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Risk assessment of the application of tissue-tolerable plasma on human skin

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ABSTRACT

The results of the risk assessment of the tissue-tolerable plasma (TTP) jet kINPen med® and first results of pilot clinical studies are presented. Producing an atmospheric pressure plasma, this plasma jet entails no risk for humans in terms of temperature increase, UV radiation or free radical formation by the plasma. The antiseptic efficacy *in vitro* on porcine skin and *in vivo* on human skin was compared to that of octenidine. TTP could significantly reduce the bacterial load in comparison to untreated skin. However, the slightly reduced antiseptic properties of TTP are attributed to the current parameter set-up and technical limitations.

Contents

1. Introduction

The reduction of microbes on healthy skin prior to surgical procedures as well as on acute and chronic wounds plays an important role in modern medicine.

The ideal antiseptic procedure rapidly targets a wide spectrum of microbes without exerting cytotoxic effects on the living cells of the organism. While this article focuses on the risk assessment of TTP on intact skin, the investigation of the effects of TTP on wounds will be a consequence of the current studies since impaired wound healing represents an increasing problem in everyday clinical practice. On the one hand, patients' life expectancy is increasing and therewith their health problems, and on the other hand microorganisms are becoming more and more resistant to antibiotics and antiseptics due to their increased use

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[1,2]. Therefore, the development of effective alternative antiseptic methods is required.

For many years, electrical plasma discharge has been utilized to sterilize surfaces of technical equipment. Often, the temperatures in the generation zone are several hundred Centigrade [3–5]. For interaction with heat sensitive surfaces the temperature had to be brought down by special technical arrangements. Recently a cold atmospheric-pressure plasma was developed, characterized by a temperature range between 40 °C and 50 °C in the interaction zone [6]. Being suitable also for the treatment of biological systems, such as human skin, this specific plasma is also called tissue-tolerable plasma (TTP). First studies could demonstrate that TTP is likewise well suited to the treatment of chronic wounds [7].

The investigations done and results achieved at the Center of Experimental and Applied Cutaneous Physiology (CCP) with the plasma jet kINPen med®, developed by the Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany, in cooperation with neoplas GmbH, Greifswald, Germany, are presented in this article [8]. Prior to the investigations in humans, a comprehensive risk assessment was performed to demonstrate that the application on volunteers and patients involves no risks. As a result of these preliminary experiments, the Ethics Committee of the Charité – Universitätsmedizin Berlin granted its approval for *in vivo* investigation of the TTP's antiseptic properties on human skin and chronic venous leg ulcers. In the present article a risk assessment of the jet kINPen med® is given, which was focused on temperature, UV radiation and radical formation in the plasma–tissue interaction zone. First results of the application of the plasma for treatment of wounds are discussed.

The device utilized for the described investigations was a kINPen med®, which resembles a pencil, except that its protruding lead is replaced by a plasma beam, which is ejected. The dimension of the plasma beam is 10 mm in length and approximately 1 mm in diameter (Fig. 1).

In the plasma–tissue interaction zone the plasma spreads on the surface, so that the diameter of the interaction zone amounts to approximately 2 or 3 mm. The discharge was operated with argon in the described investigations. The kINPen med®device passed the CE certification for electromagnetic compatibility, thus meeting the standards for electrical safety in humans and corresponding to the industrial standard. In addition, the device has passed successfully further testing procedures to fulfill all technical prerequisites for later admission as a medical product. A detailed description of the jet kINPen med®is given by Metelmann et al. [8].

2. Risk assessment of TTP application [9,10]

Previous to risk assessment, four potential risks emanating from the *in vivo* application of TTP were identified: electrical safety, unintended temperature increase [10], UV radiation and the formation of free radicals [9]. Corresponding analytical methods were determined to investigate these potential risks. As tissue model, porcine ear skin was utilized representing a highly suitable model for human skin [11].

2.1. Electrical safety

The use of the kINPen med® plasma jet does not represent any electromagnetic compatibility hazard as stated by the CE mark. The device has been proved according to industrial standard and pretested for later admission as medical product.

2.2. Ex vivo temperature measurements

Additionally, there was concern that there might be a potential risk of unintended temperature increase within the plasma–tissue interaction zone, which could potentially cause damage to the tissue. Measurement of the temperature was performed in non-contact mode using a digital thermometer GTH 1200A (Greisinger electronic GmbH, Regenstauf, Germany). The measuring spot was approximately 1 mm in diameter. As the temperature in the plasma–tissue interaction zone depends on the moving velocity of the plasma beam on the skin surface, the temperature was measured at different velocities of 2 mm/s, 4 mm/s, 6 mm/s, 8 mm/s, 10 mm/s and 12 mm/s. Subsequently the plasma–tissue interaction zone was inspected for potential structural damages to the skin surface, using laser scanning microscopy and histological investigations [10]. The histological evaluation showed minimal structural changes in the uppermost 2 or 3 cell layers of the stratum corneum when the moving velocity was between 2 and 4 mm/s [9,10], these changes having been caused by thermal damage to the lipid layers of the corneal layers. Also with laser scanning microscopy, superficial thermal damage could be observed when the moving velocity was low. In Fig. 2, the structure of the skin surface prior to and after plasma treatment at a moving velocity of 4 mm/s is shown. Whereas the intact cell structure with its honeycomb-like arrangement of the corneocytes is clearly visible in Fig. 2a, Fig. 2 b depicts an irregular brick-like cell structure as typically observed after thermal damage. However, these structural changes are restricted to the uppermost cell layers of the stratum corneum, whereas no thermal damage could be detected in deeper regions of the stratum corneum and beneath the skin barrier. Increasing the moving velocity of the plasma pen led to decreased structural skin surface changes. When the moving velocity was increased to $\geq$ 10 mm/s, no structural changes could be observed on the skin surface. The optimum moving velocity for low temperature plasma treatment ranges between approximately 8 mm/s and 10 mm/s [10]. This speed range is also used for laser surgery. For *in vivo* investigations of volunteers and patients an optimum moving velocity of $\geq$ 8 mm/s was recommended in order to exclude any thermal

Fig. 1. Application of TTP on human skin using kINPen med®.

damage. At this moving velocity the temperature in the plasma–tissue interaction zone ranges between 35 °C and 45 °C [9].

It has to be considered that these investigations were carried out on intact skin and not on wounds. The same results were obtained when the upper dermal layer, i.e., the stratum corneum, was removed prior to plasma treatment. For this purpose, a 0.3 mm strong layer of split skin was removed from the porcine ear model skin. The obtained results indicate that the optimum moving velocity determined for intact skin can be applied to wounds, too. If the kINPen med® was not moved at its optimum velocity in clinical application, which would be theoretically possible, the damages represented in Fig. 2 were only superficial. The application of the plasma jet is comparable to that of surgical laser and cautery systems; the latter systems exposing the tissue to considerably higher thermal stress, however. According to experience, even surgical lasers and cautery systems induce undesired thermal damages only in very rare cases. In addition, the kINPen med is provided with a safety function switching the device automatically off after 1 min of operation [8].

Our studies did not include the investigation of risks caused by inhalation or potential reactive species that could develop during TTP application. Further investigations will have to address this topic. However, at least no visible signs of evaporated tissue were observed compared to thermal damage by laser-treatment. The treating physician was wearing gloves during the procedure and kept a distance of approximately 30 cm between the treated area and the investigator's face.

2.3. Ex vivo spectral analysis of the plasma radiation

A voltage of 60 V and a gas pressure of 40 kPa turned out to be the optimum operating conditions for the kINPen med®. Using argon as discharge gas, the plasma spectrum displayed an intensive band at 310 nm, followed by significantly smaller bands in the spectral range between 330 nm and 450 nm [9]. The signal at 310 nm is a hydroxyl-group-band (OH band), whereas the subsequent smaller bands could be associated with nitrogen. In order to investigate the penetration of the UV band at 310 nm into the skin barrier, single tape strips were removed from the stratum corneum. These tape strips are adhesive films that were pressed onto the intact skin [12,13]. After removal, every adhesive film contains approximately one layer of corneocytes. The transmission of the plasma beam before and after the specific cell layer of the stratum corneum was analyzed using a fiber-based photospectrometer EPP 2000 (SI Scientific Instruments GmbH, Gliching, Germany). The investigations were performed on 10 volunteers and demonstrated that $28 \pm 7\%$ of the plasma beam penetrates one cell layer of corneocytes. Considering that the stratum corneum consists of approximately 20 cell layers, it is apparent that the low temperature plasma radiation is almost completely absorbed in the skin barrier. These results were confirmed by measurements of a 0.1 mm thick split skin. Comparing the intensity of the plasma radiation at 310 nm with that of the solar radiation at the same wavelength and assuming a plasma–tissue interaction time of maximally 3 s, 1 min of exposure to solar radiation on a sunny day will have a stronger effect on the skin than the exposure to plasma radiation. Consequently, the plasma–treated intact skin will not be damaged by UV radiation, if the plasma jet is operated at optimum conditions as described above [9]. These investigations were undertaken on intact skin. Since the UV radiation dose in the plasma–tissue interaction zone is distinctly lower than that generated on the skin by solar radiation, no risk is to be expected even in the case of barrier disturbances as occurring in wounds.

2.4. In vivo analysis of the formation of free radicals during low temperature plasma treatment

A protective system consisting of antioxidants defends our skin from attacks by endogenous and exogenous free radicals [14–16]. If the concentration of the free radicals within the tissue exceeds a critical value, the antioxidants are destroyed [9,17]. Consequently, the decrease of antioxidants in the human skin is a measuring scale for the amount of free radicals interacting with the tissue. The antioxidative protection mechanism in the human body consists of a compound of various antioxidants including, aside from others, β-carotene, lycopene, lutein, the vitamins A, C, D and E and a variety of coenzymes [18]. These antioxidants form protective chains in the skin defending each other against the destructive action of the free radicals. In preliminary investigations it was demonstrated that carotenoids represent marker substances for the entire antioxidant status of the body.

With regard to risk assessment, the distribution of the carotenoids within the stratum corneum as well as in deeper layers of the human skin was analyzed *in vivo* on the forearm skin of 6 volunteers before and after plasma application. Therefore, a confocal Raman microscope 3510 (River Diagnostics, Rotterdam, Netherlands) with an axial spatial resolution of 5 μm and a laser excitation wavelength of 785 nm was utilized.

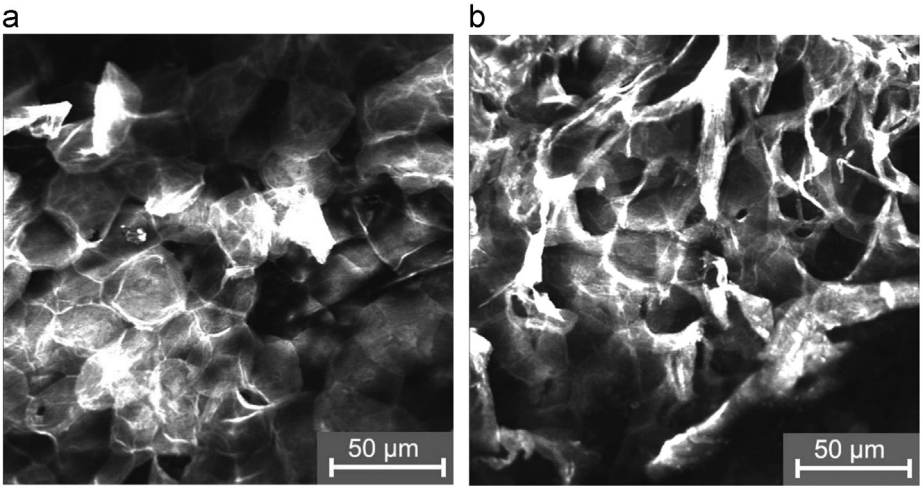

Fig. 2. Representative LSM images of the skin (a) before plasma treatment and (b) after plasma treatment with a moving velocity of 4 mm/s.

In Fig. 3, the carotenoid distribution prior to and after plasma treatment, showing the antioxidant concentration in the human skin declining from the surface of the stratum corneum towards deeper layers is demonstrated [19]. It could be clearly shown that the radical formation is an effect occurring exclusively in the upper cell layers. This effect is also expected for barrier-disturbed skin.

This physiological decrease of the antioxidants within the skin seems plausible as the skin surface represents the boundary to the environment and radical formation is mainly caused by the exposure of this boundary zone to solar radiation, requiring an optimum protection of the skin. After the low temperature plasma treatment, the distribution of the carotenoids in the skin has clearly changed. A reduction of the carotenoid concentration was observed particularly in the upper layers of the stratum corneum. Moreover, the decline of the carotenoid concentration after the plasma treatment clearly indicates the formation of high amounts of free radicals during the procedure destroying part of the antioxidants. However, this radical formation occurs only on the skin surface and in the uppermost layers of the stratum corneum. While the cells of the stratum corneum are of highest importance for formation of the barrier, these cells are void of nuclei and mitotic activities. Living cells found in deeper layers, especially stem cells within the basal layer of the epidermis, remained unaffected by this process [19] thereby minimizing the risk of permanent damage. Obviously, this radical formation might also be responsible for the desired antiseptic properties of the plasma since free radicals are known to destroy microorganisms [20–22]. Due to their distribution profile in the skin barrier, these highly reactive molecules, however, do not affect the living cells in the deeper epidermis. Further *in vivo* investigations of TTP effects on living cells in vitro and on wounds that are not protected by epidermal layers will have to show to which extent the granulation tissue will be affected by TTP-treatment. Based on these assumptions, the formation of free radicals during plasma treatment does not necessarily constitute a potential risk for volunteers or patients. As a result of in vitro experiments on the model of porcine ear skin it has been shown that during the plasma interaction with the tissue, the plasma penetrates the skin from the surface of the hairs into the hair follicles comparable to a lightning passing a lightning rod [23], which could be a further advantage of TTP application. Hair follicles are known to be an efficient long-term reservoir for microorganisms, i.e., for bacteria, viruses, and fungi [24]. Follicular antisepsis by conventional antiseptics is very difficult to achieve as they penetrate hardly into the hair follicles [25]. Using heat-sensitive fluorescent dyes could demonstrate that plasma penetrates and exerts its effects deeply in the hair follicles [23]. Thus, plasma might provide capabilities superior to standard antiseptic solutions.

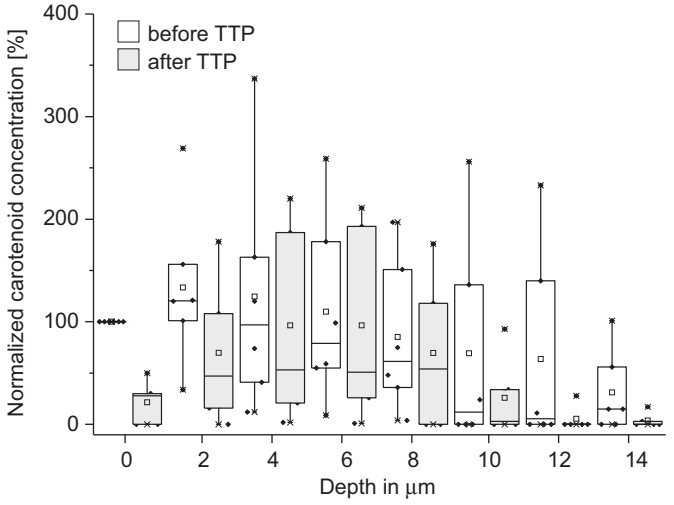

Fig. 3. Carotenoid concentrations at different depths of the stratum corneum before and after TTP application. The value at the surface on the untreated site was set as 100%, and the corresponding depths of treated and untreated sites were calculated accordingly.

3. Antiseptic efficacy of the TTP

3.1. *Ex vivo investigations on the antiseptic efficacy of TTP [26]*

Preliminary investigations to evaluate the antiseptic efficacy of TTP were performed in vitro on porcine ear skin, which represents a good model for human skin [11]. Three skin areas (A, B, and C) of 3×3 cm^2, each, were marked on each of the six porcine ears. Skin area A remained untreated; skin area B was treated with the standard antiseptic octenidine (Octenisept®; Schuelke & Mayr GmbH, Norderstedt, Germany) and area C with TTP at a moving velocity of 10 mm/s. After the respective treatment, the bacterial colonization on the skin surface was determined according to Williamson's protocol [27,28]. Briefly, 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 solution. The neutralization was validated in separate tests according to EN 1040 [29]. Assuming a value of 100% for bacterial colonization of the untreated skin, an average value of 99% of destroyed bacteria was established for the treatment with octenidine, while approximately 94% of the bacteria were destroyed by TTP treatment [26]. The slightly higher efficacy of octenidine compared to TTP was not expected since earlier studies showed that plasma also penetrates into the hair follicles leading to an intrafollicular destruction of microorganisms being responsible for the recolonization of the skin. Probably, the antiseptic effectivity of TTP could be enhanced by increasing the diameter of the plasma jet. Due to current small beam diameter of 3 mm, a homogeneous treatment of the complete skin area of 3×3 cm^2 remains difficult to realize. It is likely that small residues remained untreated. This technical limitation of the current prototype can be overcome by enlarging the diameter of the plasma jet beam. Future experiments will have to show whether this technical modification might increase the antiseptic efficiency.

3.2. *In vivo investigations on the efficiency of TTP on healthy humans [27]*

After thorough risk assessment of TTP and its effects on humans, approval of the Ethics Committee of the Charité – Universitätsmedizin Berlin was obtained for the *in vivo* investigations on volunteers and patients.

Ten healthy volunteers, aged between 26 and 42 years were included in the study. Two skin areas of 4×4 cm^2 were investigated on each forearm, whereby one area always remained untreated and one area was treated with either TTP or octenidine. The application areas were randomized for each volunteer. The bacterial colonization on the skin surface was likewise determined according to Williamson's protocol [28]. Comparable to the in vitro results, the antiseptic efficacy of octenidine-treatment was almost 100%, whereas after TTP treatment an elimination of only 74% of the bacteria was achieved. This reduced antimicrobial efficiency of TTP might also be explained by the described technical limitations of the small plasma beam diameters [4,19].

4. Discussion

In spite of intensive research, the detailed mechanisms of low temperature plasma treatment are still under discussion. Several hypotheses exist including the assumption that plasma treatment induces tissue irritations, which, in turn, stimulate dermal processes that have a strong and positive impact on wound healing. Further studies on larger study populations with improved TTP-devices or those tailored to the intended use will help to better understand TTP-related effects on the process of wound healing. Up to now, it can be stated that the antimicrobial efficacy of TTP might be explained, inter alia, by the radical formation processes in the uppermost layers of the stratum corneum. This hypothesis is supported by the decline of antioxidants following TTP treatment observed by Fluhr et al. [19]. The capacity of free radicals to destroy bacteria, viruses and fungi has been known for a long time from studies using high-temperature plasmas [4,19].

Additionally, further synergistic effects between temperature increase, UV radiation and radical formation have to be considered responsible for the overall antimicrobial activity of TTP-treatment. The TTP-induced temperature increase could potentially lead to an improved blood flow throughout the wound bed and thereby stimulate the wound healing process [4]. Moreover, an important finding of the recently described studies is that the TTP application remains without risk for humans if the treatment is performed by a trained physician according to instructions [9]. To the best of our knowledge, neither the TTP-induced UV radiation, nor the temperature increase, nor the formation of radicals pose a potential risk to the treated patients and volunteers.

Further convincing advantages of TTP are that it represents a physical method which might stimulate chemical processes in the tissue. While conventional liquid antiseptics target only a restricted spectrum of microbes that can be resistant to antiseptic substances TTP could still exert antiseptic effects. Moreover, it has to be considered that these microbes are frequently embedded in a biofilm which is protecting the microbes from any antiseptic with limited penetration capability. It has to be investigated whether TTP-treatment might be an advantage over conventional antiseptics concerning the penetration of the biofilm components. In addition, future research will show to which extent TTP potentially eliminates multi-resistant microorganisms, which can be hardly treated with conventional methods, and that TTP treatment involves the hair follicles, which represent an efficient microbial reservoir [24].

In summary it can be stated that TTP represents a novel technology which opens up promising prospects in the field of skin antisepsis and wound healing. While this technology needs improvement, yet, for large-scale clinical application, some technical requirements have still to be complied with. The plasma–tissue interaction zone, for instance, should be generously dimensioned, i.e., at least 5–8 mm in diameter, so that the tissue surface can be homogeneously treated. It is expected that these improvements will be realized soon, so that optimum wound healing systems will be accessible to both clinicians and medical practitioners before long. Future studies will have to address the potential effects of tissue stimulation on the wound healing process and the antimicrobial activities against bacteria multi-resistant to antibiotics.

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