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ORIGINAL ARTICLE

Freezing nitrogen ethanol composite reduces periprosthetic infection caused by *Staphylococcus aureus* contaminated metal implants: An animal study

Kuan-Lin Chen[a,b,c], Chao-Ming Chen[a,b,c,d], Yi-Chung Chen[a,b], Jir- You Wang[a,b,e], Cheng-Fong Chen[a,b,c], Po-Kuei Wu[a,b,c,d,*], Wei-Ming Chen[a,b,c]

[a]Therapeutical and Research Center of Musculoskeletal Tumor, Department of Orthopaedics, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [b]Department of Orthopaedics & Traumatology, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; [c]Department of Orthopedics, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC; [d]Institute of Clinical Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC; [e]Institute of Traditional Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC

Abstract

Background: Implant-associated infection remains a major complication of orthopedic surgery. The treatment of such infection is complicated by bacterial biofilm formation on the metal surfaces of implants. Biofilm surrounds and protects the bacteria against the organism's endogenous defense system and from external agents such as antibiotics and mechanical debridement. This study aims to evaluate whether freezing nitrogen ethanol composite (FNEC), the combination of liquid nitrogen and 95% ethanol in a 3 to 1 ratio, used frequently in bone tumor surgery, is capable of disinfecting *Staphylococcus aureus* contaminated implants.

Methods: The femurs of six New Zealand white rabbits were implanted with *S. aureus*-contaminated screws, half of which were treated with FNEC before implantation. The femurs were harvested 14 days after implantation. Histological analysis and TUNEL assay were conducted. The autoclaved screw, contaminated screw, and FNEC-treated contaminated screw were investigated using scanning electron microscopy to evaluate the biofilm structure.

Results: The FNEC-treated group had significantly lower relative C-reactive protein levels. An obvious periosteal reaction at the implant site was observed in all rabbits in the non-FNEC group but none was observed in the FNEC-treated group. The FNEC-treated group exhibited fewer empty lacunae, less inflammatory infiltration, and less bone necrosis. Immunohistochemical analysis showed no *S. aureus* in bone tissue from the FNEC-treated group. Scanning electron microscopy showed disruption of the biofilm on the contaminated screw treated with FNEC.

Conclusion: FNEC showed potential in disinfecting S.aureus-contaminated implants. Further investigation is warranted, such as the effect on the implant-cement-bone interface, for FNEC to be used clinically in treating implant-associated infection.

Keywords: Cryotherapy; Freezing nitrogen ethanol composite; Periprosthetic joint infection

1. INTRODUCTION

Metal implants such as bone plates and intramedullary nails have revolutionized the treatment of fractures, chronic arthritis, intervertebral disc degeneration, and many other conditions. However, the introduction of an implant into the body carries a risk of microbial infection.[1,2] Implant-associated infection remains a major complication of orthopedic surgery, with an incidence of 0.7% to 4.2% in elective orthopedic surgeries and 1% to 30% in trauma surgeries.[3–8] In limb salvage surgeries using tumor endoprosthesis, the incidence is as high as 9% to 36%.[9–14] The treatment of such infection is complicated by bacterial biofilm formation on the metal surfaces of implants. Biofilms are polymeric matrices produced and secreted by bacteria attached to a surface.[15] This structure surrounds and protects the bacteria against the organism's endogenous defense system and from external agents such as antibiotics and mechanical debridement. A variety of methods have been investigated for the eradication of biofilms. However, no golden method has been developed yet for removing biofilm intraoperatively.

Freezing nitrogen ethanol composite (FNEC), which is the combination of liquid nitrogen and 95% ethanol in a 3 to 1 ratio, has been used intraoperatively for eradicating giant cell tumors.[16] Ethanol is widely used as a disinfectant in clinical settings.[17] Liquid nitrogen, the other component of FNEC, is widely used in the preservation of cultured cells. The properties of the components of FNEC give rise to our interest in discovering the capability of FNEC in implant-related infection. This study aims to determine whether FNEC can disinfect contaminated screws with established biofilms and reduce contaminated-implant-related infection.

* *Address correspondence. Dr. Po-Kuei Wu, Division of Joint Reconstruction, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC. E-mail address: drwuvgh@gmail.com (P.-K. Wu).*

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2.METHODS

2.1. Bacterial strain and implant preparation

Six standard stainless steel screws (3 mm Ø × 12.7 mm, Synthes) were incubated in *Staphylococcus aureus* (UAMS-1, ATCC 49230) containing broth, which the absorbance optical density was 0.5, at 37°C for 2 days with shaking at 200 rpm to ensure bacterial adherence and biofilm formation.

2.2. FNEC bath preparation

A total of 600 mL of Liquid nitrogen and 200 mL of 95% ethanol was mixed in a heat isolative container. The ratio of liquid nitrogen and 95% ethanol was 3 to 1.

2.3. Animals and surgical procedure

The subjects were six healthy New Zealand White rabbits (weight, 2-2.4 kg; age, 4-5 months). They were housed in the same cage and given a week to acclimate before the trial. The rabbits were divided equally into two groups: non-FNEC group and FNEC-treated group. Each rabbit of the non-FNEC group was implanted with one *S. aureus*-contaminated screw at the metaphysis of the left distal femur. In the FNEC-treated group, the screws were implanted after being soaked in FNEC in the heat-isolative container for 15 minutes (Fig. 1).

Before surgery, all rabbits received appropriate anesthesia. The left hind limbs were shaved and disinfected with povidone-iodine. The femurs were exposed through a para-femoral incision. All rabbits were sacrificed on post-op day 14. Body weight, gross wound images, radiographs, and serum CRP levels were measured before sacrifice. Histological and immunohistochemical analyses were carried out on the harvested bone tissues. The experiments and protocols were approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital.

2.4. Radiography

Radiographs of the femurs were taken on post-op day 0, 7, and 14 (SXT-1000A; Toshiba, Tokyo, Japan). Characteristics such as bone defects, periosteal reactions, and soft tissue swelling were recorded.

2.5. Serum CRP ELISA

At day 0, pre-op and immediate post-op blood samples were drawn from the ear vein 2 hours before and 2 hours after the surgery. The other samples were collected on post-op day 3, 7, and 14. An anticoagulant was given preoperatively and postoperatively. The samples were centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and stored at −80°C until serum CRP assays were conducted. Serum CRP ELISA (LS-F4718, LSBio, Seattle, USA) was performed using the manufacturer's protocol. The relative CRP value was calculated by serum CRP divided by the preoperative CRP reading of each subject.

2.6. Histological analysis

Bone tissues of the non-FNEC group and FNEC-treated group were harvested 3 mm away from the screw hole and fixed in 10% formalin for 1 week, and decalcified with 5% formic acid for 7 days, dehydrated, and embedded in paraffin blocks. Tissues were cut into 5-μm thick sections. The sections were deparaffinized and rehydrated, then stained in Mayer's hematoxylin (MHS16; Merck) and Eosin Y (230251; Merck). Empty lacunae, leukocyte infiltration, and bone necrosis were investigated under high-power field microscopy.

2.7. Immunohistochemistry and TUNEL assay

Bone tissues were fixed in 10% formalin for 7 days, decalcified with 5% formic acid for 7 days, dehydrated, and embedded in paraffin. The paraffin-embedded sections (5 μm) were deparaffinized, rehydrated, and treated with 0.1% trypsin for 15 minutes at 37°C for antigen retrieval. Blocking reagents included 3% H_2O_2 and 1% BSA. Sections were incubated in anti-*S. aureus* polyclonal antibody (1:500 in 1% BSA) (ab20920; Abcam, Cambridge, United Kingdom) overnight at 4°C. Anti-rabbit HRP (111-035-144; Jackson ImmunoResearch Laboratories, Baltimore, USA) or Dylight 550 (ab96884; Abcam, Cambridge, United Kingdom) secondary antibodies were used to visualize *S. aureus* in bone sections.

DNA strand breaks were labeled by terminal deoxynucleotidyl transferase in bone tissues according to the manufacturer's protocol (11684817910; Merck). Double staining of apoptotic cells was performed. Cell nuclei were stained with DAPI.

2.8. SEM analysis

Three stainless steel screws (3 mm Ø × 12.7 mm, Synthes) received SEM analysis. Before analysis, one screw received standard autoclave disinfection. Two other screws were incubated in *S. aureus* (UAMS-1, ATCC 49230) containing broth at 37°C for 2 days as the protocol for the implanted screws. One of the two contaminated screws was bathed in FNEC for 15 minutes before analysis.

2.9. Statistics

All statistical analyses for histological data were done with GraphPad Prism software (GraphPad Software, San Diego, CA). For parametric data, we used a one-way ANOVA test with post hoc Bonferroni's test for analysis. For non-parametric data, we

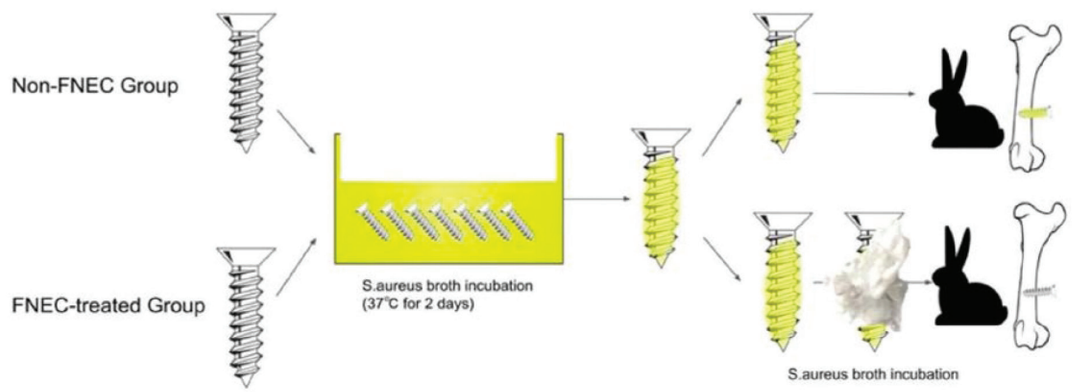

Fig. 1 Subjects in the non-FNEC group were implanted with *Staphylococcus aureus*-contaminated screws while the FNEC group was implanted with *S. aureus*-contaminated screws that were treated with FNEC for 15 minutes. FNEC = freezing nitrogen ethanol composite.

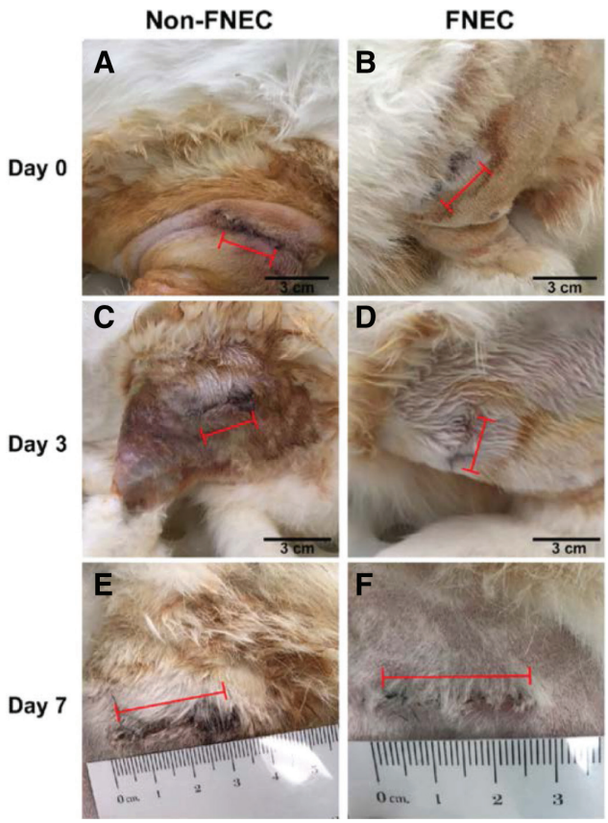

Fig. 2 Gross wound inspection on day 0, day 3, and day 7. Photo of surgical wound (red brackets) for implanting the *S. aureus* contaminated screw without (A-C) or with FNEC treatment (D-F) on day 0 (after surgery) and postoperative days 3 and 7. The non-FNEC group showed more local swelling compared to the FNEC-treated group. FNEC = freezing nitrogen ethanol composite.

used Kruskal-Wallis method and Dunn's post-comparison test for analysis. A *p*-value of the one-way ANOVA test or Kruskal-Wallis test was considered statistically significant when <0.05.

3. RESULTS

3.1. Less local and systemic inflammation in the FNEC-treated group

In the non-FNEC group, all three rabbits showed signs of wound swelling on post-op day 3. The rabbits implanted with FNEC-treated screws demonstrated less swelling in cutaneous tissue and better wound healing (Fig. 2). All three rabbits in the non-FNEC group showed a periosteal reaction at the site of implantation. No significant periosteal thickening was observed in the FNEC-treated group (Fig. 3A and B).

The reduction in body weight on post-op day 7 was more significant in the non-FNEC group than in the FNEC group. At post-op day 7, the average body weight of rabbits in the FNEC-treated group is 96.35 ± 0.37% of the weight reported pre-operatively, while the non-FNEC group reported to be 71.19 ± 1.42% of preoperative body weight (p = 0.026). Blood analysis showed an increase in CRP concentrations in both groups immediately after surgery. The CRP levels reached peak on post-op day 3 without a significant difference between the two groups. On post-op day 7, CRP levels began to drop. On post-op day 14, the relative CRP level was significantly lower in the FNEC group than in the non-FNEC group (1.16 ± 0.0005 vs 1.77 ± 0.22; p = 0.04) (Fig. 3C).

3.2. Histological examination and identification of *S. aureus*

Histologic analysis revealed fewer empty lacunae in the FNEC-treated group than the non-FNEC group (0.138 ± 0.033 vs 1 ± 0.076, respectively; p = 0.000014) (Fig. 4A-C). The area of leukocyte infiltration in the non-FNEC group was 1.75 ± 0.5%, while no leukocyte infiltration was observed in the FNEC-treated group (p = 0.01) (Fig. 4D-F). The area of necrosis was significantly smaller in the FNEC-treated group than in the non-FNEC group (0% vs 12.97% ± 3.17%; p = 0.0027) (Fig. 4G-I). Immunohistochemistry analysis revealed positive stain for *S. aureus* in the non-FNEC group, while no stain was observed in any of the three specimens from the FNEC-treated group (Fig. 5).

3.3. TUNEL assay shows less osteocyte apoptosis in the FNEC-treated group

TUNEL analysis revealed osteocyte apoptosis in the lacunae of specimens from the non-FNEC group but not from the FNEC-treated group (Fig. 6).

3.4. SEM reveals biofilm disruption in FNEC-treated screws

SEM images showed no significant biofilm layer formation on screws treated with standard autoclave disinfection. Screws incubated with *S. aureus* had an apparent biofilm layer adhering to the metal surface. Such a layer was also observed on the contaminated screws treated with FNEC baths. However, we observed a disrupted continuity of the biofilm layer on the screw treated with the FNEC bath (Fig. 7).

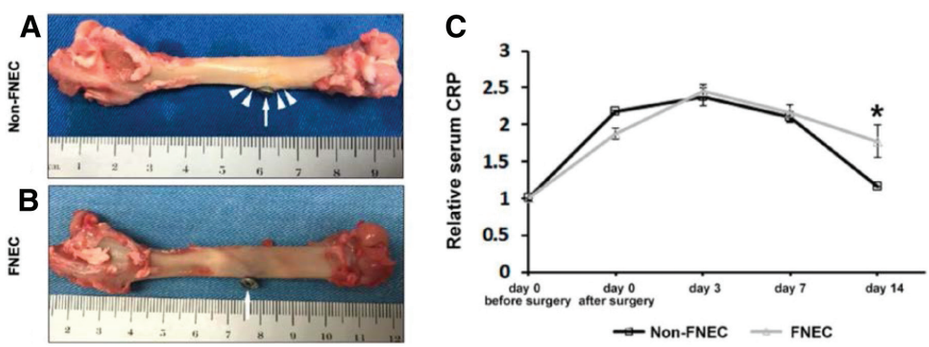

Fig. 3 Gross bone appearance and relative CRP level. A, Femur harvested from the non-FNEC group showed obvious periosteal thickening. B, Femur harvested from the FNEC group showed no obvious periosteal thickening. C, FNEC group had a lower relative serum CRP level at day 14. *p < 0.05. CRP = XXX; FNEC = freezing nitrogen ethanol composite.

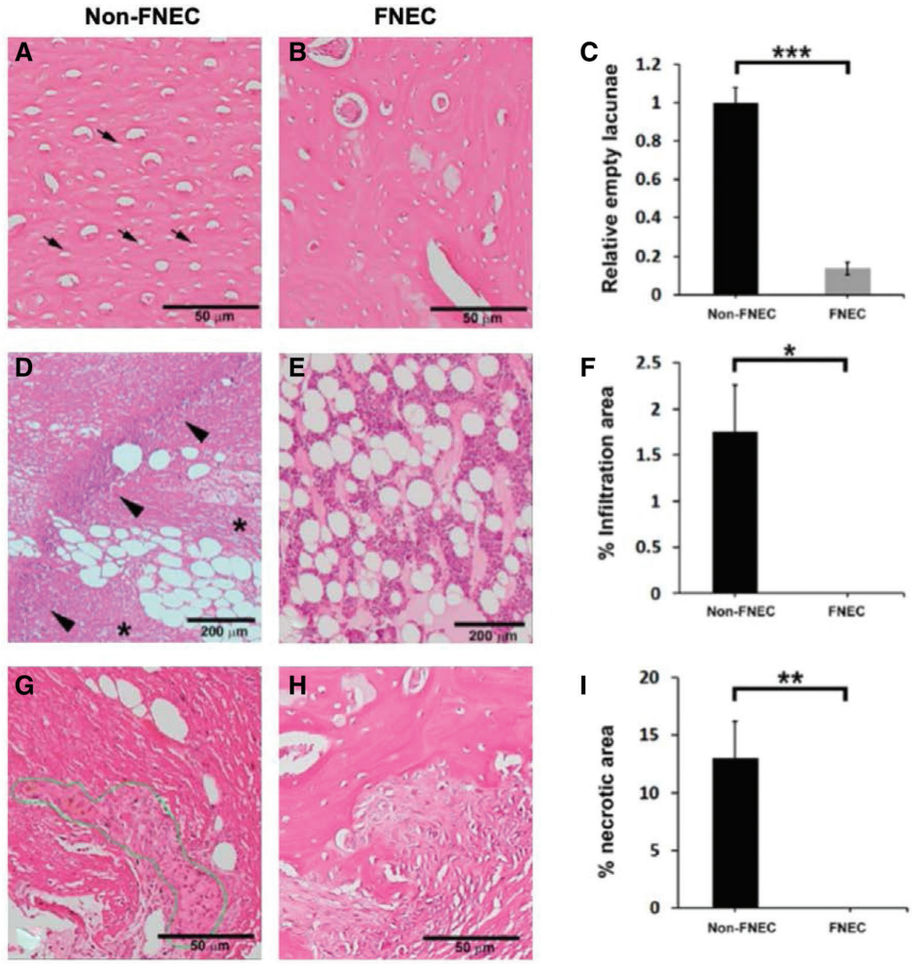

Fig. 4 H&E staining and quantitative analysis. A, Empty lacunae (black arrows) can be seen on the specimen from the non-FNEC group. B, Less empty lacunae were identified in the FNEC-treated group. C, Significant less empty lacunae were identified in the FNEC-treated group. D, Inflammatory infiltration (black arrows) and fibrotic marrow (stars) can be seen in the non-FNEC group. E, No obvious fibrotic marrow and inflammatory infiltration were seen in the FNEC-treated group. F, Less fibrotic marrow and inflammatory infiltration were identified in the FNEC-treated group. G, Bone necrosis (green dotted line) can be seen in the non-FNEC group. H, No obvious bone necrosis was seen in the FNEC-treated group. I, Significantly less area of necrosis was noted in the FNEC-treated group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. FNEC = freezing nitrogen ethanol composite.

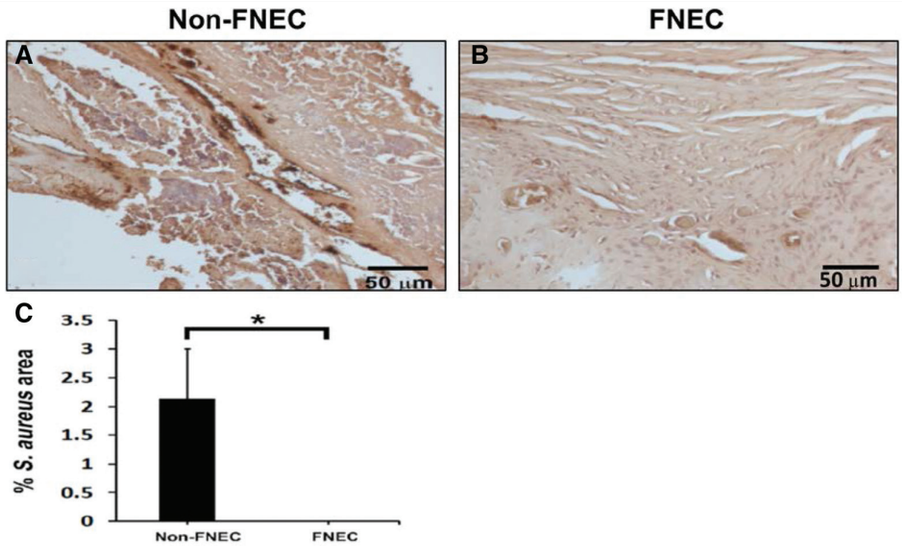

Fig. 5 Immunohistochemistry analysis of the femurs. A, Non-FNEC group revealed a positive *Staphylococcus aureus* stain. B, The FNEC-treated group showed no obvious positive *S. aureus* stain. C, The area of *S. aureus* stain was larger in the non-FNEC group. *$p < 0.05$. FNEC = freezing nitrogen ethanol composite.

4.DISCUSSION

Metal implants are essential for treating a variety of orthopedic conditions but carry a risk of microbial infection. The treatment of such infection is complicated by bacterial biofilm formation, which is a layer protecting bacteria from host defense, disinfectants, and antibiotics.[2] However, no golden method has been developed yet for eliminating biofilm intraoperatively. FNEC, which is the combination of liquid nitrogen and 95% ethanol in 3 to 1 ratio, has been used for eradicating giant cell tumors intraoperatively. Our results demonstrated that by soaking contaminated implants in FNEC for 15 minutes, the capability of *Staphylococcus*-contaminated screws to cause infection was reduced. The finding was supported by evidence from three different aspects: clinical reaction of the rabbits, histological analysis and SEM findings. The FNEC-treated group reported less local erythematous change, no periosteal thickening and a significantly lowered CRP ratio at day 14. In histological analysis, FNEC-treated group showed less apoptosis, bone necrosis and absence of *S. aureus* stain. To take a closer look on how FNEC affected the *S. aureus*-contaminated screws, an SEM analysis was done. Biofilm was identified on the screw received *S. aureus* incubation and disruption of the biofilm integrity was observed on the screw treated with FNEC bath.

The finding that FNEC can disinfect contaminated metal implants with established biofilm provides great value. Because biofilms are firmly adherent to metal surfaces and resistant to scrubbing irrigation, clearing implants of biofilms usually requires implant removal.[18,19] Removing well fixed implants can cause great loss of bone stock, which make future reconstruction more difficult and increase the chance of implant failure.

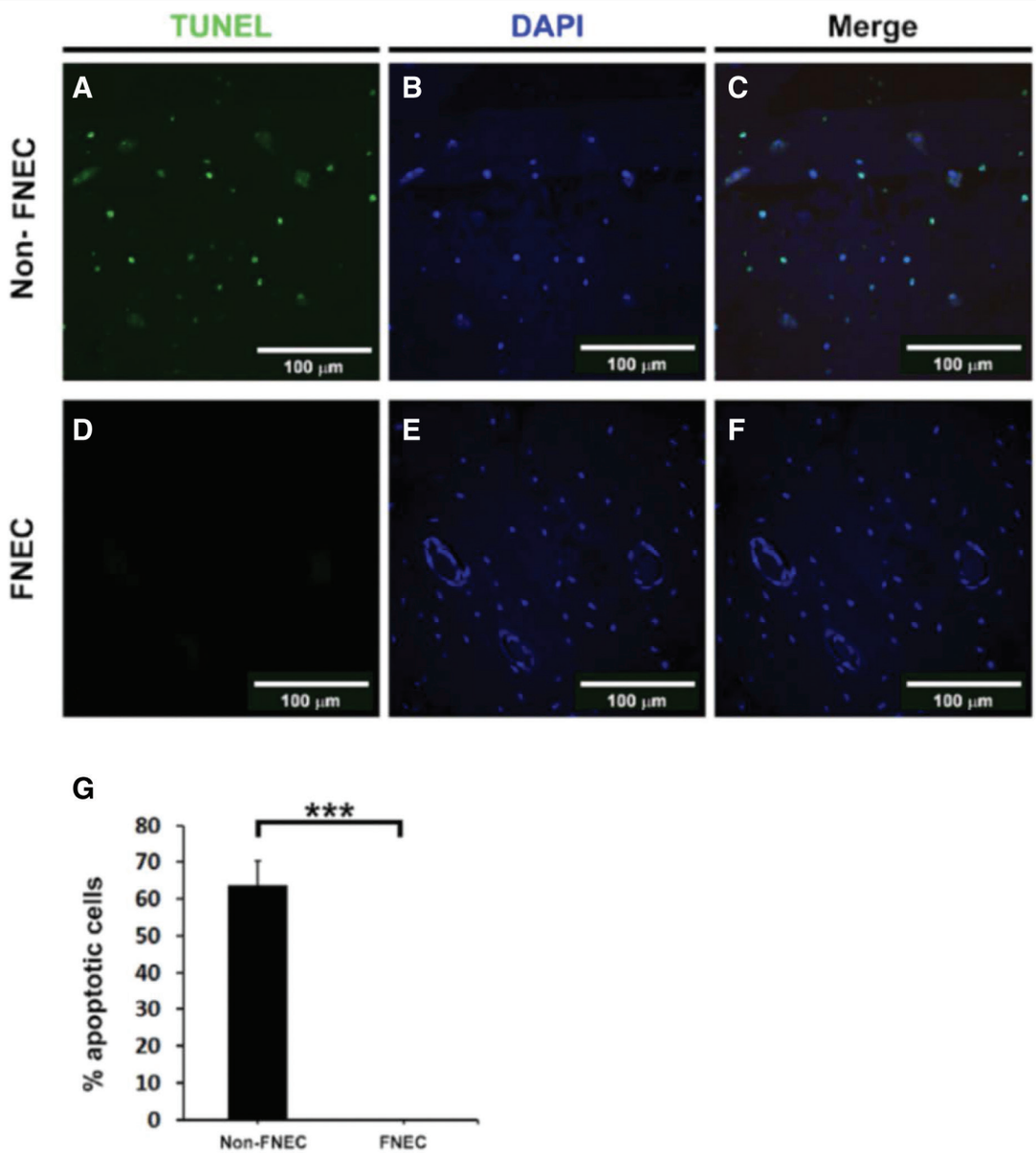

Fig. 6 TUNEL assay. A-C, Apoptotic osteocytes were identified in the non-FNEC group. (Green area in the photo. The blue area was the DAPI stained nuclei.) D–F, No obvious signal for apoptotic osteocytes noted in the FNEC-treated group. G, Quantitative analysis showed significantly decreased apoptosis in the FNEC-treated group. ***$p < 0.001$. FNEC = freezing nitrogen ethanol composite; DAPI = XXX.

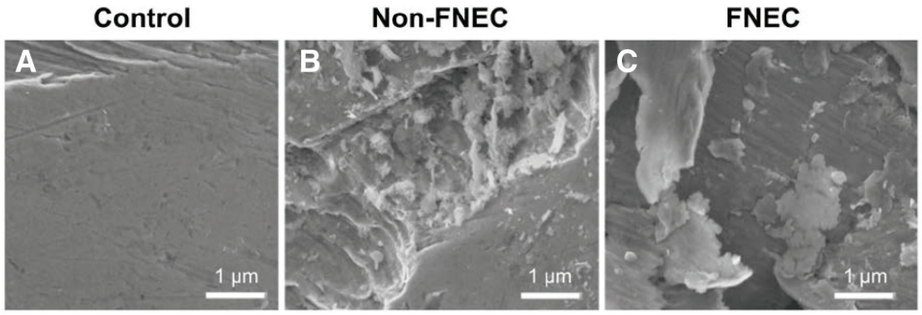

Fig. 7 SEM analysis. A, Screws disinfected by standard autoclave protocol showed no significant biofilm formation. B, The presence of biofilm on a *Staphylococcus aureus* contaminated screw. C, *S. aureus* contaminated screws treated with FNEC exhibit disruption of biofilm. FNEC = freezing nitrogen ethanol composite.

Those consequences give rise to increased morbidity. Efforts have been made in identifying ideal patients that may retain implants after being diagnosed of periprosthetic joint infection. For patients developed total knee periprosthetic joint infection within 4 weeks of onset, debridement-antibiotics-implant-retention (DAIR) can be considered as the initial management. However, for those acute patients, the success rate of DAIR is reported to be only 80%. For patients having chronic infection, the success rate is reduced to 28% to 62%.[20] A great portion of failure in DAIR arise from the inadequate removal of biofilm. Previous study reported that pulse irrigation on a cobalt chrome metal plate could not reduce biofilm signal intensity more than 10-fold, leaving a high chance of recurrence. An antiseptic agent that is applicable intraoperatively over the metal implants for eliminating biofilm may be the key to increase treatment success rate. Many methods have been investigated for the eradication of biofilm. Hoekstra et al reported that a povidone-iodine-ointment wound dressing removed all biofilms after 4 and 24 hours of application.[21] Tsang et al reported that a 20-minute treatment with 5% acetic acid eliminated 96.1% of biofilm-associated Methicillin-sensitive *S. aureus*.[22] Chlorhexidine scrub resulted in a significant reduction in bacterial colony counts when applied on *S. aureus* biofilm-coated titanium alloy disks.[19] Polonio et al reported that a combination of high concentration vancomycin and 5 mM sodium salicylate effectively kills the bacteria in biofilm.[23] However, no method has been recognized as gold standard because those methods either had operating duration that was too long or increased risk of systemic or local adverse effects. The finding that FNEC was effective in disinfecting *S. aureus*-contaminated metal implants and destruct biofilm at a reasonable operating time may play an important role in improving the success rate in treating periprosthetic joint infection while preserving metal implants.

This study is limited by its small sample size, short observation interval, and use of a single pathogen and a single implant material. The effect of FNEC on the bone-cement-implant junction is yet to be studied. Future research opportunities include finding a simple method to apply FNEC on contaminated implants while preserving its effectiveness in eliminating biofilm.

In conclusion, FNEC is capable of disinfecting metal implants with established *S. aureus* biofilm. Further investigation is required to translate the finding into clinical practice.

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