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Comparison of the Antiseptic Efficacy of Tissue-Tolerable Plasma and an Octenidine Hydrochloride-Based Wound Antiseptic on Human Skin

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Key Words
Antiseptic efficacy · Plasma treatment in vivo · Octenidine dihydrochloride · 2-Phenoxyethanol · Skin barrier · Antioxidants

Abstract
Colonization and infection of wounds represent a major reason for the impairment of tissue repair. Recently, it has been reported that tissue-tolerable plasma (TTP) is highly efficient in the reduction of the bacterial load of the skin. In the present study, the antiseptic efficacy of TTP was compared to that of octenidine hydrochloride with 2-phenoxyethanol. Both antiseptic methods proved to be highly efficient. Cutaneous treatment of the skin with octenidine hydrochloride and 2-phenoxyethanol leads to a 99% elimination of the bacteria, and 74% elimination is achieved by TTP treatment. Technical challenges with an early prototype TTP device could be held responsible for the slightly reduced antiseptic properties of TTP, compared to a standard antiseptic solution, since the manual treatment of the skin surface with a small beam of the TTP device might have led to an incomplete coverage of the treated area.

Introduction

Today, a wide range of highly efficient topical antiseptics is in use, and new antiseptic substances especially for wound care are being continuously developed [1–4]. The first steps of wound bed preparation include wound debridement, in order to reduce the amount of necrotic tissue ('cellular burden'), and thereafter antiseptic measures to reduce the formation of biofilms within the wound ('bioburden') and the wound edge [5]. The spectrum of microbes within the wound environment entails a variety of bacteria and fungi, and the composition of the biofilm is steadily shifting [6, 7]. Modern antiseptics target a wide spectrum of bacteria and fungi exerting only minimal cytotoxic effects especially on the highly proliferating wound tissue [8]. Polihexanides and octenidine dihydrochloride meet these criteria to a certain extent [9] and are commonly used for wound treatment. In addition to chemical antiseptic treatments, physical antiseptic procedures such as tissue-tolerable plasma (TTP) are being evaluated for their benefit to wound care. TTP is loosely described as an electrically neutral medium of positive and negative

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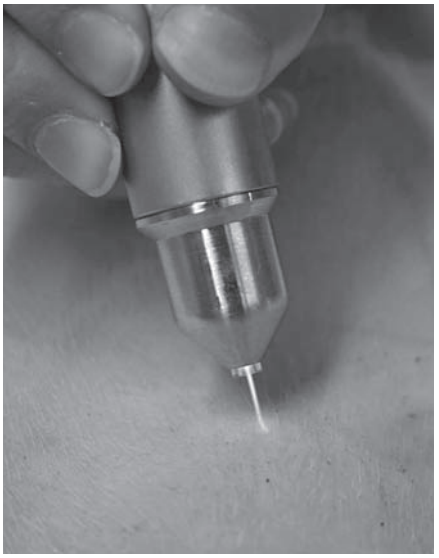

Fig. 1. Action of the plasma stream on the skin surface.

particles and represents another state of aggregation of matter next to the solid, liquid and gas state of matter [10–13]. Aside from these preliminary results of the usage of TTP for wound antisepsis [11, 14, 15], this technology is also thought to be applicable for antisepsis of intact skin [12, 13]. Recently, TTP produced by an electrical discharge in a plasma jet has been reported to efficiently reduce bacterial colonization on human skin [16]. However, these investigations were mainly performed in vitro using cell culture assays or tissue harvested from animals as a model [17]. The first in vivo study was published by Isbary et al. [15], investigating the effects of plasma treatment on lesions of patients suffering from Hailey-Hailey disease. Risk analysis of the application of TTP for antiseptic treatment in dermatology found skin damage by TTP-related UV radiation to be negligible. Moreover, thermal damage by TTP was minimal and related only to the first superficial cell layers of the stratum corneum [17]. Based on these first risk assessments, approval by the Ethics Committee had been obtained to use the TTP technology for an in vivo study on antiseptic efficacy. In the present study, the efficacy of a widely used antiseptic solution, a combination of octenidine dihydrochloride and 2-phenoxyethanol (Octenisept®), and the TTP was compared in vivo on healthy human skin. In addition, the influence of antiseptic treatment on the skin barrier and on the antioxidative network of the human skin was evaluated.

Subjects and Methods

Volunteers

For the investigations, 10 (6 male and 4 female) healthy volunteers aged between 26 and 42 years were recruited at the Department of Dermatology, Charité – Universitätsmedizin Berlin, Germany. The approval of the Ethics Committee to conduct the study had been obtained before the start of the investigations (EA1/010/10).

Antiseptic Treatments and Technical Equipment

In all experiments, an antiseptic containing 0.1% octenidine dihydrochloride and 2% 2-phenoxyethanol as active ingredients (Octenisept, Schuelke & Mayr GmbH, Norderstedt, Germany) was used.

Plasma Jet Pen

The low-temperature plasma used in the present study was generated by the plasma jet kinpen 09® developed by the Leibniz Institute for Plasma Science and Technology, Greifswald, Germany, in cooperation with Neoplas GmbH, Greifswald, Germany. A detailed description of the plasma jet is given by Weltmann et al. [16] and Hubner et al. [18]. The plasma jet consists of a base station including the power supply and the control unit, together with a handpiece containing the gas flow and the electrical discharge system. The discharge system was operated with argon, applied in the pulsed mode, with the pulses being generated at a frequency of 1.82 MHz.

The plasma stream had a length of approximately 10 mm; the plasma-tissue interaction zone was approximately 1 mm in diameter. A photo illustrating the action of the plasma stream on the skin surface is shown in figure 1.

Analysis of the Skin Barrier by in vivo Laser Scanning Microscopy

Lademann et al. [19] demonstrated that in vivo laser scanning microscopy (LSM) is well suited for the analysis of the barrier properties of the human skin. In the present study, the in vivo LSM Stratum (Optilas Ltd., Melbourne, Australia) was used for analyzing the structural changes of the uppermost layers of the skin – the stratum corneum –, which represents the skin barrier to the outer environment. A detailed description of the laser scanning microscope is provided by Meyer et al. [20]. The system consists of a base station and a handpiece. Accommodating the argon laser unit ($\lambda = 488$ nm), the power supply unit, the spectrometer and the control unit, the base is connected to the handpiece by optical fibers. The handpiece contains the optical imaging and focus control unit. The skin area analyzed by this LSM was 250 $\times$ 250 μm in size.

Determination of Antioxidants in the Human Skin

A resonance Raman spectrometer developed for the detection of carotenoids in the human skin was used to analyze the influence of the skin treatment on the antioxidative system of the human skin with both antiseptic methods [21]. The Raman system consisted of a base accommodating the Ar laser ($\lambda = 488$ nm) and the spectrometer with the detector unit. The base is connected by optical fibers to the handpiece containing the optical imaging system and the focal control unit. Using this system, the concentration of the carotenoids in the skin could be detected up to a depth

of 150 μm. The measuring spot on the skin was 8 mm in diameter. A detailed description of the measuring system is provided by Darvin et al. [22, 23].

Treatment of the Cutaneous Test Sites

The investigations were carried out on the right and left forearms of the volunteers. On each forearm two skin areas of 4 × 4 cm in size were marked. One of these two skin areas on each arm was left untreated, whilst the other area was treated with octenidine dihydrochloride/2-phenoxyethanol solution on one forearm and with TTP on the other. The application areas were randomized for each volunteer. Using the spray applicator, the octenidine dihydrochloride/2-phenoxyethanol solution was applied in the same manner as in the operating room for antiseptic treatment of the skin. The amount of octenidine dihydrochloride/2-phenoxyethanol solution applied was sufficient to ensure that the skin surface was completely and sufficiently covered. After 15 min, when the octenidine dihydrochloride/2-phenoxyethanol solution had completely evaporated, the bacterial colonization of the treated skin surface was investigated. The corresponding skin area on the other forearm was treated with TTP. For this purpose, the plasma beam was moved at an average velocity of 10 mm/s. This corresponds to the optimum moving velocity of the plasma previously reported [12]. In order to prevent residues of surviving bacteria on the untreated skin areas, the plasma beam was moved homogeneously over the entire skin area.

Harvesting and Culture of Bacteria

Immediately after the plasma treatment or 15 min after the treatment with octenidine dihydrochloride/2-phenoxyethanol solution, the amount of bacteria on the skin surface was determined in accordance with the protocol of Williamson and Kligman [24]. Using a sterile stainless-steel ring of 20 mm in diameter, 1 ml of a basic solution consisting of 50% of a phosphate buffer solution (Dulbecco's PBS; Laboratory GmbH, Graz, Austria) and 50% of egg yolk were applied onto each skin area, which were dropped inside the ring in order to neutralize the antiseptic efficacy of the octenidine dihydrochloride/2-phenoxyethanol solution. The neutralization was validated in separate tests according to EN 1040 [25]. The solution was distributed homogeneously inside the ring with a sterile applicator. The contact time of the basic solution with the tissue was 1 min. Subsequently, 0.5 ml of the supernatant was removed with a pipette and diluted with 4.5 ml basic solution (diluted solution 1:10). The solution was shaken for 30 s in a test tube shaker in order to obtain a homogeneous mixture. 0.5 ml of the solution was applied to an agar plate (Columbia Blood type, Merck AG, Darmstadt, Germany) for bacterial growth. The agar plates, used for the microbiological part of the study, contained a highly nutritious, general-purpose medium for the isolation and cultivation of nonfastidious and fastidious microorganisms of the Columbia Blood type.

The agar plate was kept in an incubator for 24 h at a temperature of 36°C. After 24 h, the agar plates were photographed and the numbers of colonies on each plate were determined by count and expressed as colony-forming units (CFU)/test field.

To correct for the interindividual changes in bacterial counts, the initial number of CFU on the untreated areas of the corresponding arms of each volunteer was standardized to 100%. The number of CFU determined after either skin antisepsis with octenidine dihydrochloride/2-phenoxyethanol solution or TTP was correlated to the respective initial value of the untreated skin, and the elimination of bacteria was expressed in percentage for each individual volunteer. Based on these values the overall elimination of bacteria and standard deviations were calculated for both treatments.

Statistics

For statistical analysis, mean values and standard deviations were calculated. For further statistical analysis, the software program SPSS® 18.0 was applied. The Wilcoxon test was utilized to compare the numbers of bacterial colonies after different treatment modes affording a significance $p < 0.05$.

Results

Antiseptic Efficacy

The number of bacterial CFU detected on the untreated skin test sites showed large interindividual differences from 16 to 601 CFU/test field. One volunteer exhibited an extremely high number of CFU and was therefore excluded from the study. The following results are related to the remaining 9 volunteers. On all test sites an average of 202.5 ± 174.2 CFU was found before either of the treatments. Because of the large interindividuality, the initial number of CFU on the untreated areas of the corresponding arms of each volunteer was standardized to 100%.

As demonstrated in figure 2, after treatment with octenidine dihydrochloride/2-phenoxyethanol solution, a 99 ± 1.4% elimination of CFU was observed, whereas after TTP treatment a 74.3 ± 24.8% bacterial reduction was detected. Both types of antiseptic procedures led to a significant reduction in the bacterial colonization ($p < 0.05$). Moreover, the differences between the groups treated with octenidine dihydrochloride/2-phenoxyethanol solution and TTP were significant ($p < 0.05$). It should be noted that the standard deviation calculated from the results in the test fields treated with octenidine dihydrochloride/2-phenoxyethanol solution was clearly smaller compared to the standard deviation in the TTP-treated test sites.

Influence on the Antioxidative Potential and Skin Barrier

The concentration of carotenoid antioxidants is given in arbitrary units prior to and after treatment with either octenidine dihydrochloride/2-phenoxyethanol solution or TTP. No differences in the carotenoid concentrations before and after treatment with either method were detected ($p > 0.05$; fig. 3).

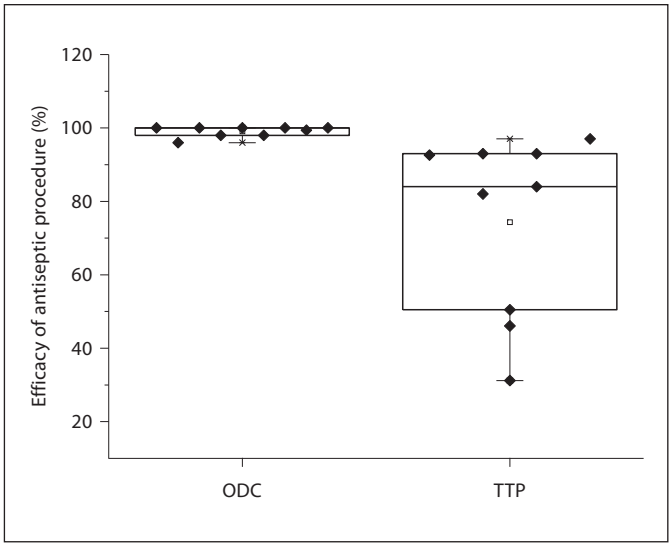

Fig. 2. Efficacy of the antisepsis after treatment with ODC (octenidine dihydrochloride/2-phenoxyethanol solution) and after TTP treatment.

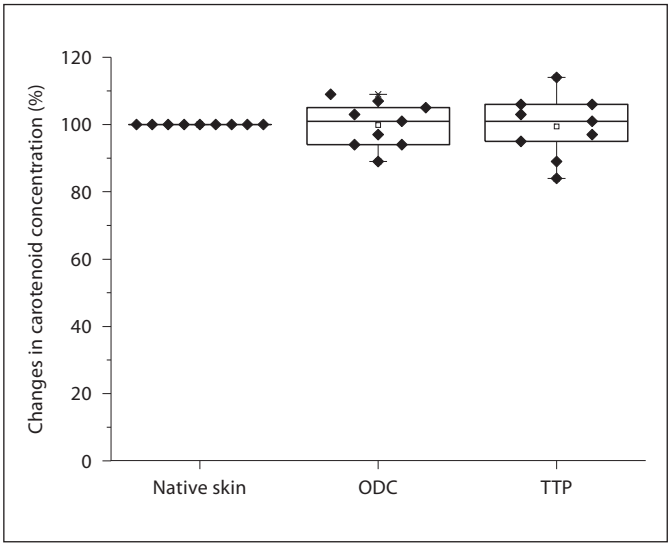

Fig. 3. Concentration of carotenoids measured by resonance Raman spectrometry prior to and after treatment using either TTP or ODC (octenidine dihydrochloride/2-phenoxyethanol).

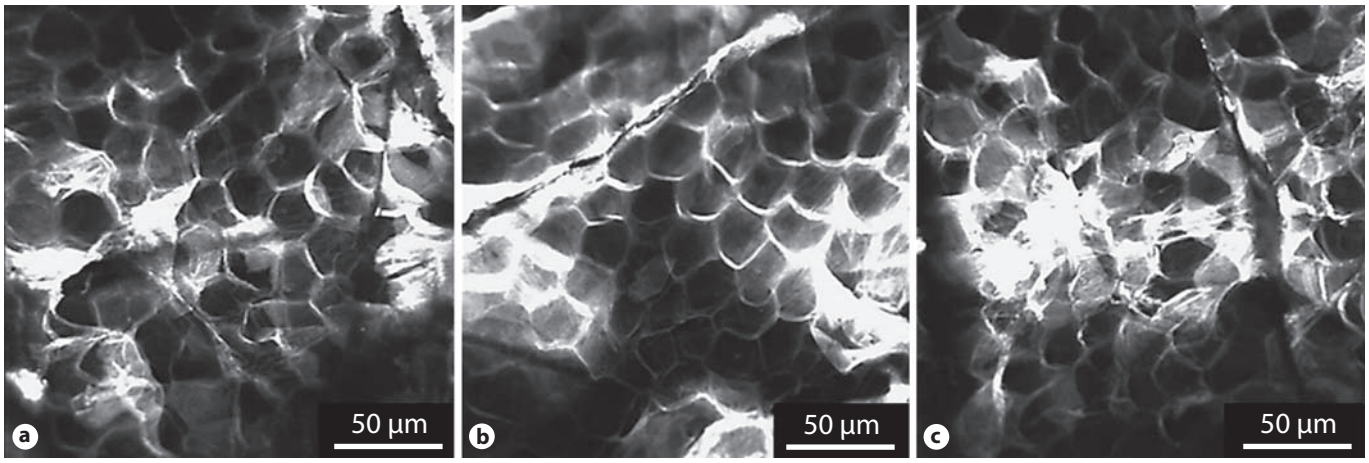

Fig. 4. Typical images of the skin surface analyzed by LSM: untreated skin (**a**); TTP treatment (**b**); octenidine dihydrochloride/2-phenoxyethanol solution treatment (**c**).

Investigation of the Epidermis and Upper Dermis Using LSM

In figure 4, typical images of the skin surface structure analyzed by LSM prior (fig. 4a) to and following TTP (fig. 4b) and octenidine dihydrochloride/2-phenoxyethanol solution (fig. 4c) application are presented. The images show a smooth skin surface with a clearly recognizable skin structure. A honeycomb corneocyte structure prior to and after both treatment methods, corresponding to an intact skin barrier, was found in the skin of all volunteers. No signs of inflammation such as dilated blood vessels, leukocyte rolling or inflammatory infiltrate within the epidermis were observed before and after both treatments. In none of the 9 volunteers could signs of skin damage be observed.

Discussion

With the increasing emergence of multidrug-resistant bacteria, the search for innovative antimicrobial strategies is most important. Apart from chemical antisepsis, physical antiseptic procedures seem to be promising tools for the antiseptic treatment of living tissue and material, since they might be able to eliminate bacteria in hair follicles [26]. Cold atmospheric plasma is a state of matter similar to gas in which a certain portion of the particles is ionized [13]. Due to the presence of a nonnegligible number of charged carriers, the plasma becomes electrically conductive; thus, it responds strongly to electromagnetic fields. It has been proposed that cold plasma is able to destroy bacteria by damaging the microbial DNA and surface structures without being harmful to human tissues [13, 15, 27].

The aim of our study was to evaluate whether the already hypothesized effect of TTP on bacterial growth in vitro could be reproduced in vivo and to compare the bactericidal efficacy of TTP to the clinically well-established antiseptic octenidine dihydrochloride/2-phenoxyethanol solution [28].

The results revealed that the number of bacterial colonies on the skin surface was strongly reduced by both methods of treatment. Complete elimination of bacteria from the test site by octenidine dihydrochloride/2-phenoxyethanol solution could be achieved in 5 out of 9 volunteers. For the 4 remaining volunteers, only small numbers of CFU per test field were detected after treatment. Inadequate antiseptic treatment, as well as a recolonization from the follicular orifices, has to be considered to be a reason for the incomplete elimination of bacteria from the skin.

TTP treatment can also provide a highly efficient antiseptic effect on the skin surface. The results are in agreement with the data presented in different studies, in which a significant reduction of the bacterial load after plasma treatment was reported [12, 14, 15, 17, 26]. Lademann et al. [17] suggested that the main reason for the antiseptic efficacy might be the formation of free radicals in the plasma-tissue interaction zone. Additionally, synergistic effects with the TTP-related UV irradiation and the slight temperature increase have to be discussed. Recently, the highly efficient antiseptic effect of the plasma on Gram-positive and Gram-negative bacteria, biofilm-producing bacteria, viruses, fungi and spores has been reported [10]. Advantages of plasma treatment are that bacteria cannot develop a resistance to the plasma [10] and that plasma treatment does not evoke allergies or toxic reactions as reported for conventional antiseptics, since plasma treatment is a physical antiseptic process.

Recently, it has been reported that plasma can also reduce the bacterial load in hair follicles and in this regard might actually be superior to liquid antiseptics. Lange-Asschenfeldt et al. [29] showed that hair follicles are an efficient reservoir for bacteria. Once successfully treated with local antiseptics, the skin surface is recolonized by bacteria and fungi, which are conveyed with the sebum onto the skin surface. Most topically applied liquid substances only reach the follicular orifices, but do not penetrate into the deeper regions of the hair follicle [19, 26, 30]. Consequently, the efficacy of plasma treatment should be superior or at least equal to that of liquid antiseptics. The results of the present study show, however, that plasma has a slightly reduced efficacy compared to octenidine dihydrochloride/2-phenoxyethanol solution.

One might speculate that one reason for this phenomenon may be the difficulty to scan a skin area of 4 × 4 cm manually with a thin plasma beam without leaving isolated 'islands' void of plasma-tissue interaction. This limitation for the plasma jet application can be easily overcome by enlarging the plasma beam to a diameter of 5–10 mm, or by the introduction of technical scanning devices as already used in therapeutic laser devices.

Fluhr et al. [31] demonstrated the formation of free radicals on the skin surface and in the hair follicles during the plasma treatment. Increased levels of free radicals normally lead to a decrease in antioxidants due to neutralization of the free radicals. In the present study, however, no changes in the carotenoid concentration, which is one of the major antioxidants in the skin, could be detected after treatment with both methods. This discrepancy can be explained by the fact that in the present study resonance Raman spectroscopic measurements were applied where a tissue volume of 8 mm in diameter and approximately 150 μm in depth was integrally analyzed. Thus, the measurements of the carotenoid concentration under the present study confirm that the radical formation is restricted exclusively to the skin surface and the uppermost layers of the stratum corneum, i.e. the effect of the plasma is restricted to those dermal structures, which accommodate the bacterial colonies.

Another indication for the tissue tolerability of TTP is that the properties of the skin barrier remain unchanged as demonstrated by the LSM analysis. In case of irritation of the skin by the antiseptic procedures, LSM features similar to acute dermatitis, including spongiosis, exocytosis, vesicle formation and blood vessel dilation as well as disruption of the stratum corneum and epidermal ne-

crosis [32], could be expected. However, in the present study no signs of alteration of the skin as studied by LSM were found after using both antiseptic procedures.

In summary, it can be stated that plasma treatment of the skin may be a promising alternative to skin antisepsis using liquid antiseptics. The lower efficacy of plasma treatment compared to the treatment with liquid antiseptics might be attributed to current technical limitations of the used prototypes that have already been overcome by newer devices. For locally restricted treatments (e.g. in dentistry) a plasma jet with a thin plasma beam may be particularly suited. However, large skin areas should be treated with plasma beams with larger diameters (preferably 5–10 mm) or scanning devices, which ensure complete coverage of the treated area.

The present study shows that TTP treatment is concentrating its efficacy on the uppermost layers of the stratum corneum and the first cell layers of the corneocytes without harming the treated skin.

An advantage of TTP treatment is that it obviously exerts antiseptic effects on the hair follicles, where liquid antiseptics show only limited efficacy. The investigations described herein are of preliminary nature. At the present time, the observed results cannot be transferred 1:1 to chronic wounds as the individuals included in the study were healthy volunteers. Therefore, further studies are necessary to evaluate the capabilities of TTP on skin diseases and wounds. Time will show whether TTP will gain comparable importance as in the case of medical laser technology today.

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Disclosure Statement

The authors declare no conflict of interest.

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