, therefore, if adequate aseptic techniques are applied during arthroplasty surgery, there would be no need to disinfect the wound via irrigation. In real-world clinical situations, however, implant contamination does occur due to suboptimal application of standard aseptic practices during surgery, the presence of residual pathogens on the wound, or other unidentified variables. For these reasons, in the present study, we attempted to reproduce these scenarios in our three animal models. In the intra-articular implant model, for example, the implant was exposed to the bacteria for one hour, which is the average length of a

typical arthroplasty procedure.[43] In the skin defect and bone-exposed models, the wounds were exposed to the bacteria for six hours, which is the approximate time it would take for a patient to be transferred to the trauma centre and wait for surgery preparation.

In the present study, we identified a range of effective EDTA-NS concentrations to reduce infections in three kinds of wounds, showing that EDTA-NS was superior to NS alone for disinfecting intra-articular implants in a rat model. Also, this study was adequately powered (n = 30 for each concentration) to produce reliable results. However, the study had some limitations. Firstly, we contaminated a model wound in rats, which may be inadequate compared to humans. Rats definitely have different reactions compared to humans during bacterial infections. Before clinical trials can be performed, similar studies on larger animals with an immune system more similar to that of humans are warranted. Secondly, we used representative gram-positive and gram-negative bacteria (*S. aureus* and *E. coli*, respectively) to infect the wound experimentally. These two species are the primary pathogens that occur in bone and joint infections.[33] However, besides these two primary pathogens, there are still many other bacteria that could cause orthopaedic disease, and the optimal concentration of EDTA solution may be different for different pathogens. The third limitation of the study is the technique used for pathogen detection: in our research we applied direct culture on petri dishes, which arguably underestimates the presence of pathogens and consequently lowers the detected rate of infection. A more accurate method therefore needs to be applied in future studies–evidence elsewhere has shown that pre-enrichment and molecular techniques are more sensitive.[44,45]

In conclusion, results from the present study provide new data on the range of effective EDTA-NS concentrations for wound irrigation, which have the potential to be translated into clinical trials if further research in larger animals with more comparable immune systems takes place.

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Supplementary material

The ARRIVAL checklist demonstrating that the animal experiments in this research followed appropriate guidelines.

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