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Residual Antimicrobial Effect of Chlorhexidine Digluconate and Octenidine Dihydrochloride on Reconstructed Human Epidermis

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Key Words

Antiseptics · Chlorhexidine digluconate · Octenidine dihydrochloride · *Pseudomonas aeruginosa* · *Staphylococcus aureus* · Reconstructed human epidermis · Residual antimicrobial effect

Abstract

The objective of the present investigation was to examine the residual antimicrobial activity after a topical exposure of reconstructed human epidermis (RHE) to equimolar solutions of either chlorhexidine digluconate (CHG, 0.144% w/v) or octenidine dihydrochloride (OCT, 0.1% w/v) for 15 min. RHE-associated antiseptic agents were more effective on *Staphylococcus aureus* than on *Pseudomonas aeruginosa*. *S. aureus* was not detected after 24 h of contact, which demonstrated a microbicidal efficacy of greater than 5-$\log_{10}$ reduction. In contrast, *P. aeruginosa* was reduced by approximately 2 $\log_{10}$ at the same incubation time, which parallels the growth of the initial inoculum. This result could be interpreted either as a microbiostatic effect or as an adherence of *P. aeruginosa* to a low positively charged surface. Small amounts of CHG and OCT can penetrate the stratum corneum. Using these antiseptic agents, the viability of keratinocytes was reduced to 65–75% of that of the untreated RHE control following 24 h incubation in the presence of test microorganisms. With consideration of antimicrobial activity and cytotoxic effect, OCT corresponds better to a biocompatible antiseptic agent than CHG.

Introduction

Reconstructed human epidermis (RHE) equivalents have been adapted as an in vitro alternative to replace animal experiments, especially in the field of evaluating cosmetics. RHE equivalents such as skin pharmacological models have been successfully applied to in vitro characterization of skin barrier functions [1–3], skin absorption processes [4, 5], drug metabolism [6] and corrosion/irritation predictions [7–10]. Validated models are commercially available as EpiSkin® (SkinEthic Laboratories, France), EpiDerm® (MatTek Cooperation, USA), and EST-1000 (Cell Systems, Germany) [11] but also in-house models have been utilized [12–14] for the assessment of in vitro skin corrosion of chemicals. Excellent reviews have been published to allow correct classification of corrosive chemicals and those likely to be severe irritants [10, 15, 16]. In vitro evaluations of skin sensitivity of antiseptic agents using RHE models are relatively rare [13], although these substances can dam-

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age basal keratinocytes after having been topically applied in excess. The main task of an antiseptic agent is to destroy or inhibit the growth of microorganisms in or on living tissue [17]. Cytotoxic effects on mammalian cells are usually accepted because the injured tissues can regenerate within a few days. However, prophylactic or therapeutic antiseptic treatment of skin might cause the antiseptic agent to adhere to the stratum corneum. The produced high density of cationic charges topically present may be responsible for a residual antimicrobial activity on skin similar to the one found after disinfecting hands with chlorhexidine-containing preparations [18]. On the other hand, superficially attached cationic, positively charged substances with low charge density may be responsible for a preferential adherence of the negatively charged bacteria.

Similar to surfactants, the antiseptic agent may be accountable for removing and damaging the protective barrier of the stratum corneum [19]. Thus, it can enhance the diffusion of the antiseptic agent into the epidermis, resulting in cytotoxic effects on keratinocytes.

Utilizing our in-house model of RHE, we started investigating a residual antimicrobial effect on the test microorganisms, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, after applying equimolar concentrations of either chlorhexidine digluconate (CHG) or octenidine dihydrochloride (OCT). Further, the resulting irritation potency was evaluated and the biocompatibility of the two antiseptic agents was deduced from assays of cytotoxicity and microbicidal activity.

Materials and Methods

Cell Culture

Normal adult human keratinocytes were purchased from PromoCell (C-12003, lot: 0012601.2) and for the reconstruction of the epidermis second- to third-passage proliferating keratinocytes were used. Keratinocyte growth medium KGM-2 (PromoCell, C-20011) was applied. It was completed with the attached supplement mix (PromoCell, C-39016) resulting in final concentrations of 0.004 ml/ml bovine pituitary extract, 0.125 ng/ml recombinant human epidermal growth factor, 5 μg/ml recombinant human insulin, 0.33 μg/ml hydrocortisone, 0.39 μg/ml epinephrine, 10 μg/ml human transferrin and 0.06 mM $CaCl_2$. Proliferating keratinocytes were harvested by trypsinization at a confluence of maximally 80–90% using a detach kit for human primary cells (PromoCell, C-41220).

Production of RHE and Validation

RHE equivalents were produced in 12-mm-diameter Millicell-PCF polycarbonate culture inserts (0.63 cm^2 growth area) with 0.4-μm-diameter pore size (Millipore, PIHP01250), essentially as described by Poumay et al. [14] with minor modifications. 150–200 μl EpiLife medium (Invitrogen, Cascade Biologics, M-EPI-500-CA), supplemented with HKGS (Invitrogen, Cascade Biologics, S-001-5) containing 3×10^5 keratinocytes were seeded into each culture insert, which had been placed into 6-well culture plates (TPP, Biochrom) prefilled with 1 ml of the same medium. After 24 h of incubation at 37°C in a humidified atmosphere with 5% CO_2 in air, the media in inserts were substituted by 0.5 ml of fresh medium, and simultaneously 0.5 ml of fresh medium was added into each well. The medium was changed the next day. On day 3 of culture, the keratinocytes were exposed to the air-liquid interface by removing the medium of the inserts. In the 6-well culture plates, 1.5 ml of EpiLife medium containing HKGS, 1.5 mM $CaCl_2$, and 50 μg/ml ascorbic acid were added to each well to feed the cells only from the bottom of the polycarbonate filter. The modified EpiLife medium was then changed every 2 days and the air-lift culture was continued for another 14 days to obtain culture models with characteristics of epidermis demonstrating the four different stratified layers [19]. All cell culture media were used without antibiotics to avoid possible interactions.

RHE samples were topically exposed to 1% Triton X-100 (positive control) and PBS (negative control) for 1–3 h. The required time of exposure to reduce the viability of keratinocytes to 50% of that of control was determined for each setup of RHE and amounts to 2.5–3 h.

Measurement of Residual Antimicrobial Effect

OCT (0.5 ml of 0.1%, 1.6 mM, Schülke & Mayr, lot: 1170700) or CHG (0.144%, 1.6 mM, Sigma, C9394) were applied topically to the RHE for 15 min at room temperature. Sterile water was used as negative control. After incubation, the control and antiseptic solution were completely removed and the tissue surface was washed 5 times with 0.5 ml of sterile balanced salt solution, each time interrupted by carefully drying the tissue surface using a sterile cotton tip swab.

The test microorganisms, *P. aeruginosa* (ATCC 15442) and *S. aureus* (ATCC 6538), were adjusted to 10^6 colony-forming units (CFU)/ml in Eagle minimal essential medium with Earle's salts with 10% fetal bovine serum; 0.5 ml bacterial dispersion was applied to the pretreated and washed tissue surface. Incubation up to 4 h was carried out in 24-well culture plates at room temperature in a humidified atmosphere. For 24 h of incubation the inserts were transferred back into 6-well plates containing culture medium. As an additional negative control, 1 ml of test microorganisms per well without RHE was used. Incubation was performed in two different test setups for each microorganism. Microbicidal effect was determined after either 15 or 30 min and either 15 min or 24 h for *Staphylococcus aureus*, and after either 2 or 4 h and either 2 or 24 h for *P. aeruginosa*. After the demanded incubation time, 0.1 ml of the incubation mix was transferred into 0.9 ml of the inactivator solution TSHC (3% tween 80, 3% saponin, 0.1% L-histidine and 0.1% L-cysteine) for *P. aeruginosa* or TSLS (4% tween 80, 3% saponin, 0.4% lecithin from soy bean and 1% sodium dodecyl sulfate) for *S. aureus*. The validation of the inactivation mixtures of either TSHC or TSLS on CHG and OCT was additionally verified in separate experiments. After 5 min inactivation, serial dilutions were prepared in the inactivator and 0.1 ml of each dilution was plated in triplicate on Trypticase soy agar. The CFU of the test microorganisms were counted after 48 h of incubation

at 37°C. The $\log_{10}$ microbicidal reduction value for each contact time was calculated according to the formula:

$$\log_{10} \text{ microbicidal reduction value} = \log_{10} n_c - \log_{10} n_d$$

where n_c is the number of CFU of viable cells in the inoculum after contact with the RHE treated with sterile water and n_d is the number CFU of viable cells in the inoculum after contact with the RHE treated with the antiseptic agent.

All experiments were repeated at least 3 times.

Measurement of Viability of Keratinocytes in RHE

After removing the test microorganisms the tissue inserts were rinsed from the inside with 5 × 0.5 ml sterile balanced salt solution, carefully dried with a sterile cotton tipped swab and transferred into 24-well culture plates containing 0.5 ml (0.5 mg/ml) preincubated MTT. The MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] tests [20] have been standardized for RHE [8]. Incubation was exactly for 3 h at 37°C. The MTT medium was gently removed and the RHE was rinsed at least 3 times with 2 ml PBS and dried carefully with cellulose swabs. Tissue inserts were embedded in 2 ml extractant solution (0.04 M HCl in propan-2-ol) for overnight incubation at 4°C in the dark in new 24-well plates. After extraction the inserts were pierced with the help of a scalpel and the solution was pipetted up and down until it appeared homogeneous. After the transfer of two 200-µl aliquots of the purple formazan solution into a 96-well flat-bottom microtiter plate, the optical density was measured at 560 nm against a blank (extractant) solution and calculated as percent tissue viability relative to the untreated negative control, which was defined as 100%.

Statistical Analysis

Data are expressed as mean ± standard deviation. Comparisons between two different groups were performed by the Mann-Whitney U test, and between more than two groups by nonparametric one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test using GraphPad software Prism 5.00 for Windows. Results were considered statistically different when $p < 0.05$.

Results

In-House RHE Model

RHE equivalents produced from normal adult human keratinocytes were used after 14 days of culture at the air-liquid interface. Histological examinations of hematoxylin and eosin-stained cryosections demonstrated a reproducible epidermis-like structure including a multilayered stratum corneum. The stratum corneum was sufficiently robust to resist the rapid penetration of the cytotoxic marker chemical Triton X-100. The recommended exposure time to reduce cell viability by 50% was greater than 2 h [21]. For the negative control OD values were in an acceptable range of 0.9–1.3 [22].

Measurement of Residual Antimicrobial Effect

The $\log_{10}$ bacterial reduction was assessed after topically applying 10^6 CFU of test microorganisms to the RHE models, which had been pretreated for 15 min with equimolar concentrations of either CHG or OCT. The results of the quantitative suspension tests for the different incubation times are represented in figure 1a–c for *P. aeruginosa* and in figure 2a, b for *S. aureus*. The inactivation mixture TSHC was effective for CHG and OCT in the presence of *P. aeruginosa* and TSLS inactivated both antiseptic agents in the presence of *S. aureus*. After 2 h of incubation, the $\log_{10}$ reduction was 0.3 for *P. aeruginosa* using OCT pretreated RHE, which was significantly different from that of the control and the CHG-RHE, which demonstrated low antimicrobial effect (fig. 1a). The $\log_{10}$ reduction of *P. aeruginosa* was increased from 0.8 after 4 h (fig. 1b) to 1.6 after 24 h (fig. 1c) incubation utilizing CHG pretreated RHE. In contrast, the antiseptic agent OCT was significantly more effective on *P. aeruginosa* than CHG. The $\log_{10}$ reduction of the OCT pretreated RHE was 1.3 after 4 h (fig. 1b) and 2.6 after 24 h (fig. 1c) incubation. Both antiseptically exposed RHE demonstrated approximately 2-$\log_{10}$ reduction in the presence of *P. aeruginosa,* which parallels with the increase of 2 $\log_{10}$ of test microorganisms in the control RHE. *S. aureus* is reduced more effectively by the antiseptically treated RHE than *P. aeruginosa*. The test microorganism *S. aureus* was not detectable after 24 h of incubation after contact with both CHG- and OCT-RHE, which is equal to a $\log_{10}$ bacterial reduction of more than 5.6 (not shown). After 15 min of contact, the $\log_{10}$ reduction was 1.2 for *S. aureus* utilizing CHG pretreated RHE, which was significantly different from that of 3.6 of OCT-RHE (fig. 2a). The $\log_{10}$ reduction of *S. aureus* was scaled up to either 3.3 for CHG-RHE or 4.2 for OCT-RHE after 4 h incubation (fig. 2b).

Measurement of Viability of Keratinocytes in RHE

Viability of keratinocytes in RHE was assessed immediately after testing the microbicidal effect for each experimental setup. The tissue inserts were subjected to MTT assays after thoroughly removing test microorganisms from the RHE. The cytotoxic effect was detected after either 4 or 24 h for *P. aeruginosa* (fig. 3) and either 30 min or 24 h for tests in the presence of *S. aureus* (fig. 4). Both after 4 and 24 h the viability of keratinocytes in the CHG- and OCT-treated RHE models was significantly reduced after testing the microbicidal effect on *P. aeruginosa* compared to the control group of unexposed RHE. In the presence of bacteria a significant cytotoxic effect was only evident after 24 h of contact with RHE water

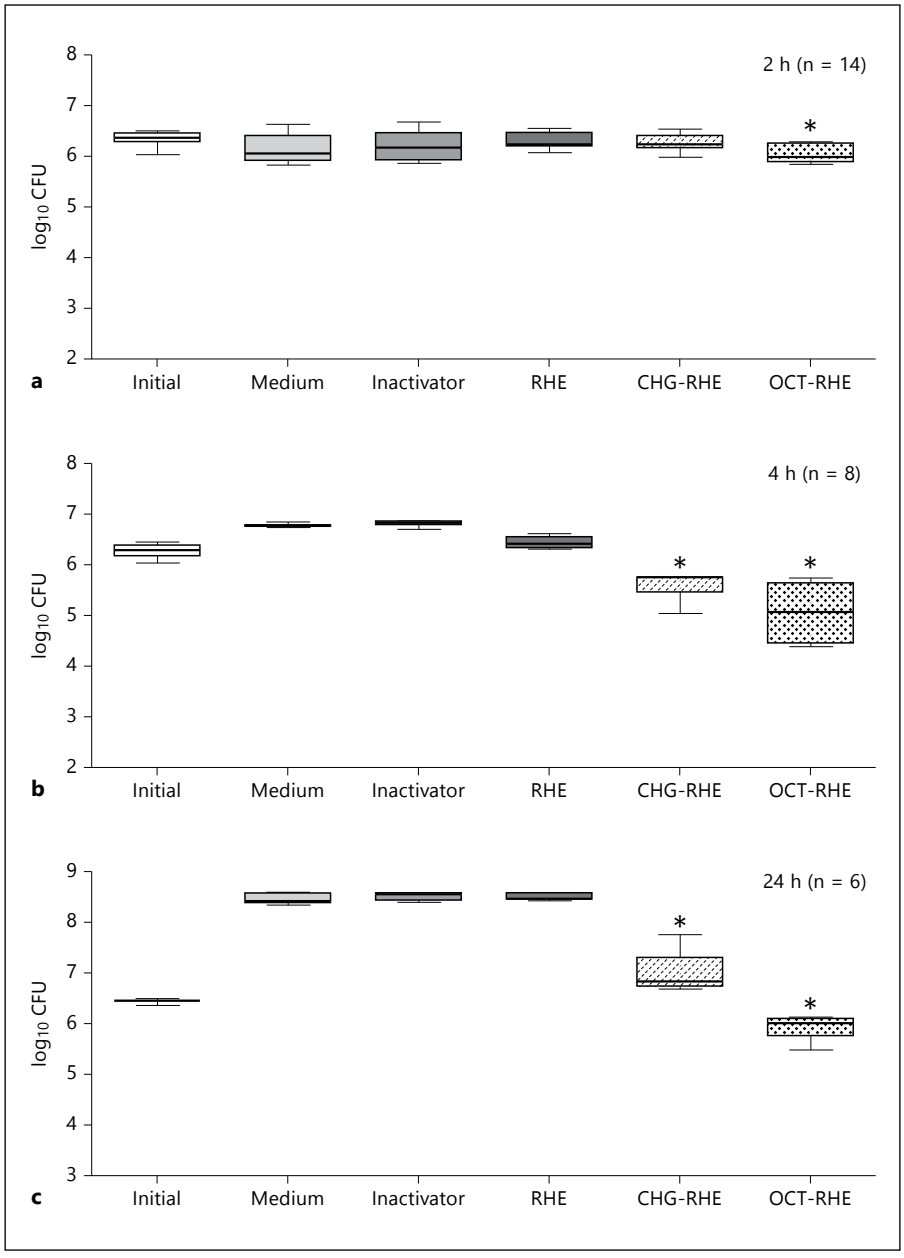

Fig. 1. Log_{10} CFU after 2 (**a**), 4 (**b**), and 24 h (**c**) incubation of *P. aeruginosa* topically applied on RHE equivalents, which had been exposed to water (RHE) or 0.16 mmol/l of either CHG (RHE-CHG) or OCT (RHE-OCT) for 15 min, compared with the initial inoculum and controls (medium and inactivator). * $p < 0.05$ compared to the control group (RHE).

models (viability of 83.5 ± 4.0%) and was enhanced after pretreatment of RHE with either CHG (viability of 71.8 ± 3.8%) or OCT (viability of 74.5 ± 4.5%) using the same incubation time with this test microorganism. After 4 h of contact with *P. aeruginosa* the viability of keratinocytes amounted to 91.8 ± 6.2% of that of the control and was significantly reduced by about 10% in either CHG-RHE equivalents (86.7 ± 12.1%) or OCT pretreated RHE models (87.6 ± 9.9%) compared with the control group (fig. 3). There was no cytotoxic effect evident assessing the viability of keratinocytes in RHE after testing the microbicidal effect for 30 min in the presence of *S. aureus*. After 24 h of contact with this microorganism the viability of keratinocytes was significantly decreased in RHE water models to 80.4 ± 3.2%, and also in RHE equivalents, which had been pretreated with either CHG (viability of 64.0 ± 0.9%) or OCT (viability of 67.1 ± 2.2%), compared to the control group of unexposed RHE (fig. 4). This cytotoxic effect is more notable in RHE equivalents following antiseptic pretreatment.

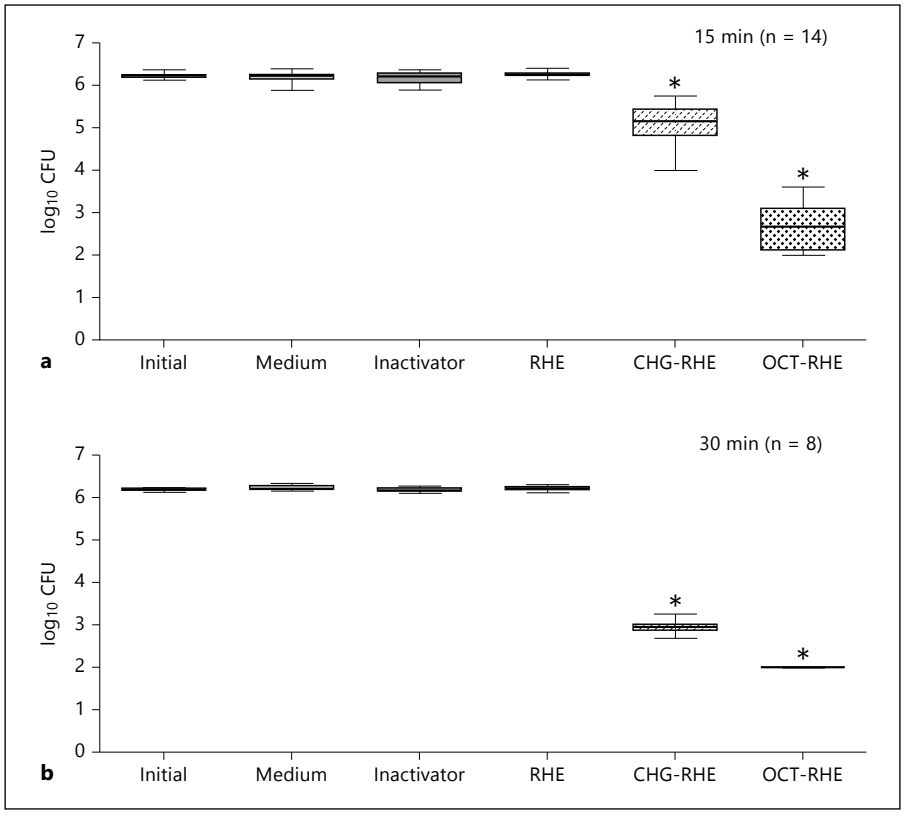

Fig. 2. Log_{10} CFU after 15 (**a**) and 30 min (**b**) incubation of *S. aureus* topically applied on RHE equivalents, which had been exposed to water (RHE) or 0.16 mmol/l of either CHG (RHE-CHG) or OCT (RHE-OCT) for 15 min, compared to the initial inoculum and controls (medium and inactivator). * $p < 0.05$ compared to the control group (RHE).

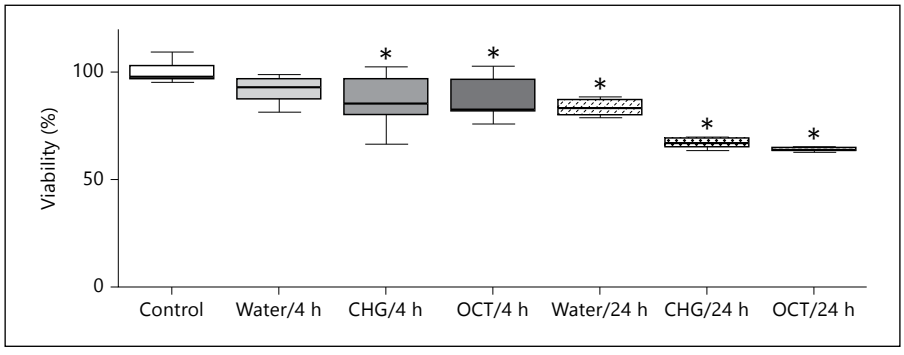

Fig. 3. Viability of keratinocytes in RHE equivalents, which had been exposed to water or 0.16 mmol/l of either CHG or OCT for 15 min, after testing microbicidal effect for either 4 h (n = 8) or 24 h (n = 6) with *P. aeruginosa* compared to that of unexposed negative control (RHE). * $p < 0.05$ compared to the control group.

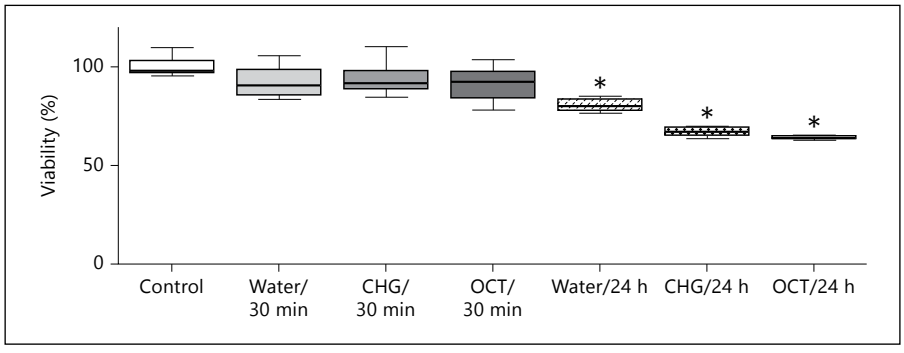

Fig. 4. Viability of keratinocytes in RHE equivalents, which had been exposed to water or 0.16 mmol/l of either CHG or OCT for 15 min, after testing microbicidal effect for either 30 min (n = 8) or 24 h (n = 6) with *S. aureus* compared to that of unexposed negative control (RHE). * $p < 0.05$ compared to the control group.

Discussion

RHE equivalents produced from normal adult human keratinocytes were used for the characterization of topical adherence of either CHG or OCT. These antiseptic agents are frequently used in the medical field, especially as skin antiseptic [23]. Both are cationic antimicrobial substances and bind strongly to the cell walls and membranes of bacteria because of their negative charge. The outer membrane is destabilized, leading to nonviable cells [24]. The cytotoxic effect of CHG and OCT is also produced by the interaction with lipophilic substances and phospholipids of human and mammalian cell membranes. Therefore, constituents of the cell envelope of corneocytes and lipids present in the stratum corneum of the epidermis provide potentially binding partners for CHG and OCT. Indeed, a residual antimicrobial activity was present after topical pretreatment of our in-house RHE models with these antiseptic agents (fig. 1a–c, 2a, b). This effect was suspected because earlier investigations on CHG [25–27] and OCT [28] demonstrated a residual activity on the skin. In addition, a residual antimicrobial effect was also evident in either CHG or OCT cell combinations using murine fibroblasts and human epithelial cells [29]. The microbicidal effect of RHE-associated OCT is superior to that of CHG comparing equimolar initial concentrations (fig. 1a–c, 2a, b). This result is supported by others comparing CHG and OCT in different antimicrobial tests [30, 31]. RHE-associated antiseptic agents are more effective on *S. aureus* than on *P. aeruginosa*. The Gram-positive test microorganism was not detected after 24 h of contact with the antiseptically pretreated surface of RHE equivalents. In contrast, the Gram-negative *P. aeruginosa* was only reduced by approximately 2 $\log_{10}$ at the same incubation time. In parallel, the initial inoculum was also increased by approximately 2 $\log_{10}$ (fig. 1c). These results can be interpreted either as a microbiostatic effect or as an attachment of *P. aeruginosa* to the surface of antiseptically pretreated RHE models. Antimicrobial substances immobilized to surfaces can kill bacteria upon contact. For an optimum efficiency of a biocidal cationic surface a charge-density threshold must be fulfilled [32, 33]. Too low positively charged stratum cornea may be responsible for the attraction and adherence of negatively charged bacteria, only resulting in low antimicrobial efficacy, which may be detected by a microbiostatic effect. It cannot be excluded that antiseptic treatment of the skin results immediately or at a later point in time in a low-density positively charged surface, which favors the adherence of negatively charged cellular material of bacteria. Low-charge density regions that might allow living bacterial cells to adhere can act as a basis which may propagate the growth of colonies on top of a monolayer of dead cells. This possibility should be further evaluated both in vitro and in vivo using normal skin. Coating polystyrene of cell culture plates with either CHG or OCT in a concentration range of between 5 and 50 μg/ml demonstrated that OCT prevented adherence of *S. aureus* following 48 h of contact but not that of *P. aeruginosa*. In contrast, CHG-coated polystyrene preferred the attachment of both bacteria. Coating concentrations ≥100 μg/ml of CHG prevented the attachment of *S. aureus* but not that of *P. aeruginosa* (unpublished data).

In general, it is recommended to incubate the RHE equivalents for a further 42 h after an exposure period of between 15 and 60 min [8, 22]. The test chemical is considered to be an irritant to the skin if the tissue viability after exposure and posttreatment incubation is ≤50%. In our experiments, the viability of keratinocytes in RHE equivalents was assessed immediately after testing the antimicrobial efficacy to prevent possibly microbial growth. In all tests, the tissue viability was greater than 50% (fig. 3, 4). After 30 min of contact with either *S. aureus* only or in combination with the antiseptically pretreated stratum corneum, the resulting tissue viability was not reduced, but a cytotoxic effect was evident after 24 h of exposure (fig. 3). This parallels with the results in the presence of *P. aeruginosa* (fig. 4). Pretreatment of RHE equivalents with either CHG or OCT increases the cytotoxicity after 24 h of incubation compared with that of the control with only bacteria, which may be the result of the penetration of the stratum corneum by small amounts of either CHG or OCT. Cytotoxicity of 0.5% CHG was also demonstrated using a three-dimensional skin model after 10–30 min exposure and 15–18 h posttreatment incubation [13]. Other investigations revealed minor concentrations of CHG [34] and OCT [35] in different layers of the skin. The study on CHG was performed using vertical diffusion cells to evaluate the delivery of the antiseptic agent through excised full-thickness human skin. It was demonstrated that the CHG permeation was not linear. The top layer of the skin (100 μm thick) including the stratum corneum (10–20 μm thick) and other epidermal layers (50–100 μm thick) contained the largest amount of the active agent following 30 min exposure to 2% CHG [34]. The estimated low CHG concentration may have produced the minor cytotoxic effect on keratinocytes in the RHE model in our study. Diffusion cell experiments were conducted over 28 h for 0.1% OCT using intact skin of different animals in vitro. Ap-

proximately 1–4 μg/cm^2 OCT was found in the stratum corneum and the dermis of all split skin samples [35]. These small amounts of OCT can produce cytotoxicity in RHE equivalents similar to that of CHG. However, it cannot be excluded that the viability of keratinocytes in antiseptically exposed RHE models will be either positively or negatively influenced by an elongated incubation period. However, both antiseptic agents demonstrated poor permeation into the deeper layers of the epidermis. Pretreatment of RHE equivalents with equimolar concentration of either CHG or OCT produces a similar tissue viability of 65–75%, which can be neglected.

To assess the suitability of an antiseptic agent, both the microbicidal activity and the cytotoxic effect must be taken into consideration to derive biocompatible antibacterial agents [36]. From the results of the presented study the microbicidal effect of OCT is superior to that of the RHE-associated CHG comparing the same applied molar concentrations of active agent. With simultaneous consideration of tissue cytotoxicity, OCT applies better to the properties of biocompatibility than CHG. However, further investigations to establish the proper application time for antisepsis are warranted.

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Disclosure Statement

The authors have no conflicts of interest that are directly relevant to the content of this study. One author (J.S.) is an employee of Schülke & Mayr GmbH and contributed to the design of the study.

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