In Vitro Killing of Clinical Fungal Strains by Low-Temperature Atmospheric-Pressure Plasma Jet

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***Abstract*—Plasma medicine is an expanding focus and offers new aspects of therapy combining potent physical partial efficacies, like such as ultraviolet, infrared, and reactive species and particles, and nowadays, many successful treatments of different illnesses have been described. Fungal skin and nail infections pose significant therapeutic and economical problems. To test the plasma susceptibility of clinical strains of the most frequently encountered fungal species involved in dermatomycosis, clinical isolates of *Trichophyton interdigitale*, *Trichophyton rubrum*, *Microsporum canis*, and *Candida albicans* were irradiated by a cold atmospheric pressure plasma jet. Punctual plasma irradiation eradicated fungal growth of all species with the largest inactivation zones with most progress in the first 15 s of treatment, treating *C. albicans* and least progress in that of , the lowest being *M. canis*. No isolate exhibited resistance to plasma treatment. Plasma treatment also completely eradicated reproductive fungal elements of *T. interdigitale* in dandruff of patients with tinea pedis *ex vivo* and in the environment in contaminated shoes. Accordingly, cold plasma seems suited to antifungal *in vivo* treatment of fungal skin infections and decontamination of environmental infective material.**

***Index Terms*—Atmospheric pressure plasma, *Candida*, dermatomycosis, *Microsporum*, plasma medicine, tinea pedis, *Trichophyton*.**

I. Introduction

NONTHERMAL atmospheric pressure plasma has been introduced in medical and biologic applications since it demonstrated well-characterized antimicrobial *in vitro* efficacy, as well as medically important biochemical effects [1]–[3]. In the last years, the first results of clinical plasma applications were undertaken to treat acute and chronic infectious diseases and wounds with successful treatments, e.g., of eye infections, bacterial skin infections, chronic ulcer wounds, and pulmonary tuberculosis [4], [5]. One restriction to the general, unlimited, and nonprofessional application of plasma is the conscientious conformance of the requirements of the safe medical use of a treatment.

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Since we were able to demonstrate high log-step reductions of the yeast *Candida albicans* by cold plasma on agar -treating whole plates [6], the plasma potency toward clinical isolates of the most frequently encountered causative pathogens of dermatomycoses *Trichophyton rubrum*, *Trichophyton interdigitale*, and *Microsporum canis* were now tested to estimate the plasma treatment *in vivo*.

Dermatophyte infections and onychomycosis are very common infectious diseases and may have significant clinical consequences, such as secondary bacterial infections, chronicity, therapeutic difficulties, and esthetic disfigurement, in addition to serving as a reservoir of infection. Nearly 30% of Europeans suffer from tinea pedis, often combined with nail infection (onychomycosis) [7], [8]. The most common causative fungi of tinea pedis and onychomycosis are the dermatophytes *T. rubrum* and *T. interdigitale* (former *T. mentagrophytes var. interdigitale*), and to a lesser extent, *C. albicans* and, seldom, molds (e.g., *Aspergillus niger*).

Tinea pedis and onychomycosis are worldwide- increasing diseases, which are, in part, due to the increasing prevalence of elder people and the progress of modern medicine using more immunodepressing therapies (chemotherapy, irradiation, and biologics). Immune depression favors the development of fungal infections, like such as tinea and onychomycosis. Other risk factors are genetic disposition, humid environment (bathing or sports), diabetes mellitus, psoriasis vulgaris, and vascular insufficiency. A particular problem is the recalcitrant tinea pedis and onychomycosis [9], [10].

Nowadays, tinea pedis and onychomycosis can be treated by modern systemic in combination with topic therapy with clinical improvement but often cannot be cured. The main reasons for this therapeutic dilemma are a high relapse rate due to reinfections in a highly contaminated environment, the humid milieu (interdigital humid chamber phenomenon) promoting fungal growth, the synergistic role of invading bacteria (*Staphylococcus aureus*) and yeasts (*C. albicans*), and the thickness of the invaded corneocyte layer. Another fact is the impossibility for the antimycotic to reach inhibitory concentrations (minimal inhibitory concentration) in the affected skin or nail tissue. The treatment of tinea pedis with onychomycosis can take one year or more and is accompanied by possible adverse effects of the systemic antimycotics [11].

Another problem is the lack of adequate disinfection of fungal spores in the environment. This is of crucial importance since tinea pedis is a contagious infection disease, which is propagated mainly from human to human, environment to human, and, not seldom, pet (cats and dogs) to human by skin,

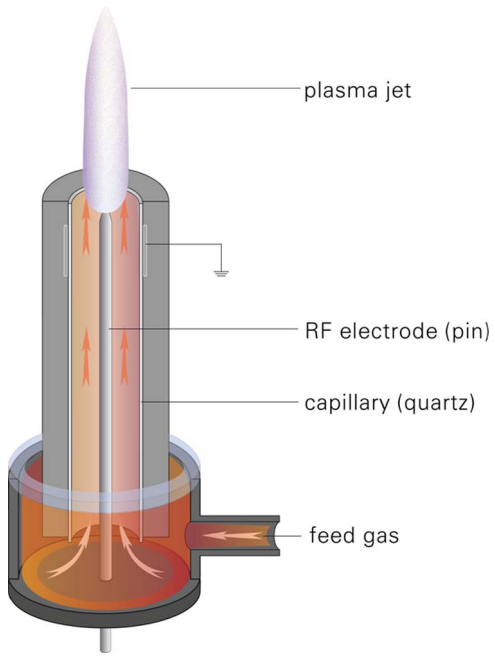

Fig. 1. Schematic setup of the APPJ device [12].

hair and dandruffs, towels and clothes. Arthrospores of dermatophytes are very resistant and can survive in the environment, and on furniture, and on equipment for several years, providing a long-term source of infection.

A. Plasma Option

Plasma, in medicine, offers a unique combination of different physicochemical efficacies and may overcome the therapeutic dilemma when it succeeds in inactivating the reproductive fungal elements in the tissue without affecting the neighbored tissue and when painful and multiple treatment sessions can be avoided.

II. Methods

The effects of plasma against different fungal isolates and species were tested with the suspension of the strains being irradiated on agar, following increasing time spans. To evaluate the antifungal potency of the plasma, the following steps were performed: 1) The eradication zones and the corresponding time spans were compared. 2) The results of the tests served to approximate the time to inactivate definite areas of fungal cultures on agar. A third technique was used to test the potency of the atmospheric pressure plasma jet (APPJ) to inactivate fungal elements embedded in 3) dandruffs and 4) arthrospores *ex vivo*.

Plasma source: APPJ

The schematic setup of the APPJ device used in this paper is given in Fig. 1. For a detailed characterization of the APPJ, see [12]. Briefly, in the center of a quartz capillary (with an inner diameter of 1.6 mm), a pin-type electrode (with 1-mm diameter) is mounted. Argon, as the feed gas, flows through the capillary (with a gas flow rate of about 8 L/min). A radio-frequency voltage (1–5 kV, 1.5 MHz) is coupled to the center electrode. The plasma is generated from the top of the center electrode and expands to the surrounding air outside the nozzle. The axial temperature profile of the plasma was obtained by fiber-optic temperature measurement (FOT Lab Kit Fluoroptic Thermometer, Luxotron model 755). A temperature-dependent fluorescent signal of luminescent magnesium fluorogermanate, which was excited with a Xe flash lamp, was monitored. To avoid killing effects (and skin damage for future application) by constructional reasons, the temperature at the tip of the beam with a length of up to 12 mm did not surpass 37 °C as a result of the cooling effect by the argon stream, together with the electronic regulation. The tip of the beam is defined as the distal end of the visible pointed discharge. The temperature profile of the flame is shown in Fig. 2.

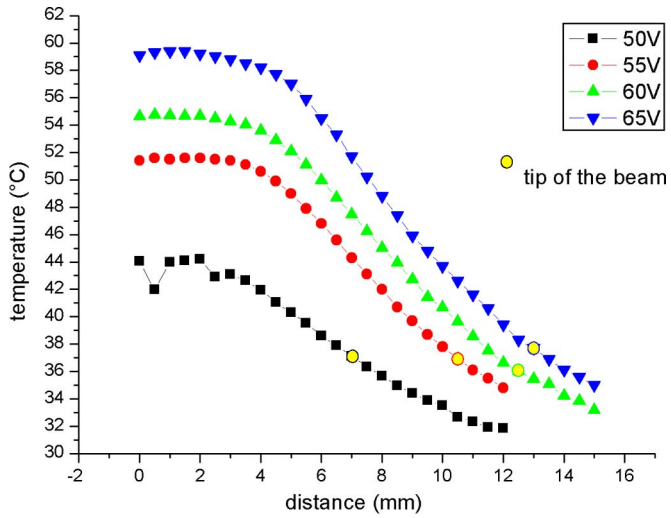

Fig. 2. Temperature profile of the plasma beam (60 V).

Optical emission spectroscopy in the UV, visible (VIS), and near infrared (NIR) regions of the plasma from 250 up to 1000 nm was performed using a fiber spectrometer (StellarNet EPP2000-UVN). The spectra measured at different axial positions of the plasma jet at 3 W and an Ar gas flow rate of 5 slm in the continuous working mode are shown in Fig. 3. In the VIS/NIR region, between 700 and 1000 nm mainly, emission lines of excited argon atoms are found. Lines of nitrogen emission have been measured in the UV-A region between 300 and 400 nm. Their relative intensity increases along the plasma jet according to the decrease in argon emission at the tip of the jet because of the onward mixing of the feed gas argon with the surrounding ambient air. The most significant is the emission of excited OH radicals at 309 nm. However, there was no detectable emission in the UV-C range between 200 and 280 nm [12].

A. Testing Punctual Antifungal Effects on Agar

Fresh suspensions of clinical fungal isolates [10^6 colony-forming unit (cfu)/mL] in sterile saline (0.9%) were diluted until nearly confluent growth of the colonies on Sabouraud

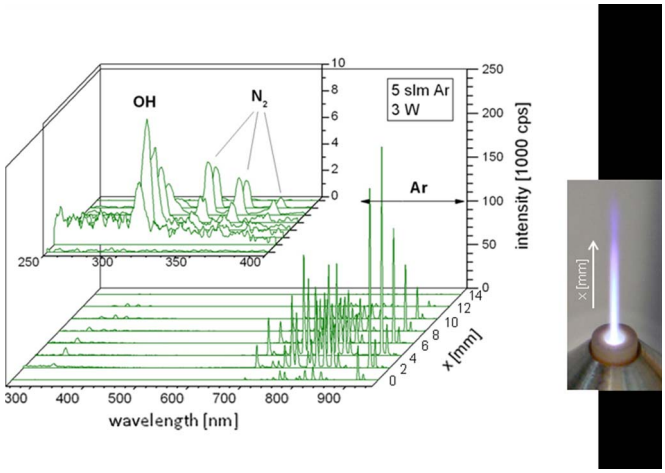

Fig. 3. Optical emission spectra measured at different axial positions of the Ar plasma jet [12].

dextrose agar (4% glucose, Heipha, Heidelberg, Germany) and resp. Taplin agar (4% glucose, Heipha) was obtained. The dermatophytes were cultured on Taplin or Sabouraud dextrose agar over 3–7 d at 30 °C until full growth developed; *C. albicans* was cultured on Sabouraud dextrose agar over 3 d at 36 °C [13].

The following species were tested: 1) Yeasts: *C. albicans*, ($n = 5$ strains). 2) Dermatophytes: *T. rubrum*, *T. interdigitale*, and *M. canis* (5 strains each). All strains were isolated in our clinic from patients with mycotic lesions (tinea pedis, tinea corporis, and onychomycosis).

1) Plasma treatment: The APPJ device was fixed in a rack to assure a constant distance between the tip of the plasma jet and the surface of the agar with the plated microorganisms, allowing direct contact of the tip of the jet with the agar surface over definite time spans (0, 3, 6, 9, 12, 16, 20, 24, 28, and 30 s). After plasma treatment, the agar plates were incubated and visually screened for inhibition zones at the irradiated areas after 3–7 d. In order to evaluate the kinetics of the antifungal efficacy over time for each species, we measured the diameters of the inhibition zones resulting after increasing the irradiation time and calculated the amount of inactivated cfu's at each time using the area of the corresponding eradication zone.

B. Irradiation of Fungal Colonies Grown on Agar

To approximate clinical use with the need to effectively eradicate larger areas of mycotic skin lesions by the plasma, fungal suspensions on whole agar plates (56.7 cm^2) were treated by the APPJ. One isolate of *T. rubrum* and two of *T. interdigitale* were tested. Suspensions of the respective fungal strains were streaked onto the Sabouraud dextrose agar or the Taplin agar before being treated with the jet. The treatment differed from the former (punctual) by passing the jet over the agar plate following meandric lines over 10 min (5 × 2 min), according to the experimental setup described in [6]. The irradiated culture dishes were incubated directly after irradiation aerobically at 30 °C over 7 d (dermatophytes) resp. 36 °C over 3 d (*Candida*) and compared with untreated control plates.

C. Irradiation of Fungal Elements Embedded in Dandruffs From Tinea Lesion

Dandruffs were scrubbed from tinea lesions of patients from our clinic setting by scalpel, were directly placed on the Sabouraud dextrose agar (every sample size about 1 mm × 1 mm) and were immediately irradiated over 2 min by plasma (APPJ). Nonirradiated dandruffs from the same lesions served as controls. Nearly 50% of culture positivity for dermatophytes can be expected in the dandruffs of patients with tinea pedis, as we could demonstrate in preliminary tests (data not shown). After irradiation of the dandruffs, the agar plates were aerobically incubated over four weeks at 30 °C, and the number of grown cfu's of *T. interdigitale* was compared with controls (nonirradiated dandruffs).

In total, we tested 15 series of sampling dandruffs from patients before antifungal treatment of tinea pedis caused by *T. interdigitale*. There were 12 (min) to 32 (max) dandruff samples (see Table I) taken at each serial sampling; half of the samples were plasma treated (2 min punctual), and the remaining half served as (not irradiated) control. Every dandruff sample (irrespective of prior irradiation) was cultured on the Sabouraud dextrose agar (with 4% glucose, Heipha) over six weeks at 30 °C. Cultured colonies were biochemically and microscopically differentiated, following the national guidelines for good laboratory practice. After culturing, the number of positive samples (cultural fungal growth) of irradiated and not irradiated dandruff samples was compared, and the percentage of reduction was calculated.

D. Treatment of Environmental Arthrospores of Dermatophytes

Three pairs of shoes (one pair of sports shoes and two pairs of leather shoes) of a patient with chronic tinea pedis of both feet were examined for spores of *T. interdigitale*, which was the causative microorganism of the tinea. The insoles of all shoes were cultural positive for *T. interdigitale*, resulting in 1–5 cfu/swab and shoe on the Sabouraud dextrose agar (data not shown). Directly after swabbing, the insole of a shoe, every inner shoe was irradiated by the APPJ by moving the jet over the complete inner surface over 3 min. Thereafter again, the insoles of all shoes were swabbed (one insole, one swab), and the swabs were streaked onto the Sabouraud dextrose agar. The agar was incubated aerobically over six weeks at 30 °C with weekly visual control for fungal growth.

III. Results

A. Effects of Plasma Treatment

The punctual plasma treatment of the fungal suspensions on agar was followed by inhibition of the growth of any tested species and isolate *in vitro*. The plasma irradiation produced a growth-free circle around the point of contact with the plasma jet during irradiation. The inhibition zones were analyzed for mycotic growth by swabbing the irradiated areas and cultivating the samples over eight weeks on selective media. As no more growth could be obtained on all visually fungus-free

TABLE I
ANTIFUNGAL PLASMA TREATMENT (2 min) OF SKIN-SCRUBBED DANDRUFF FROM PATIENTS WITH TINEA PEDIS CAUSED BY *T. interdigitale* AND POSTTREATMENT POSITIVITY FOR FUNGAL GROWTH (*T. interdigitale*)

sample	n dandruff samples evaluated	n plasma treated dandruff samples	n dandruff samples with fungal* growth after APPJ	n dandruff samples non treated with fungal* growth (controls)	reduction efficacy by plasma treatment (%)
22/09	14	7	0	3	100
23/09	20	10	0	2	100
24/09	24	12	0	0	-
25/09	28	14	0	14	100
26/09	32	16	0	0	-
27/09	20	10	0	7	100
28/09	26	13	0	0	-
29/09	16	8	0	0	-
37/09	16	8	0	8	100
41/09	24	12	0	0	-
68/09	12	6	0	0	-
884/08	16	8	0	0	-
891/08	14	7	0	0	-
12/08	16	8	0	8	100
30/08	16	8	0	3	100

* *T. interdigitale*

plasma-treated areas (data not shown), the term inhibition was replaced by eradication. The eradication zones varied between isolates and species and increased with treatment time [see Fig. 4(a)–(d)]. The maximal inhibition zones were obtained with *C. albicans* with 200 mm^2, followed by *T. rubrum* (123 mm^2) and *T. interdigitale* (107 mm^2). The lowest reduction was found with treating *M. canis* (2.3 mm^2). Compared with the other species, the treatment of *M. canis* was related to the lowest number of killed fungal cfu (0.3 cfu/cm^2, see Fig. 5). The highest progression of the fungi-free area over time was found with *C. albicans* [see Fig. 4(a)], with 50% of the reduction area obtained after 6 s of plasma treatment; the lowest was found with *M. canis* [see Fig. 4(d)], with a delay of 28–30 s until 50% maximal area was reached (see Table II).

B. Treatment of Fungal Suspensions on Larger Areas (Agar Plates of 56.7 cm^2)

To verify if the time necessary to effectively eradicate larger areas by a moving jet can be derived from the data from our experiences [see Fig. 4(a)–(d)], we tested the plasma jet to eradicate three clinical strains (one *T. rubrum* and two *T. interdigitale* strains) on 56.7 cm^2 of agar (corresponds to the surface of conventional agar plates). As 15 s were shown to be able to eradicate $> 50\%$ of the fungal growth on agar, we chose 10 min of treatment for one plate. To test the influence of larger growth (> 1000 cfu/plate) on the eradicating plasma potency, we additionally irradiated one plate with excessive growth (1572 cfu/plate on the control plate).

Ten-minute (5×2 min) irradiation of the agar plates was followed by complete eradication of the fungal growth. The excessive amount of fungal cfu on a plate with > 1500 cfu's on its surface did not shrink the eradicating potency (see Table III).

C. Irradiation of Dandruffs Ex Vivo

Seven of the 15 sample series showed fungal growth of *T. interdigitale*, the highest positivity was found with 14 positive cultures from 28 dandruff samples (sample series 25/09, see Table I), the positive ranking scaled from 2 to 14 positive samples per series. All irradiated dandruff samples failed to exhibit the cultural growth of *T. interdigitale* after 8 weeks of cultivation compared to non irradiated control dandruffs. Two minutes of plasma treatment was shown to effectively eradicate reproductive fungal elements in dandruffs of patients with tinea pedis (*T. interdigitale*).

D. Irradiation of Shoes

All six plasma-treated insoles from the 3 pairs of contaminated shoes failed to exhibit any fungal (or bacterial) growth after 6 weeks of incubation in the agar plates (data not shown).

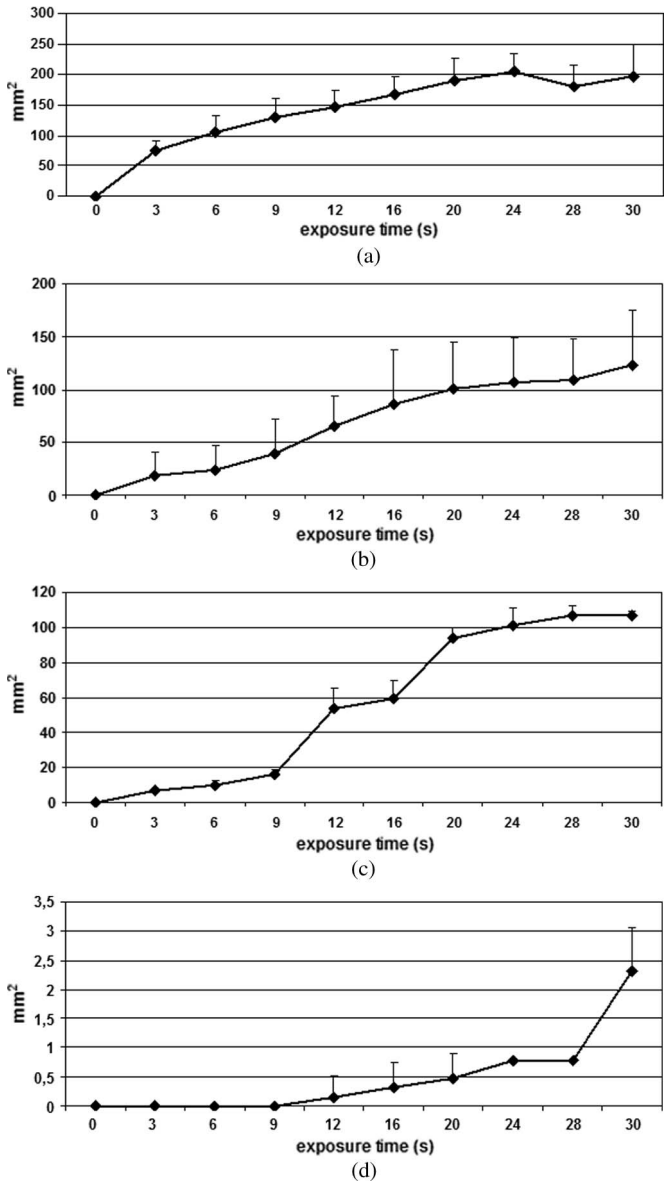

Fig. 4. (a) Inhibition zones of irradiated strains of *C. albicans* ($n = 5$). (b) Inhibition zones of irradiated strains of *T. rubrum* ($n = 5$). (c) Inhibition zones of irradiated strains of *T. interdigitale* ($n = 5$). (d) Inhibition zones of irradiated strains of *M. canis* ($n = 5$).

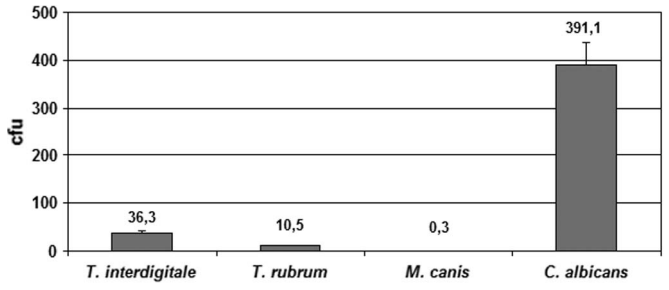

Fig. 5. Maximum of the killed fungal cfu (mean, $n = 5$) obtained during plasma treatment of 30 s.

The plasma irradiation was shown to eradicate environmental fungal contamination in heavily contaminated shoes.

TABLE II
APPJ TREATMENT OF CLINICAL FUNGI. MAXIMUM OCCURRING CLEARED AREAS $A_{\max}$ (mm^2), CORRESPONDING TREATMENT TIME $t_{\max}$ (s), AND CORRESPONDING TREATMENT TIME TO 50% AND 90% REDUCTION

pathogen	A_{max} (mm^2)/SD	t_{max} (s)	$t_{50\%}$ (s)	$t_{90\%}$ (s)
T. interdigitale	107 / 5.3	28	12	20
T. rubrum	123 / 52.3	30	12	28
M. canis	2,3 / 0.7	30	28-30	28-30
C. albicans	204 / 29.7	24	6	16-20

* after treatment of max. 30 s; SD standard deviation

TABLE III
PLASMA ACTIVITY TOWARD FUNGAL SUSPENSIONS ON AGAR SURFACE TREATED OVER 10 min BY THE APPJ

pathogen	control plate (cfu/plate)	cfu after APPJ (cfu/plate)	treatment time (min)	reduction %
T. rubrum (Taplin)	277	0	5 x 2	100 %
T. interdigitale (Sabouraud)	1572	0	5 x 2	100 %
T. interdigitale (Sabouraud)	352	0	5 x 2	100 %

IV. DISCUSSION

In the last few years, therapeutic plasma applications have moved into the focus of the newly developing field of plasma medicine. The multivalent biophysical and -biochemical features of the plasma discharge and, the anti-inflammatory, antimicrobial, and antiproliferative effects make plasma an ideal tool not only for the treatment of dermatological diseases. Direct killing of pathogens on animal skin by plasma could be demonstrated with mice, whose skin of which could be disinfected by plasma without affecting the animals [1]. At the moment, reports upon antifungal plasma treatment are rare, and since the APPJ proved high activity against relevant wound pathogens and contaminants [5], a similar approach to inactivate dermatophytes on solid media appeared realistic. Data clearly show that representative species of the most commonly encountered fungal species in human mycology responsible for tinea corporis, tinea pedis, and onychomycosis could effectively be inactivated by *in vitro* plasma treatment. From 3 s up to 30 s of treatment, all isolates exhibited increasing distinct zones completely free of fungal growth. It was remarkable and corresponds to our antibacterial results [6], in which that, for most species, the eradication zone significantly surpassed the zone of direct irradiation by the beam (direct plasma effect), indicating that other than direct irradiation effects were responsible for killing the microbial cells in this area (indirect plasma effect). Since no cells could be recultivated after prolonged time spans, even after longtime liquid cultivation (data not shown), the plasma action against fungi can be considered mycocidal.

In our study the enlargement of the eradication zone around the nozzle tip generated maximum diameters of max 19 mm (*C. albicans*), 12 mm (*T. interdigitale* and *T. rubrum*),

and 2 mm (*M. canis*). These data demonstrate potent but different antifungal effectiveness by the plasma with highest efficacy against *C. albicans*. The same plasma dose (defined time span of irradiation under identical conditions) clears different areas of growth of different fungi, but the effect in any case remains mycocidal. This is of fundamental clinical relevance, since the plasma power toward the species did not differ in the dimension of the killing effect per surface, which would result in a diminished amount of cfu/cm^2 case of less activity but in the absolute amount of cleared surface only.

Accordingly, a greater clearing effect can be expected by longer irradiation (not for *M. canis*). As a consequence, independently from the area which could be cleared by the plasma treatment of individual fungus strain, the treatment in any case reliably achieves irreversible (mycocidal) inactivation of the complete fungal growth in a defined area, which differs from species to species and even in case of relatively tiny eradication zones, like those obtained with *M. canis* $(d = 2$ mm$)$. This treatment was highly effective (all fungal growth eradicated). Interestingly, the relatively small diameters (of the corresponding area) obtained by irradiation of *M. canis* exactly reflect the geometry of the tip of the plasma beam (diameter of 2 mm), and it can be hypothesized that, in controversy to all other tested fungal species (also bacteria, data not shown), the inactivation of *M. canis* is restricted to direct plasma effects and can be designated as susceptible to direct and resistant to indirect plasma effects. The nature of the antimicrobial properties of plasma is still not fully understood. Based on the plasma characterization data given in Fig. 3, as well as in [12], temperature effects can be excluded as the main reason of the antifungal plasma activity just as UV radiation in the biologically active UV-C region around 254 nm. Because of the lack of substantial emission in the UV-C range, the presence of excited NO can be widely excluded [14]. However, as demonstrated by the significant emission at 309 nm (see Fig. 3), there is a substantial amount of OH radicals within the plasma, which are produced by plasma– chemical dissociation and excitation reactions from water molecules present in the feed gas, as well as in ambient air above the wet agar plates. These OH radicals are supposed to be, together with other reactive oxygen species, are supposed to be the lethal plasma components toward living cells [6], [14], [15].

Since plasma treatment proved high effectiveness *in vitro* against the most relevant causative agents of human and partial veterinary fungal species, *T. rubrum*, *T. interdigitale*, *M. canis*, and the worldwide most relevant yeast *C. albicans*, it can be assumed that *in vivo* treatment can eradicate these pathogens in mycotic lesions. Additionally, the reported plasma effects affecting wound healing, like amelioration of tissue oxygenisation, as proved with Laser Doppler Fluximetry (LDF) [16], could further support the antifungal therapy *in vivo*. A handicap of the APPJ is the unsuitability in the treatment of larger areas. As we could demonstrate that areas of 56.7 cm^2 can be effectively eradicated by the APPJ during 10 min, this time could be substantially shortened when plasma sources with larger application area can be applied. Such treatment could also provide more homogenous and accurate irradiation. Sources with larger application areas are now under investigation, with (one source with an application area of 18 cm^2 [dielectric barrier discharge (DBD), Leibniz Institute of Plasma Science and Technology e.V. (INP Greifswald e.V.)] and one with 3.14 cm^2 (DBD, Cynogy GmbH, Duderstadt, Germany). These tools are currently under investigation in the *in vivo* treatment of tinea pedis and onychomycosis.

V. Conclusion

Plasma irradiation of strains of the most important causative agents of dermatomycosis, i.e., the dermatophytes *Trichophyton T. rubrum*, *Trichophyton T. interdigitale*, and *Microsporum canis* and the yeast *Candida albicans*, was able to kill $>$ 90% of the organisms during 30 s *in vitro*. Plasma has also inactivated reproductive fungal elements in dandruffs *ex vivo* and in the environment (shoes). The high antifungal efficacy of the plasma is crucial for suited clinical application. Plasma, in the future, may serve as a supportive and/or alternative antimycotic treatment tool.

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