

In vivo skin treatment with tissue-tolerable plasma influences skin physiology and antioxidant profile in human stratum corneum

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Abstract: The antimicrobial treatment of wounds is still a major problem. Tissue-tolerable electrical plasma (TTP) is a new approach for topical microbial disinfection of the skin surface. The aim of the present study was to investigate the influence of TTP on a carotenoid profile in relation to skin physiology parameters (epidermal barrier function, stratum corneum (SC) hydration, surface temperature and irritation parameters). We were interested in the interaction of TTP and the antioxidative network, as well as the consequences for skin physiology parameters. These parameters are also indicative of TTP safety *in vivo*. For plasma application, 'Kinpen 09' was used (surface exposure 30–43°C) for 3 s. Beta-carotene and water profiles were assessed by *in vivo* Raman microspectroscopy (skin composition analyzer 3510). Skin physiology parameters were measured with Tewameter TM 300, Corneometer CM 825, skin thermometer and Chromameter CR 300. All parameters were assessed non-

invasively on seven healthy volunteers before and after plasma application *in vivo*. We could show that TTP application leads to a decrease in beta-carotene especially in the superficial SC. Skin-surface temperature increased by 1.74°C, while the transepidermal water loss (TEWL) increase indicated an impaired barrier function. SC hydration decreased as seen in water profile especially in the superficial layers and capacitance values. A slight increase in skin redness was measurable. The induction of reactive oxygen species is probably the major contributor of TTP efficacy in skin disinfection. Skin physiology parameters were influenced without damaging the skin or skin functions, indicating the safety of TTP under *in vivo* conditions.

Key words: cold plasma jet – *in vivo* raman microspectroscopy – antioxidants – transepidermal water loss – hydration – disinfection

Accepted for publication 4 November 2011

What is already known about this topic?

The tissue-tolerable electrical plasma (TTP) is a new approach for topical treatment of the skin surface, for example, for microbial disinfection of the skin surface.

What does this study add?

The decrease in beta-carotene as a function of increased free radicals induced by TTP is probably the major contributor of TTP efficacy in skin disinfection. Skin physiology parameters were influenced by plasma application, but not to an extent that would lead to damage to the skin or skin functions. Thus, skin physiology parameters indicate the safety of TTP under *in vivo* conditions.

Introduction

The physiology and antiseptical care of wounds is still a clinical challenge (1–3). The treatment of the wounds could become ineffective, because of deep-bedded infections and the limited penetration depth of topically applied antiseptics into the wound tissue (4,5).

Plasma is defined as ionized gas, and cold plasma is a plasma at a temperature that can be applied to the skin surface *in vivo*. The plasma jet used in our study is generated at normal pressure under ambient temperatures and moderate operating voltages. The tissue-tolerable electrical plasma (TTP) is a new approach for topical treatment of the skin surface (6,7). Recently, it has been reported that TTP can be used under *in vitro* conditions for an effective disinfection of the human skin surface (8). So far, TPP

has been studied in wound therapy, in comparison with two wound antiseptics on artificial bacterially contaminated eyes of commercially slaughtered pigs: its action against *Pseudomonas aeruginosa* biofilms grown on polystyrene and silicone materials could be demonstrated (6,9).

The mechanisms of action of TTP on the skin have not yet been studied. In principle, three different effects could be responsible, acting separately or in combination inducing a highly efficient disinfection: the primary effect is the increase in temperature of the plasma–tissue interaction zone. In multiple studies, it could be demonstrated that this increase in temperature is low, resulting in a surface temperature between 30 and 43°C. The thermal damage of the skin could be detected only in the first upper layers of corneocytes in the stratum corneum (SC), but not in the epidermis (8). The second effect, which must be considered in the plasma action mechanism, is the production of UV radiation during plasma emission. The spectral characteristics of the plasma emission depend on the composition of the applied gas stream, in which the charge takes place on the electrical discharge system. Depending on these parameters, UV radiation in the UVB, UVA and under special conditions, also in the UVC, can be produced (10,11). In the risk assessment (12) for the application of the plasma jet used in the present study, it could be demonstrated that the UVB radiation occurs at 310 nm, which is related to the OH radical formation (13).

The UV dose, to which the skin is exposed during plasma treatment, is significantly lower than the dose produced over a 10-min period of sunbathing during the summer months (12). Nevertheless, the high efficacy of disinfection could be demonstrated for plasma treatment under these conditions (4,14,15).

The third effect, which could be responsible for the efficiency of the disinfection, is the formation of free radicals in the plasma discharge and, consequently, in the plasma–tissue interaction zone.

The major part of the effect is based on free radical transfer onto the upper part of the SC, as only a minor part is related of the direct photon–skin interaction (16–18). This effect was investigated in the present study.

In previous experiments, it could be demonstrated that the carotenoids in the human skin could represent valid marker substances for the complete antioxidative network of the human organism (19,20), because of the high antioxidative activity in the reactions of neutralization of free radicals (21–23).

Recently, it was reported that carotenoids are non-invasively detectable under *in vivo* conditions in the human skin by means of Raman microspectroscopy (24,25). The investigations in the present study were performed under *in vivo* conditions, as the radical formation *in vitro* (e.g. excised human skin) is strongly reduced under equal conditions, on account of the reduced oxygen concentration in dead tissue. Oxygen is essential for the radical formation of the tissue, because the first cascade of produced radicals consists of reactive oxygen species (26,27).

It is known that the influence of exogenous stress factors on human skin, such as environmental pollutants and sun irradiation, produces free radicals (28–30) and subsequently depletes the cutaneous antioxidative network *in vivo* (31,32). Non-invasive methods to assess skin physiology parameters have been used in clinical studies, specifically in efficacy testing of antioxidants (33–36).

The aim of the present study was to investigate the influence of TTP on the carotenoid profile assessed non-invasively by Raman spectroscopy, and skin physiological parameters, such as epidermal barrier function, SC hydration, surface temperature and surface pH.

Material and methods

Plasma jet

In the present study, a plasma jet was utilized, which had been developed at the Institute of Plasma Physics, Greifswald (Greifswald, Germany) manufactured and commercialized as ‘Kinpen09®’ (Neoplas GmbH, Greifswald, Germany). The compact, miniaturized, atmospheric plasma jet represents an innovative technology with possible application for different skin-surface treatments. There are two specific features of the plasma jet: (i) the handpiece, a small-sized and lightweight unit for plasma generation allowing fast and almost free 3D motions. This makes the plasma jet applicable to any anatomical location of the human skin; and (ii) the contracted and relatively cold plasma jet allows focused small-spot treatments, even in heat-sensitive objects, such as tissue and cells with temperature surface exposure between 30 and 43°C. The plasma is therefore also termed tissue-tolerable plasma (TTP). The device ‘Kinpen09®’ has passed CE certification (electromagnetic compatibility), thus fulfilling the standards for electrical safety in humans (CE number 609.003.1). Argon gas was used as a dis-

charge medium in the plasma jet. The argon gas jet carries free radicals that are produced inside the device by means of an electrical discharge.

The emission spectrum of the applied plasma device consists of a band at a high intensity of 310 nm and a series of significantly lower intensity bands in a spectral range between 330 and 450 nm. The risk assessment of the application of the plasma jet in dermatology has recently been published by Lademann et al. (12).

Measurements of physiological parameters in human skin

Non-invasive assessment of epidermal barrier function was performed using a Tewameter TM 300 attached to a central unit MPA 9 and a standard PC. Stratum corneum (SC) hydration was measured with the capacitance-based Corneometer CM 825. The skin-surface temperature was measured with a contact-free skin thermometer. The above-listed probes were attached to a central MPA 9 unit and linked for data storage to a PC (all biophysical instruments were purchased from Courage & Khazaka, Cologne, Germany). Skin colour was assessed utilizing the Chromameter CR 300 (Minolta, Munich, Germany). The published guidelines on transepidermal water loss (TWEL), SC hydration, surface pH and skin colour were taken into account (37–40).

In vivo confocal Raman microspectroscopy

Confocal Raman microscopic (CRM) measurements were performed using the skin composition analyzer (Model 3510), designed for *in vivo* skin measurements (River Diagnostics, Rotterdam, the Netherlands). The CRM has been granted a CE guarantee. The axial spatial resolution is 5 µm, the laser excitation wavelength is set at 785 nm, and the measurement stage includes confocal sampling optics. All measurements were performed on the volar forearm of the volunteers. The test area was placed on a fused silica window mounted during the measurement stage. Laser light was focused onto the skin with a microscope objective located under the window. The location of the laser focus relative to the skin surface could be accurately and automatically adjusted at variable depths. Raman fingerprint spectra (400–1800/cm) were recorded from the skin surface down to a depth of 24 µm (35 µm for water profiles), in a 2-µm steps. In this way, detailed Raman profiles were acquired across the SC. The measurement time for one spectrum was 10 s. Relative carotenoid concentrations were calculated from the Raman profiles, following the method described by Caspers et al. (24,25). Several reference spectra of the major skin constituents were fitted to the Raman spectra of the volar forearm. To correct for variations in the absolute Raman intensity, which decreases at a greater depth distance to the skin surface, the fit coefficients were normalized for the Raman signal of keratin, which is the dominant dry mass fraction in the SC. The procedure resulted in the local relative concentration profiles of carotenoids in the SC, relative to the amount of keratin (41,42). The carotenoid concentration in the skin was measured at baseline and after the application of the plasma jet. In one volunteer, a follow-up was performed 24 and 48 h after plasma application. All measurements were carried out on the same skin areas (volar forearm).

Study design

A definite area of 16 cm² was marked on the forearms of the volunteers. The biophysical parameters were determined on the skin before plasma treatment at baseline values. All measurements were

performed after 30 min of adaptation to the room conditions (average room temperature $20.7 \pm 0.3^\circ\text{C}$; average relative humidity $49.0 \pm 0.1\%$). Two TEWL values and three Corneometer, pH-meter and Chromameter values were recorded. The plasma treatment was carried out at an average moving velocity of the plasma stream on the skin surface of approximately 10 mm/s. The TEWL measurements were performed immediately after plasma treatment, followed by the hydration and at least the colour measurements. All biophysical measurements were completed 5 min after plasma treatment.

Volunteers

The investigations were performed on seven healthy volunteers (four males, three females), aged between 27 and 53 years; mean 42.4 years (range: male: 27–53 years; females: 34–49 years) with skin type II or III according to the Fitzpatrick classification (43). All measurements were performed on the volar forearm of the volunteers. Permission for the investigations had been granted by the Ethics Committee of the Charité University Hospital. The volunteers signed a written informed consent prior to the start of the study. Before the first measurements, the skin surface was carefully cleaned with a dry paper towel.

Statistics

Statistical analyses were performed using Prism 3 software (Graph Pad Software Inc., San Diego, CA, USA). Normal distributions were tested before calculating a paired *t*-test. Values are given as mean \pm standard deviation (SD). A statistically significant difference was set at $P < 0.05$ (*). *P*-values < 0.01 were labelled as (**) and $P < 0.001$ as (***)

Results

Clinical observation

The volunteers reported a mild sensation of pain comparable to a superficial needle puncture during the plasma application, which subsided within 1 min after the end of the treatment. In several volunteers, mild erythema was observed temporarily, occurring approximately 60 s after the end of the TTP treatment, which normalized within a short period of time.

Biophysical measurements

After plasma treatment, the TEWL values of the volunteers increased in an average of more than 50% (Fig. 1). The distribution of the water content in the SC is demonstrated in Fig. 2: After plasma treatment, the water concentration was reduced in the different cell layers down to a depth of approximately $24 \mu\text{m}$. The highest reduction was again observed on the skin surface, where on average a 30% reduction of the original water concentration values could be detected after plasma treatment by Raman microspectroscopy (Fig. 2). The reduction was detectable down to a depth of $25 \mu\text{m}$. In deeper parts of the SC, water content did not differ before and after TTP application. These measurements were confirmed by the SC hydration measurements using the Corneometer. The capacitance was significantly ($P < 0.0001$) reduced from $46.4 (\pm 6.5)$ to $34.4 (\pm 4.8 \text{ SD})$. The average skin temperature increased significantly ($P < 0.0001$) from $28.4 (\pm 0.9 \text{ SD})$ to $30.2^\circ\text{C} (\pm 1.0 \text{ SD})$. The skin colour was assessed by colorimetric measurements for redness, for example, *a**-value increased from $8.83 (\pm 1.86 \text{ SD})$ to $9.79 (\pm 1.96 \text{ SD})$ without reaching significance ($P > 0.05$). *L**-values (white-black axis) remained unchanged ($P > 0.05$) before $66.6 (\pm 1.51 \text{ SD})$ and after treatment $66.23 (\pm 1.65 \text{ SD})$.

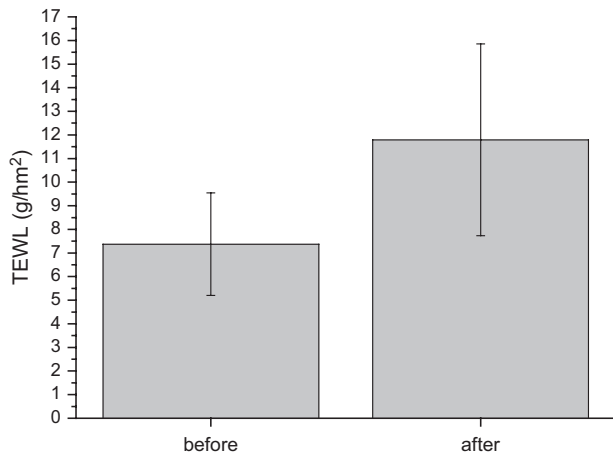


Figure 1. Transepidermal water loss before and after the application of the plasma jet on human skin assessed with a Tewameter TM 300. The values are expressed as mean \pm SD; $n = 7$.

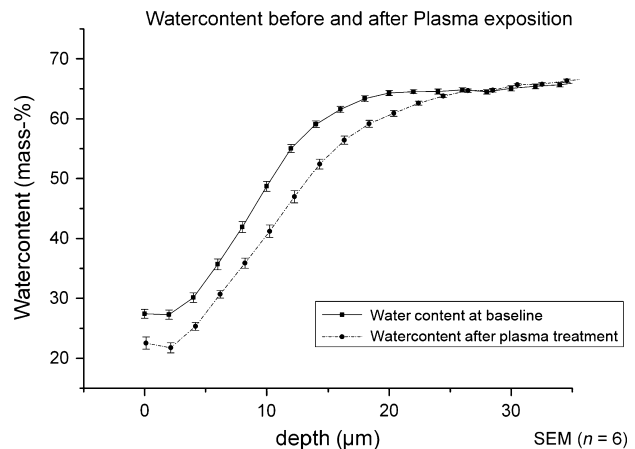


Figure 2. Water profile analysed in vivo by confocal Raman microscopy. The values are expressed as mean \pm SD; $n = 7$.

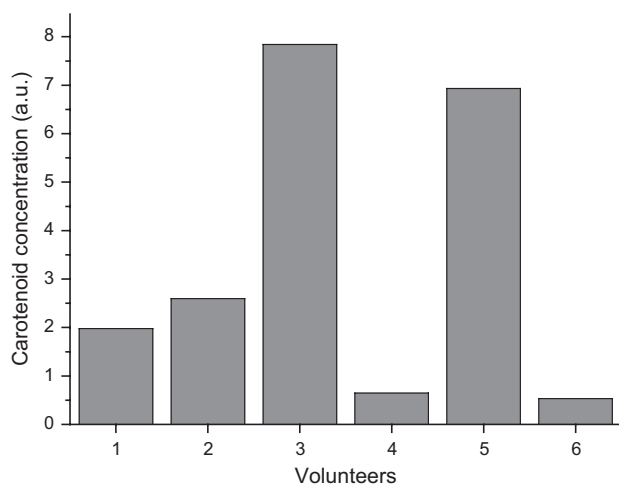


Figure 3. Baseline concentration of the superficial carotenoid levels of all volunteers. Measurements were performed on the volar forearm with the use of confocal Raman microscopy.

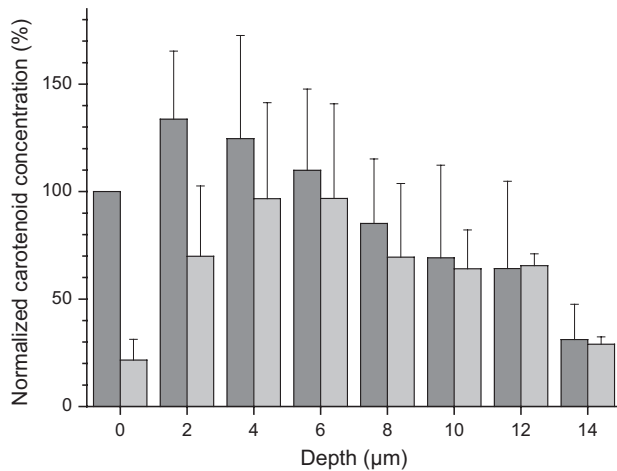


Figure 4. Carotenoid concentration in different depths of the stratum corneum before (dark grey column) and after (light grey column) plasma jet treatment. Measurements were performed on the volar forearm with the use of confocal Raman microscopy. The value at the surface on the untreated site was set as 100%, and the corresponding depths of treated and untreated sites were calculated accordingly.

Carotenoid profile

The basal carotenoid concentration in the SC of the six volunteers is demonstrated in Fig. 3. Different volunteers showed different initial carotenoid concentrations in the uppermost part of the SC. For better comparison of the results of the treatment effect, the baseline carotenoid concentration (before treatment) was normalized to 100%. In Fig. 4, the carotenoid concentration in different depths of the SC, before and after plasma treatment, is shown. The carotenoid level was markedly reduced in the upper part of the SC. This reduction is considerably stronger in the upper than in the lower part of the SC (0–18 µm). On the skin surface, the reduction is observed at approximately 70%.

Discussion

Recently, it could be demonstrated that the thermal damage after tissue-tolerable plasma (TTP) treatment of the skin occurs only to a minimal degree in the upper cell layers of the SC (12). However, the thermal effect does not lead to irreversible damage. In deeper parts of the SC, in the dermis and epidermis, no cell damage could be observed as assessed by *in vivo* laser scanning microscopy. The superficial damage appears to be the reason for the increase in TEWL values, occurring subsequent to plasma treatment (Fig. 1). The consequences of a TTP treatment are in accordance with the reported enhanced penetration of topically applied substances after plasma treatment of the skin (44). These substances penetrate highly efficiently through the skin barrier, reaching the epidermis and dermis. An acute penetration enhancement of topically applied substances is mostly associated with a damaged skin barrier.

In Fig. 2, it can be seen that the water content in the SC is reduced after plasma treatment, in particular in the upper skin layers. The Raman microspectroscopic measurements presented in Fig. 2 are confirmed by the capacitance-based Corneometry measurements, concerning the hydration of the upper part of the skin. This integral value demonstrated that the water content in the skin was reduced after plasma treatment. The decrease in SC

hydration especially in the upper part is in agreement with the findings of Lademann et al., (8,12) stating that the thermal damage after plasma treatment is located only in the upper layers of the corneocytes in the skin barrier. The evaporation of the water in the skin during plasma treatment as a function of increased skin-surface temperature could be excluded, as the measured surface temperature only increased by 1.8°C after plasma treatment, which is clinically irrelevant (from 28.4 to 30.2°C). The reduced water content is probably because of the secondary effects inducing a temporary water evaporation from the superficial SC by TTP as a slight increase in TEWL values could be observed (as demonstrated in Fig. 1). Furthermore, the reduction of carotenoid concentration leads to an increase in free radicals that in consequence might have a negative influence on barrier-related lipids and the natural moisturizing factor (NMF).

Recently, it was demonstrated that Raman microspectroscopy is suited for the determination of carotenoid levels in different layers of the human SC (41). This non-invasive measurement technique, used in the present study for the analysis of carotenoid profiles in superficial skin layers, showed a variable carotenoid content in different individuals. This finding is in agreement with the results reported by Darvin et al., (19) who stated that the carotenoid concentration in the skin reflects the individual lifestyle of the volunteers. To achieve a more stringent analysis of the carotenoid concentration after plasma treatment in different depths of the SC, the initial carotenoid concentration of the volunteers was standardized to 100%. The highest reduction in carotenoid level was observed in the superficial skin layers (Fig. 4). The initial carotenoid concentration in the superficial SC layers was almost reduced down to 70%. In accordance with the findings of Darvin et al., (31) a reduction in cutaneous carotenoids is caused by an interaction with high amounts of free radicals, produced by an external source, for example, during UV irradiation caused by plasma treatment. An actual carotenoid reduction occurs when free radicals increase over a critical level, whereby the natural antioxidant network is no longer able to neutralize the free radicals (45–47). Consequently, the amount of radicals produced by the plasma treatment in the skin, especially in the upper layers of the SC, must be higher than this critical value. As the detected carotenoid concentration decreases after plasma application, it can be assumed that the free radical induction is sufficient enough to destroy, not only carotenoids in the lipid compartments, but can also destroy bacteria (6) and fungi located on the skin surface and in the upper layers of the SC.

In analogy to the changes in the carotenoid concentration, the SC lipid structure is also altered, as recently demonstrated by Lademann et al., (44) showing that topically applied substances penetrate better through the skin barrier following plasma treatment. In the present study, it could be established that the barrier integrity was impaired by plasma treatment, resulting in an increase in TEWL, as well as a reduction in SC hydration and a reduction in the antioxidative network of SC.

It should be taken into consideration that obtained degradation of superficial carotenoids could be re-established by the use of a carotenoid-rich supplementation (48–50). The recovery time is dependent on the influenced stress factor and its intensity, which usually lasts for 1–3 days before levelling (31,49). This parameter is an individual characteristic, which depends on the carotenoid

reserves of the organism and on the nutrition. Moreover, it could be expected that superficial carotenoids will be recovered from outside to inside probably delivering by sweat and sebaceous glands (51).

In summary, the results of this study and recently published data showed that the main process for the high efficacy of the plasma treatment in skin disinfection is based on the production of free radicals. The data of this study could further demonstrate that skin physiological parameters including barrier function, SC hydration, skin temperature and carotenoid level concentration were influenced by plasma application, but not to an extent that would lead to damage to the skin or skin functions. In consequence, TTP could be demonstrated as safe in regard to skin physiology under clinical conditions.

Acknowledgements

We would like to thank the Foundation 'Skin Physiology' of the Donor Association for German Science and Humanities for financial support. The

authors also acknowledge that this work was supported by a grant funded by the German Ministry of Education and Research (BMBF, Grant No, 13N9779).

Joachim W. Fluhr was an employee of Bioskin® at the time when the study was performed. SS, OL, SS, HR, MED and JWF performed the research; JF, MED, AK, WS and JL designed the research study; SS, JF, JL and MED analysed the data; JWF, MED, WS and JL wrote the paper.

Funding sources

We would like to thank the Foundation 'Skin Physiology' of the Donor Association for German Science and Humanities for financial support. The authors also acknowledge that this work was supported by a grant funded by the German Ministry of Education and Research (BMBF, Grant No, 13N9779).

Conflict of interests

Joachim W. Fluhr was an employee of Bioskin® at the time when the study was performed.

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