Efficacy of EDTA-NS irrigation in eradicating *Staphylococcus aureus* biofilm-associated infection

an in vitro and in vivo study

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Aims

To investigate the efficacy of ethylenediaminetetraacetic acid-normal saline (EDTA-NS) in dispersing biofilms and reducing bacterial infections.

Methods

EDTA-NS solutions were irrigated at different durations (1, 5, 10, and 30 minutes) and concentrations (1, 2, 5, 10, and 50 mM) to disrupt *Staphylococcus aureus* biofilms on Matrigel-coated glass and two materials widely used in orthopaedic implants (Ti-6Al-4V and highly cross-linked polyethylene (HXLPE)). To assess the efficacy of biofilm dispersion, crystal violet staining biofilm assay and colony counting after sonification and culturing were performed. The results were further confirmed and visualized by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). We then investigated the efficacies of EDTA-NS irrigation in vivo in rat and pig models of biofilm-associated infection.

Results

When 10 mM or higher EDTA-NS concentrations were used for ten minutes, over 99% of *S. aureus* biofilm formed on all three types of materials was eradicated in terms of absorbance measured at 595 nm and colony-forming units (CFUs) after culturing. Consistently, SEM and CSLM scanning demonstrated that less adherence of *S. aureus* could be observed on all three types of materials after 10 mM EDTA-NS irrigation for ten minutes. In the rat model, compared with NS irrigation combined with rifampin (Ti-6Al-4V wire-implanted rats: 60% bacteria survived; HXLPE particle-implanted rats: 63.3% bacteria survived), EDTA-NS irrigation combined with rifampin produced the highest removal rate (Ti-6Al-4V wire-implanted rats: 3.33% bacteria survived; HXLPE particle-implanted rats: 6.67% bacteria survived). In the pig model, compared with NS irrigation combined with rifampin (Ti-6Al-4V plates: 75% bacteria survived; HXLPE bearings: 87.5% bacteria survived), we observed a similar level of biofilm disruption on Ti-6Al-4V plates (25% bacteria survived) and HXLPE bearings (37.5% bacteria survived) after EDTA-NS irrigation combined with rifampin. The in vivo study revealed that the biomass of *S. aureus* biofilm was significantly reduced when treated with rifampin following irrigation and debridement, as indicated by both the biofilm bacterial burden and crystal violet staining. EDTA-NS irrigation (10 mM/10 min) combined with rifampin effectively removes *S. aureus* biofilm-associated infections both in vitro and in vivo.

Conclusion

EDTA-NS irrigation with or without antibiotics is effective in eradicating *S. aureus* biofilm-associated infection both ex and in vivo.

Bone & Joint Research

Article focus

- The effectiveness of irrigation with ethylenediaminetetraacetic acid-normal saline (EDTA-NS) in removing bacterial biofilms, particularly those caused by *S. aureus*, in different materials commonly used in orthopaedic implants.

Key messages

- Irrigation with EDTA-NS at concentrations of 10 mM or higher for ten minutes is effective in removing *S. aureus* biofilms on different materials used in orthopaedic implants.
- In vivo studies show that EDTA-NS irrigation in combination with antibiotic therapy is effective in eradicating *S. aureus* biofilm-associated infections.

Strengths and limitations

- The study used a comprehensive approach to evaluate the efficacy of EDTA-NS irrigation in removing *S. aureus* biofilms, including various in vitro and in vivo methods.
- The study only focused on *S. aureus* biofilms, and the efficacy of EDTA-NS irrigation on other types of biofilms remains unclear.
- The study used animal models, which may not fully represent the complex and heterogeneous nature of biofilm-associated infections in humans.

Introduction

Staphylococcus aureus frequently and asymptomatically colonizes 30% to 50% of all individuals in the general population, and can opportunistically cause sepsis, surgical site infection, bone and joint infection, endocarditis, pericarditis, osteomyelitis, and septic arthritis in individuals (especially hospital patients) with compromised immune defenses.[1-3] In the last decade, hospital costs associated with *S. aureus* infections were estimated to be more than $45 billion per year.[4,5] Because *S. aureus* can successfully escape the host immune system and undermine antibiotic treatments by various mechanisms, *S. aureus* infections are clinically challenging, being characterized by a long disease course and high recurrence rate.[6,7] Many studies demonstrate that biofilm formation plays a pivotal role in the persistence of *S. aureus* infections.[8-10]

Biofilm is an aggregated and structured community of bacteria residing in a polymer-based extracellular matrix composed of proteins, DNA, and polysaccharides.[9,11] Besides soft-tissue, *S. aureus* can also attach to surfaces of medical implants and host tissue; if a mature biofilm develops, it becomes resistant to antibiotic therapy and hinders the infiltration of immune cells, making it difficult to clear the bacteria.[12,13] *S. aureus* embedded in biofilm is less susceptible to sensitive antibiotics that are normally effective against planktonic bacteria.[14-16] As a result, even minimal inhibitory concentrations of *S. aureus* in biofilm might not be tolerated by a host, limiting the efficacy of antibiotics and contributing to treatment failure.[17,18]

Because of this limitation of antibiotic therapy, surgical procedures to remove infected tissues, when advisable, are usually necessary to remove *S. aureus* biofilm, especially in chronic cases.[19] Irrigation and debridement can physically remove, or at least disrupt *S. aureus* biofilm, thus enhancing the efficacy of subsequent postoperative antibiotic therapies.[20,21] However, eradicating biofilms using surgical and antibiotic therapies is considered to be an 'all-or-nothing' treatment, because any residual bacteria-laden biofilm can cause the infection to reoccur.[16,22] As a result, repeated surgeries and prolonged antibiotic therapies are usually needed for biofilm-associated infections.[23-25] Thus, strategies for improved disruption of *S. aureus* biofilms during irrigation and debridement are of great interest to clinicians and researchers.

Metal ions, including Ca^{2+}, Zn^{2+}, and Mg^{2+}, are essential elements for bacterial adhesive molecules to properly function.[26-28] Approaches that target adhesive molecules through deprivation of metal ion cofactors might be a feasible strategy for disrupting biofilm.[29] A low-toxicity binding agent is needed, one that has a well-established safety profile and that can strongly bind to and then form a complex with metal ions; ethylenediaminetetraacetic acid (EDTA) is one such molecule.[28,30-32]

Previous studies demonstrated that EDTA-supplemented irrigation solutions can effectively reduce the adhesion of planktonic bacteria on host tissue and medical implants.[33,34] Irrigation with EDTA solutions has also been shown to successfully prevent *S. aureus* infection in several preclinical models of contaminated wounds.[28,33,35,36] It remains unclear, however, whether irrigation with EDTA solutions is effective in disrupting biofilm wherein bacteria are more tightly attached to the matrix, host tissues, and implants.

Methods

Bacterial growth and preparation of bacterial stock solution for inoculum

A stock of *S. aureus* (ATCC 43300; American Type Culture Collection, USA) was maintained on tryptic soy agar (TSA) with 5% sheep blood serum (TSA II, Cat. No. 254,053; Becton-Dickinson, Germany). To create the inoculum for the experiments, we used a sterile plastic loop to randomly collect one colony of bacteria, which was then transferred to tryptic soy broth (TSB) (Solarbio, China) and incubated overnight at 37°C. A Varioskan LUX multifunctional microplate reader (Thermo Fisher Scientific, USA) was used to measure bacterial cell concentration, which was then adjusted a cell concentration of 1×10^8 CFU/ml using a standard optical density (OD) curve obtained at 595 nm.[37]

Preparation of irrigation solution

To obtain different concentrations of EDTA-normal saline (EDTA-NS) solution, EDTA (Sinopharm Chemical Reagent, China) was dissolved at concentrations of 1, 2, 5, 10, and 50 mM in 0.9% NaCl (Sinopharm Chemical Reagent). The pH of the EDTA-NS irrigation solution was adjusted with 0.1 M NaOH to pH 7.4. Before using the EDTA-NS irrigation solutions in experiments, we filtered the solutions through microfilters (pore size: 0.22 μm; Sigma-Aldrich, USA), and then stored the solutions at 37°C in an incubator for at least one hour until needed.

Biofilm formation on simulated host tissue and orthopaedic materials

Matrigel matrix is a man-made substance that is widely used in biomedical research to mimic the extracellular matrix of animal and human tissues; it consists of laminin, collagen IV, heparan sulfate, and other basement membrane proteins.[38]

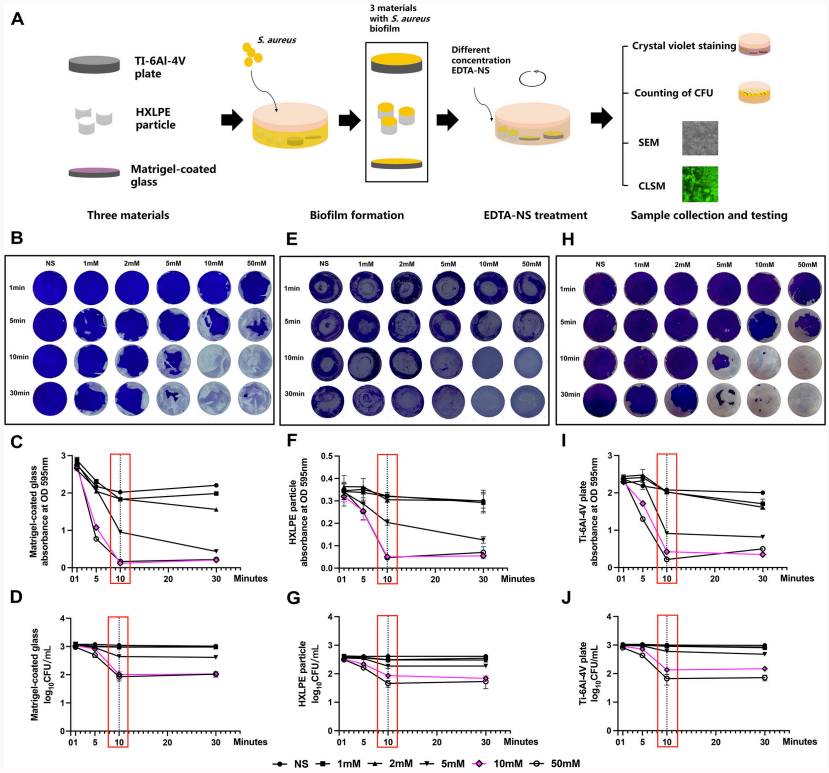

Fig. 1

Efficacy of varying concentrations of ethylenediaminetetraacetic acid-normal saline (EDTA-NS) irrigation in eradicating *Staphylococcus aureus* biofilm in vitro. a) Schematic diagram of experimental setup. b), e), h) Bright-field images of crystal violet-stained (purple-blue colour) *S. aureus* biofilms on Matrigel-coated culture glass, highly crosslinked polyethylene (HXLPE) particles, and Ti-6Al-4V titanium alloy orthopaedic plates after simulated irrigation with different concentrations (0, 1, 2, 5, 10, or 50 mM) at different durations (1, 5, 10, or 30 minutes) of EDTA-NS. c), f), i) Quantification of remaining biomass of *S. aureus* biofilms on Matrigel-coated glass, HXLPE particles, and Ti-6Al-4V plates measured by optical density (OD) (absorbance at 595 nm) on a multifunction plate reader; lower OD mean values indicate greater efficacy. Boxed areas in each graph indicate minimum best duration and solution concentrations ($p < 0.01$). d), g), j) Mean bacterial burden (colony-forming unit (CFU)/ml) of *S. aureus* biofilms on Matrigel-coated glass, HXLPE particles, and Ti-6Al-4V plates measured after different concentrations and durations of EDTA-NS simulated irrigation. Data are plotted as means and standard deviations. CLSM, confocal laser scanning microscopy; SEM, scanning electron microscopy.

To mimic host tissue in these experiments and different implanted medical devices,[39] we coated cell culture glass with Matrigel matrix (Corning Life Sciences, USA) or used samples of highly cross-linked polyethylene (HXLPE) particles (Dow Chemical, USA), and/or Ti-6Al-4V titanium alloy orthopaedic plates (Yitai Metal Manufacturing, China). Before biofilm formation, all experimental materials were immersed in TSB for 30 minutes, and then placed into an incubator at 37°C for 30 minutes. The three materials were then incubated in culture with 1×10^8 CFU/ml *S. aureus* in TSB (with 0.25% glucose) in 12-well plates (Corning Life Sciences) at 37°C for 48 hours.

EDTA-NS treatment and biofilm quantification

After 48 hours in culture, each experimental material with the accompanying biofilm was placed into separate sterile 10 cm culture dishes (Corning Life Sciences). Each concentration (1, 2, 5, 10, and 50 mM) of EDTA-NS was separately applied to the biofilms on the three materials for 1, 5, 10, or 30 minutes in a volume of 20 ml/dish. To simulate the irrigation process, we placed the 10 cm culture dishes with the materials and EDTA-NS solutions on a shaker table set at 50 rpm/min. The three materials were then transferred to fresh culture wells after two gentle washes with 0.9% NaCl in a volume of 20 ml/dish. The materials with adherent bacteria were then stained with 0.1% crystal violet (Beijing Solarbio Science & Technology, China) for five minutes and washed gently three times with water. After air drying the stained materials for 15 minutes at room temperature, the staining was resolved by adding 1 ml/well of 33% acetic acid (Sigma-Aldrich) so that we could quantify the remaining adherent biomass.

Crystal violet staining of biofilms on adherent samples in culture is a reliable and straightforward method to quantify total biomass.[40] OD of dye absorbance remaining on the experimental materials was measured at 595 nm with a multifunction plate reader (Thermo Fisher Scientific). The biofilm removal assay was performed in triplicate for each experimental material and EDTA-NS irrigation concentration and irrigation duration. We also used digital bright-field photomicrographs to document the extent of dye remaining on the surfaces.

Scanning electron microscopy

The sample experimental materials and accompanying biofilms were removed from the different wells after irrigation and gently washed two times with 0.9% NaCl. Each sample was fixed with 2.5% glutaraldehyde for four hours and then placed into a lyophilizer until complete lyophilization was achieved. For scanning electron microscopy (SEM) imaging, the materials were then mounted on aluminium stubs with double-sided adhesive

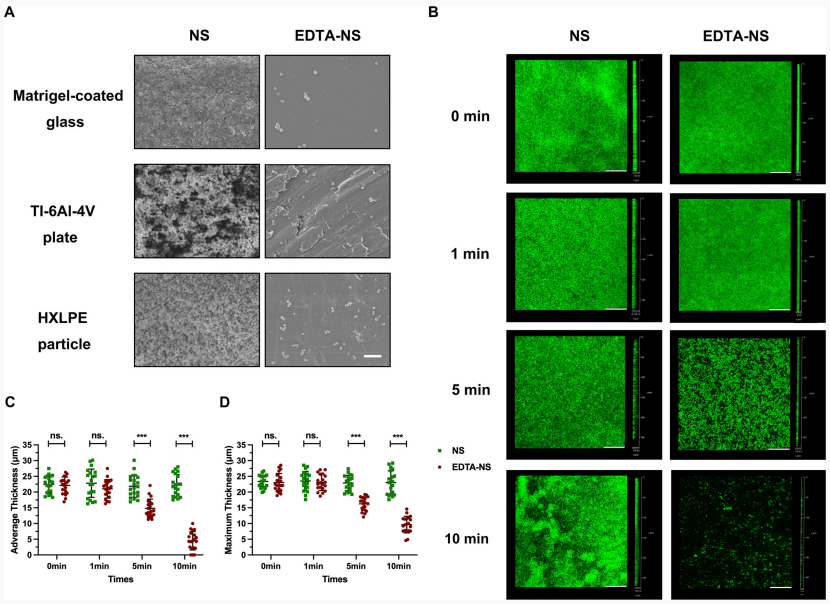

Fig. 2

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) analysis of *Staphylococcus aureus* biofilms on three materials commonly used for prosthetic devices after ethylenediaminetetraacetic acid-normal saline (EDTA-NS) and NS irrigation. a) Representative SEM images of *S. aureus* biofilms on the three prosthesis materials after 10 mM EDTA-NS or NS irrigation alone for ten minutes. b) Representative CLSM images of *S. aureus* biofilms on Matrigel-coated glass after 10 mM EDTA-NS or NS irrigation at the indicated durations. Quantitative analysis of the c) mean and d) maximum thickness of *S. aureus* biofilm on Matrigel-coated glass after irrigation with 10 mM EDTA-NS at the indicated irrigation durations (0, 1, 5, and 10 minutes). Same conventions as in Figure 1; scale bars: 50 μm. ns, not significant, $p > 0.05$; *** $p < 0.001$. Statistical evaluations by Mann-Whitney U test. HXLPE, highly crosslinked polyethylene.

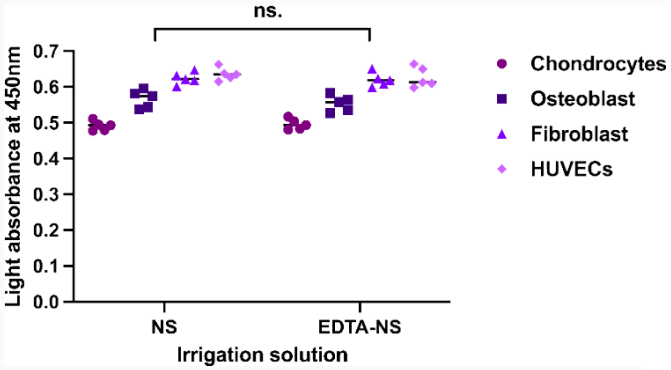

Fig. 3

Cytotoxicity of four different cell types after treating with different concentration and duration of ethylenediaminetetraacetic acid-normal saline (EDTA-NS) (ns, no significant difference; p 0.05). HUVECs, human umbilical vein endothelial cells.

tape, sputtered with 10 nm thick Au/Pd (Bal-Tec, Germany) coating, and then examined by a JEOL scanning electron microscope (model JCM-7000; JEOL, USA).

Confocal laser scanning microscopy

Biofilm grown on the glass was also studied using confocal laser scanning microscopy (CLSM) to assess any morphological alterations of biofilms after EDTA-NS treatment. The biofilm-covered glass samples were removed from the different wells and then washed gently with 0.9% NaCl to remove any loosely attached bacterial cells. For visualization, the samples were stained with SYTO9 (Thermo Fisher Scientific). The stained biofilms were then imaged using a Leica confocal microscope (Leica, model TCS SP5; USA). Several features of the biofilms were measured by CLSM: mean average thickness and mean maximum thickness. A total of 20 random areas on the biofilm images were assessed and contributed to the mean values. Each experiment was repeated three times for reproducibility.

Cell viability analysis

Granulation tissue that forms on the surfaces of a wound during the healing process comprises multiple cell types, including fibroblasts and endothelial cells. Osteoblasts and chondrocytes are considered to be the primary cells of the musculoskeletal system.[28,41,42] Therefore, four cell types were used to test the toxicity of the experimental irrigation solutions: mouse fibroblasts (cell line L929), human umbilical vein endothelial cells (HUVECs), rat chondrocytes, and mouse osteoblasts (cell line M3T3). L929, M3T3, and HUVEC cells were purchased from the Cell Resource Centre, Peking Union Medical College (National Infrastructure of Cell Line resource, NSTI, China). Rat chondrocyte extraction and culturing of chondrocytes were carried out according to procedures outlined in Lin et al.[43]

To evaluate cell viability, we used a Cell Counting kit-8 (CCK8) (Dojindo, Japan) assay. Briefly, cells were plated (5,000 cells/well, 96-well plates; Corning Life Sciences) in different cell-appropriate media (αMEN for fibroblast and endothelial cell cultures, DM/F12 for chondrocyte cultures, and DMEM-high glucose for M3T3 cultures (all Gibco-Thermo Fisher Scientific)), and then maintained in an incubator at 37°C and 5% CO_2. All the culture mediums contained 10% foetal bovine serum (Gibco-Thermo Fisher Scientific) and 1% penicillin (Gibco-Thermo Fisher Scientific).

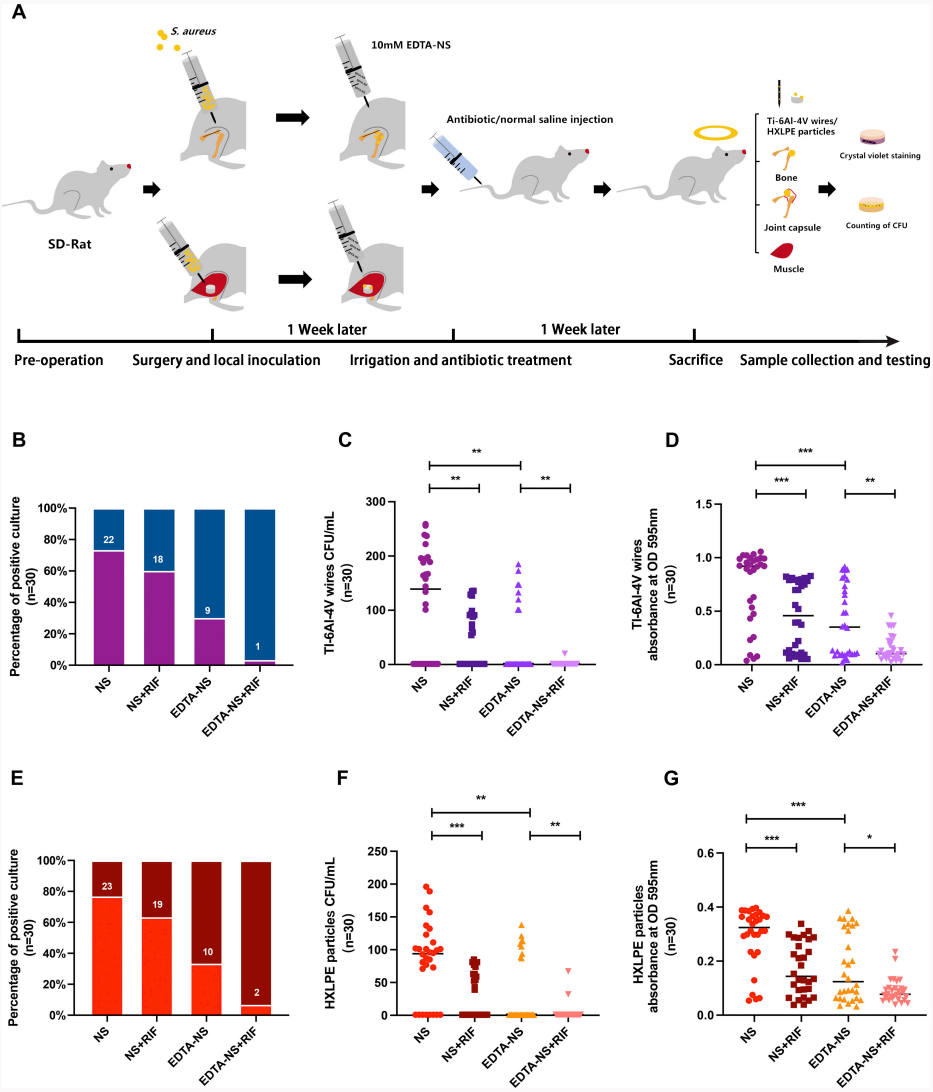

Fig. 4

Efficacy of *Staphylococcus aureus* biofilm removal by ethylenediaminetetraacetic acid-normal saline (EDTA-NS) irrigation in rat. a) Schematic diagram of experimental setup. b) Mean percentage positive cultures (filled purple-coloured bar) from whole joint samples derived from Ti-6Al-4V wire-implanted rats. Horizontal white-coloured bars and numbers indicate median of group and absolute number of positive cultures, respectively. c) Bacterial burden (colony-forming unit (CFU)/ml) in cultures derived from Ti-6Al-4V wire-implanted rats. Filled symbols are data from individual rats in each group; horizontal lines are medians. d) Quantification of *S. aureus* biofilm on Ti-6Al-4V wires by crystal-violet staining in cultures by optical density (OD) (595 nm). e) Mean percentage positive cultures (filled red-coloured bars) derived from whole implant sites of highly crosslinked polyethylene (HXLPE) particle-implanted rats. The same symbol conventions apply as above. f) Bacterial burden (CFU/ml) in samples cultured from HXLPE particle-implanted rats. g) Quantification of *S. aureus* biofilms on HXLPE particles by crystal-violet staining by OD (595 nm). NS, normal saline; RIF, rifampin. ***p < 0.001, **p < 0.01; *p < 0.05; statistical evaluations by Fisher's exact test or Mann-Whitney U test.

After the cell cultures reached 80% confluence, they were treated with 10 mM EDTA-NS solution for ten minutes at 37°C. Then the EDTA-NS solution was removed, fresh medium was added to each well containing 10% CCK-8, and the plates were incubated (37°C, 5% CO_2). After two hours of incubation, the 96-well plates were read in a multifunction plate reader to measure absorbance (OD) of each well at 450 nm. The ambient temperature was maintained at 18°C to 25°C and humidity at 60%.

Experimental animals and ethical approval

Female Sprague-Dawley rats aged eight to ten weeks (brought from Shanghai Jiao Tong University) were used for this study. Yorkshire research pigs (brought from Jia Gan Biotechnology), with a mean weight range of 35 to 40 kg (aged 9 to 12 months), were also used in this study. The animals were accommodated in a controlled environment with stable temperature (20°C to 23°C) and humidity (50%), adhering to a 12-hour light/dark cycle. They were given unrestricted access to both food and water. All animals were given at least one week to acclimate to the laboratory conditions before undergoing any testing procedures. All experimental interventions were carried out under aseptic conditions. To minimize research bias, animals were randomly assigned to various treatment conditions using a randomization table, and the process was blinded.

All experimental procedures and protocols used in this research were approved by our hospital's Institutional Animal Care and Use Committee. These procedures and protocols follow China's regulations on experimental

Table I. Quantitation and statistical evaluation of *S. aureus*-positive cultures derived from samples irrigated with 10 mM ethylenediaminetetraacetic acid-normal saline, normal saline, and rifampin in rat.

Cultured sample type	NS, n (%)	NS + RIF, n (%)	EDTA-NS, n (%)	EDTA-NS + RIF, n (%)
Ti-6Al-4V wire-implanted rats				
Ti-6Al-4V wires	18 (60)*	13 (43)*	8 (27)*†	1 (3)†
Bone	21 (70)*	17 (57)*	7 (23)†	1 (3)†
Joint capsule	20 (67)*	16 (53)*	8 (27)*†	1 (3)†
Whole joint‡	22 (73)*	18 (60)*	9 (30)*†	1 (3)†
HXLPE particle-implanted rats				
HXLPE particles	22 (73)*	13 (43)*	9 (30)*†	2 (7)†
Muscle	19 (63)*	18 (60)*	8 (27)†	1 (3)†
Whole implant site§	23 (76)*	19 (63)*	10 (33)*†	2 (7)†

Group differences evaluated by Fisher's exact test.
*$p < 0.05$ versus EDTA-NS + RIF.
†$p < 0.05$ versus NS alone.
‡Any positive cultures from Ti-6Al-4V wires, bone, and joint capsule.
§Any positive cultures from HXLPE particles and muscle.
EDTA-NS, ethylenediaminetetraacetic acid-normal saline; HXLPE, highly crosslinked polyethylene; NS, normal saline; RIF, rifampin.

animal usage, and they are consistent with the guidelines of the Animal Research: Reporting of In Vivo Experiments (ARRIVE). We have adhered to the ARRIVE guidelines and have supplied the Checklist.[44]

Animals and surgery

To evaluate the effectiveness of EDTA-NS irrigation in removing biofilms in vivo, we used established bone and joint implantation infection animal models.[45,46] Rats were anaesthetized with vaporized isoflurane mixed with 5% O_2. For anaesthesia induction, we used isoflurane (5%), and to maintain anaesthesia during surgery we used 2% isoflurane, all through inhalation. Pigs were anaesthetized using an intramuscular injection cocktail of 2 mg/kg azaperone, 0.025 mg/kg atropine, and 16 mg/kg ketamine. Anaesthesia during pig surgery was maintained by 0.5% to 1.5% inhalation isoflurane (RWD Life Science, China).

Details of the rat surgery are described in previous studies.[28,33,45,47–49] Briefly, for the rat model, we surgically implanted Ti-6Al-4V wires (0.88 mm dia; 20 mm length) into the femoral canal and knee joint;[35,48,49] HXLPE particles (Dow Chemical, USA) were implanted into the longissimus muscle.[46,50] Each rat was then injected with 25 µl of inoculum solution (1×10^7 CFU/ml) at the implant surgical sites (Ti-6Al-4V wires or HXLPE particles). All the rats survived after surgery. Overall, 480 rats were meticulously categorized into two distinct groups based on the type of implants used. Each animal model, featuring either Ti-6Al-4V wires or HXLPE particles, was further subdivided into four subgroups. These subgroups encompassed rats subjected to NS irrigation alone, NS irrigation combined with rifampin treatment, EDTA-NS irrigation alone, and EDTA-NS irrigation combined with rifampin therapy, with each subgroup containing 60 rats. Half of the Ti-6Al-4V wires or HXLPE particles from infected rats were used for microscopy and crystal staining, while the other half was used to evaluate the positive rate and bacterial burden.

For the pig model, two Ti-6Al-4V titanium alloy plates (Zhejiang Guangci Medical Apparatus and Instruments, China) were fixed onto the medial side of pig's tibia with four screws; these titanium plates are commonly used clinically. Two HXLPE bearings (Beijing Chunlizhengda Medical Instruments, China) were placed in the biceps femoris muscle; these bearings are a component of knee prostheses used in the clinic for humans. Each surgical site with the experimental implants was injected with 1 ml of inoculum solution (1×10^7 CFU/ml). All the pigs successfully survived the surgery. In total, 48 pigs were randomly divided into two groups based on the type of implants used. Within each animal model, which included Ti-6Al-4V plates or HXLPE bearings, there were further subdivisions into three subgroups. These subgroups consisted of pigs that underwent non-debridement, NS irrigation combined with rifampin therapy, and EDTA-NS combined with rifampin therapy, with each subgroup comprising eight pigs.

All model animals were subjected to daily rectal temperature checks for the early detection of signs of infection. Daily wound assessments were conducted to evaluate surgical site conditions and monitor healing progress, while regular weight measurements were taken to track any unexpected changes in body weight.

Debridement and irrigation procedures

Debridement and irrigation were performed according to standard clinical methods, as previously described.[28] The surgical site was reopened under anaesthesia via the previous incisions, and infected and necrotic tissues were then removed for analysis. For the rat model, 300 ml of 10 mM EDTA-NS or 0.9% NaCl (NS control) was used to irrigate the surgical site for ten minutes. For the pig model, 5 l of 10 mM EDTA-NS or 0.9% NaCl (NS control) was used to irrigate the surgical site for ten minutes.

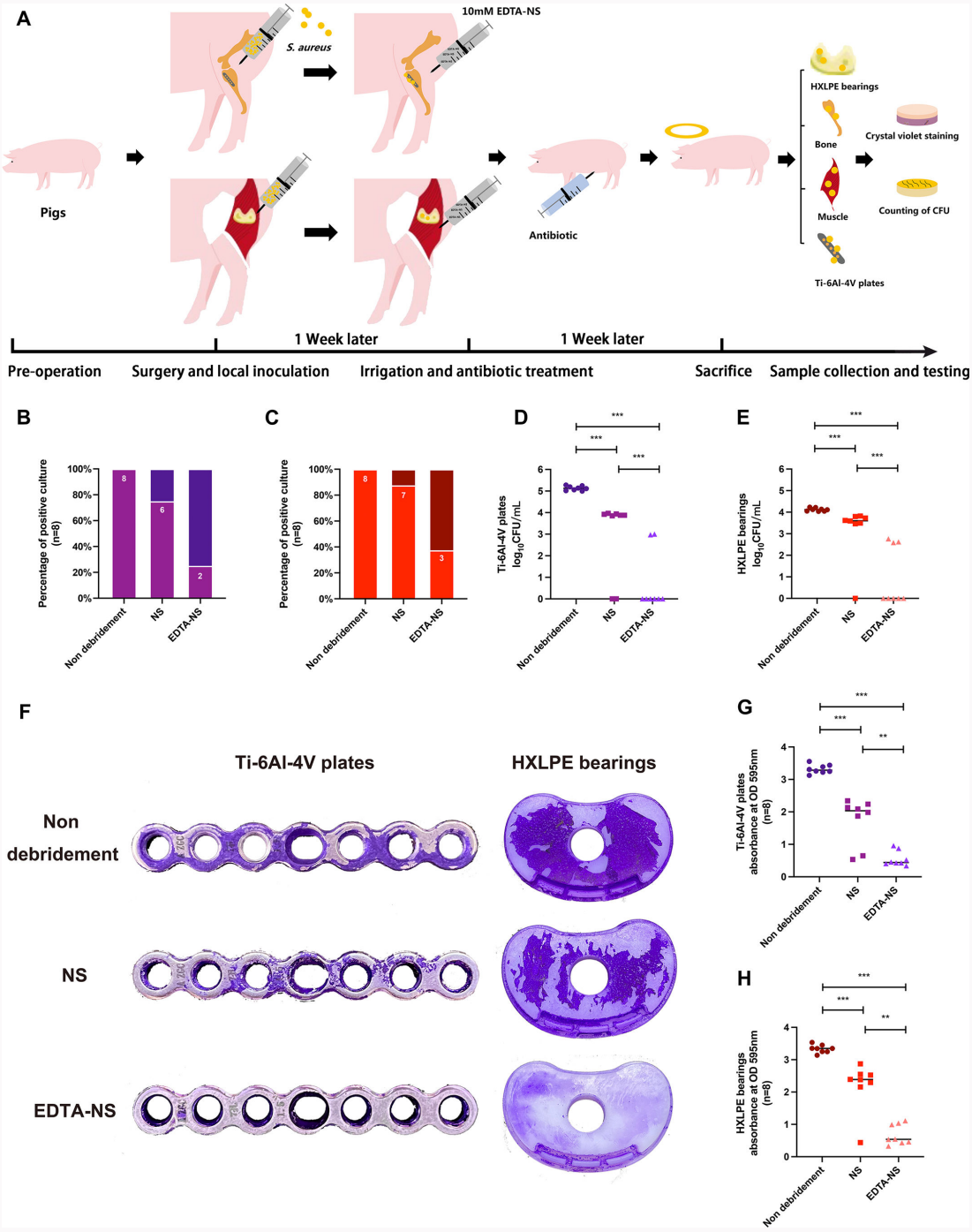

Fig. 5

Efficacy of *Staphylococcus aureus* biofilm removal by ethylenediaminetetraacetic acid-normal saline (EDTA-NS) irrigation in pigs. a) Schematic diagram of experimental setup. b) Mean percentage of positive cultures (filled purple-coloured bar) from whole joint samples derived from Ti-6Al-4V plate-implanted pigs. Same conventions apply as in Figure 3. c) Mean percentage of positive cultures (filled orange-coloured bar) from whole implant site cultured specimens derived from highly crosslinked polyethylene (HXLPE) bearing-implanted pigs. d) Bacterial burden (colony-forming unit (CFU)/ml) in cultures derived from Ti-6Al-4V plate-implanted pigs. e) Bacterial burden (CFU/ml) in cultures derived from HXLPE bearing-implanted pigs. f) Macroscopic bright-field images of crystal violet-stained Ti-6Al-4V plates and HXLPE bearings derived from implanted pigs. g) Quantification of *S. aureus* biofilm on Ti-6Al-4V plates by crystal violet staining in cultures by optical density (OD) (595 nm). h) Quantification of *S. aureus* biofilm on HXLPE bearings with same method. Horizontal bars indicate medians. ***p < 0.001; **p < 0.01; statistical evaluations by Fisher's exact test or Mann-Whitney U test.

All the debridement and irrigation procedures were performed by the same researcher (JS) to maintain consistency and reproducibility. Finally, all the wounds were irrigated with NS (100 ml for rat model; 1 l for pig model) to remove any residual EDTA-NS or as a control. After the initial implantation surgery, two groups of rats (NS irrigation combined with rifampin and EDTA-NS combined with rifampin) were administered with rifampin at 10 mg/kg (intramuscular) every day for seven days,[51–53] and for the control groups, 0.9% NaCl was given in an identical manner and route as the experimental solutions. All pigs were administered 12.5 mg/kg (intraperitoneally) every 12 hours for four days.[51–54]

Table II. Quantitation and statistical evaluation of *Staphylococcus aureus* positive cultures derived from samples irrigated with 10 mM ethylenediaminetetraacetic acid-normal saline or normal saline without debridement in pig model.

Cultured sample type	No debridement	NS	EDTA-NS
Ti-6Al-4V plate-implanted pigs			
Ti-6Al-4V plates	8 (100)	6 (75)	2 (25)*
Screws	8 (100)	5 (63)	1 (13)*
Surrounding tissue	8 (100)	6 (75)	1 (13)*†
Whole implant site‡	8 (100)	6 (75)	2 (25)*
HXLPE bearing-implanted pigs			
HXLPE bearings	8 (100)	7 (88)	3 (38)*
Surrounding tissue	8 (100)	6 (75)	2 (25)*
Whole implant site§	8 (100)	7 (88)	3 (38)*

Value are counts (percentages) of *S. aureus* positive cultures derived from different samples and eradication type.
Evaluation using Fisher's exact test.
*$p < 0.05$ versus no debridement.
†$p < 0.05$ versus NS.
‡Any positive cultures of Ti-6Al-4V plates, screws, and surrounding tissue.
§Any positive cultures of HXLPE bearings and surrounding tissue.
EDTA-NS, ethylenediaminetetraacetic acid-normal saline; HXLPE, highly crosslinked polyethylene ; NS, normal saline.

Bacterial cultures and biofilm bacterial identification

All the animals were euthanized one week after the irrigation procedure in order to harvest surgical samples for analysis. We obtained samples of the joint capsule and distal femur, and removed the Ti-6Al-4V wires and surrounding muscle tissues. We also harvested the implanted HXLPE particles from the rat tissues, the Ti-6Al-4V plates from the pig tissues, screws, HXLPE bearings, and the surrounding tissues were removed for the pig model.

Bone, joint capsule, and muscle were first homogenized using a tissue grinder (Merck, Germany). Ti-6Al-4V wires (n = 30 per group), HXLPE particles (n = 30 per group), Ti-6Al-4V plates (n = 8 per group), and HXLPE bearings (n = 8 per group) were sonicated in 50 ml of NS to release the adherent bacteria from the biofilm. Then, we collected the supernatant and added it to a dish containing TSA agar with 5% sheep blood serum (Becton-Dickinson), followed by incubation at 37°C for 24 hours to allow the bacteria to grow.[55] Culture plates were then photographed with a digital camera, and the bacterial colonies were quantified using ImageJ software (National Institutes of Health, USA).[56] After incubating the plate for 24 hours, we inspected the plates for any bacterial growth and documented them; the presence of one or more bacterial colony was considered a positive instance. When bacterial growth was noted, 16S ribosomal DNA sequencing with a MicroSeq 500 microbial identification system (Thermo Fisher Scientific) was used to confirm the bacterial species. Ti-6Al-4V wires (n = 30 per group), HXLPE particles (n = 30 per group), Ti-6Al-4V plates (n = 8 per group), and HXLPE bearings (n = 8 per group) were stained separately with 0.1% crystal violet for five minutes and resolved with 30% acetic acid for subsequent quantification by OD dye absorbance.

Sample size calculation and experimenter blinding

We set the type I error (α) equal to 0.05 and the power (1-β) equal to 80% in calculating the in vivo study sample size. The superiority margin was set at 0.1. According to a previous study, to detect a 45% difference between 10 mM EDTA-NS (5%) and NS (50%), we required 26 rats per group when using the sample size calculation method of Charan and Kantharia.[57] Given the possibility that a small number of rats could die because of infection and post-surgical complications, we set the sample size higher to 30 rats per group.

Statistical analysis

We used the Pearson's chi-squared statistic or Fisher's exact probability test to evaluate differences between EDTA-NS and NS irrigation groups (i.e. dichotomous variables). The Mann-Whitney U test was used to evaluate differences in continuous variables, because we suspected that the assumption of normality may not hold for our data.[58] Statistical analyses were performed and summary descriptive statistics were calculated using GraphPad Prism version 8 for Mac (GraphPad, USA). A p-value < 0.05 was considered to be a statistically significant difference.

Results

Irrigation with EDTA-NS disrupts *S. aureus* biofilms in vitro

Figure 1a shows the experimental setup we used to evaluate whether irrigation with EDTA-NS can effectively disperse *S. aureus* biofilm that develops on materials commonly used in orthopaedic implants. Varying concentrations and irrigation durations of EDTA-NS (1, 2, 5, 10, or 50 mM for 1, 5, 10, or 30 minutes) were effective in disrupting *S. aureus* biofilms on Matrigel-coated glass, HXLPE particles, and Ti-6Al-4V titanium alloy plates (Figures 1b, 1e, and 1h). We found that 10 mM EDTA-NS of simulated irrigation for at least ten minutes eradicated over 99% of *S. aureus* biofilm formed on all three types of materials. After quantifying the remaining biomass of *S. aureus* biofilms on different implants, measured by OD (absorbance at 595 nm), it was evident that compared to NS irrigation, the application of 10 mM EDTA-NS for ten minutes was superior: Matrigel-coated glass, EDTA-NS 0.121 (standard deviation (SD) 0.0161) versus NS 2.022 (SD 0.0167) (p 0.001, Mann-Whitney U test); HXLPE particles, EDTA-NS 0.0507 (SD 0.0012) versus NS 0.3667 (SD 0.0163) (p 0.001, Mann-Whitney U test); Ti-6Al-4V plates, EDTA-NS 0.424 (SD 0.0066) versus NS 2.173 (SD 0.0575) (p 0.001, Mann-Whitney U test) (Figure 1c,f,i). Simulated irrigation for longer durations and with higher EDTA-NS concentrations did not significantly increase the efficacy of biofilm removal over what was accomplished with 10 mM EDTA-NS for at least ten minutes. EDTA-NS irrigation also significantly reduced *S. aureus* colony counts (CFU/ml), reaching a plateau when 10 mM EDTA-NS was used for irrigation for ten minutes: Matrigel-coated glass, EDTA-NS 100 (SD 30.05) versus NS 1099 (SD 17.93) (p 0.001, Mann-Whitney U test); HXLPE particles, EDTA-NS 86.33 (SD 16.04) versus NS 391.7 (SD 10.07) (p 0.001, Mann-Whitney U test); Ti-6Al-4V plates, EDTA-NS 134 (SD 17.58) versus

NS 997 (SD 34.6) (p 0.001, Mann-Whitney U test) (Figure 1d,g,j).

To bolster the above conclusions about the most effective EDTA-NS concentrations and irrigation durations, we used SEM (Figure 2a) and CLSM (Figure 2b) to assess any morphological alterations of the biofilms. These images and analyses demonstrated that almost all the *S. aureus* biofilm was detached from all three materials after EDTA-NS (10 mM/10 min) irrigation. CLSM revealed a significant time-dependent reduction in biofilm biomass after being irrigated in vitro with 10 mM EDTA-NS, and the thickness (μm) of biofilm (NS vs EDTA-NS, mean thickness: 22.16 (SD 3.42) vs 4.51 (SD 2.92); maximum thickness: 23.03 (SD 3.73) vs 9.65 (SD 2.65)) were significantly reduced after optimal irrigation procedure of EDTA-NS irrigation (Figures 2b to 2d).

As shown in Figure 3, the viability results using the CCK8 assay verified that 10 mM EDTA-NS applied for ten minutes was not cytotoxic to four representative types of cells that comprise granulation tissue: HUVECs, fibroblasts, osteoblasts, and chondrocytes.

Efficacy of *S. aureus* biofilm removal by EDTA-NS irrigation in a rat model

A schematic illustration of the experimental setup for this rat model is shown in Figure 4a. To evaluate the efficacy of EDTA-NS irrigation in removing *S. aureus* biofilm in vivo, implants previously infected with *S. aureus* during surgery were irrigated with 10 mM EDTA-NS or NS for ten minutes. In Ti-6Al-4V wire-implanted rats, irrigation with a combination of 10 mM EDTA-NS and rifampin in the postoperative period produced a significantly lower positive rate of *S. aureus* colonies (3.33% (1/30)) compared with NS irrigation alone (73.33% (22/30), $p < 0.001$, Fisher's exact test), NS irrigation combined with rifampin (60.00% (18/30), $p < 0.001$), or EDTA-NS irrigation (30.00% (9/30), $p = 0.012$, Fisher's exact test). A similar result was observed in HXLPE particle-implanted rats (Figure 4e). Irrigation with 10 mM EDTA-NS combined with rifampin in the postoperative period produced a significantly lower positive rate (6.67% (2/30)) compared with NS alone (76.67% (23/30), $p < 0.001$, Fisher's exact test); NS irrigation combined with rifampin in the postoperative period (63.33% (19/30), $p < 0.001$); or EDTA irrigation (33.33% (10/30), $p = 0.021$, Fisher's exact test).

Similarly, irrigation with 10 mM EDTA-NS resulted in a lower mean colony count in the culture sample after injection with *S. aureus*. The biofilm bacterial burden and crystal violet staining further showed that rats treated with rifampin after irrigation and debridement had a biomass of *S. aureus* biofilm that was much smaller (Figures 4c and 4d for Ti-6Al-4V wires, Figures 4f and 4g for HXLPE particles). Table I shows positive rates of *S. aureus* cultures derived from Ti-6Al-4V wire-implanted and HXLPE particle-implanted rats after irrigation with 10 mM EDTA-NS and rifampin. EDTA-NS combined with rifampin was significantly more effective than NS alone, NS combined with rifampin, and EDTA-NS without rifampin.

Efficacy of biofilm removal by EDTA-NS irrigation in pig model

A schematic of the experimental setup for tests in the pig model is shown in Figure 5a. In Ti-6Al-4V plate-implanted pigs (Figure 5b), irrigation with 10 mM EDTA-NS produced a significantly lower positive *S. aureus* culture rate (25% (2/8)) compared with NS irrigation alone (75% (6/8), $p = 0.131$, Fisher's exact test) or no debridement (100% (8/8), $p = 0.007$, Fisher's exact test). In HXLPE bearing-implanted pigs (Figure 5c), irrigation with 10 mM EDTA-NS produced a significantly lower positive rate (37.5% (3/8)) compared with NS irrigation alone (87.5% (7/8), $p = 0.119$, Fisher's exact test), or no debridement (100% (8/8), $p = 0.026$, Fisher's exact test). Irrigation with 10 mM EDTA-NS results in the lowest *S. aureus* counts compared with NS irrigation and no debridement in both Ti-6Al-4V plate-implanted (Figure 5d) and HXLPE bearing-implanted pigs (Figure 5e).

Crystal violet-stained images of Ti-6Al-4V plates and HXLPE bearings derived from the pig model are shown in Figure 5f. EDTA-NS (10 mM) irrigation for at least ten minutes eradicated *S. aureus* biofilms formed on Ti-6Al-4V plates and HXLPE bearings better than non-debridement (absorbance measured at 595 nm) (Figures 5g and 5h). Positive rates in cultures derived from Ti-6Al-4V plate-implanted and HXLPE bearing-implanted pigs are shown in Table II.

Discussion

Over the past few decades, there has been an increasing awareness that *S. aureus* biofilms are a major cause of multiple bone and joint infections.[8] Although there have been several notable progressions in developing treatments for biofilm infections, no single effective therapy is currently available for patients suffering from *S. aureus* biofilm infection.[18] Therefore, antibiotic therapies remain the first choice of treatment. With the increasing number of *S. aureus* cells exposed to antibiotics, the selection pressure is associated with the development of antibiotic resistance.[14] The rise of antibiotic resistance has led to a decrease in the efficacy of traditional treatment. Antibiotic therapy with vancomycin is one of the most frequently used drugs against biofilm, however clinicians are cautious about the administration of these drugs due to the propensity of *S. aureus* to develop resistance.[15] Meanwhile, rifampin is considered to be the only biofilm-active antibiotic for implant-associated infections caused by staphylococci. It is regrettable that, despite rifampin's high efficacy in eliminating staphylococci biofilm, it only achieves a 60% to 70% inhibition of the biofilm.[59] Given the difficulty of biofilm achieving eradication with antibiotics alone, surgical debridement continues to serve as the cornerstone of infection control, with wound cleansing representing a vital component of this essential procedure.

Wound cleansing with an irrigation solution is an effective strategy to physically remove bacteria from contaminated and infected wounds. Currently, irrigation with NS is the first-line method for wound management to prevent infection due to its non-cytotoxicity, and because antiseptic irrigation solutions can be toxic. However, for situations where mature biofilm has developed, NS alone is ineffective in disrupting biofilm and eradicating bacterial infections.[60,61] Thus, for treating biofilm-associated infections, antiseptic irrigation solutions like benzalkonium chloride (BZK) and povidone iodine (PVP-I) are frequently recommended, even though they have low therapeutic effectiveness and can potentially induce wound healing problems.[62,63] As antimicrobial wound management remains a major global problem, it is critical to identify possible

irrigation solutions with a low cytotoxicity and high efficacy for biofilm disruption.[31,64] To the best of our knowledge, no previous study has evaluated the efficacy of EDTA-NS solution on disrupting biofilms. Here, we provide both in vitro and in vivo evidence that 10 mM EDTA-NS irrigation applied for ten minutes, combined with rifampin, effectively eradicates *S. aureus* biofilm-associated infections on materials commonly used in orthopaedics.

The efficacy of EDTA-NS solution in reducing adhesion of planktonic bacteria on host tissue and orthopaedic implants has been well established in previous studies.[28,33–35,65] What is new about our results is that EDTA-NS irrigation removed a large mass of biofilm from the surface of two common orthopaedic materials and Matrigel, which mimics host extracellular matrix. Furthermore, when combined with the antibiotic rifampin and debridement, irrigation with EDTA-NS virtually eradicated *S. aureus* infection in both rat and pig models in our study. These results underscore the potential for EDTA-NS irrigation as a promising approach in combating biofilm-associated challenges in orthopaedic and biomaterial applications, offering exciting prospects for improved patient outcomes and infection control strategies in clinical practice.

Although EDTA-NS was effective at disrupting biofilm, we showed that it was important to also use antibiotics after surgical debridement. Clearly, after disrupting the adherence of biofilm and facilitating its detachment, host tissues and implants were still susceptible to reattachment of *S. aureus*. However, EDTA itself had no bactericidal effect, even when its concentration was extremely high, which was consistent with a previous study.[66] Our in vivo data confirmed this notion that debridement and irrigation with EDTA-NS are the most effective when combined with a biofilm-targeted antibiotic in treating biofilm-associated infection.

Over the past few decades, it has become increasing clear that biofilm formation is a major cause of persistent *S. aureus* infections.[8] Many biofilm-disrupting strategies, including chemicals, phages, and enzymes, have been proposed and developed.[67] Adverse effects caused by such strategies, including but not limited to transmission of virulent genes, endotoxin release, and autoimmune responses, have largely limited translation of these strategies into clinical practice.[68,69] Our results, and those of several previous studies,[28,33,35] have demonstrated that EDTA is non-toxic to host cells within a certain concentration range, opening the door to clinical translation. Whether chemicals, phages, and enzymes result in a better outcome and few adverse effects when added to EDTA-NS irrigation solution is worth exploration in future studies.

Several potential adverse effects merit consideration in the context of EDTA-NS irrigation. Prolonged irrigation can deplete local metal ions, such as calcium, although a prior rat study showed limited impact on blood calcium levels.[35] Caution is advised during clinical use, especially with extensive limb wounds. Previous studies used a 1 mM EDTA-NS concentration, while this study employed a 10 mM EDTA-NS irrigation solution.[28,33–35] Although in vitro testing revealed no cytotoxicity, the in vivo interaction of the EDTA-NS irrigation solution with tissue may differ. While no adverse reactions were reported in this study, the findings should be interpreted with caution.

The current work had several limitations. First, only one strain of *S. aureus* was tested; different strains and different bacterial species could lead to biofilms with different characteristics. Therefore, the optimal concentrations and durations of EDTA-NS irrigation in this study might be slightly different for those situations. The optimal concentration and duration for other strains and bacterial species need to be explored in future studies. Another limitation of our study is the lack of measurements regarding the adhesion force between *S. aureus* biofilm and implants after EDTA-NS irrigation in our in vitro experiments. The question of whether the adhesion strength of free bacteria to the internal plant surfaces decreases following irrigation with EDTA-NS remains unanswered. To ascertain the level of adhesion strength within the implant's internal environment and bacteria after irrigation, further research will be necessary. Furthermore, due to limited research funding, the number of pig subjects we used might be insufficient, resulting in large absolute but statistically insignificant differences in infection rate. Finally, the mechanism of EDTA irrigation efficacy in biofilm disruption was left unexplored and should be investigated in the future. Deprivation of metal ions from the biofilm microenvironment may underpin our results, but at this point this is a speculative rather than a substantially confirmed mechanism.

In conclusion, irrigation with EDTA-NS in combination with rifampin is effective in eradicating *S. aureus* biofilm-associated infections both in vitro and in vivo. Therefore, EDTA-NS is considered to be a potential future clinical therapy, with high biosafety and efficacy in treating biofilm-associated infections. More comprehensive preclinical studies are warranted to confirm the efficacy of EDTA-NS in biofilm eradication, along with a more detailed examination of potential mechanisms for EDTA-NS in biofilm eradication.

Supplementary material

ARRIVE checklist.

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Data sharing

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Ethical review statement

All procedures performed in this study were approved by the local ethics committee (Shanghai Sxith People's hospital, No. 2021-0430), and complied with the ethical standards of the Declaration of Helsinki.

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