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Healing of *Pseudomonas aeruginosa*-infected wounds in diabetic db/db mice by weakly acidic hypochlorous acid cleansing and silver nanoparticle/chitin-nanofiber sheet covering

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ABSTRACT

Weakly acidic hypochlorous acid (HClO; 200 ppm, pH 6.5) is effective against a broad range of microorganisms. We have previously reported a study of developing antimicrobial biomaterials made up of chitin-nanofiber sheet (CNFS) -immobilized silver nanoparticles (CNFS/Ag NPs) and showed that either cleansing with HClO or covering with CNFS/Ag NPs daily for more than 7 days resulted in delayed wound healing. This study aimed to evaluate disinfection and wound healing by a combination of cleansing with HClO and covering with CNFS/Ag NPs daily for 3 days. Applying HClO + CNFS/Ag NPs daily for 3 days and then cleansing with just pure water and covering with CNFS alone daily for 9 days were performed for *Pseudomonas aeruginosa*-infected wounds in *db/db* diabetic mice. We found a significant enhancement of wound healing and a reduction of bacteria counts compared to the controls. Histological examination showed significantly advanced granulation tissue and capillary formations in the wounds on Day 12. These results suggest that limited disinfection to 3 days with HClO + CNFS/Ag NPs may be sufficient to avoid negative effects on wound repair.

1. Introduction

Wound healing is the end result of a series of interrelated cellular processes initiated by humoral factors such as cytokines and growth factors [1]. These cellular processes are inhibited by high bacterial burden of the wounds [2], which can contribute to degradation of the cytokines and growth factors [3]. Multiple studies have shown that high bacterial burden of the wound can exceed 1×10^6 bacteria per gram of tissue [4]. Such high levels of tissue bacteria can be present without clinical signs of infection and can deleteriously affect wound healing [5]. Notably, *Pseudomonas (P.) aeruginosa* is a major nosocomial microbe and opportunistic pathogen that can infect wounds and is known to play a role in impaired wound healing [6]. Systemically administered antibiotics do not effectively decrease the level of bacteria in a chronic granulating wound because of an increased risk of developing allergies and the potential for selection of bacteria resistant to the drug [6,7] Antiseptics and nonantibiotic antimicrobials such as povidone-iodine, silver sulfadiazine, silver nanoparticles, mafenide acetate cream, and weakly acidic hypochlorous acid (HClO) solution have been demonstrated to be cytotoxic to the cellular components of wound healing [8–10]. Therefore, a topical antimicrobial that can decrease high bacterial burden of the without inhibiting the wound healing process is a therapeutic imperative [6].

The advantages in biochemical activities of chitin/chitosan-based materials include: anti-infectious activity [11,12]; stimulation of angiogenesis/wound repair; and stabilization/activation of growth factors [13–15]. Since chitin nanofiber sheets (CNFSs) are biodegradable and have large surface-to-mass ratios, CNFSs are widely applied in pharmaceuticals as composite materials. On the other hand, Ag nanoparticles (Ag NPs) have strong antimicrobial activity against most microorganisms, including bacteria, fungi, and viruses. We recently demonstrated that CNFS/Ag NP composites have enhanced antimicrobial activities against microbial pathogens, including bacteria (*Escherichia coli*), fungi (*Aspergillus niger*), and viruses (H1N1 influenza

Abbreviations: CFU, Colony forming units; CNFS, Chitin-nanofiber sheet chitin-nanofiber sheet; CNFS/Ag NPs, Chitin-nanofiber sheet-immobilized silver nanoparticles; FGF, Fibroblast growth factor; HClO, Hypochlorous acid; AgNPs, Silver nanoparticles

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A virus) [16,17].

Several techniques using water or saline at a pH between 5.5 and 6.5 and at temperatures between 35 and 45 °C have been developed for the cleansing of chronic wounds with infection [18–20]. At the same time, weakly acidic hypochlorous acid (HClO) solution at a pH between 5.5 and 6.5 has been demonstrated to have excellent *in vitro* bactericidal properties [21,22]. However, the cleansing efficacy of HClO solution, especially in the context of the *in vivo* high bacterial burden of the chronic wounds, has not (to the best of our knowledge) been reported in the literature.

Our previous studies on repair in healing-impaired wounds were performed using healing-impaired diabetic (*db/db*) mice [23,24], mitomycin C-treated wounds [25], and radiation-induced wounds [26]. We observed significant induction of the formation of granulated tissue and capillaries and accelerated wound healing in healing-impaired diabetic mice with the application of several materials for wound dressing [23,24,27]. However, those studies were performed without any consideration of high bacterial burden of the wound.

In the present study, HClO solutions and CNFS/Ag NP composites were prepared, and the combined cleansing effects of HClO and covering with CNFS/Ag NP composites daily for 3 days were investigated using *P. aeruginosa*-infected wounds on the backs of healing-impaired *db/db* mice. The aim of this study was to propose both cleansing with HClO solution (pH 6.5) and covering with CNFS/Ag NP composites (HClO + CNFS/Ag NPs), which may allow for limited use in a clinical situation, such as prevention and treatment of infection in chronic wounds in healing-impaired patients without delaying wound healing.

2. Materials and methods

2.1. Preparation of test HClO solution

HClO solution (200 ppm, pH 6.5) was prepared by addition of 1/25 (vol/vol) of 0.5 % NaClO (Yoshida Pharmaceutical Corp., Tokyo, Japan) to sterilized pure water. The pH of 200 ppm HClO was then adjusted to pH 6.5 with 1 N HCl or NaOH. The concentration of HClO was measured as residual chlorides (free available chloride) using ClO (HClO and ClO^-)-selective test papers (high concentration, 25 - 500 ppm; low concentration, 1 - 25 ppm; Kyoritu Check Lab. Corp., Tokyo, Japan) [21].

2.2. Preparation of CNFS/Ag NPs

A suspension of size-controlled Ag NPs was prepared as previously described [17]. Briefly, 0.5 g of Ag-containing glass powder was dispersed in 50 mL of an aqueous solution of 0.8 wt% glucose in a 100 m L-glass vial. The mixture was autoclaved at 121 °C and 200 kPa for 20 min and then gradually cooled to room temperature; the mixture then was centrifuged at 1500*g* for 10 min. The resulting brown supernatant containing the Ag NP suspension (about 70 μg/mL) was stored in the dark at 4 °C.

CNFS (1 cm × 1 cm) was submerged in a 1.5-mL ClickFit polypropylene microcentrifuge tube (TreffLab AG, Degersheim, Switzerland) containing 1 mL of Ag NP suspension (at about 35 μg/mL) and shaken well for 30 min using a shaker (Mild Mixer PR-36; TAITEC, Tokyo, Japan). It was estimated that 8.5 μg of AG NPs were immobilized on 1 cm^2 of CNFS [17].

2.3. Cleansing P. aeruginosa-infected wounds with HClO solution in vivo

All animal experiments were approved by the National Defense Medical College, Tokorozawa, Saitama, Japan #17,007, and were carried out following the relevant guidelines for animal experimentation. Diabetic *db/db* mice (C57BLKS/J lar-+Lepr db/+Lepr db; 7- to 10-week-old males) were obtained from Japan SLC, Inc. Hamamatsu, Shizuoka, Japan. Animals were maintained under appropriate conditions (26 °C, 55 % humidity). On the nominal Day 0 of the study, mice were placed under general anesthesia by intraperitoneal injection with pentobarbital sodium (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The dorsal area was totally depilated, and full-thickness-round wounds were created on the back of each mouse using a sterile 8-mm dermal punch (Kai Industries Co., Ltd., Oyana, Japan) and a pair of sterilized sharp scissors [23,24]. To generate infected wounds were generated, *P. aeruginosa* ATCC 27853 American Type Culture Collection ATCC, Manassas, VA, USA, which was stored at −80 °C in Luria-Bertani (LB) broth containing 50 % sterile glycerol, with freshly growing cells (100 μL) at a density of 1.0×10^6 colony forming units (CFU)/mL was applied to the surface of each freshly generated wound, and each wound was covered first with a piece of CNFSs (degree of deacetylation ≈ 30 %) obtained as a commercial product (BeschitinW, Nipro Corp., Osaka, Japan). The animals were then returned to their cages for 24 h. Starting 24 h after the *in vivo* infection and repeating once daily, each infected wound was gently rubbed 10 times with a strip of gauze (1 cm^2) wetted with 10 mL of HClO solution or pure water that had been pre-warmed to 35 - 40 °C, and it was then covered with CNFS/Ag NPs (HClO + CNFS/Ag NP, pure water + CNFS/Ag NP) or CNFS (HClO + CNFS, pure water + CNFS) daily for the first 3 days. Thereafter, all wounds were cleansed with pure water and covered with CNFS daily during days 4 - 12. The non-cleansing group was covered with CNFS without any wound cleansing during each of the 12 days of the experimental periods. Both before and after the cleansing of wounds on Days 1, 2, 3, and 6, the bioburden was collected from each infected wound by wiping with a strip of sterile gauze (1 cm^2). The resulting cell suspension was subjected to 10-fold serial dilutions, and 100-μL samples of diluted suspensions were plated onto 90 mm × 15 mm petri plates of *P. aeudomona* isolation agar; plates were then incubated at 37 °C for 24 h, and viable cells, after confirming that the resulting bacteria were indeed *P. aeruginosa* by observation of the morphology, were enumerated [28]. Furthermore, digital photographs were taken on Days 1, 2, 3, 6, 9, and 12; these images were used to measure the rate of wound closure and confirm no signs of complications, including acute inflammation, abscess formation, and seroma accumulation.

2.4. Histological examination

Following the post-cleansing collection of wound contents on Day 12, animals were euthanized by pentobarbital sodium. The skin surrounding each infected wound was then removed from each mouse for histological examination only on Day 12 after wound creation (N = 6). For each treatment, the removed skin samples including wound tissue were fixed in a 10 % formaldehyde solution, embedded in paraffin, and sectioned in 4-μm increments (Yamato Kohki Inc., Asaka, Saitama, Japan). Sections were made perpendicular to the anterior-posterior axis and perpendicular to the surface of the wound. These sections (about 10 × 1.5 mm^2) were transferred to glass slides and stained with hematoxylin-eosin (H&E) reagent. Following coverslipping, tissues were evaluated microscopically. In each section (N = 8), the microscopic field showing the wound was photographed, and the number of capillary lumens (diameter, 5 - 50 μm or containing 2 - 20 erythrocytes) was counted microscopically in the microphotograph.

2.5. Statistical analyses

Results are expressed as means ± SDs. The Student's *t*-test was used to determine the probability of significant differences with two-tailed tests adjusted by the Bonferroni method using statistical software JMP® (SAS Institute Japan Ltd. Tokyo, Japan Inc. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Cleansing P. aeruginosa-infected wounds with HClO solution and covering with CNFS/Ag NPs in vivo

Male diabetic mice are a well-established model for increased susceptibility to infection and impaired wound healing. *P. aeruginosa*-infected wounds on the diabetic mice were gently cleansed by once-daily gentle rubbing 10 times with gauze impregnated with 1 mL of either HClO solution or pure water (total 10 mL) and covered with either CNFS/Ag NPs or CNFS daily for the first 3 days. All wounds in the 4 groups were cleansed with pure water and covered with CNFS daily for 4 - 12 days. The no cleansing group was covered with CNFS without wound cleansing during each of the 12 days of the experimental period. Both before and after the cleansing of wounds on days 1, 2, 3, and 6 and only after the cleansing on days 9 and 12 in the 4 groups, the wound contents were collected by wiping with a strip of sterile gauze from each infected wound, and viable cells were counted. In the non-treatment group, after removal of CNFS, viable cells were counted. All animals tolerated the creation of *P. aeruginosa*-infected wounds and the daily cleansings without any complications. No signs of acute inflammation, abscess formation, or seroma accumulation were seen at the infected wound sites through the 12th day after surgery and infection.

On Day 1 before cleansing, about 1.0×10^6 CFU of *P. aeruginosa* in the suspension generated at wiping were detected from each infected wound. After wounds were cleansed with either HClO solution or pure water on Day 1, the mean viable cell counts were about 3.2×10^3 and 9.8×10^3 CFU, respectively, representing decreases from the Day-1 pre-cleansing bioburden. However, when the wounds were again covered with either CNFS/Ag NPs or CNFS for another 24 h, the Day-2 pre-cleansing cell counts from the infected wounds rebounded to levels of about 1.0×10^5 CFU (HClO + CNFS/Ag NP group), 9.2×10^5 CFU (pure water + CNFS/Ag NP group), 1.0×10^5 CFU (HClO + CNFS group), and 6.7×10^6 CFU (pure water + CNFS) (Fig. 1). On Day 12, there were no significant differences in the mean viable cell counts among the 4 groups. On the other hand, the mean viable cell count in the no cleansing group showed no decrease from the Day-1 pre-cleansing bioburden.

3.2. Repair of P. aeruginosa-infected wounds by cleansing with HClO solution and covering with CNFS/Ag NPs in vivo

Digital photographs in each group were taken to permit quantification of wound closure rates on Days 1, 2, 3, 6, 9, and 12 (Fig. 2). Wound closures were not observed in all groups. There were no significant differences during the first 3 days. Wounds in the no cleansing group showed a significant delay in wound closure compared to those in the other 4 groups (Fig. 3), and generation of pus with biofilm was observed on Days 3 - 12. The percentage of open wounds in the HClO + CNFS/Ag NP group on Days 9 and 12 was significantly reduced, compared to those in the other 4 groups.

3.3. Histological analyses

Histological examinations showed that wounds in the no cleansing group had significantly less granulation tissue formation (less than 23 %) on Day 12 than those cleansed with either HClO or pure water (Fig. 4). Furthermore, significantly higher granulation tissue formation was observed in the HClO + CNFS/Ag NP group on Day 12 than in the pure water + CNFS/Ag NP, HClO + CNFS, and pure water + CNFS groups (Fig. 4B).

Vascularization was examined in the wounds of each group by microphotographic observation (× 100, magnification) on Day 12. Fig. 5 shows representative microphotographs of the wounds in the HClO + CNFS/Ag NP (A) and no cleansing (B) groups. The formation of capillaries (diameter, 5 - 50 μm or containing 2 - 20 erythrocytes) in the wounds of each group is shown in Fig. 5. The results indicated that wounds in the no cleansing group had significantly less capillary formation on Day 12 than the other groups. In particular, the effects of treatment of the infected wound with HClO + CNFS/Ag NP were highest among all those groups.

4. Discussion

We previously reported that treatment of *P. aeruginosa*-infected wounds on *db/db* diabetic mice for 12 days with once-daily cleansing with HClO solution (200 ppm, pH 6.5) resulted in a significantly decreased *P. aeruginosa* bioburden, albeit with a minor delay in wound repair [28]. On the other hand, our previous study suggested that CNFS/Ag NPs have strong cytotoxicity identical to that of Ag NPs due to

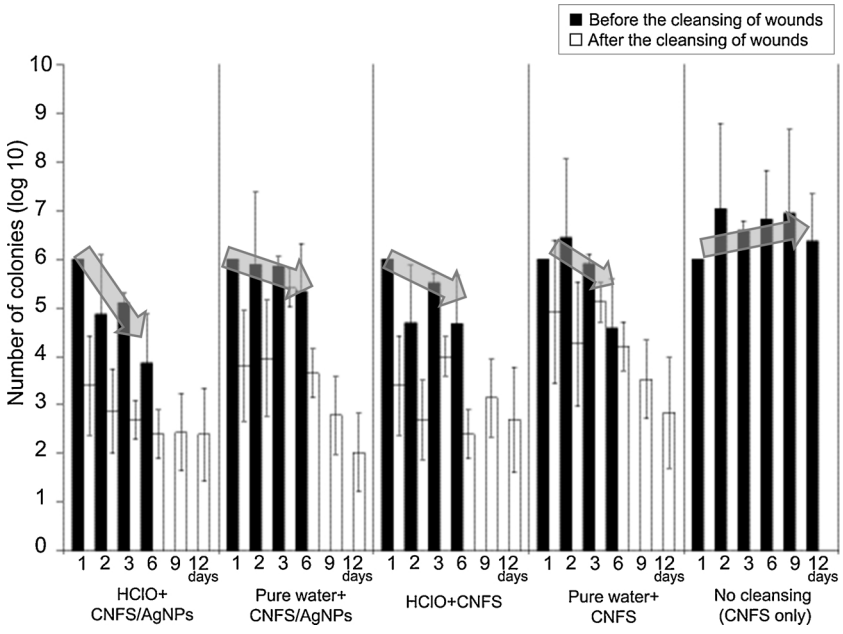

Fig. 1. Reduction of *P. aeruginosa* colonies from each treated wound. *P. aeruginosa*-infected wounds on diabetic mice were cleansed by gentle rubbing, once daily, with gauze impregnated with either HClO solution or pure water and covered with either CNFS/Ag NPs or CNFS (HClO + CNFS/Ag NPs, pure water + CNFS/Ag NPs, HClO + CNFS, pure water + CNFS, and no cleansing (CNFS only)). Both before (■) and after the cleansing of wounds (□), on the indicated day after wound creation, wound contents were collected from each wound, and viable cells in each wound were counted. The arrows represent average decrease of *P. aeruginosa*-colonies. Data are presented as means ± SD (N = 7).

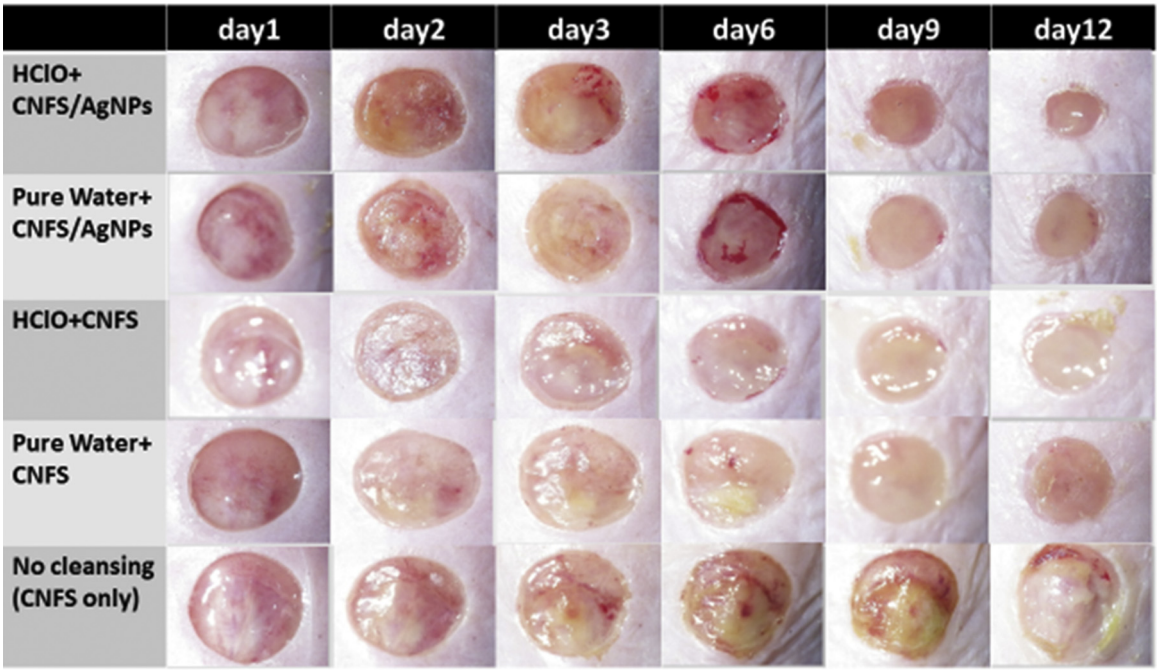

Fig. 2. Digital photographs of open areas of infected wounds. *P. aeruginosa*-infected wounds on diabetic mice were cleansed once daily with either HClO solution or pure water and covered with either CNFS/Ag NPs or CNFS (HClO + CNFS/Ag NPs, pure water + CNFS/Ag NPs, HClO + CNFS, pure water + CNFS, and no cleansing (CNFS only)). Wounds were photographed before cleansing on Days 1, 2, 3, 6, 9, and 12 after wound creation to permit assessment of the percentage of open wound. Images are representative of wounds (N = 7) for the indicated treatment group on the indicated day.

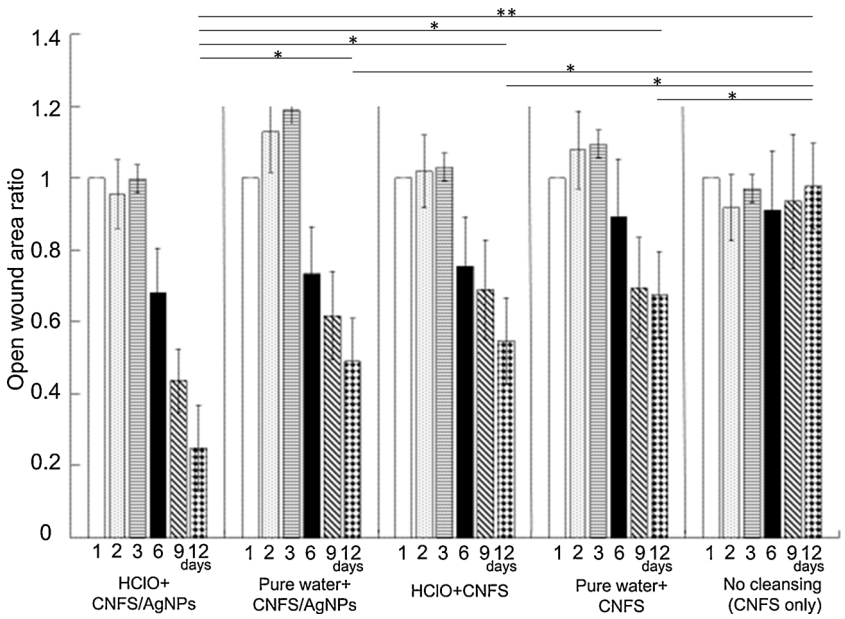

Fig. 3. Percentages of open wounds. The area of open wounds on Day 1 is defined as 100, and the relative open wound in each group is calculated using the digital photographs in Fig. 2. Data are presented as means ± SD (N = 7). *P < 0.05, **P < 0.01 (Student's *t*-test).

generation of oxidative stress [9,17]. Although the cytotoxicity is distinguished from delay of wound repair, continuous CNFS/Ag NPs treatment of the wound for more than 7 days resulted in significant delay of repair [9]. However, the wound repair in fact could be recovered by limiting its usage for 3 days.

In the present study, cleansing with HClO and/or covering with CNFS/Ag NPs daily for 3 days and then cleansing with just pure water and covering with CNFS daily for 9 days were performed to *P. aeruginosa*-infected wounds on *db/db* diabetic mice to evaluate antibacterial activity and wound repair. The results suggested that HClO + CNFS/Ag NPs treatment for 3 days significantly enhanced disinfection, as well as wound healing, *in vivo* compared to the other treatments (pure water + CNFS/Ag NP, HClO + CNFS, pure water + CNFS/Ag NPs, and no cleansing). The histological examinations showed significantly advanced granulation tissue and capillary formation in HClO + CNFS/Ag NPs-treated wounds on Day 12. Furthermore, no signs of complications were observed in animals harboring wounds treated with HClO + CNFS/Ag NPs for 3 days; the absence of signs extended to histological analysis of wound skin harvested on Day 12. Thus, topically treated wounds with HClO + CNFS/Ag NPs for 3 days showed no delay of wound healing. The present results suggest that limiting disinfection to 3 days with HClO + CNFS/Ag NPs-treatment may be appropriate for

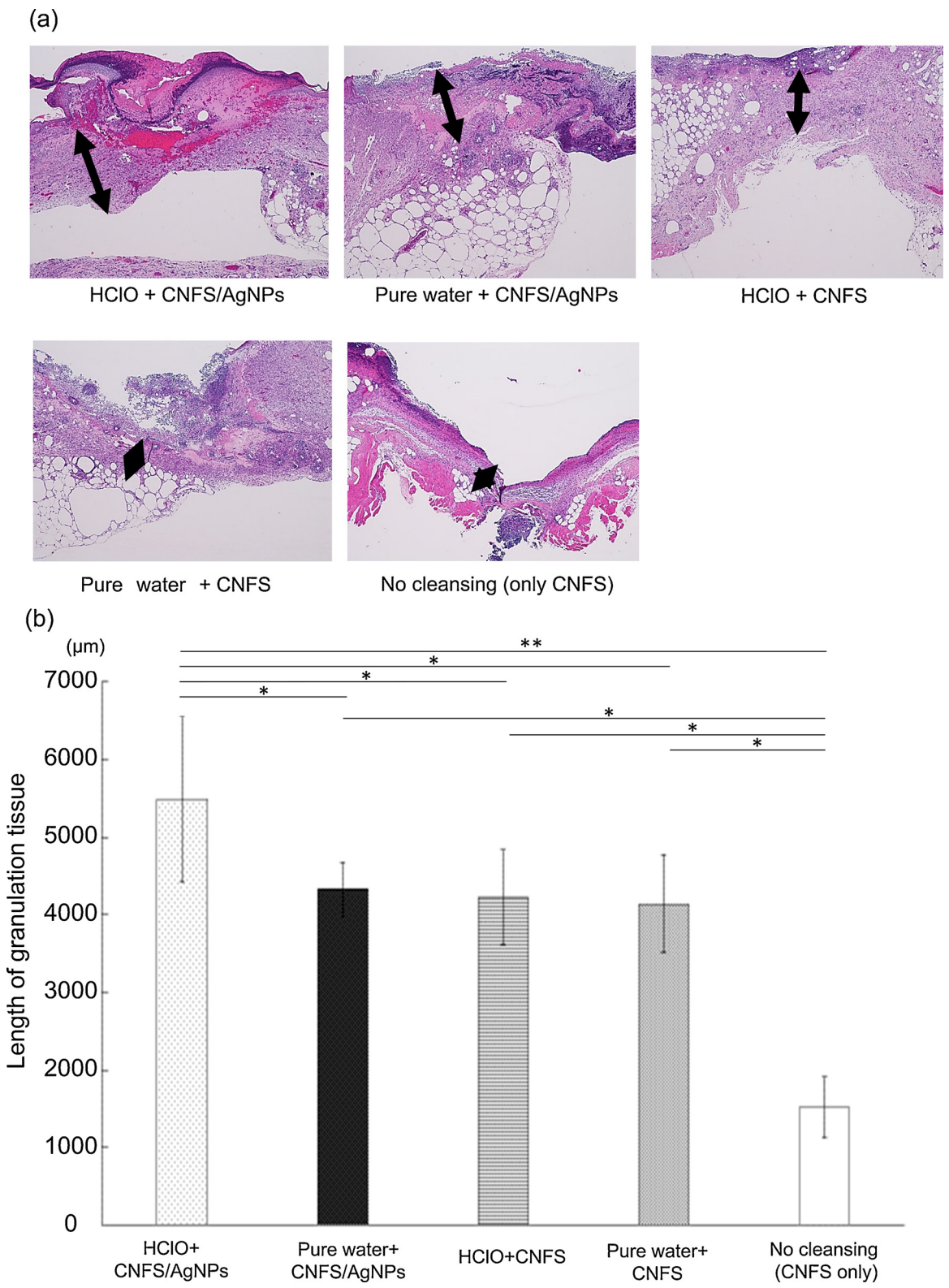

Fig. 4. Histological examination of granulation tissue formation in each treated group on Day 12. The skin surrounding the infected wounds of each treated group was harvested, processed for H&E staining, and microphotographed (N = 6) (A). The lengths of granulation tissue formation in the infected wounds are indicated by double-headed arrows. Microphotographs are representative of wounds (N = 6) for the indicated treatment group (A). The granulation tissue formation on Day 12 is significantly higher in the HClO + CNFS/Ag NPs group than in the other groups (N = 6) (B). The data represent the means ± SD. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

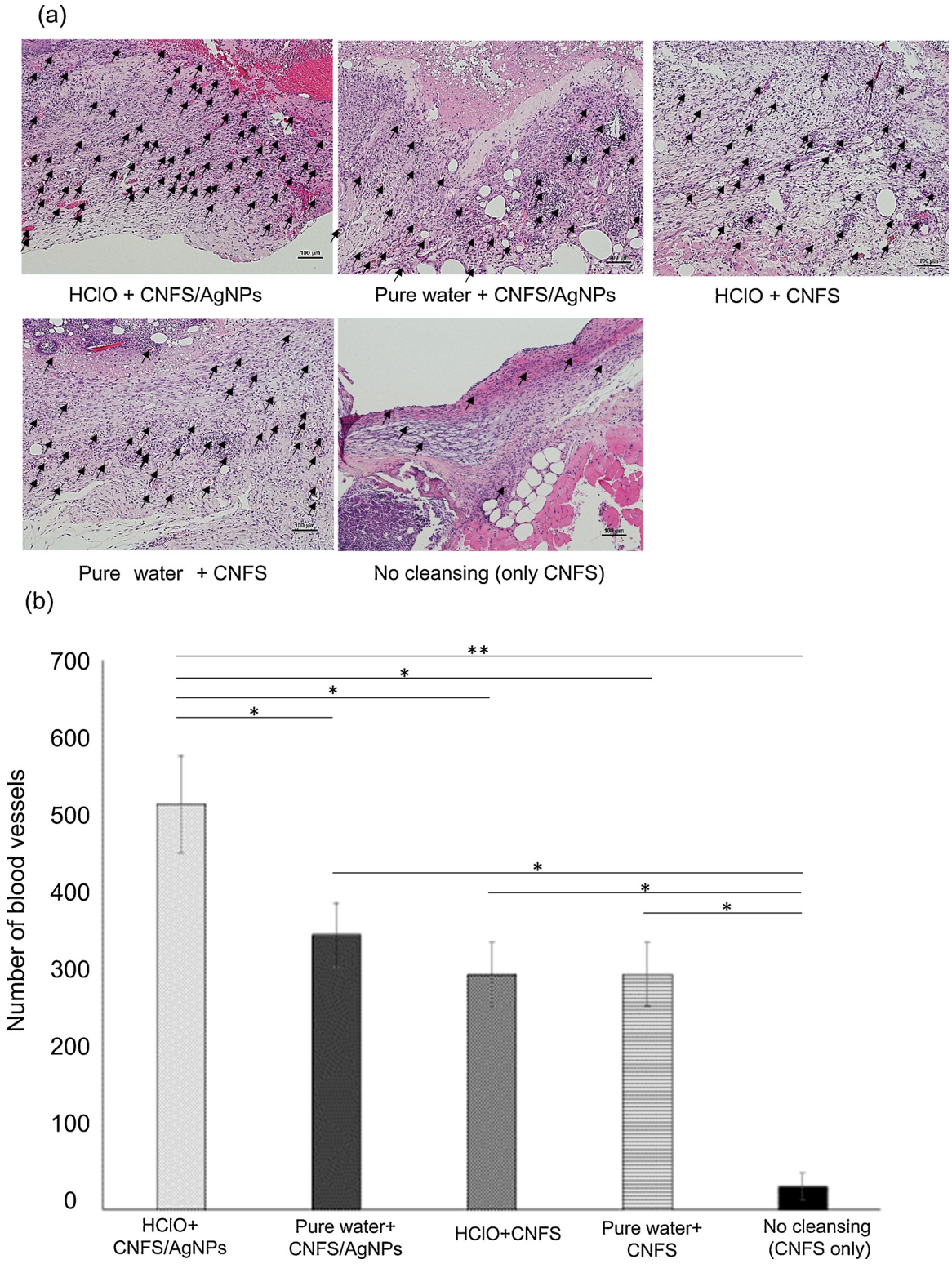

Fig. 5. Vascularization in each treated group on Day 12. The skin surrounding the infected wounds of each treated group was harvested, processed for H&E staining, and microphotographed. The arrows show small and medium blood vessels. Microphotographs are representative of wounds (N = 6) for the indicated treatment group (A). Formations of small and medium vessels in the wound bed were counted using photomicrographs in Fig. 4. Vascularization on Day 12 is significantly higher in the HClO + CNFS/Ag NPs group than in the other groups (N = 6). The data represent the means ± SD. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

use in a clinical situation, such as prevention and treatment of infection in chronic wounds in healing-impaired patients.

An efficacious broad-spectrum antimicrobial agent is increasingly becoming a therapeutic imperative [22,29]. In the context of wound healing, such an agent should be effective as a topical preparation, yet not be too cytotoxic to the relevant cell types. *P. aeruginosa* infections of wounds in clinical settings are major complications [30]. In our previous study, when *P. aeruginosa*-infected wounds on diabetic mice *in vivo* were gently cleansed for 12 days by daily rubbing 10 times with gauze impregnated with 1 mL of either 200 ppm HClO solution (pH 6)

or pure water, wounds cleansed with HClO solution showed significantly lower bacterial loads. HClO is relatively stable under weakly acidic conditions (pH 5 - 7) and serves as the predominant microbicidal component in chlorine-based disinfectants [31,32]. However, HClO reacts readily with various NH_2- or CHO-containing organic (*e.g.*, protein, amino acid, and carbohydrate) and inorganic compounds, resulting in rapid consumption of HClO by oxidation reactions, significantly decreasing HClO's microbicidal activity [21,31]. Thus, HClO is likely rapidly neutralized in the infected wound environment.

On the other hand, CNFS was used to adsorb and to stabilize Ag NPs, to remove the caramel generated during autoclaving, and to prevent aggregation and precipitation of Ag NPs [33]. Ag NPs were homogeneously dispersed and stably adsorbed onto CNFSs with nanoscale fiber-like surface structures [33]. Subsequent studies showed that bactericidal, antifungal, and antiviral activities of CNFS/Ag NPs increased with Ag NP adsorption, indicating potential applications of CNFSs as novel stabilizers and carriers for Ag NPs [17,33]. Although CNFS/Ag NPs and Ag NPs have been shown to have various degrees of *in vitro* and *in vivo* cytotoxicity [9] resulting in significant delay of wound healing, it has been suggested that the delay could be mitigated by cleansing the covered wound and limiting the application of CNFS/Ag NPs to 3 days.

HClO and CNFS/Ag NP have not been approved by the Pharmaceuticals and Medical Devices Agency of Japan for use as a pharmaceutical or medical device, despite the approval of NaClO for such purpose. Additional systemic studies on safety and efficacy of HClO and CNFS/Ag NP including their systemic uptake are required to establish their efficacy, safety, and stability for medical use.

5. Conclusions

P. aeruginosa-infected wounds on *db/db* diabetic mice for 7 days or more with once-daily cleansing with HClO solution resulted in a significantly decreased *P. aeruginosa* burden of wound, albeit with a minor delay in wound repair by the cytotoxicity [28]. In addition, continuous CNFS/Ag NPs treatment of the wound for 7 days or more resulted in significant delay of wound repair by cytotoxicity of Ag NPs due to generation of oxidative stress [9]. In contrast, this study showed that combined treatment of *P. aeruginosa*-infected wounds on *db/db* diabetic mice by 3 days of once-daily cleansing with HOCl solution and covering with CNFS/Ag NPs (HClO + CNFS/Ag NPs) resulted in a significantly decreased *P. aeruginosa* bioburden, as well as enhanced wound repair.

Ethical statement

All animal experiments were approved by the National Defense Medical College, Tokorozawa, Saitama, Japan.

Declaration of Competing Interest

None of the authors has any commercial associations or financial interest to declare.

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