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INTRODUCTION TO DIN-SPECIFICATION 91315 BASED ON THE CHARACTERIZATION OF THE PLASMA JET kINPen® MED

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Standardization is an important tool to reduce costs and risks of research. For the innovative field of plasma medicine, no national or international standards could be identified until now. The present study introduces the first German DIN (German Institute of Standardization)-specification 91315 named "General requirements for medical plasma sources" which was published in June 2014. This developable specification describes obligatory basic criteria for the characterization of different biocompatible cold atmospheric pressure plasma (CAP) sources which are intended supposed for medical applications. With these test methods it is possible to obtain information about performance characteristics as well as effectiveness and safety of medical plasma sources. The suggested biological and physical test methods have been carried out with the CAP jet kINPen® MED. We demonstrate that both test methods described in the DIN-SPEC 91315 are easily to adapt and that the plasma device kINPen® MED is safe and effective with regard to its physical and biological requirements.

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1. Introduction

During the last years the use of physical plasma for medical applications has grown rapidly establishing a new research field called plasma medicine. One of the most popular fields in plasma medicine today is the application of CAP sources in dermatology. It is characterized by growing evidence of favorable plasma effects especially on damaged dermatological biological systems based on basic research [1]. This evidence has been obtained with a wide variety of different plasma sources and biological systems [2], impeding comparability of scientific data. Furthermore, plasma medicine is in a transition state, where devices currently are under development by small-and-medium-sized companies. Some companies already offer plasma sources for medical applications without presenting generally accepted practical criteria. However, scientists and developers of innovative medical plasma sources bear a high degree of responsibility to fulfill high expectations of patients and give therefore no hasty promises. In this particular situation, basic criteria for the performance characterization of plasma devices are crucial to establish this innovative technology for medical applications. Moreover, such basic criteria are important to estimate the benefits and potential side effects of CAP sources in therapeutic applications. They also may help to identify devices which are not useful for future medical applications. Additionally, such information will enhance the safety for investigators, patients, and therapists.

One possibility to define generally accepted basic criteria for medical plasma sources are national and international standards. Such standardizations are important tools for both research and economic success. Regularly, national and international standards are consensus-based and are developed with the participation of
all stakeholders and for the benefit of the society as a whole. In contrast, informal or consortia standardization may be developed by a selected consortium. This special standardization tool is defined as specification (DIN-SPEC). This document determines material and immaterial issues or findings, data etc. from standardization or scientific approaches. A DIN-SPEC can be developed either by temporarily appointed committees advised by DIN e.V. (Berlin, Germany) and their advisory boards or in the course of CEN (European Committee for Standardization) workshops. Furthermore, such specifications are the basis for the DIN Standard and they are publicly available. Due to the fact that no consensus based process is necessary a fast transfer of knowledge and technology is possible.

After careful research, no national or international standards covering the field of plasma medicine could be identified. Therefore, and because of the previously mentioned reasons the authors created with the collaboration of DIN e.V. a novel German DIN-SPEC 91315 with the title “General requirements for medical plasma sources” summarizing the state-of-the-art of basic research and technical development [3]. The DIN-SPEC 91315 includes descriptions of obligatory basic criteria for the characterization of CAP applications on biological systems. Simple and generally applicable biological test procedures (inactivation of microorganisms in-vitro, cytotoxicity, and detection of chemical species in liquids) and physical test methods (temperature, thermal capacity, optical emission spectrometry, UV-irradiance, gas emission, and leakage current) are provided to obtain first and basic information about performance characteristics as well as effectiveness and safety of medical plasma sources. It must be noted that the presented assays alone are not qualified for a complete safety evaluation of plasma sources with regard to medical usage. This has to be done according to DIN EN 60601-1 “Medical electrical equipment - Part 1: General requirements for basic safety and essential performance” [4]. Hence, the DIN-SPEC 91315 should be viewed as a complement to the existing standards and rules for medical devices concerning specific aspects of atmospheric-pressure plasma devices for medical use. However, the document does not limit the scientific freedom and work. It offers an option to characterize plasma devices suitable as therapeutic tools. We know that further different biological and physical techniques are implemented in the scientific community. However, such a catalogue of test methods could be the basis to compare different CAP sources by fundamental and obligatory performance parameters. Accordingly, we chose simple assays which are easy to adapt, if common laboratory equipment is available. Furthermore, described test methods are easy to reproduce but do not cover all possible applications in medicine. Since there are a variety of different plasma geometries the described assays have to be adjusted to every unique plasma source with regard to their individual treatment conditions. The DIN-SPEC 91315 can and should be developed further during the next three years. Ideas and suggestions are welcome because it is not the intention to impose fixed rules within plasma medicine. Therefore, experts from science, medicine, and economy are invited to develop an international standard for specific plasma sources and their application fields in a subsequent step. Also, this DIN-SPEC 91315 is a good basis for a further consensus based international standardization process in the field of plasma medicine.

In the present study we performed a physical and biological characterization of the CAP jet kINPen® MED (INP Greifswald/neoplas tools GmbH, Greifswald, Germany) [5-7] based on the DIN-SPEC 91315.

2. Material and methods

2.1. Plasma device: kINPen® MED

The CAP jet kINPen® MED (INP Greifswald/neoplas tools GmbH, Greifswald, Germany) is commercially available as a medical device (Fig. 1). The kINPen® MED consists of a pen-sized hand-held unit for plasma generation at atmospheric pressure, a DC power supply (maximum system power 50 VA at 230 V, 50 Hz), and a gas supply unit. The plasma of the CAP jet is generated at the tip of a pin-type electrode (1 mm diameter) on high-voltage potential which is surrounded by a capillary (inner diameter 1.6 mm) and a grounded electrode. The discharge ignites primarily in argon which is fed through the capillary with a flow rate of 5 standard liters per minutes (slm) [5-8]. The excited gas then expands into the surrounding air at the end of the capillary nozzle and appears there as plasma-jet. The kINPen® MED is applicable primarily for small-scaled spot-like treatments. To treat the target surface (e.g. liquids, agar plates, human or animal skin), the distance between surfaces and nozzle should be 7–10 mm. Such treatments result in a direct contact between plasma and target surfaces. For treatment of larger areas the kINPen® MED has to be moved over the surface of the target in a defined manner.

2.2. Measurements of physical-technical efficiency

2.2.1. Temperature

The gas temperature of the visible plasma jet was measured at different axial distances from the capillary nozzle of the plasma source up to the end of the visible plasma jet using a fiber optic temperature sensor (FOT Labor Kit, LumaSense Technologies, Inc. GmbH, Santa Clara, USA).

2.2.2. Thermal output

For the determination of thermal output a calorimetric measurement is necessary. Therefore, a defined copper plate

![Fig. 1. Schematic setup (a) and photograph (b) of the plasma-jet device kINPen® MED (INP Greifswald/neoplas tools GmbH, Greifswald, Germany).](image-url)
(dimensions: $10 \times 10 \times 0.09 \text{mm}^3$, weight: $0.12 \text{g}$) is connected to a temperature sensor (FOT Labor Kit, LumaSense Technologies, Inc. GmbH, Santa Clara, USA). The calculation of the thermal output of the plasma is based on a time-dependent heating of the substrate according to Eq. (1) ($P_{\text{thermal output}} = m \times \frac{dT}{dt}$; $m$ = mass of the substrate; $cw$ = specific heat of the substrate [$\text{J/(kg \times K)}$]; $dT/dt$ = change of the substrate temperature in relation to a defined time period):

\[
P = m \times cw \times \frac{dT}{dt}
\]

(1)

2.2.3. Optical Emission Spectroscopy (OES)

With the help of OES it is possible to determine the spectral composition of optical plasma radiation. Here, OES was performed in the ultraviolet (UV), visible (VIS), and near infrared (NIR) region with a calibrated fiber optic spectrometer (AvaSpec-3648-USB2, Avantes, Apeldoorn, Netherlands). Plasma emission was detected with a cosine corrector connected to the optical fiber of the spectrometer. To protect the cosine corrector from direct plasma contact, a quartz window ($d = 2 \text{ mm}$) was mounted in front of it. Supplementary information regarding OES can be found in DIN 51008-2 [9].

2.2.4. UV-radiation

UV irradiance was analyzed within the following ranges: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). Therefore, the UV-radiation was detected with the fiber optic spectrometer system (mentioned above) which was calibrated by means of a deuterium-halogen radiation source (Avantes AvaLight-DH-BAL-Cal).

The effective irradiance $E_{\text{eff}}$ for a wavelength range $\lambda_1$ to $\lambda_2$ is determined by spectral irradiance $E_\lambda$ ($\lambda$) and relative spectral sensitivity $S(\lambda)$:

\[
E_{\text{eff}}(\lambda_1, \lambda_2) = \int_{\lambda_1}^{\lambda_2} E(\lambda) \times S(\lambda) \, d\lambda
\]

or

\[
E_{\text{eff}} = \sum_{\lambda} E_\lambda \times S(\lambda) \times d\lambda
\]

The effective radiant exposure $H_{\text{eff}}$ results from integration over the exposure time $t$ [29]:

\[
H_{\text{eff}} = \int E_{\text{eff}} \times dt
\]

2.2.5. Gaseous formation

General information and guidelines for the measurement of the formation of toxic gases are given in DIN EN ISO 12100 - Safety of machinery - General principles for design - Risk assessment and risk reduction [10]. To detect ozone, an ultraviolet absorption method (Ambient O3 monitor, Horiba APOA-360, HORIBA Ltd., Kyoto, Japan) was used. To simulate application-oriented situations, different working distances and angles around the nozzle were varied. Supplementary information regarding OES can be found in DIN 51008-2 [9].

2.2.6. Leakage current

In order to prevent humans or animals from electric shocks, and to guarantee basic isolation properties of medical devices, thresholds for several types of leakage currents had been defined in DIN EN 60601-1-4 [4]. More specific, usability and safety related aspects of electricity and optical radiation are treated in collateral standards DIN EN 60601-1-6 and DIN EN 60601-2-57. As CAP sources are driven by electricity they may transfer unwanted leakage currents to the body of a patient or the operator. It is highly recommended to check regularly for these currents during the development process of the plasma source. A very simple, standard-conform setup makes use of just a voltmeter and a frequency weighting filter. Alternatively, various commercial devices for safety tests are on the market. The kINPen® MED has been tested with a UNIMET® 800ST (Bender GmbH & Co. KG).

2.3. Measurements of biomedical efficiency

2.3.1. Chemical analysis

For the chemical analysis of plasma produced reactive species in liquids physiological saline (0.85% NaCl [w/v]) was used as liquid. 2 ml of saline was treated in 6 well plates for 1–5 min with plasma. Then, the formation of nitrite ($\text{NO}_2^-$), nitrate ($\text{NO}_3^-$), and hydrogen peroxide ($\text{H}_2\text{O}_2$) was photometrically detected (UV/VIS Spectrophotometer SPECORD® S600, Analytik Jena GmbH, Jena, Germany). $\text{NO}_2^-$ and $\text{NO}_3^-$ concentrations were detected according to DIN EN 26777 [11] and DIN 38405-9 [12], respectively, with the help of color forming reactions using commercially available test kits (Spectroquant®, Merck, Darmstadt, Germany). $\text{NO}_2^-$ can be detected by the formation of diazonium salt in the presence of sulphanilic acid in an acidic solution. In that case, diazonium salt reacts with $n$-(1-naphthyl)-ethyldiamindihydrochloride to a red violet azo dye. The detection of $\text{NO}_3^-$ is based on 2,6-Dimethylphenol (DMP), which reacts with nitrite ions to 4-nitro-2,6-dimethylphenol. $\text{H}_2\text{O}_2$ concentration was measured according to DIN 38409-15 [13]. In the presence of $\text{H}_2\text{O}_2$ titanyl sulfate reacts to the yellow colored peroxotitanyl sulfate which can be detected at 405 nm. Furthermore, pH was estimated after plasma treatment according to DIN EN ISO 10523 [14] with the help of a semi-micro pH-electrode ($\phi 4.5 \text{ mm}$; SNTek P13, Sentek Ltd., Essex, UK).

2.3.2. Inactivation of microorganisms

Five different microbial strains that represent common skin pathogens were used for the analysis of the inactivation efficiency of the kINPen® MED device (source: DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany):

- Staphylococcus aureus ATCC 6538/DSM 799 (S. aureus)
- Staphylococcus epidermidis ATCC 14990/DSM 20044 (S. epidermidis)
- Escherichia coli K-12 NCTC 10538/DSM 11250 (E. coli)
- Pseudomonas aeruginosa ATCC 10,145/DSM 50,071 (P. aeruginosa)
- Candida albicans ATCC 10321/DSM 1386 (C. albicans)

These microbial strains all refer to risk group II according to Section 3 the biological agents regulations and annex III of the directive 2000/54/EG. Therefore, they can be used in microbiological labs without additional restrictions.

For overnight cultures, microbial strains were cultured in tryptic soy bouillon (liquid medium A [pH. Eur. 7.0], Carl Roth GmbH & Co. KG, Karlsruhe, Germany) under aerobic conditions at 37 °C (bacterial strains) or at 30 °C (yeast strain). For stock culture preparation, Columbia blood agar plates (VWR International GmbH, Darmstadt, Germany) were used.

2.3.3. Microbial inhibition zone assay

Overnight precultures of each microbial test strain were centrifuged (5000g) and the pellets were resuspended in physiological saline (0.85% NaCl [w/v]) to get a stock suspension with a concentration of $10^8$ colony forming units per milliliter (cfu/ml). Agar plates (diameter of 90 mm filled with tryptic soy agar (Merck, Darmstadt, Germany) were inoculated with 100 µl of each diluted microbial suspensions. Afterwards, localized spot-like plasma treatments (1–5 min) were carried out on the inoculated agar plates. After an appropriate incubation time (bacterial strains: 24 h

...
at 37 °C; yeast strain: 48 h at 30 °C) diameters of the inhibition zones were determined by measuring the lengths of the longest and of the shortest diameter with a commercial ruler. Based on these values, a mean diameter was calculated in millimeters (mm).

2.3.4. Treatment of microbial suspensions

For these experiments overnight precultures and stock solutions of each microbial strain were prepared as described above. 5 ml of each microbial suspension were then plasma-treated in 6 well plates for 1–5 min. Afterwards the treated suspensions were diluted to obtain different concentrations (10⁻¹ to 10⁻⁵ cfu/ml). 100 µl of each concentration was plated on an agar plate and incubated aerobically for 24 h at 37 °C (bacterial strains) or 48 h at 30 °C (yeast strain) prior to colony counting. The upper detection limit was set to 350 cfu/ml and 1 cfu/ml was the lowest limit. Controls were not treated with plasma. Based on the numbers of viable microorganisms on non-treated \( N_0 \) cfu/ml and countable plasma-treated samples \( N_i \) cfu/ml, the logarithmic reduction factors \( \log R \) were calculated:

\[
\log R = \log N_0 - \log N_i
\]

Semi-logarithmic plots were chosen as graphical presentation for inactivation kinetics of microorganisms.

2.3.5. Treatment of human cells

The SV40 virus-transformed adherent skin fibroblast cell line GM00637 as a paro pro toto for skin cell toxicity was purchased from the Coriell Institute (Camden, New Jersey, USA). The cells were cultured at 37 °C (5% CO₂, 90% humidity) in DMEM High Glucose w/L-Glutamine (PAA, Coelbe, Germany) medium supplemented with 10% fetal bovine serum (FBS; Biochrome AG, Berlin, Germany) and 1% Penicillin/Streptomycin (PAA, Coelbe, Germany). Twice a week the fibroblasts were divided in 175 cm² cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany).

The day before plasma treatment, 2.5 × 10⁵ cells were seeded in Falcon® 35 mm cell culture dishes (Corning, Tewksbury, Massachusetts, USA) with 2 ml of DMEM. The next day medium was removed, cells were washed twice with 1 × PBS (phosphate buffered saline, pH 7.4), and covered with 1 ml of 1 × PBS. Cells were exposed to the plasma jet for 10, 30, 60, 90, or 180 s (duplicate samples were treated for each application time). Directly after punctual plasma applications (at most 5 min after treatment) 3 ml of fresh DMEM with 13% of FBS were added and the cell culture dishes were incubated for 48 h before performing the MTS assay as previously described.

To analyze the MTS results, raw absorbance data from the ELISA reader were corrected for background fluorescence by subtracting all absorbance values from a no-cell control including only DMEM and MTS solution. The corrected data were then normalized to the negative control (as 100% cell vitality). The standard deviation (SD) was calculated.

3. Results and discussion

3.1. The importance of the first DIN-SPEC in plasma medicine

To ensure a continual economic growth, it is not only sufficient to promote research and development, but to broadly disseminate new knowledge so that as many institutions and companies as possible may take benefit of it. Therefore, international standardization may be regarded as a strategic instrument for scientific and economic success.

In particular, standards that are prepared with the participation of companies involved in the respective field are suitable for a broad provision of innovative knowledge. This is of special importance as information laid down in standards is publicly available, in contrast to patents or licenses.

Based on current publications on cold atmospheric plasma research as well as on own research work, general biological, physical, and technical parameters were identified. The parameters were discussed among renowned experts from academic research and industry to determine standards which should be fulfilled by cold atmospheric plasma sources. In addition, research was done on current national and international specifications, which might be related to medical devices designated to produce and develop medical plasma sources. Relevant and present specifications were taken into account to prepare the DIN-SPEC 91315. In close cooperation with the German Institute for Standardization (DIN e.V., Berlin, Germany), a manuscript of informal or consortia standardization was prepared as a PAS (Publicly Available Specification), reviewed accordingly, and finally released as DIN-SPEC 91315. It should be noted that this specification is a first idea which can be amended.

3.1.1. Temperature

The temperature is one of the major key parameters to characterize plasma sources for medical applications. In the case of skin treatment a traumatization of skin and soft tissues must be excluded. A slight elevation of temperature (up to 38.5 °C) induces an increasing proliferation of living keratinocytes [15]. Therefore, plasma treatment can support wound healing and tissue regeneration if a threshold of 40 °C is not exceeded. It is also well known that higher treatment temperatures can cause denaturation of proteins and destruction of membranes.

The temperature of the CAP jet kINPen® MED was measured as axial temperature profile along the visible effluent outside the capillary nozzle (Fig. 2). As it is demonstrated in Fig. 2, the plasma temperature at the tip of the visible plasma jet, i.e. 7–10 mm outside the nozzle, is between 35 and 40 °C. This is the recommended application-oriented treatment distance of the kINPen® MED, which can be adjusted in practice by an exchangeable plastic spacer (see Fig. 1b). Therefore, a plasma temperature of 39 ± 1 °C is guaranteed at the contact point with the surface to be treated [5,6]. The determined plasma temperatures are within the biological tolerance range. Furthermore, treatments of tissues are carried out by moving the plasma jet over the target surfaces. In this way, a possible harmful heating of various target points is excluded.
3.1.2. Thermal output

As the temperature, the thermal output varies in the axial area of the visible plasma jet. The determined values decrease with increasing axial distance [8]. Because of the adjusted treatment distance the thermal output at the visible tip of plasma jet is important. At this point a relatively constant power transfer between 145 and 160 mW was detected with the kINPen MED. This result relates to the low temperature differences at the tip of the visible plasma jet. Therefore both properties indicate relatively constant energetic conditions at the tip of visible plasma jet.

3.1.3. UV-radiation and Optical Emission Spectroscopy (OES)

UV radiation is another important biologically active plasma compound but also a potential risk for living tissues. UV-A and UV-B have the ability to enter the epidermis and can cause damages to DNA, proteins and lipids [2]. Furthermore, different studies have shown that UV-C has a strong antimicrobial effect. This is due to the fact that UV-C radiation destroys DNA molecules of living microorganisms, spores, and viruses [16,17]. Moreover, UV emission during plasma treatment has different effects on treated skin areas. Therefore, it is possible to use UV-A and UV-B for special dermatological applications, like treatment of various inflammatory skin diseases [18–20], eczema [21,22], or connective tissue diseases [23–25]. On the other hand, strong UV irradiation is characterized as a human carcinogen. Acute effects of intensive UV exposure are inflammation, erythema, tanning, and local or systemic immunosuppression [26]. Furthermore, it is known that DNA damages are caused by high doses of UV-C and UV-B radiation. This procedure induces mutagenic and cytotoxic DNA lesions like 6–4 photoproducts and cyclobutane pyrimidine dimers (CPDs), which can be repaired by the nucleotide excision repair (NER) pathway [27,28]. If the DNA damages are not properly repaired in cells, carcinogenic or lethal effects may result [27]. Chronic effects of intensive UV exposure include photoaging of human skin, immunosuppression, and photocarcinogenesis [29].

For these reasons it is very important to characterize UV emission of medical plasma sources to exclude or minimize such risk factors in plasma therapy. Therefore, the optical emission spectrum was measured in the UV region from 200 nm up to 450 nm (Fig. 3).

In the UV-A region (315–380 nm) emission of nitrogen has been detected. Moreover, emission of OH radicals were measured in the UV-B region at 309 nm. No distinct emission lines could be measured in the UV-C range between 200 up to 280 nm [5]. The International Commission on Non-Ionizing Radiation Protection defined guidelines for non-therapeutic and non-elective ultraviolet radiant exposure of the skin [30]. In the spectral region from 180 to 400 nm the radiant exposure should not exceed 30 J/m² or 3 mJ/cm² as a daily dose. In the case of kINPen MED a maximum total irradiance of around 35 ± 5 µW/cm² were measured in the UV region of 100–400 nm at 10 mm application-oriented distance from the nozzle [5]. Fig. 3 shows the optical emission spectra between 200–450 nm. To keep the DIN-SPEC 91315 as simple as possible, technically challenging measurements in the VUV region < 200 nm are not included.

A punctual treatment in a working distance of 7–8 mm leads to an irradiated area of about 5 mm². Therefore, an area of 1 cm² consists of 20 surface elements. To treat an area of 1 cm², the nozzle has to be moved in a meandering pattern for 60 s. Hence,
each surface element is irradiated for about 3 s which corresponds to an effective irradiance of $105 \pm 15 \mu J/cm^2 = 1.05 \pm 0.15 J/m^2$ in the spectral region 100–400 nm. Therefore, 1/30 of the acceptable daily dose would be achieved [5]. This means, that there are absolutely no health risks due to ultraviolet irradiation when the kINPen® MED is used therapeutically. Furthermore, application of the kINPen® MED in practice will be done by moving the plasma jet over the area to be treated. This results in short contact times at one point. Therefore, unwanted side effects of UV irradiation can be avoided. Nevertheless, it should be considered that injured skin barriers are more sensitive against external influences, like UV irradiation. Therefore, examination of additional biological test systems in relation to plasma treatment should be performed.

3.1.4. Gas emission

The formation of reactive species also plays a major role in biological effects of plasma treatment. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by reactions with surrounding air can induce the production of toxic gases like ozone, nitrogen dioxide (NO2), and nitrogen monoxide (NO) [8,31]. High concentrations of these gases have a negative impact on the environment and pose a risk for patients, therapists and laboratory experts. Ozone, for example, is characterized as an air pollutant with harmful effects on the respiratory system of humans or animals. Long-term ozone exposure is associated with the development of asthma in humans [32]. Generally, the value of odor threshold in air is set at 0.02 ppm or 40 μg/m3. In addition, the EU determines values for non-health risk concentrations. The directive 2002/3/EG describes that ozone concentrations up to 0.055 ppm or 120 μg/m3 as a daily (8 h) average cause no health risk. Ozone examinations were done at several measuring points around the plasma jet. In the close vicinity of the visible effluent of the plasma jet, high ozone concentrations above 0.2 ppm were found. At a realistic distance representing the application conditions, i.e. more than 20–30 cm away from the plasma jet, concentrations were not higher than 0.055 ppm (Fig. 4, [5]). Therefore, the permitted exposure limit is not exceeded during the plasma treatment period.

NO2 is also characterized as an air pollutant with harmful effects on human or animal respiratory systems. NO2 is a product of NO, which was oxidized in the presence of oxygen or other oxidants. In case of plasma treatment in an atmospheric air environment, NO2 is emitted into the surrounding treatment area. Its odor threshold is set at 0.44 ppm or 0.9 μg/m3. The EU guideline 2008/50/EG specifies a daily limit value of 40 μg/m3, which should not be exceeded for the safety of human and animal health. However, nitrogen dioxide was not detectable around the visible plasma jet. To sum up, no health risks for patients and therapists are to be expected from the generation of toxic gases during plasma therapy with the kINPen® MED.

3.1.5. Leakage current

Electrical currents between plasma and the surface to be treated could at least partially account for biological plasma effects. For medical devices in general, the determination of leakage currents, like the ground leakage current, contact current and patient leakage current, are demanded to prevent dangerous electric shocks. In the case of the kINPen® MED, the leakage current value varies with the distances between nozzle and the treated surface (Fig. 5). In practice, the variations are minimized with the help of an exchangeable plastic spacer (working distance of 7 mm). The device has proven conformity to the safety related requirements of medical devices described in DIN EN 60601-1 - Medical electrical equipment - Part 1: General requirements for basic safety and essential performance [4]. In rare cases, sensitive people have the ability to perceive the electric current despite the statutory threshold values. The sensing threshold is individually and depends on the age and gender. From a physiological point of view, the measured current values here do not pose a health problem [33].

3.1.6. Chemical analysis

As already pointed out, ROS and RNS play a major role in biological effects of plasma treatment [34]. Usually, living cells, tissues and microorganisms are surrounded by liquid environments, both under experimental in-vitro conditions and also in living tissue environment. One of the fundamental insights of basic research in plasma medicine of the last years is that biological properties of tissue elements can be effectively changed by plasma treatment.
The pH value as well as nitrite, nitrate, and hydrogen peroxide are considered to be representative markers for complex reaction chains important for biological plasma effects. The pH value as well as nitrite, nitrate, and hydrogen peroxide are easy to detect and allow conclusions on main reactive processes in plasma-liquid interaction and rough estimations of biological performance of plasma sources.

Using the kINPen® MED for a spot-like treatment of physiological saline, i.e. without moving the plasma jet via the liquid surface, NO$_2^-$, NO$_3^-$, and H$_2$O$_2$ were detectable in different amounts (Fig. 6). The values of these species increased with longer treatment time. After five minutes of treatment 6.3 ± 0.9 mg/l H$_2$O$_2$, 2.0 ± 0.9 mg/l NO$_3^-$ and 0.8 ± 0.2 mg/l NO$_2^-$ were detected. At the same time, the pH shifts from 7.0 ± 0.006 (initial value) to 6.0 ± 0.02.

In summary, measuring the pH regime and detecting the concentrations of NO$_2^-$, NO$_3^-$, and H$_2$O$_2$ of a plasma treated liquid are ideal combinations to get first and basic information about chemical reactivity and resulting biological plasma effects and are therefore very useful as comprising output parameters for medical plasma sources.

### 3.1.7. Microbial inhibition zone assays

It is already known that plasma treatment results in an inactivation of various kinds of microorganisms [8,37–45]. These data are estimated using different plasma sources as well as different microbiological test approaches. However, a more or less consistent and broadly applicable antimicrobial test system to compare technically different plasma sources is required. Therefore, the microbial inhibition zone assay represents a simple and easy reproducible test procedure to detect the antimicrobial efficiency of different CAP sources. This assay represents an ideal test system for the inactivation of microorganisms in a wet environment being representative for in-vivo conditions, e.g. in infected wounds [39]. Furthermore, the size of inhibition zone after plasma treatment gives a rough orientation about the size of the active zone of the tested plasma device. Therefore, a comparison of spot-like and plane plasma sources is possible in the broadest sense. If the tested plasma source has a treatment zone bigger than 90 mm of diameter, agar plates with larger diameter have to be used.

The clear inhibition zone is characterized as a circular area without visible microbial growth after plasma treatment. The assay can be performed without moving the plasma source over the inoculated agar plate, i.e. both spot light and more plane plasma sources can be tested. Furthermore, no special equipment, like a xyz-table and specific software solutions are necessary. Different preliminary tests examined the most suitable initial concentrations of different microorganisms for the inhibition zone assay. It could be shown that a concentration of 10$^5$ cfu/ml of each microbial strain are the best for inhibition zone assays corresponding to about 16 cfu/cm$^2$ if 90-mm-diameter agar plates are used. If lower concentrations than 10$^5$ cfu/ml are used inhomogeneous microbial spreading may occur and thus, results would be difficult to evaluate. Higher concentrations than 10$^5$ cfu/ml lead to a compact microbial growth which is characterized by an overlap of living microorganisms on nutrition agar plates and inhibit the formations of sharply defined plasma inhibition zones. A set of five different microorganisms were chosen to perform the microbial inhibition zone assay: S. aureus, S. epidermidis, E. coli, P. aeruginosa and C. albicans. These five microorganisms represent a broad spectrum of medical relevant pathogens. Moreover, they are relatively easy to handle so that challenging and expensive laboratory equipment is not required. Therefore, they are ideal for a basic plasma source characterization if the practicability for medical applications and/or life-science experiments has to be identified.

Fig. 7 shows that the inhibition zones increased with longer plasma treatment times for all tested microorganisms. The inhibition zones of the gram-positive Staphylococcus strains showed
lowest initial diameter sizes compared to all other examined microbial strains. In the case of S. epidermidis an increase of 33% and in the case S. aureus an increase of 36% during the entire treatment time was identifiable. The examined gram-negative strains (P. aeruginosa and E. coli) showed higher initial diameter sizes. With longer treatment times significant bigger inhibition zones were detectable. For both P. aeruginosa and E. coli a total increase of 52% during treatment time was found. In contrast, C. albicans showed the biggest inhibition zones and an increase of 58%. To sum up, the kINPen® MED has the ability to inactivate all tested different microorganisms but in diverse dimensions. Nevertheless, the spot-like plasma treatment with the source resulted in an active zone bigger than the visible effluent. 

Using this inhibition zone assay different sensitivities and diameter sizes of inhibition zones could be estimated for the different test strains. Likely, the different cell wall structures of gram-positive and negative bacteria are an explanation for that. The cell wall of gram-negative bacteria contains a thin peptidoglycan layer, whereas gram-positive bacteria cell wall consists of multilayered peptidoglycan constructed with the help of peptide and glycan chains. Therefore, gram-positive bacteria are more resistant to physical influences than gram-negative bacteria, hence gram-positive bacteria are more difficult to inactivate with CAP [46]. Our results correspond to this inactivation behaviour, since the tested gram-positive strains (S. aureus and S. epidermidis) showed smaller inhibition zones than the gram-negative strains (E. coli and P. aeruginosa). Daeschlein et al. [45] could also show that treatment of gram-positive and gram-negative bacteria with different CAP sources leads to divergent results. Therefore, the susceptibility of microorganisms against plasma treatment differ from species to species with better susceptibility of gram-positive species [45]. This underlines the necessity of a global microbial proof for an in-vitro characterization of medical plasma sources.

It is assumed that yeast strains are more robust against plasma treatment than bacterial strains [47]. These results are in contrast to our findings as the yeast C. albicans shows the biggest inhibition zones after kINPen® MED treatment. Daeschlein et al. could also demonstrate that C. albicans showed the biggest inhibition zones after kINPen® MED treatment in contrast to other tested fungal strains [40]. This phenomenon illustrates again, that a proof of a broad spectrum of microorganisms for an in-vitro characterization of medical plasma sources is necessary.

3.1.8. Treatment of microbial suspensions

In a further experiment the antimicrobial efficiency of different CAP sources was tested with the help of microbial suspensions. This assay represents an ideal test system for the inactivation of microorganisms in a liquid environment and is sufficient to estimate any bulk effect of the plasma treatment. During preliminary investigations it was found that 10^5 cfu/ml are also the best microbial concentration to characterize different atmospheric plasma sources related to their antimicrobial efficiency in liquids. To simplify the test system a punctual plasma treatment of cell suspensions in 6-well plates were performed. If the tested plasma source has a treatment zone bigger than a cavity of a 6-well plate, plates or petri dishes with larger diameter have to be used. As described in the previous chapter, a set of five different microorganisms (S. aureus, S. epidermidis, E. coli, P. aeruginosa and C. albicans) was tested to cover a wide spectrum of plasma medical relevant pathogens.

No significant inactivation effects were found for a kINPen® MED treatment up to 5 min of the microbial suspensions (Fig. 8). Usually, the antimicrobial efficiency of a CAP source can be proven with the help of microbial suspensions [35,48]. In case of the kINPen® MED treatment the argon gas flow certainly blows the microbial suspension into the edges of the wells. On the one hand this should result in an intensive mixing of the microorganism suspension during treatment. On the other hand, the treated suspensions have no direct contact to the plasma jet. To solve this problem, a shaker can be used to keep the well plates moving in a reproducible manner so that the treated suspension rotates during plasma treatment. Still, for the treatment with the kINPen® MED no improved antimicrobial effect was found under these conditions. However, this assay was included in the DIN-specification to check the bulk effects of different plasma sources. Also, this assay was chosen to keep the test regulations as general and simple as possible and adaptable to all variable geometries of different medical plasma sources. For the revision of the DIN-SPEC 91315 three years after publication it must be kept in mind that this assay needs to be reviewed in the case of small-scaled and spot-like plasma devices.

3.1.9. Treatment of human cells

The intended use of plasma devices as medical instruments requires the knowledge about the direct effect of plasma on human cells. At the present, one major focus of plasma medicine is the plasma application in dermatology. Hence, adherent human skin cells are recommended as test cells. A very common and simple in-vitro test procedure for the investigation of cytotoxicity effects after plasma treatment is the MTS assay, a cell vitality assay. As described in the DIN-SPEC 91315 we chose an immortalized skin fibroblast cell line (GM00637) for these experiments.

Fibroblasts were treated punctually for up to 180 s, whereby cells were covered with 1 ml of 1× PBS. As expected the cell vitality decreases with longer treatment time. After 30 s of plasma application the cell vitality already decreased to about 56%, thus the median lethal dose (LD50) is presumably around an application time of 35 s. After 90 s of constant treatment almost no cell vitality was detected and after 180 s of plasma treatment cells were completely eliminated (Fig. 9, black bars). The strong and fast cell death after kINPen® MED application is not only the result of a direct lethal effect of plasma, but can also be explained at least partially by the strong gas pressure. Plasma treated GM00637 cells are growing in a monolayer and the mechanical force of the gas
pressure of 5 slm is able to detach the cells from the bottom of the cell culture dish. In addition, exactly at the point of plasma jet contact with the culture dish, cells were not covered with PBS anymore. This certainly led to dehydration of cells at that plasma contact point during the plasma treatment. This phenomenon can cause lethal damages of the cells. For the determination of the gas pressure depending injury of the cells additional experiments were performed. In this experiment the argon gas without igniting the plasma were tested. These experiments were performed with the kINPen\textsuperscript{\textregistered} 09 device as the predecessor of the kINPen\textsuperscript{\textregistered} MED. Therefore, the physical properties of these devices are very similar and experimental conditions like the gas pressure were adapted to the experiments with the kINPen\textsuperscript{\textregistered} MED. Results showed that treatment time independent cell loss of about 15% occurs after gas application alone (Fig. 9, grey bars). Subtracting the cell loss due to the gas pressure from the plasma application data, the actual plasma effect on GM00637 cells after 30 s is about 71.5% and the LD50 value is around 45 s for kINPen\textsuperscript{\textregistered} MED application.

Because of the hypothesis that ROS as well as RNS are mainly responsible for the biological plasma effects, different concentrated H\textsubscript{2}O\textsubscript{2} solutions were used as positive control. Cells were incubated in 6 \textmu M, 7.5 \textmu M or 9 \textmu M H\textsubscript{2}O\textsubscript{2} solution for 30, 60, 90 and 180 s. The MTS tests revealed that the vitality of GM00637 cells decreased with higher H\textsubscript{2}O\textsubscript{2} concentrations. For the incubation with the 6 \textmu M H\textsubscript{2}O\textsubscript{2} solution the vitality was about 77% (data not shown), 54% for 7.5 \textmu M (Fig. 9, white bars) and 42% for 9 \textmu M (data not shown) H\textsubscript{2}O\textsubscript{2} solution. Different incubation times seemed to have a minor impact on the cell vitality, as results showed only a slightly decreasing vitality with higher concentrations of H\textsubscript{2}O\textsubscript{2} (Fig. 9, white bars). The DIN-SPEC 91315 defines the incubation with the 7.5 \textmu M H\textsubscript{2}O\textsubscript{2} solution as positive control since this concentration is the most suitable one for the detection of the LD50 value.

Other studies also revealed that the severity of the skin cell damage depends on the treatment time. In the study of Haertel et al.\textsuperscript{[49]} HaCaT keratinocytes were treated for 10 or 30 s with the kINPen\textsuperscript{\textregistered} 09 device\textsuperscript{[49]}. Suspended HaCaT cells were treated in full cell culture medium and 24 h after plasma treatment the ability of the cells to re-attach to the cell culture dish and the expression levels of different adhesion molecules were analyzed. Treatments for 10 s had no effects on the keratinocytes, whereas 30 s of argon plasma treatment led to a decreased number of cells which could re-attach and furthermore the expression levels of some adhesion molecules changed. However, a time-dependent decrease of cell vitality was also shown 2012 by Hoentsch et al.\textsuperscript{[50]}. They also treated suspended murine epithelial cells (mHepR1) in complete cell culture medium for 30, 60 and 120 s with the kINPen\textsuperscript{\textregistered} 09. The vitality of the cells and cell-cell connections via tight junctions were analyzed. The results revealed that a 30-s plasma treatment caused changes of the cell morphology; from flat to a round cell shape. And even 72 h after treatment these rounded cells were not able to adhere again. In addition the cell-cell attachment degraded with longer treatment times until large openings between these cells occurred. The cell vitality was measured using the MTS assay and results show a decreasing vitality with longer treatment times (\textsim 90\% after 30 s treatment, \textsim 60\% after 60 s of treatment and \textsim 10\% after 120 s of treatment). These findings are comparable to data shown in this study (decreasing cell vitality with longer application times).

Nevertheless, the vitality of GM00637 cells is apparently stronger affected by plasma treatment than the vitality of murine epithelial cells (compare cell vitality after a 60-s plasma treatment: 23\% for GM00637 and 60\% for mHepR1). One explanation for that is that different cell lines were used whereas GM00637 seems to be more sensitive to plasma application than mHepR1. Also, the weaker gas pressure used in the study of Hoentsch et al. with 1.9 slm compared to 5 slm in the present study can be an explanation\textsuperscript{[50]}. Still, our results reveal a strong and fast cytotoxic effect of the tested plasma jet. Therefore, it should be clarified at this point that these results cannot be transferred to or compared with plasma applications of the human skin. In these in vitro tests cells are growing in monolayers, thus are more sensitive to external physical or biological influences. Skin cells that are capable of cell division are growing in the complex tissue structure of the skin and are protected by the stratum corneum (layers of dead corneocytes). Consequently it is reasonable to assume that cells of the human skin are way more resistant against plasma treatments.

However, the MTS assay and the previously described inhibition zone test procedure of different microbial strains are reliable, simple and well reproducible test systems for a fast and basic investigation of plasma dependent toxic effects on eukaryotic and prokaryotic cells. But since the results are not completely transferrable to living skin models, further standardization processes are needed which include more specific test assays, like skin or animal models.

In this study, we introduce the usage of a plasma jet for these experiments. For the use of other plasma sources where the production of the plasma is based on other physical principles as it is the case for dielectric barrier discharges (DBD), these experiments have to be adjusted. In general, experimental conditions should be chosen with regard to the application form of the device. When using the kINPen\textsuperscript{\textregistered} MED for example, the length of the effluent determines the distance between the nozzle of the device and the treated surface because the tip of the visible effluent has to touch the target. DIN-SPEC 91315 describes that plasma devices have to be tested by a punctual treatment of the agar plates and cell culture dishes. In the case of DBDs where the ignition of the plasma is based on other physical principles as it is the case for dielectric barrier discharges (DBD), these experiments have to be adjusted. In general, experimental conditions should be chosen with regard to the application form of the device. When using the kINPen\textsuperscript{\textregistered} MED for example, the length of the effluent determines the distance between the nozzle of the device and the treated surface because the tip of the visible effluent has to touch the target. DIN-SPEC 91315 describes that plasma devices have to be tested by a punctual treatment of the agar plates and cell culture dishes. In the case of DBDs where the ignition of the plasma requires a small distance to the target, it must be ensured that all cells in the dish are equally exposed to the plasma for the correct investigation of the cell vitality when performing MTS assay. In these cases it can be achieved by circular movement of the cell culture dishes.
4. Conclusion

The first German specification DIN-SPEC 91315 “General requirements for medical plasma sources” describes generally and easily reproducible assays which define basic criteria for plasma sources for medical applications. With the results it is possible to characterize the basic performance of medical plasma sources, evaluate their efficiency and improve the safety for users (e.g. user, patients, and therapists/investigator). Based on kINPen® MED investigations, the presented study demonstrates that the general assays described in DIN-SPEC 91315 are easily to adapt. DIN-SPEC 91315 is a developing specification which constantly improves by suggestions and legwork of the plasma medical community. In joint cooperation further efforts should be made to create an international standard for specific plasma sources and their individual application fields. More specific test assays should be transformed for a better transferability to living skin models. With this attend it is possible to fulfill the high expectations of patients and ensures the functionality and safety of medical plasma sources.

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