ORIGINAL INVESTIGATION

The In Vitro Antimicrobial Activity of Wound and Skin Cleansers at Nontoxic Concentrations

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ABSTRACT

OBJECTIVE: To determine in vitro antibacterial activity of commercially available skin, wound, and skin/wound cleansers at cell-safe (nontoxic) concentrations.

DESIGN: Saline and 19 other commercial wound and skin cleansers were evaluated for cytotoxic effects on mouse dermal fibroblasts. Cells were exposed to serial 10-fold dilutions of each cleanser until treatment-induced cytotoxicity was comparable to the baseline cytotoxicity of unexposed control fibroblasts. Antimicrobial "time-kill" kinetics of these test concentrations of cleansers was tested against methicillin-resistant *Staphylococcus aureus*.

RESULTS: The experimental design allowed calculation of relative cytotoxicity indexes ranging from 0 to 100,000. Shur-Clens Restore Wound Cleanser (ConvaTec, Skillman, New Jersey) and saline were found to be the least toxic (toxicity index: 0); Hibiclens (Mölnlycke Health Care, Norcross, Georgia), Restore Skin Cleanser (Hollister Inc, Libertyville, Illinois), and Betadine Surgical Scrub (Pursue Products LP, Stamford, Connecticut) were found to be the most toxic (toxicity index: 10,000). At noncytotoxic concentrations, NeutroPhase (NovaBay Pharmaceuticals Inc, Emeryville, California) was the most rapidly bactericidal, achieving a 4-log reduction in colony-forming units in less than 1 minute. Puracyn (Innovacyn Inc, Rialto, California) was next at 30 minutes, whereas most of the agents tested required more than 24 hours.

CONCLUSIONS: Wound healing depends on controlling bacterial balance while maintaining the viability of the healing tissues. In vitro toxicity indexes provide helpful guidelines subsequent to in vivo evaluations and clinical applications. The study findings suggest that NeutroPhase, in contrast with many commercially available wound cleansers, has rapid bactericidal activity at concentrations that are safe for human cells.

KEYWORDS: wound cleansers, hypochlorous acid, chronic nonhealing wounds

ADV SKIN WOUND CARE 2014;27:65–9

INTRODUCTION

Chronic nonhealing wounds, such as venous ulcers, diabetic ulcers, and pressure ulcers, cause tremendous patient suffering. Treatment of such wounds presents a serious unmet medical need. Strategies that optimize the tissue repair have evolved with advances in understanding of the wound healing process.[1]

Successful wound healing begins with proper wound bed preparation. There are 4 components to wound bed preparation, all of which address the different pathophysiological abnormalities underlying chronic wounds. These components form a framework that has been named TIME (tissue management, inflammation and infection control, moisture imbalance and epithelial [edge] advancement).[2] Infection control is an important part of the TIME framework. Evidence shows that a bacterial burden of 10^6 microorganisms or more per gram of tissue seriously impairs healing.[3] Bacteria may stimulate a persisting inflammation leading to the production of inflammatory mediators and proteolytic enzymes. Among many other effects, this causes extracellular matrix degradation and inhibition of re-epithelialization.[4] Recently, there has been increased interest in the role of biofilms in impaired healing.[5,6] A wound cleanser without antimicrobial and antibiofilm activity, such as saline, may not be ideal for wound care.[7] Epithelial advancement, another critical component of the TIME framework, requires activity of fibroblasts[8,9] and keratinocytes,[10] which may be hampered by aggressive and toxic wound cleansers. For example, Cetrimide-based cleansing agents are not recommended as their cytotoxic action may impede healing.[11] When such products are used, epithelial cells are killed alongside bacteria. The problem arises from the fact that bacteria recolonize and multiply every 30 to 60 minutes,[12] whereas epithelial cells can only reproduce every 24 hours.[13] As a result, bacteria always win, and wound healing is delayed.

An ideal wound cleanser provides periodic reduction of bacterial contamination and removal of debris without adversely impacting cellular activities vital to the wound healing process.[14]

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Therefore, one of the first steps in a comprehensive strategy for evaluating wound care products is to study their potential cytotoxicity for relevant cell types. In vitro models for cytotoxicity evaluation have included monolayer cultures of human fibroblasts,[15–19] mouse fibroblasts,[20] keratinocytes,[15,21,22] and polymorphonuclear leukocytes.[23]

In this study, the authors determined the noncytotoxic, safe concentration of all 20 important and widely used skin/wound cleansers and compared the microbicidal activity of these cleansers at their noncytotoxic concentrations.

MATERIALS AND METHODS

Test Agents

Twenty commercial skin, wound, and skin/wound cleansers were evaluated. Materials were obtained from manufacturers or distributors (Table 1). For the initial test, materials were used in their original concentrations.

Cells and Testing

L929 mouse fibroblasts were obtained from the American Type Culture Collection (ATCC CCL-1). Cytotoxicity was evaluated by modification of methods described by Wilson et al.[15] L929 cells were briefly seeded into 96-well plates at a density of 20,000 cells/well, allowed to adhere in α-minimum essential medium (α-MEM) (containing 10% fetal bovine serum and 2 mM l-glutamine for 24 hours), and cultured under the same conditions until they were ready for use. After 24 hours, media was removed from the cells by aspiration. The cells were then exposed to the various test agents for 30 minutes at 37°C and assayed for viability. Cell viability was determined using CellTiter 96 nonradioactive cell proliferation assay (Promega, Madison, Wisconsin). The agents were serially diluted 1:10 with phosphate-buffered saline (PBS), and each dilution was tested for cytotoxicity until the results of the cells exposed to the diluted test solutions were similar to those of cells exposed to PBS alone. Testing at each dilution was performed in duplicate.

The CellTiter 96 proliferation assay is composed of a tetrazolium compound, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS), and an electron coupling reagent phenazine methosulfate. MTS is reduced by cells into a formazan product, which is soluble in tissue culture medium. The absorbance of formazan at 490 nm can be measured directly without additional processing.

Time-Kill Bacterial Assay

Time-kill kinetics of each test agent at nontoxic dilution in PBS was tested against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591 (10^8 colony-forming units [CFU]/mL). The nontoxic dilution for each product was based on MTS cell proliferation/viability assay results and defined as the dilution required for generating experimental cell viability to be 85% of controls (cells exposed only to α-MEM medium). Time points included 1, 5, 15, 30, and 60 minutes and 4 and 24 hours. At each time point, bacteria were serially diluted, plated, incubated at 37°C, and enumerated for CFU counts. A time point at which at least 10,000 reduction in CFU counts was observed (from 10^8 CFU/mL to 10^4 CFU/mL) was defined as time to 4-log kill and determined for each skin/wound cleanser.

Table 1.

SKIN AND WOUND CLEANSERS

Cleanser	Use	Manufacturer
Restore Wound Cleanser	Wound	Hollister Woundcare, Libertyville, Illinois
Saline (0.9% sodium chloride)	Wound	
Shur-Clens	Wound	ConvaTec, Skillman, New Jersey
Puracyn OTC	Wound	Oculus Innovative Sciences for Innovacyn, Inc, Rialto, California
WoundClenz OTC Wound Cleanser	Wound	Century Pharmaceuticals, Inc, Indianapolis, Indiana
3M Wound Cleanser	Wound	3M Health Care, St Paul, Minnesota
Dermagran Wound Cleanser	Wound	Derma Sciences Inc, Princeton, New Jersey
NeutroPhase	Wound	NovaBay Pharmaceuticals, Inc, Emeryville, California
Biolex Wound Cleanser	Wound	C. R. Bard, Inc, Covington, Georgia
CarraKlenz Dermal Wound Cleanser	Wound	Carrington Labs, Irving, Texas
SAF-Clens AF	Wound	ConvaTec, Skillman, New Jersey
Prontosan Wound Irrigation Solution	Wound	B. Braun Medical Inc, Bethlehem, Pennsylvania
Allclenz Wound Cleanser	Wound	Healthpoint, San Antonio, Texas
Hydrogen peroxide (3%)	Wound	Hydrox Laboratories, Elgin, Illinois
Dermal Wound Cleanse	Wound	Smith & Nephew, St. Petersburg, Florida
Hibiclens (chlorhexidine gluconate solution 4.0% wt/vol)	Wound/Skin	Mölnlycke, Norcross, Georgia
Johnson & Johnson's Baby Shampoo	Skin	Johnson & Johnson, Skillman, New Jersey
Restore Skin Cleanser	Skin	Hollister Woundcare, Libertyville, Illinois
Betadine Surgical Scrub (povidone-iodine, 7.5%)	Skin	Purdue Products LP, Stamford, Connecticut
Elta Perineal Wash	Skin	Swiss-American Products, Inc, Dallas, Texas

RESULTS

The cytotoxicity dilution and antibacterial time–kill results are shown in Table 2. In order to reach a nontoxic concentration for mouse fibroblasts, each skin/wound cleanser had to undergo 0- to 10,000-fold dilutions (Table 2). Shur-Clens Restore Wound Cleanser (ConvaTec, Skillman, New Jersey) and saline were found to be the least toxic to fibroblasts, requiring no dilution to maintain viable cells (a toxicity index of 0). Several agents (NeutroPhase [NovaBay Pharmaceuticals Inc, Emeryville, California], Puracyn [Innovacyn Inc, Rialto, California], WoundClenz OTC [Century Pharmaceuticals Ltd, Gujarat, India], Biolex [Biolex Therapeutics, Chapel Hill, North Carolina], CaraKlenz [Medline Industries Inc, Mundelein, Illinois], 3M Wound Cleanser [3M, St Paul, Minnesota], and Dermagran [Derma Sciences, Princeton, New Jersey]) required only 1 ''10-fold'' dilution (with a toxicity index of 10). SAF-Clenz AF (ConvaTec), Prontosan (B. Braun Medical, Bethlehem, Pennsylvania), and Allclenz (Smith & Nephew, London, United Kingdom) each had a toxicity index of 100. Dermal Wound Cleanser (Smith & Nephew), Johnson & Johnson's Baby Shampoo (Johnson & Johnson, New Brunswick, New Jersey), Elta Perineal Wash (SteadMed Medical, Fort Worth, Texas), and hydrogen peroxide (Hydrox Laboratories, Elgin, Illinois) had indices of 1000, whereas the toxicity index of Betadine Surgical Scrub (Purdue Products LP, Stamford, Connecticut), Hibiclens (Mölnlycke Health Care, Norcross, Georgia), and Restore Skin Cleanser (Hollister Inc, Libertyville, Illinois) was 10,000.

The time to kill at the nontoxic dilution of NeutroPhase (10-fold dilution) was less than 1 minute, followed by Puracyn (10-fold dilution) at 30 minutes. The time to kill at nontoxic dilutions of all other commercially available wound cleansers was greater than or equal to 24 hours (Table 2).

DISCUSSION

An ideal wound cleanser should have minimal cytotoxicity together with potent and rapid antimicrobial activity. Potent wound cleansers with a high toxicity index (eg, Betadine, chlorhexidine, polyhexamethylene biguanide) will likely have deleterious effects to living tissue. At the same time, a nontoxic wound cleanser (eg, saline, Shur-Clens, Restore Wound Cleanser) without antimicrobial activity will likely provide minimal reduction in bacterial burden (Table 3).

This in vitro study demonstrates that many wound and skin cleansers may be toxic to fibroblasts—one of the key cells in wound repair—and suggests that these cleansers might also be toxic to other cells. When diluted to ''cell-safe'' concentrations, most of the cleansers' lost antibacterial activity was reflected by the length of time needed to reduce the growth of *S aureus*. Although there is not a well-defined rule to quantify the relationship between the in vitro cell toxicity of a skin/wound cleanser and its effects on healing wounds, it has been shown that in vitro cell toxicity correlates with retardation of healing.[15] For example, application of SAF-Clens AF and Shur-Clens into a full-thickness guinea pig dorsum skin wounds resulted in a healing process that did not differ from healing in wounds in which saline was applied. The application of Betadine Surgical Scrub resulted in significantly slower dermal and epidermal healing.[24] These findings correlate with the results of in vitro fibroblast model where SAF-Clens AF and Shur-Clens were found to be nontoxic

Table 2.

DILUTIONS NONTOXIC TO L929 CELLS AND TIME TO LOG_{10} CFU REDUCTION in *S AUREUS* FOR THE TEST AGENTS

Agents Tested	Nontoxic Dilution	Toxicity Index	Time to 4 Log_{10} Kill
Restore Wound Cleanser	10^{0}	0	>24 h
Saline (0.9% sodium chloride)	10^{0}	0	>24 h
Shur-Clens	10^{0}	0	>24 h
Puracyn OTC	10^{-1}	10	30 min
WoundClenz OTC Wound Cleanser	10^{-1}	10	>24 h
3M Wound Cleanser	10^{-1}	10	>24 h
Dermagran Wound Cleanser	10^{-1}	10	>24 h
NeutroPhase	10^{-1}	10	<1 min
Biolex Wound Cleanser	10^{-1}	10	>24 h
CarraKlenz Dermal Wound Cleanser	10^{-1}	10	>24 h
SAF-Clens AF	10^{-2}	100	24 h
Prontosan Wound Irrigation Solution	10^{-2}	100	>24 h
Allclenz Wound Cleanser	10^{-2}	100	24 h
Hydrogen peroxide (3%)	10^{-3}	1000	>24 h
Elta Perineal Wash	10^{-3}	1000	>24 h
Dermal Wound Cleanser	10^{-3}	1000	>24 h
Johnson & Johnson's Baby Shampoo	10^{-3}	1000	>24 h
Hibiclens (chlorhexidine gluconate solution 4.0% wt/vol)	10^{-4}	10,000	>24 h
Restore Skin Cleanser	10^{-4}	10,000	>24 h

Table 3.

CYTOTOXICITY AND ANTIBACTERIAL ACTIVITY OF SKIN AND WOUND CLEANSERS

	Low Cytotoxicity Toxicity Index 0–100	High Cytotoxicity Toxicity Index: 1000–10,000
Antibacterial activity at "cell-safe" concentration < 10 minutes	NeutroPhase	none
Antibacterial activity at "cell-safe" concentration > 10 minutes	Puracyn OTC	none
	SAF-Clens AF	
	Allclenz Wound Cleanser	
Non-antibacterial in 24 hours at "cell-safe" concentration	Restore Wound Cleanser	Hydrogen peroxide (3%)
	Saline (0.9% sodium chloride)	Elta Perineal Wash
	Shur-Clens	Dermal Wound Cleanser
	WoundClenz OTC Wound Cleanser	Johnson & Johnson's Baby Shampoo
	3M Wound Cleanser	Hibiclens (chlorhexidine gluconate solution 4.0% w/v)
	Dermagran Wound Cleanser	Restore Skin Cleanser
	Biolex Wound Cleanser	Betadine Surgical Scrub (povidone-iodine, 7.5%)
	CarraKlenz Dermal Wound Cleanser	
	Prontosan Wound Irrigation Solution	

to fibroblasts at commercial concentrations, whereas the povidone-iodine (Betadine Surgical Scrub) showed the highest cytotoxicity.

The results of these studies offer some guidance for wound care in the complex circumstances encountered in most wounds. Several of the cleansers studied are not toxic to cells even in vitro, whereas a single 10-fold dilution is sufficient to render another group nontoxic. Depending on the goals envisioned for the cleanser treatment, these groups might well be considered best from a safety point of view. In this category, based on their microbiologic effect in these studies, 2 agents, NeutroPhase and Puracyn (or the similar product Dermacyn), separate out as the others required over 24 hours to reach a 4-log reduction in *S aureus*. NeutroPhase required a less than 1-minute exposure, and Puracyn or Dermacyn required a 30-minute exposure to reach the same reduction. NeutroPhase is a pure hypochlorous acid (HOCl, 0.01%) solution in 0.9% saline at pH 4, whereas Dermacyn and Puracyn, according to their labels, contain electrolyzed water (99.97%), sodium chloride (NaCl) 0.023%, sodium hypochlorite (NaOCl) 0.004%, and hypochlorous acid 0.003%. Hypochlorous acid is a naturally occurring well-known broad-spectrum,[25,26] fast-acting[27] antimicrobial agent produced by neutrophils and monocytes[28] as part of the innate immune system's response to infection. In addition to being able to directly penetrate bacteria, spores, and amoeba cysts, hypochlorous acid has been shown to disrupt biofilm.[29–32] Hypochlorous acid has also been described as being 80 to 100 times more potent as a germicide than the equivalent molar ratio of hypochlorite anion.[33] This is due to the fact that pure hypochlorous acid as an uncharged species can penetrate microbial cells and spore walls, whereas the charged hypochlorite anion cannot penetrate cell walls. Previous reports show that hypochlorous acid has broad-spectrum antibacterial activity against gram-positive and gram-negative pathogens including drug-resistant bacteria such as MRSA, vancomycin-intermediate resistant *S aureus*, and mupirocin-resistant *S aureus* with a minimal bactericidal concentration ranging from 0.1 to 2.8 μg/mL and also demonstrated fungicidal activity against *Candida albicans* and *Aspergillus niger*.[17] Higher concentration of free HOCl in NeutroPhase compared with Dermacyn or Puracyn likely explains faster antibacterial activity of NeutroPhase. The rapid activity of NeutroPhase seen in these studies is reflective of its potent in vivo activity in a rat chronic wound model.[34]

Although safe at concentrations offered in NeutroPhase, hypochlorous acid and hypochlorite anion may cause tissue necrosis and/or apoptosis at higher concentrations found in Dakin solution (0.5%).[35–37]

In this study, in vitro methods were used to evaluate the potentially deleterious effects of cleansers on wound healing as well as the likely antimicrobial activities of cleansers. The in vitro findings correlated with many in vivo studies and with clinical advice. The antimicrobial activity of 2 of the 19 agents studied—NeutroPhase and Puracyn—stood out. Both cleansers contained hypochlorous acid, a particularly-rapidly acting antimicrobial produced endogenously as part of the body's innate immune system. These studies should prove useful to clinicians developing wound care strategies and to those wishing to develop and expand in vitro methods to evaluate the potential effects of agents used for wound care. ●

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