Hypochlorous Acid: An Ideal Wound Care Agent With Powerful Microbicidal, Antibiofilm, and Wound Healing Potency

Serhan Sakarya, MD; Necati Gunay, MS; Meltem Karakulak, MS; Barcin Ozturk, MD; Bulent Ertugrul, MD

Abstract and Introduction

Abstract

Introduction Chronic wounds and the infections associated with them are responsible for a considerable escalation in morbidity and the cost of health care. Infection and cellular activation and the relation between cells are 2 critical factors in wound healing. Since chronic wounds offer ideal conditions for infection and biofilm production, good wound care strategies are critical for wound healing. Topical antiseptics in chronic wounds remain in widespread use today. These antiseptics are successful in microbial eradication, but their cytotoxcity is a controversial issue in wound healing.

Objective The aim of this study was to investigate the effect of stabilized hypochlorous acid solution (HOCl) on killing rate, biofilm formation, antimicrobial activity within biofilm against frequently isolated microorganisms and migration rate of wounded fibroblasts and keratinocytes.

Materials and Methods. Minimal bactericidal concentration of stabilized HOCl solution for all standard microorganisms was 1/64 dilution and for clinical isolates it ranged from 1/32 to 1/64 dilutions.

Results All microorganisms were killed within 0 minutes and accurate killing time was 12 seconds. The effective dose for biofilm impairment for standard microorganisms and clinical isolates ranged from 1/32 to 1/16. Microbicidal effects within the biofilm and antibiofilm concentration was the same for each microorganism.

Conclusion. The stabilized HOCl solution had dose-dependent favorable effects on fibroblast and keratinocyte migration compared to povidone iodine and media alone. These features lead to a stabilized HOCl solution as an ideal wound care agent.

Introduction

Wound healing is a sequence of complex and well-orchestrated events. Although the precise mechanism of wound healing is not fully understood, 3 interrelated phases—inflammation, migration, and remodeling—require coordinated activity for successful wound healing, which is a progressive series of events facilitated by platelets, leukocytes, fibroblasts, and keratinocytes. Platelets facilitate homeostasis and the release of growth factors, then leukocytes participate in the inflammatory process. Fibroblasts and keratinocytes have a critical role in wound healing by enhancing reepithelization and the remodeling of the extracellular matrix (ECM).[1–3]

Most chronic wounds are related to diabetes mellitus, venous stasis, peripheral vascular diseases, and pressure ulcerations. An open wound is a favorable niche for bacterial colonization and infection. Infection in chronic wounds starts with contamination, then colonization and critical colonization take place before an infection forms.

Biofilm formation is now recognized as a serious problem in chronic wound infections.[4] Biofilm is a complex structure of microorganisms that generate a protective shell, allowing bacteria to collect and proliferate.[5] Most of the microorganisms that form biofilms can also be found growing in microbial infections. The same species of bacteria have significant differences in existence that range either free floating and living within the biofilm. The biofilm structure of microorganisms renders phagocytosis difficult,[6] increases resistance to antibiotics,[7] and adheres to unfavorable niches such as chronic wounds.[8]

One of the remarkable features of the immune system against invading pathogens is its ability to generate an effective and rapid response by developing a group of highly reactive chemicals, such as reactive oxygen species (ROS). The mitochondrial membrane-bound enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) is a primary enzyme responsible for ROS production.[9] During the activation of neutrophils, respiratory bursts generate hydrogen peroxide (H_2O_2) and the activated granule enzyme myeloperoxidase converts H_2O_2 to hypochlorous acid (HOCl) in the presence of Cl- and H+.[10] Hypochlorous acid leads to cell death by the oxidation of sulfhydryl enzymes and amino acids, ring chlorination of amino acids, loss of intracellular contents, decreased uptake of nutrients, inhibition of protein synthesis, decreased oxygen uptake, oxidation of respiratory components, decreased adenosine triphosphate production, breaks in DNA, and depressed DNA synthesis.[11–15] Hypochlorous

acid is highly active against all bacterial, viral, and fungal human pathogens[16] and a small amount of HOCl can kill spore-forming and non-spore bacteria in a short time period.[17,18]

Since most of the etiologic factors in chronic wound infections are forming biofilm,[19] and most of the topical antiseptics impair wound healing with their cytotoxic effect, therapeutic strategies against biofilm with high microbial eradication and good wound healing effects will decrease the morbidity and mortality rates of patients and reduce the economic burden. The aim of this study was to investigate the effect of stabilized HOCl solution on microbial and biofilm eradication, antimicrobial activity within biofilm against frequently isolated microorganisms, and migration rate of wounded fibroblasts and keratinocytes.

Materials and Method

Reagents

Hypochlorous acid is generated from sodium hypochlorite and hydrogen peroxide reverse reaction. The concentration used in this study was 218 ppm, pH 7.1, ORP 871 MV and its stability was 24 months (NPS Biosidal, Istanbul, Turkey).

Cell Lines and Microorganism Strains

Skin fibroblast cell line (BJ ATCC CRL-2522, American Type Culture Collection, Manassas, VA) was grown in Eagle's Minimum Essential Medium with 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel). Human skin keratinocyte cell line (CCD 1101 quarter, American Type Culture Collection, Manassas, VA) was grown in keratinocyte serum-free medium (Life Technologies, Carlsbad, CA). Slime producing *Staphylococcus aureus* (ATCC 35556) and *Pseudomonas aeruginosa* (ATCC 15692-PAO-1) were obtained from the Leibniz-Institut DSMZ GmbH (Braunschweig, Germany) and *Candida albicans* (ATCC 90028) from the American Type Culture Collection. Clinical isolates were obtained from the clinical microbiology laboratory of Adnan Menderes University Hospital Aydın, Turkey.

Minimum Bactericidal Concentration and Time Kill Assay

Inocula were prepared following the described guidelines of Clinical and Laboratory Standards Institute.[20] The stabilized HOCl solution inactivated with organic materials and HOCl solution with serial dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 (109, 55, 22.5, 11, 5.5, and 2.75 ppm, respectively) were prepared in sterile phosphate buffered saline (PBS) solution. Sterile PBS alone was used as a control. Hypochlorous acid dilutions and control were prepared in 10 ml tubes and inoculums were added with a final cell density of 10^5 cfu/ml and incubated at 37°C for 60 minutes for minimum bacterial concentration (MBC). A 10 µl sample was removed and plated on Mueller Hinton (MH) or Sabouraud dextrose agar (SDA) plates. After incubating for 24 hours at 37°C, the growths were observed. The concentration at which there was a complete absence of colony growth was determined to be the MBC.

In time-kill (TK) studies, MBC concentrations of HOCl were inoculated with 105 cfu/ml of each organism and incubated for 0, 5, 10, 15, 20, 30, 60, and 90 minutes at 37°C. For each time point, 10 µl of inoculum was transferred to agar and incubated at 37°C for 24 hours. The time at which there was a complete absence of colony growth was determined to be the TK.

To determine the accurate killing time of bacteria, green fluorescent protein (GFP)-transfected *P. aeruginosa* were exposed to 1/32 dilution (5.5 ppm) of HOCl and video recorded through fluorescence microscopy (Olympus, Tokyo, Japan).

Antibiofilm and Microbicidal Effects of Hypochlorous Acid Within Biofilm

The ability of microorganisms to form biofilm on abiotic surfaces was quantified as described in Christensen et al.[21] Briefly, microorganisms were grown overnight in Triptic soy broth (Sigma-Aldrich, St. Louis, MO) or Sabouraud dextrose broth (Sigma-Aldrich, St. Louis, MO) with 0.25% glucose at 37°C. The culture was diluted 1:40 in proper media, and 200 µL of this cell suspension was used to inoculate into wells of 2 groups of sterile 96-well polystyrene U-bottom microtiter plates. After 48 hours incubation at 37°C, wells were gently washed 3 times with 200 µL of sterile PBS and a 2-fold serially diluted HOCl and sterile PBS alone were added to each well and incubated for 24 hours at 37°C.

One group of wells was used for biofilm eradication and the other for the microbicidal effect within the biofilm. After incubation, a biofilm-eradication group of wells was washed 3 times with PBS, dried in an inverted position and stained with 200 µL of 1% crystal violet for 15 minutes. Wells were rinsed and dried, and crystal violet was solubilized in ethanol-acetone (80:20, vol/vol). The optical density (OD) of well contents was determined at 595 nm using a microplate reader (Thermo Fisher Scientific, Milford, MA). A group of wells used to evaluate the bactericidal effect within the biofilm group was washed 3 times and each well was scraped and sonicated for 5 minutes in 100µl sterile PBS. Then, 10 µl of the contents of each well was plated on Mueller-Hinton or Sabouraud dextrose agar plates. Growth was observed after incubating for 24 hours at 37°C.

Skin Fibroblast and Keratinocyte Cell Migration in a Wound Healing Assay

Cells were cultured to confluence in the wells of 24-well plates (Corning Inc, Corning, NY). After 48 hours, a sterile 10 µl pipette tip was used to create a single wound across the diameter of each monolayer, and the medium was replaced with povidone iodine and HOCl solutions with serial dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32 with PBS and PBS alone as a control, as previously described.[22] The cells were then incubated at 37°C and 5% CO_2. Images of each monolayer were captured at 0, 4, 8, and 24 hours.

For determining the short time exposure effect of povidone iodine and HOCl, cells were treated with the serial dilutions of povidone iodine and HOCl solution for 10 minutes and rinsed with PBS. The cells were incubated in proper media at 37°C and 5% CO_2 and images were captured at the time interval indicated above. Cell migration into the wound was calculated using the publicly available Image J software. Cell migration into each wound after 4, 8, and 24 hours was compared to the wounded monolayer at 0 hours.

Statistical Analysis

All experiments were repeated 3 times and each experimental and control condition assayed in triplicate. Analysis of variance (ANOVA) was used to compare the mean responses among experimental and control groups. A *P* value below 0.05 was considered statistically significant.

Results

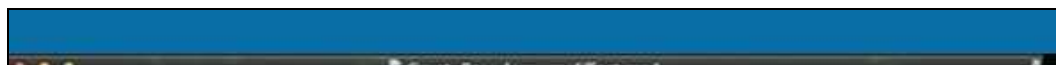**Minimal Bactericidal Concentration and Time Kill**

Minimum bactericidal concentration of HOCl for all standard microorganisms was 1/64 dilution and for clinical isolates ranged from 1/32 to 1/64 dilutions (). All the microorganisms were killed within 0 minutes. The accurate killing time of HOCl on green fluorescent protein (GFP) expressing *P. aeruginosa* was 12 seconds (Figure 1).

Table 1. Minimum bactericidal concentration of stabilized hypochlorous acid (HOCl) solution for a microorganism tested at 37°C for 60 minutes.

Microorganisms	MBC (dilution)
S. aureus (ATCC 35556)	1/64
S. aureus (CI 3)	1/64
S. aureus (CI 12)	1/32
S. aureus (CI 23)	1/64
S. aureus (CI 64)	1/64
S. aureus (CI 463)	1/32
P. aeruginosa (ATCC 15692)	1/64
P. aeruginosa (CI 1)	1/64
P. aeruginosa (CI 1112)	1/64
P. aeruginosa (CI 47)	1/64
C. albicans (ATCC 90028)	1/64
C. albicans (CI 4)	1/64
C. albicans (CI 5)	1/64
C. albicans (CI 11)	1/32

The minimum bactericidal concentration of stabilized HOCl (1/64 dilution = 3ppm and 1/32 dilution = 6 ppm) for a microorganism tested at 37°C for 60 minutes.
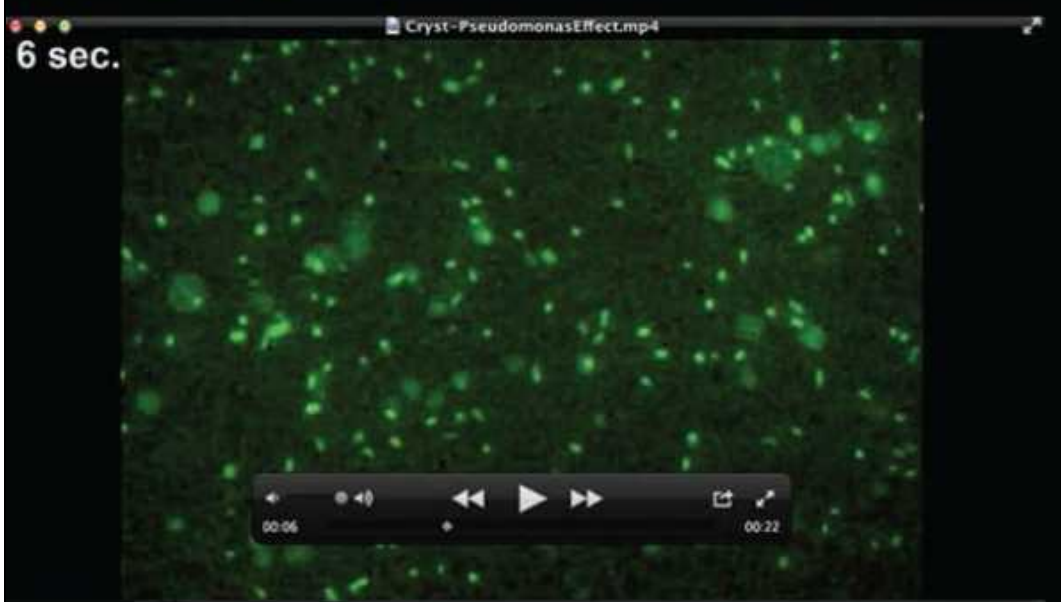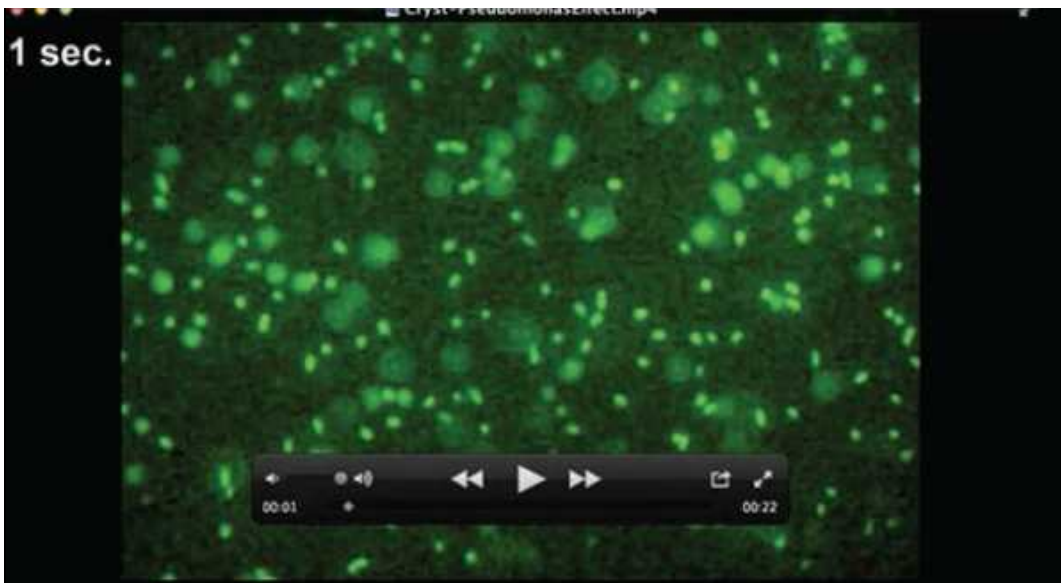ATCC: American Type Culture Collection; CI: clinical isolates; MBC: minimum bactericidal concentration.

1 sec.

6 sec.

12 sec.

Figure 1.

Screen capture of a video microscopic image of the time kill of green fluorescent protein-transfected *P. aeruginosa* with 1/32 dilution of stabilized HOCl solution at 1, 6, and 12 seconds.

Antibiofilm and Bactericidal Effects of Hypochlorous Acid Within Biofilm

The treatment of microorganisms with stable HOCl solution decreased the amount of biofilm, and the amount of microorganisms within the biofilm with the dose-dependent manner depending on species.

Although the effective concentration of the stable HOCl solution was varied between species, microbial killing rates within the biofilm was relevant to the biofilm impairment concentrations for each species. The effective dose of HOCl on biofilm impairment of *S. aureus, P.aeruginosa*, and *C. albicans* was 1/32, 1/16, and 1 dilution, respectively. The microbicidal effects within biofilm and antibiofilm concentration was the same for each of the microorganisms. These data showed HOCl has antibiofilm activity and actively penetrated through the biofilm and killed the microorganism within the biofilm (Figure 2).
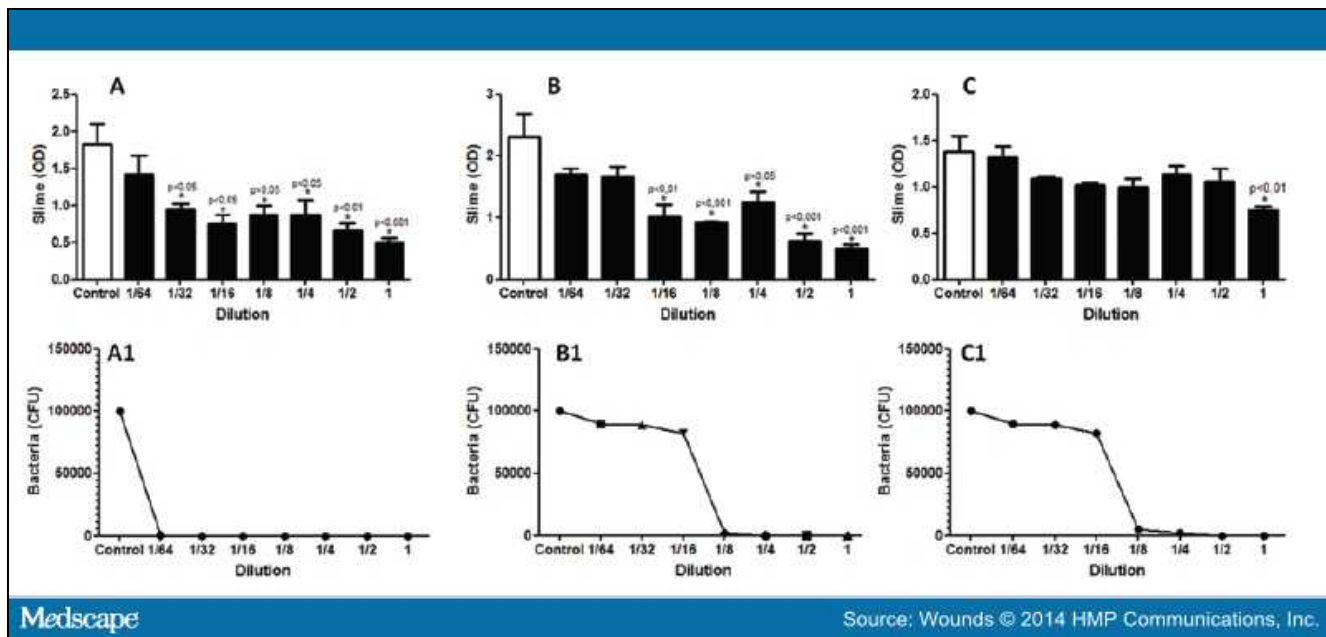
Figure 2.

Antibiofilm and microbicidal effect within biofilm of stabilized hypochlorous acid (HOCl) solution on *S. aureus* (A, A1), *P. aeruginosa* (B, B1), and *C. albicans* (C, C1).
*Significantly decreased in stabilized HOCl solution-treated microorganism biofilm compared to the media control at $P < 0.05$.

The Effect of Povidone Iodine and Stabilized Hypochlorous Acid Solution on the Skin Fibroblast and Human Skin Keratinocyte Cell Line Migration in Wound Healing Assay

Since antiseptic solutions have harmful effects on wound healing, the authors compared the effects of povidone iodine and stabilized HOCl on the injury-induced migratory response. Migration in wound healing assay with povidone iodine-treated skin fibroblast cells was disrupted and cells detached from the matrix in all concentrations and times. Contrastly, stabilized HOCl solution treatment migration was decreased in high concentrations (1/2 and 1/4 dilution), but increased in low concentrations when compared to the media control (Figures 3 and 4). In human skin keratinocyte cells, povidone iodine treatment decreased migration after 4 hours. Migration with a stabilized HOCl solution was the same when compared to the media control at 4 and 8 hours at all concentrations, but increased at 1/16 and 1/32 dilution at 24 hours (Figures 5 and 6). The 10-minute treatment results were the same as the long term treatment results (data not shown). These results suggest that a stabilized HOCl solution enhances wound healing in contrast to povidone iodine.
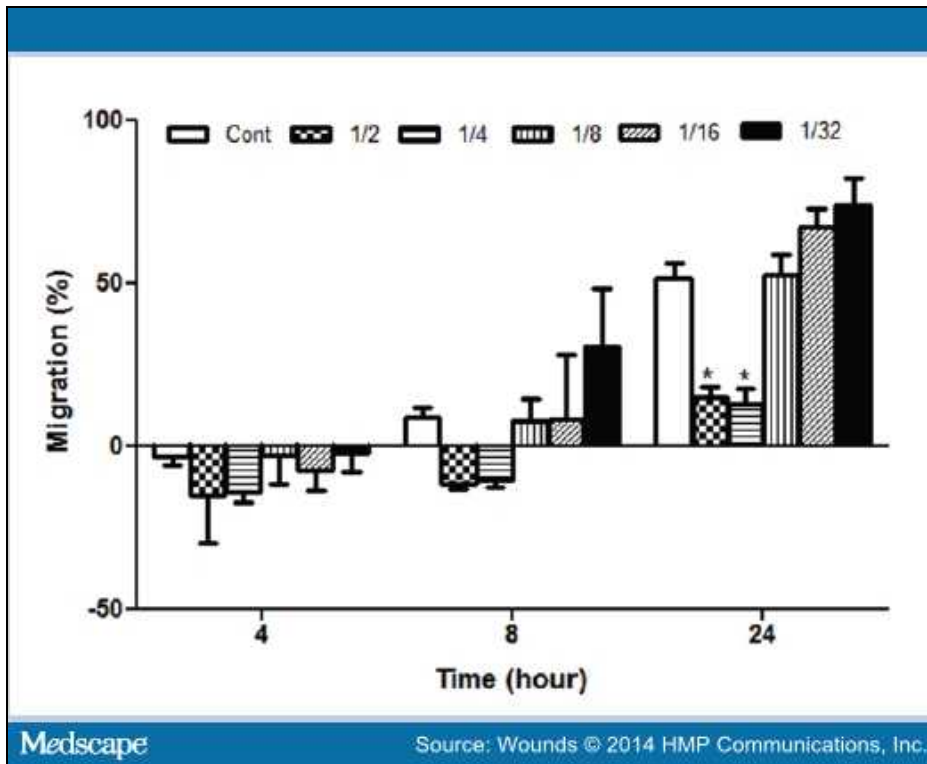
Figure 3.

Effect of stabilized hypochlorous acid (HOCl) solution treatment on skin fibroblast cell migration in a wounding healing assay. Skin fibroblast cells were cultured to confluence, and a single wound was made across each monolayer. At 0, 4, 8, and 24 hours, images of each monolayer were captured and cell migration into the wound after 4, 8, and 24 hours was compared to the monolayer at 0 hours.

*Significantly decreased in stabilized HOCl solution treated skin fibroblast cells migration compared to the media control at $P <$ 0.05.
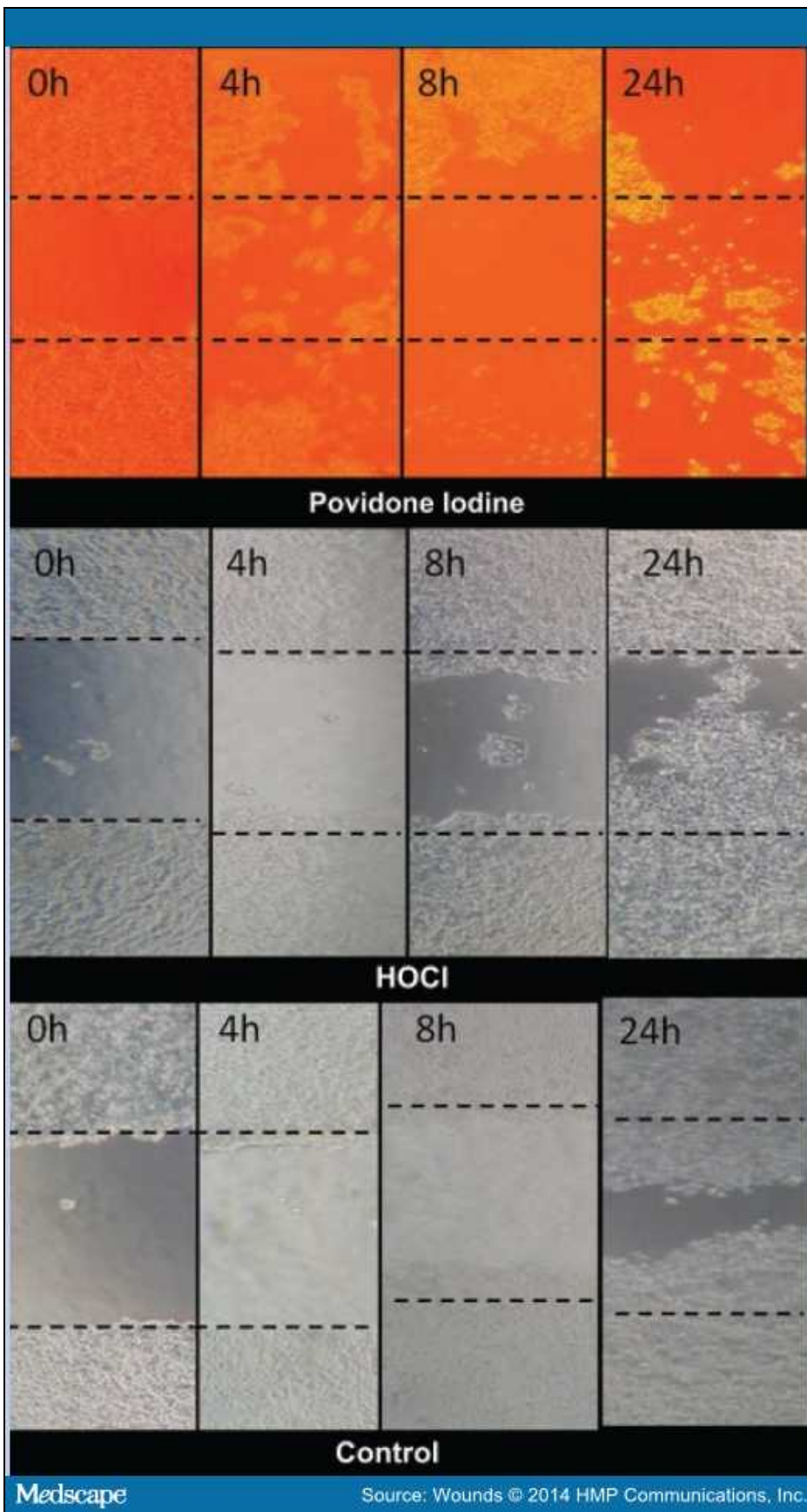
Figure 4.

Images of wounded skin fibroblast cell monolayer with 1/32 dilution of povidone iodine and hypochlorous acid solution at 24 hours. Dotted lines indicate wound boundaries at 0 hours. h: hours
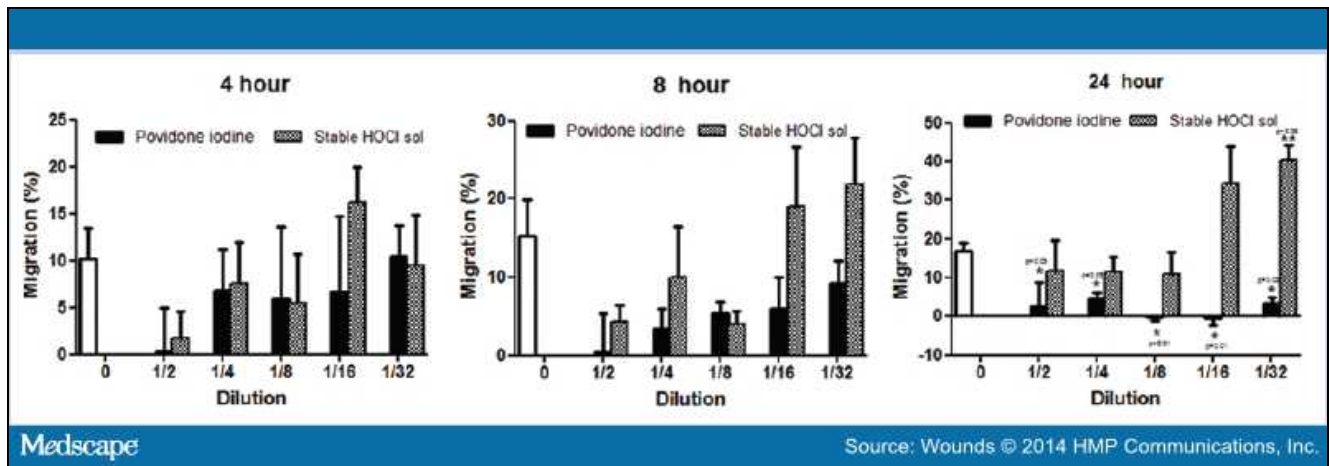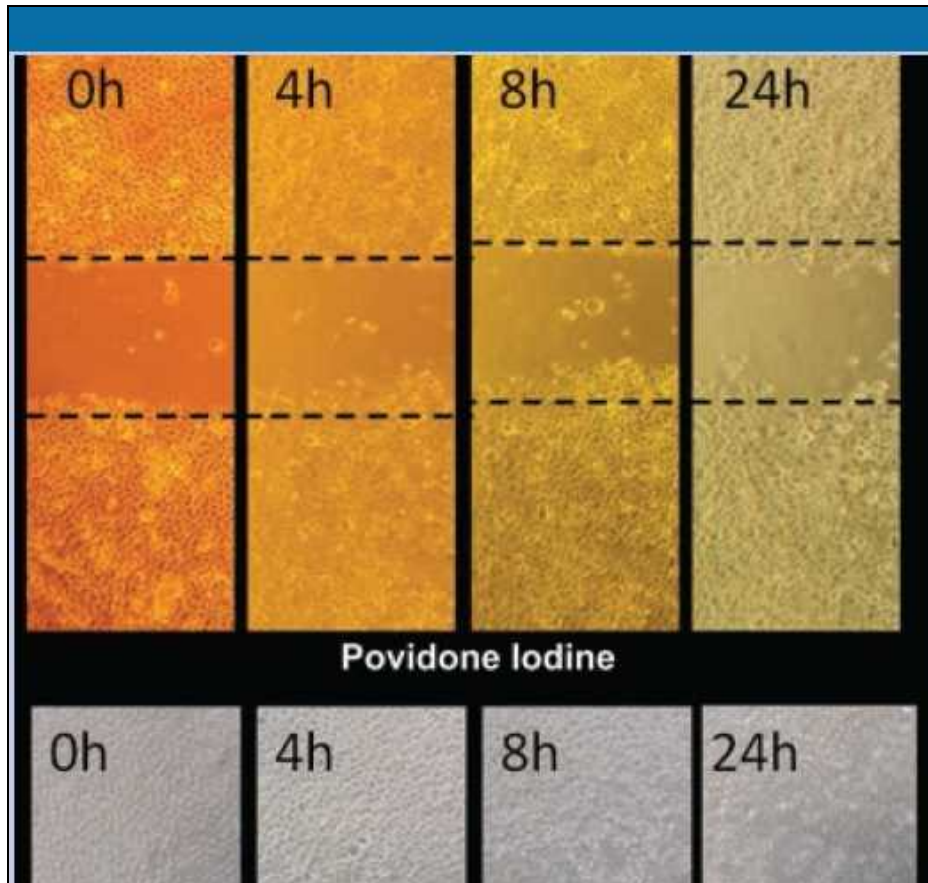

Figure 5.

Effect of stabilized hypochlorous acid (HOCl) solution and povidone iodine treatment on human skin keratinocyte cell migration in a wound healing assay. Human skin keratinocyte cells were cultured to confluence, and a single wound was made across each monolayer. At 0, 4, 8, and 24 hours, images of each monolayer were captured and cell migration into the wound after 4, 8, and 24 hours was compared to the monolayer at 0 hours.

*Significantly decreased in povidone iodine treated human skin keratinoyte cell migration; ** Significantly increased in stabilized HOCl solution compared to the media control at $P < 0.05$.
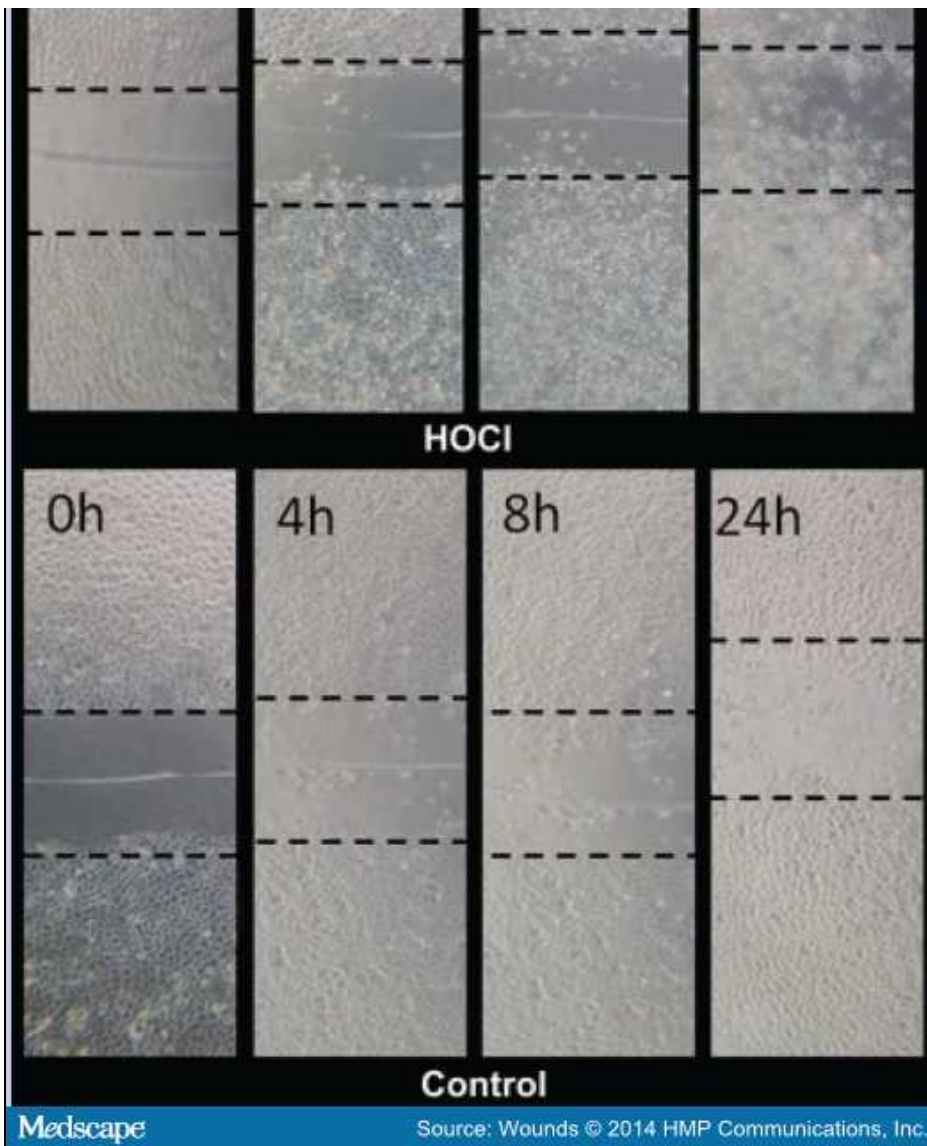
Figure 6.

Images of wounded human skin keratinoyte monolayer with 1/32 dilution of povidone iodine and hypochlorous acid at 24 hours. Dotted lines indicate wound boundaries at 0 hours. h: hours

Discussion

Chronic wounds increase morbidity and mortality of patients and cause a significant burden to health care systems. Most chronic lesions are related to diabetes mellitus, venous stasis, peripheral vascular disease, and pressure ulcerations. Two critical factors important in wound healing are infection and cellular interaction. Infection is a major problem in wound healing, and chronic wounds can become contaminated by surrounding skin, local environment, and endogenous sources. Theoretically, chronic wounds offer ideal conditions for biofilm production. Biofilm is a primary impediment to the healing of chronic wounds[23] as it forms a physical barrier to bacteria from an external environment; provides a means for bacteria to communicate with each other, leading to an increase in virulence and antibiotic resistance; and provides an escape for bacteria from immune recognition.[24] In the current study, the authors demonstrated stabilized HOCl solution had powerful () and rapid (Figure 1) killing effects on common etiologic microorganisms and had antibiofilm and microbicidal effects within biofilm (Figure 2). Although it has been shown that many antiseptic solutions have microbicidal activities against various microorganisms, their activities varied between the type of microorganisms (eg, gram negative, gram positive, or yeasts).[25–28] Previous studies have estimated that 10^6 neutrophils stimulated in vitro can produce 0.1μM HOCl and that this amount of HOCl can kill 1.5×10^7 *E. coli* in less than 5 minutes.[17] Recently, Wang et al[27] demonstrated that stabilized HOCl had antimicrobial activity against *S. aureus, P.aeruginosa*, and *C.*

albicans at concentrations ranging from 0.1 to 2.8 µg/mL and its TK values for these organisms was less than 1 minute.[28] These data correlated with the current study data but TK in this study was 0 minutes for all microorganisms.

Table 1. Minimum bactericidal concentration of stabilized hypochlorous acid (HOCl) solution for a microorganism tested at 37°C for 60 minutes.

Microorganisms	MBC (dilution)
S. aureus (ATCC 35556)	1/64
S. aureus (CI 3)	1/64
S. aureus (CI 12)	1/32
S. aureus (CI 23)	1/64
S. aureus (CI 64)	1/64
S. aureus (CI 463)	1/32
P. aeruginosa (ATCC 15692)	1/64
P. aeruginosa (CI 1)	1/64
P. aeruginosa (CI 1112)	1/64
P. aeruginosa (CI 47)	1/64
C. albicans (ATCC 90028)	1/64
C. albicans (CI 4)	1/64
C. albicans (CI 5)	1/64
C. albicans (CI 11)	1/32

The minimum bactericidal concentration of stabilized HOCl (1/64 dilution = 3ppm and 1/32 dilution = 6 ppm) for a microorganism tested at 37°C for 60 minutes.
ATCC: American Type Culture Collection; CI: clinical isolates; MBC: minimum bactericidal concentration.

Many antiseptic agents have more potent antibiofilm effects than antibiotics.[29–31] But the correlation between antibiofilm effect and microbicidal effect within biofilm has not been well described. In this study, the HOCl concentration for antibiofilm and microbicidal effects within biofilm was the same for each microorganism (Figure 2).

Although the biofilm eradication concentrations varied for each microorganisms, this was not correlated with the amount of biofilm. Since organic materials inactivate the stabilized HOCl solution activity, the difference in biofilm eradication concentration may be due to the diversity of biofilm structures of microorganisms.

Wound healing is a continuous sequence of 3 overlapping phases—inflammatory, proliferative, and maturation. At the inflammatory stage of injury, leukocytes have a critical role in the progress of the normal healing process.[32,33] Granulation tissue formation in the wound initiates with the proliferative phase and consists of cellular elements such as fibroblasts, keratinocytes, and inflammatory cells. Fibroblasts, derived from local mesenchymal cells, are the primary synthetic cells in the repair process of most structural proteins used during tissue reconstruction.[33] Although fibroblasts have a critical role in wound healing, its proliferation highly relates to keratinocyte proliferation and mediators secreted from keratinocytes and fibroblasts. Proinflammatory mediators, such as keratinocyte-derived IL-1 and inflammatory cell-derived IL-1, dominate keratinocyte-fibroblast interactions. Factors, such as keratinocyte growth factor, IL-6, endothelin-1 (ET-1), heparin-binding epidermal growth factor, and granulocte-monocyte colony stimulating factor, are upregulated in fibroblasts in response to IL-1. These factors stimulate keratinocyte proliferation and differentiation. Basement membrane constituents are also expressed by both keratinocytes and fibroblasts.[34] The defects on this mechanism or the proliferation of these cells impairs healing and causes chronic wounds.

Topical antiseptics in chronic wounds remain in widespread use today. These antiseptics are successful in microbial eradication, but at typical concentrations, they are cytotoxic and impair wound healing.[25–27] To demonstrate the effect of a stabilized HOCl solution on wound healing, the authors compared the migration of a wounded monolayer with povidone iodine and media alone in a cell culture model. The povidone iodine treatment disrupted fibroblast migration and detached cells after 4 hours. Stabilized HOCl solution decreased fibroblast migration with high doses, but it increased with a low dose when compared to the media control

(Figures 3 and 4). Stabilized HOCl solution-treated keratinocyte cells and migration increased after 8 hours of treatment with the dose-dependent manner compared to the media control. In contrast, povidone iodine decreased migration at all dilutions after 4 hours of treatment (Figures 5 and 6). Moreover, short term treatment had the same effects on fibroblasts and keratinocyte migration.

Recent in vitro and clinical studies with super-oxidized solutions (SOS), supported these findings. In vitro studies with fibroblasts have shown that SOS is significantly less cytotoxic than hydrogen peroxide and does not induce genotoxicity or accelerated aging. [35] In clinical studies with chronic diabetic foot ulcers, osteomyelitis, and pressure ulcer treatment, HOCl has been impeded by infectious complications without application of systematic antibiotic therapy and a shortened healing period without any local side effects such as allergy or dermal irritation.[36,37]

Conclusion

These findings support stabilized HOCl solution as an ideal wound care solution with a powerful and rapid killing effect on different types of microorganisms, antibiofilms, and microbicidal effect within the biofilm. Foremost, it has dose-dependent favorable effects on fibroblast and keratinocyte migration. These features lead to a stabilized HOCl solution as an ideal wound care agent. Randomized, prospective clinical trials are required to determine in vivo relevance of these findings.

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