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Microbicidal Activity of Octenidine Hydrochloride, a New Alkanediylbis[Pyridine] Germicidal Agent

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The potential of octenidine hydrochloride (WIN 41464-2) as a topical microbicide was measured both by in vitro death kinetics and reductions in numbers of bacteria on the skin of cynomolgus monkeys. Semilogarithmic survial curves were plotted to measure the microbicidal activity of various concentrations of octenidine against *Staphylococcus aureus*. The microbicidal activity of octenidine was also determined for *Staphylococcus epidermidis*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Candida albicans*. Death rates for the same microbial strains were compared with those obtained by using chlorhexidine gluconate. Octenidine concentrations of <1.5 μM (0.94 μg/ml) caused a greater than 99% reduction of each microbial population within 15 min. *Staphylococcus epidermidis* was the most susceptible of the test organisms, and *E. coli* and *C. albicans* were the least susceptible. Octenidine was more active than chlorhexidine against each test strain. Skin-degerming activities of aqueous and formulated octenidine and formulated chlorhexidine were compared in single and multiple applications of these agents to the hand and foot surfaces of monkeys by using a glove-juice extraction procedure to measure the skin microflora. Aqueous octenidine, at a concentration of 0.2 to 1.6% reduced resident microflora populations from 90 to 99.98%, depending on the concentration and number of applications. Octenidine formulated at 2% in a surfactant-based vehicle exhibited significantly better skin-degerming activity than did either a nonmedicated vehicle or the Hibiclens brand of 4% chlorhexidine gluconate.

Octenidine hydrochloride (OCT) (Fig. 1), WIN 41464-2; [*N,N'*-(1,10 decanediyldi-1[4H]-pyridinyl-4-ylidene)bis-(1-octanamine)dihydrochloride], was developed at the Sterling-Winthrop Research Institute as a potential topical antimicrobial agent (2). Previous studies with this compound have shown it to be effective in inhibiting the growth of plaque-forming bacteria (16, 20, 21) and in reducing development of plaque in both experimental animals (6, 16) and humans (13). The purpose of this study was to determine the degerming capacity of OCT on skin and to estimate its potential as a skin antiseptic by examining its microbicidal activity against common nosocomial pathogens which might be encountered as transient skin microbes.

Selection of OCT for this study was based on earlier studies which showed that the compound exhibited broad-spectrum antimicrobial activity as measured by MICs against a variety of gram-positive and gram-negative bacteria (2). This activity was comparable to that exhibited by chlorhexidine gluconate (CHG) (Fig. 1), a bisbiguanide germicidal agent currently used in various commercial skin-degerming formulations. The present study was designed to determine whether OCT exhibited microbicidal activities in vitro and on skin tissue and how these activities compared with those of CHG.

In vitro microbicidal kinetics were measured by calculating death rates of organisms exposed to different concentrations of each compound. Skin-degerming activities of OCT and CHG were measured as reductions in resident microflora populations on the treated skin of cynomolgus monkeys.

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MATERIALS AND METHODS

In vitro microbicidal activity. Compounds tested. For testing purposes, OCT was dissolved in 23 mM potassium phosphate buffer (pH adjusted to 7.0) and stored as a 5 mM stock solution. CHG was prepared at the Sterling-Winthrop Research Institute as a 0.22 M aqueous solution, which is equivalent to 20% (wt/vol) and conforms to British Pharmacopoeia standards. This solution was diluted in 23 mM phosphate buffer and stored, protected from light, as 1.0 and 1.6 mM stock solutions. All solutions were stored at room temperature and diluted to desired concentrations immediately before use.

Microbial cultures. The microbicidal profiles of OCT and CHG were obtained from studies with nine bacteial strains. These included: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 17917, *Streptococcus pyogenes* ATCC 12384, *Escherichia coli* Vogel, *Klebsiella pneumoniae* SWRI No. 87, *Serratia marcescens* ATCC 8195, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* MGH-1, and *Candida albicans* ATCC 10231. All were grown on Trypticase soy agar (BBL Microbiology Systems Inc., Cockeysville, Md.) slants at 37°C for 18 to 24 h before testing except *Streptococcus pyogenes*, which was grown on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with 10% horse serum, and *C. albicans*, which was grown on Sabouraud maltose agar (BBL). Cells were harvested with 2 to 3 ml of sterile 23 mM potassium phosphate buffer and adjusted before use to an absorbance of 0.1 U with a Spectronic 20 spectrophotometer (λ = 650 nm; Bausch & Lomb, Rochester, N.Y.). From this suspension, a 1:100 final dilution of each culture was tested.

Microbicidal kinetics assay. The bactericidal activities of OCT and CHG against *Staphylococcus aureus* were mea-

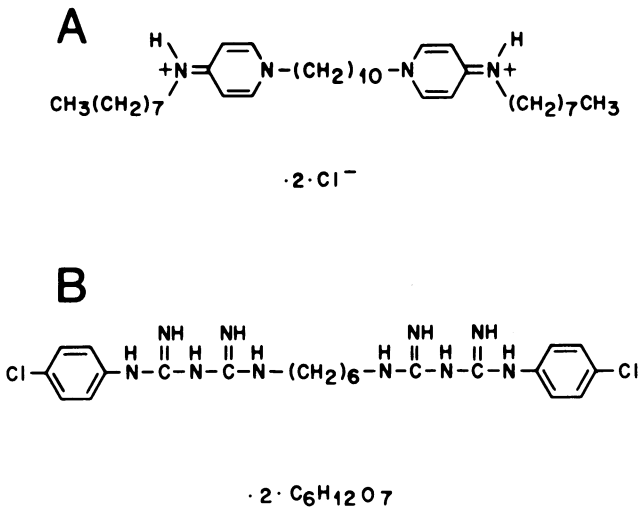

FIG. 1. Chemical structures of OCT (A) and CHG (B).

sured by exposing cultures to several different concentrations of each compound for various lengths of time. OCT was diluted from the 5 mM stock solution and tested in phosphate buffer at levels ranging from 0.5 to 5.0 μM. CHG was diluted from the 1.6 mM stock and tested in buffer from 1.0 to 160.0 μM. The test was begun by adding the bacterial culture to the test solution containing OCT or CHG; samples were taken at 5, 15, 30, and 60 min afterward. The inoculum size was approximately 10^6 CFU/ml. Each test was performed in duplicate.

The microbicidal profiles of OCT and CHG were examined by using three different compound concentrations for each of the nine species. In these tests, OCT was prepared from the 5 mM stock solution and tested in phosphate buffer at 0.5, 1.5 and 2.0 μM; CHG was prepared from the 1.0 mM stock solution and tested at 1.0, 5.0 and 40.0 μM. Use of a higher range of concentrations of CHG was based on data derived from the comparison of the bactericidal activity of OCT and CHG against *Staphylococcus aureus*. Each experiment was begun by adding test solution to the microbial culture. Samples were taken at zero time and after 15 min of incubation with compound; microbicidal activity was calculated as the log reduction from initial population. The zero-time value was obtained by diluting the test solution 1:2 in culture containing 2% Tamol-N Micro (Rohm & Haas Co., Philadelphia, Pa.) and plating immediately. Tamol-N Micro is an anionic dispersant consisting of the sodium salt of condensed naphthalene sulfonate, which we have found to be an effective neutralizer for the antimicrobial activities of OCT and CHG and also nontoxic to normal skin microflora at levels as high as 2% (Table 1).

For the duration of these tests, each microbial culture was maintained at 30°C to approximate the normal temperature of the skin surface. After initiation of the timed experiment, samples were taken at the specified times and (except for the preneutralized zero time sample from the second set of tests) diluted 1:2 in a neutralizer solution containing 2% Tamol-N Micro in 23 mM phosphate buffer (pH 7.2). Subsequent dilutions of all samples were made in lecithin phosphate buffer containing 3.5 mM phosphate with 0.22% (wt/vol) lecithin and 1.55% (wt/vol) polysorbate 80 (pH 7.2). This diluent, also used in the in vivo tests described below, has been shown to be a nontoxic holding solution for skin microflora (1). The number of surviving bacteria was determined by pour plating with agar media as defined above for each culture. The plates were incubated at 37°C for 48 h.

In vivo skin-degerming activity of OCT. Dose-ranging study with aqueous OCT. Four solutions of OCT were prepared by dissolving OCT in distilled water to final concentrations (wt/wt) of 0.2, 0.4, 0.8 and 1.6%, equivalent to 3.2, 6.4, 12.8 and 25.6 mM, respectively. These solutions ranged in pH from 6.0 to 6.8 and were maintained at 32 to 37°C for the duration of the test. Since they were unbuffered, the pH range had no effect on recovery of microflora from the skin (22) or on the normal pH of the skin (9).

Thirty-six nondepilated cynomolgus monkeys (*Macaca fascicularis*) were used to examine the skin degerming activity of these preparations using a procedure described previously (5). The animals were anesthetized by intramuscular injection with a 35:3 mixture (based on milligrams of compound) of ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) and xylazine (Cutter Laboratories, Inc., Shawnee, Kans.). This mixture provided adequate anesthesia at a dose of 10 to 12 mg/kg of body weight. The animals were maintained under anesthesia for approximately 6 h by using supplemental intramuscular injections of either the above mixture or ketamine hydrochloride alone. Their hands and feet were scrubbed by technicians who were blinded as to the identity of the four OCT solutions; the labeling of the solutions was coded to provide random assignment to each appendage.

Before application of the OCT solutions, each appendage underwent a prewash with a nonmedicated cleansing agent to remove transient microflora (11). A sample was then taken to recover resident microflora so that a population baseline could be established for each appendage. After this sample was taken, the assigned OCT solution was applied and scrubbed over the appendage. The limb was rinsed, dried, and again sampled to recover viable bacteria. Four scrubs were conducted per appendage, with a minimum of 1 h between each application. The contact time for each scrub was 3 min.

Sampling via the glove-juice method (12) was carried out after the prewash procedure and again after the first, second, and fourth scrubs. The samples were taken by adding 100 ml of sampling solution (0.1% Triton X-100 [Rohm & Haas] in 75 mM phosphate buffer at pH 7.8) to gloved appendages and thoroughly massaging for 1 min. The use of this detergent solution for sampling skin microflora has been described by others (7, 10, 22). It has cell dispersant properties while being nontoxic to extracted microbial cells. Each sample was neutralized by 1:2 dilution in phosphate-buffered 2% Tamol-N Micro solution. Decimal dilutions of each neutralized sample were prepared in lecithin phosphate buffer, and 1-ml volumes of these solutions were pour plated, in tripli-

TABLE 1. Effects of Tamol-N Micro on viability of *Staphylococcus epidermidis* ATCC 17917 and as a neutralizer for OCT and CHG

Antimicrobial agent	Log CFU after 36-h exposure to the following concn (%) of Tamol-N Micro[a]:				
	2	1	0.5	0.25	None
None	6.8	6.8	6.7	6.8	7.1
OCT (200 μg/ml)	7.3	7.3	7.4	5.5	0.0
CHG (400 μg/ml)	7.1	7.0	5.5	0.0	0.0

[a] The incubation solution consisted of 0.1% Triton X-100 in potassium phosphate buffer (pH 7.8); samples were then plated in Trypticase soy agar.

cate, with Trypticase soy agar. The plates were incubated aerobically at 37°C for 72 h.

Evaluation of OCT skin-degerming formulation. A prototype skin-degerming formulation was prepared containing 2% OCT in a surfactant-based vehicle. The skin-degerming activity of this preparation was compared with that of the vehicle formulation without OCT and that of the Hibiclens brand of 4% chlorhexidine gluconate (Stuart Pharmaceuticals, Wilmington, Del.). The OCT formulation contained tricocoamidopropyl alkonium phosphate and polyethylene glycol-78 glyceryl cocoate as skin-cleansing surfactants. Hibiclens was used as the positive reference standard; at the time of this study, it was the only chlorhexidine gluconate skin-cleansing product approved by the U.S. Food and Drug Administration for use as a surgical scrub and handwash for health-care personnel.

Twenty-four animals were used for this study and treated as described above, with the exception that the formulations were randomly assigned only to the hands of each animal. This was due to the use of a different randomization code to assign the three formulations in this study. In addition, since the OCT and vehicle formulations were a different color from Hibiclens, technician blinding was precluded during the scrub phase. Technicians conducting the plate counts, however, were unaware of treatment assignment.

Skin-degerming activity parameters. Microbial recovery data from each treated appendage were used to characterize the skin-degerming efficacy of each solution. Rapid and cumulative activities were estimated by calculating log bacterial reductions from baseline after one, two, and four scrubs with the test solutions.

RESULTS

Microbicidal Activity. As measured by their effects on *Staphylococcus aureus*, the death rate kinetic profiles of OCT and CHG were similar (Fig. 2 and 3). There was, however, an approximately 40-fold difference in activity between the two agents, OCT being considerably more active than CHG.

A 15-minute exposure to 0.5 μM OCT effected a greater than 99% reduction in populations of *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Serratia marcescens* and *Pseudomonas aeruginosa* (Table 2). At 1.5 μM OCT, the viability of all test organisms was reduced 99% or more. At 2.0 μM, a reduction of at least 99.999% was attained with all test organisms except *C. albicans*; however, this may have been an artifact due to the low inoculum size of the yeast. Among the gram-positive bacteria, *Staphylococcus epidermidis* was the most susceptible to OCT; *Staphylococcus aureus* was the least susceptible. Among the gram-negative organisms, OCT was most active against *K. pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa* and least active against *E. coli*. Activity against *C. albicans* was similar to that against *E. coli*.

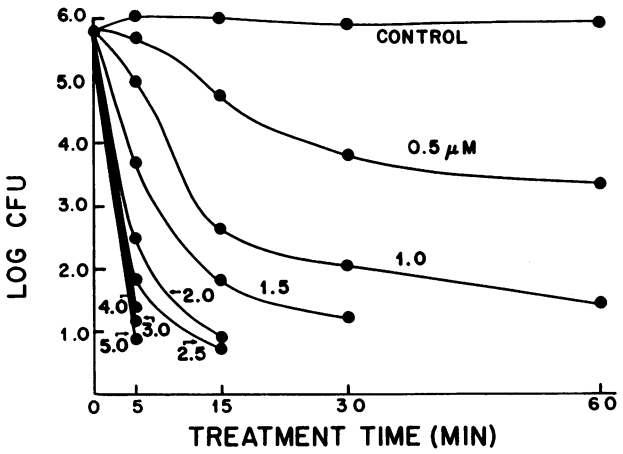

FIG. 2. Bactericidal activity of OCT against *Staphylococcus aureus* ATCC 6538. OCT was tested at concentrations from 0.5 to 5.0 μM. Samples labeled control were incubated without OCT.

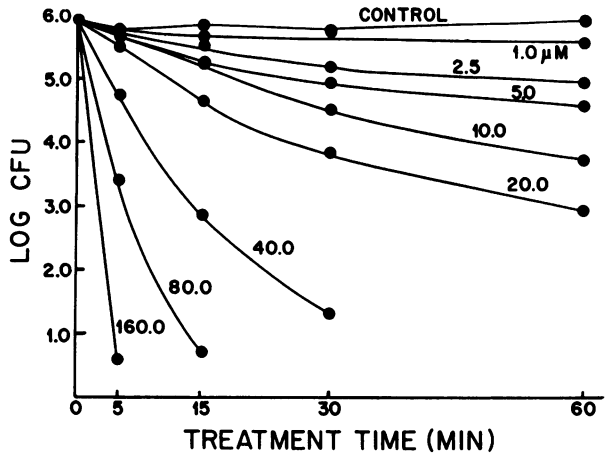

FIG. 3. Bactericidal activity of CHG against *Staphylococcus aureus* ATCC 6538. CHG was tested at concentrations from 1.0 to 160.0 μM. Samples labeled control were incubated without CHG.

Much higher levels of CHG were required for comparable microbicidal activity (Table 2). With most test organisms, a 40 μM concentration was necessary for a 99.999% reduction in viability; even at this concentration, such reductions were not attained with *Staphylococcus aureus*, *Streptococcus pyogenes*, and *C. albicans*. At 1.0 μM CHG, no culture population was reduced by more than 98%. Of the various organisms, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were the most susceptible to CHG; *Staphylococcus aureus* and *Proteus mirabilis* were the least susceptible. The activity of CHG against *C. albicans* was low.

Skin-degerming activity of OCT. The antimicrobial activity of OCT was maintained when applied to the skin of the hands and feet of cynomolgus monkeys (Table 3). Much higher concentrations of OCT were needed to achieve reductions in bacterial numbers comparable to those seen in vitro. Baseline resident microflora numbered approximately 10^7 CFU per appendage. Increased degerming activity was observed with increasing concentrations of OCT and increasing numbers of applications. The log microbial reduction increased proportionately with the log concentration of OCT. A 90% population reduction was observed after a single scrub with a 0.2% OCT solution, and a 97.5% reduction was obtained with a 1.6% solution. After four scrubs, reductions ranged from 99.6 to 99.98% over the various concentrations of OCT.

Studies with a prototype formulation showed that the skin-degerming activity of OCT could be maintained in the presence of surfactants (Table 4). While lower degerming values were observed for the formulated OCT than for aqueous preparations, the former exhibited significantly greater ($P < 0.05$) activity than either the vehicle or Hibiclens. Similar to the results with aqueous OCT, both

TABLE 2. Microbicidal activity of OCT and CHG

Microbial culture	Log inoculum[a]	Log reduction of inoculum after 15-min treatment with:					
		OCT (μM)			CHG (μM)		
		0.5	1.5	2.0	1.0	5.0	40.0
Staphylococcus aureus ATCC 6538	5.8	1.6	3.8	5.0	0.0	0.7	2.1
Staphylococcus epdiermidis ATCC 17917	5.5	3.2	>5.2	5.0	1.8	>5.2	>5.2
Streptococcus pyogenes ATCC 12384	5.9	2.2	>5.6	>5.1	0.7	2.7	3.4
Escherichia coli Vogel	6.2	1.1	2.1	>6.1	NC[b]	1.3	>6.1
Klebsiella pneumoniae SWRI no. 87	6.2	1.3	4.8	>5.0	0.4	2.6	>5.0
Serratia marcescens ATCC 8195	6.2	2.3	4.6	5.8	0.2	3.9	>5.9
Pseudomonas aeruginosa ATCC 9027	6.2	2.3	3.4	>5.8	1.6	4.0	>5.9
Proteus mirabilis MGH-1	6.3	1.2	2.9	>6.2	NC	0.3	4.3
Candida albicans ATCC 10231	4.2	0.9	2.1	>4.0	NC	1.2	2.7

[a] Values were calculated from zero time preneutralized samples.
[b] NC, No calculation due to missing values.

rapid and cumulative degerming activity was observed for the OCT and Hibiclens formulations. Both of these formulations were superior in this respect to the nonmedicated vehicle.

DISCUSSION

OCT was originally developed as a potential broad-spectrum topical antimicrobial agent. Earlier studies demonstrated its activity against dental plaque microflora (16, 20, 21); this study shows that OCT is also effective against skin microflora.

The current standard agent for measuring skin degerming activity, CHG, is fast-acting and has a broad antimicrobial spectrum (4, 14, 17). By using a very sensitive means of evaluating antimicrobial compounds (15), we showed not only that OCT effected rapid reductions in numbers of a broad spectrum of microorganisms but that OCT without exception was superior to CHG in activity. It was noteworthy that OCT effected measurable reductions in culture viability at very low concentrations and that slight changes in the concentration of OCT caused significant changes in microbicidal activity. Much larger changes in the concentration of CHG were required to evoke similar changes in activity.

In earlier studies, the MICs of OCT and CHG against a variety of test bacteria were quite similar (2, 20). In this study, the bactericidal activity of OCT was superior to that of CHG, especially against *Staphylococcus aureus* and *Streptococcus pyogenes*. Both OCT and CHG were most active against *Staphylococcus epidermidis* and least active against *C. albicans* (and *Staphylococcus aureus* for CHG). Differences in response among the genera tested are probably due to the mechanism of action of these two compounds as antimicrobial agents. CHG exerts its principle effect on the cytoplasmic membrane of the bacteria, destabilizing it and causing cell death (8). While studies have not yet been conducted to determine the mode of action of OCT, its chemical structure would indicate a mechanism of action similar to that of CHG. The cell envelope differences among the various microbial species tested could account for the differences in death rates measured for these microorganisms.

Since in earlier studies the MICs of OCT and CHG were essentially the same against a variety of microorganisms, the results of this study, showing consistently better microbicidal activity by OCT, were unexpected. This phenomenon could be due to the effect of time on the activity of the two compounds. In this study, antimicrobial activity was measured over a short time period, no longer than 60 min for the in vitro tests and 6 h for the animal tests. This is in contrast to a 48-h incubation for the MIC determinations reported in earlier studies. A greater rate of activity, possibly through greater permeation by OCT than by CHG through the cell envelope, could account for the differences in microbicidal activity seen in this study. It is also possible that prolonged incubations with these compounds could equalize their activity.

The in vitro studies were conducted at an incubation temperature of 30°C to simulate the apparent normal skin surface temperature of the test animals. For a random sample of nine anesthetized cynomolgus monkeys, temperatures ranged from 27.4 to 33.8°C and from 26.2 to 33.6°C on

TABLE 3. Skin-degerming activity of aqueous OCT

Measurement	Mean log reduction from skin microflora baseline after treatment with the following concn (%) of OCT[a]			
	0.2	0.4	0.8	1.6
Baseline	6.93 ± 0.08	6.90 ± 0.08	6.91 ± 0.08	6.85 ± 0.11
Baseline minus measurement after:				
Scrub 1	0.99 ± 0.08	1.27 ± 0.08	1.46 ± 0.10	1.61 ± 0.16
Scrub 2	1.44 ± 0.09	1.87 ± 0.13	1.98 ± 0.11	2.50 ± 0.15
Scrub 4	2.38 ± 0.14	2.85 ± 0.15	3.38 ± 0.15	3.71 ± 0.15

[a] Values represent mean of 36 samples ± standard error of the mean.

TABLE 4. Skin-degerming activity of OCT and CHG formulations compared with that of nonmedicated vehicle[a]

Measurement	Mean log reduction from skin microflora baseline after treatment with[b]:		
	2% OCT formulation	Hibiclens	Vehicle
Baseline	6.99 ± 0.11	6.97 ± 0.09	7.15 ± 0.13
Baseline minus measurement after:			
Scrub 1	1.11 ± 0.11	0.95 ± 0.10	0.77 ± 0.10
Scrub 2	1.70 ± 0.14	1.54 ± 0.15	1.21 ± 0.13
Scrub 4	2.72 ± 0.24	2.28 ± 0.21	1.66 ± 0.17

[a] Analysis of overall degerming activity by using Duncan's Multiple Range Test revealed that the result obtained with each treatment was significantly different ($P < 0.05$) from results obtained the other two.
[b] Values represent mean of 24 samples ± standard error of the mean.

hand and foot surfaces, respectively. These values agree very well with those of normal human skin (18). The skin temperature of these animals was not measured before anesthesia, but it is highly unlikely that the anesthetic had any effect on the skin degerming parameters measured, especially since this model has been shown to correlate well with results of tests with normal humans (5).

The amount of OCT required for a reduction of skin microflora was significantly greater than that observed in the in vitro studies. This could be due to the nature of skin as a substrate for microbial colonization and growth (9). The morphology and physiology of this organ is such that the resident microflora are sequestered in micropockets created by the dermatoglyphics of the epidermis. Very large numbers of organisms are present in hair, sebaceous follicles, cuticles, and other isolated regions where penetration of any skin degerming agent is difficult.

A comparison of the skin-degerming activity of the formulated OCT with those of the aqueous preparations suggests that the surfactants in the formulation may interfere with the activity of this compound. Such interference also occurs with CHG (3), which is why a level of 4% CHG is necessary for effective formulations. Although the exact nature of this interaction is unknown, it has been attributed with other agents to micelle formation (19).

This study demonstrated that OCT is a broad-spectrum antimicrobial agent which is an active biocide at low concentrations. The compound is bactericidal as well as candidicidal and is effective against resident skin microflora. The in vivo activity of OCT is rapid and cumulative over successive applications. Increasing concentrations of aqueous OCT to 1.6% caused a proportional increase in skin microflora reductions. Comparison of a 2% OCT formulation with Hibiclens demonstrated that at a somewhat lower drug concentration (32 mM for 2% OCT versus 45 mM for 4% CHG), the surfactant-based OCT formulation exhibited improved skin-degerming activity over the reference product. Based on the data generated in this report, it is possible that OCT formulated in a skin-cleansing vehicle might exhibit improved degerming efficacy over existing products when examined in humans.

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