Dose- and Time-Dependent Cellular Effects of Cold Atmospheric Pressure Plasma Evaluated in 3D Skin Models

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Abstract

Background: Application of cold atmospheric pressure plasmas (CAPs) in or on the human body was termed ‘plasma medicine’. So far, plasmas were utilized for sterilization of implants, other heat-sensitive products, or employed for chemical surface modifications. By now, CAPs are further used effectively for wound treatment. The present study analyses the effect of a plasma jet with air or nitrogen as process gas, previously evaluated for antimicrobial efficacy, on human cells using a 3D skin model. Methods: CAP treatment of 3D skin models consisting of a keratinocyte-containing epidermal layer and a fibroblast/collagen dermal matrix was performed using the Tigres plasma MEF technology. To evaluate the effects on the 3D skin models, the following plasma parameters were varied: process gas, input power, and treatment time. Results: Low CAP doses exhibited good cell compatibility. Increasing input power or elongating treatment intervals led to detrimental effects on 3D skin model morphology as well as to release of inflammatory cytokines. It was further observed that air as process gas was more damaging compared to nitrogen. Conclusions: Treatment of 3D skin models with the plasma MEF nozzle using air or nitrogen is reported. A clearly dose- and time-dependent effect of CAPs could be observed in which the CAP based on nitrogen exhibited higher cell compatibility than the CAP generated from air. These settings might be recommended for medical in vivo applications such as wound decontamination.

Key Words
Biocompatibility · Cold atmospheric pressure plasma · Fibroblast · Keratinocyte · 3D skin model

Introduction

Plasma medicine is a current research field with great promise. In the past plasmas have been mainly used for material surface activation or to create functional surfaces on diverse fabrics. Cold atmospheric plasmas (CAPs) have also been applied for sterilization of heat-sensitive medical equipment due to their bactericidal effectiveness [1]. Hence, most biomedical studies on the effect of CAPs have focused on the bacteriostatic and bactericidal properties of this new technology [2–4].
ferent physical and chemical processes, like thermal radiation, (V)UV radiation, formation of free radicals as well as other charged particles and chemical products, add simultaneously to the action of plasma. Studies on CAPs suggested the following mechanisms of plasma-cell interaction: (1) direct destruction by UV irradiation through microbial DNA damage, (2) micro-organism erosion by intrinsic photodesorption and the resulting break of chemical bonds, (3) erosion of the micro-organism through etching by atomic or molecular radicals, (4) diffusion of oxygenated species through cell wall- or spore material-ensuing oxidative damage to the cytoplasmic membrane, microbial proteins, and the DNA, and (5) lysis of the micro-organisms as a result of membrane rupture due to accumulation of charged particles [5–7]. The contributing atoms, positive and negative ions, free radicals, excited molecules and photons [8] are also known to independently influence cellular proliferation, differentiation and viability [9]. For example, UV radiation induces DNA damage leading to cell death [10, 11]. Superoxide and hydrogen peroxide as examples of charged and neutral components of plasma-generated species are known to stimulate apoptosis at certain concentrations [12, 13]. In addition, recent studies have highlighted the effects of CAPs on tissue regeneration, clearly demonstrating a direct influence of CAPs on the cellular level [14–16]. Hence, it is of interest to analyse the effects of cold atmospheric pressure plasmas (CAPs) on cells and tissues correspondingly to the antimicrobial activity. This can help to optimize treatment protocols for future clinical use.

Previously, we have looked at the antimicrobial activity of CAPs generated by the plasma MEF technology against different bacteria strains (Staphylococcus aureus, Pseudomonas aeruginosa) and yeast (Candida albicans). The studies showed that CAPs exhibit profound bactericidal and fungicidal properties in vitro depending on the chosen plasma parameters, in particular on the process gas used, the input power, and the number of treatments performed [17]. The aim of this paper is to investigate the interaction of CAP generated by the plasma MEF nozzle with human skin cells. The cell compatibility of the CAP generated is analysed depending on process gas (air/nitrogen) as well as increase in input power and treatment time. To evaluate the effects, appropriate 3D skin models comprised of a dermal fibroblast collagen matrix with an epidermal keratinocyte layer on top are employed.

Materials and Methods

3D Skin Models – Cell Culture and Organotypic Cocultures

Human normal epidermal fibroblasts (Promocell, Germany) were cultured in Dulbecco’s modified Eagle’s medium (Promocell) supplemented with 5% fetal bovine serum (Promocell), 1% gentamycin (Life Technologies, USA), and human epidermal growth factor (5 ng/ml) at 37°C in a 5% CO2 atmosphere. Human normal epidermal keratinocytes (Promocell) were cultured in a keratinocyte growth medium kit classic with low bovine pituitary extract (Pelorbiotech, Germany) at 37°C and in a 5% CO2 atmosphere. For the generation of the skin equivalent, fibroblasts and keratinocytes were harvested by trypsin-EDTA (Life Technologies) treatment.

For the preparation of the 3D skin models, rat tail collagen (Fraunhofer IGB, Germany) was mixed at a ratio of 1:1 with gel-neutralizing solution (Fraunhofer IGB). Freshly harvested human normal epidermal fibroblasts were seeded into the collagen solution, giving a final concentration of 1 × 106 fibroblasts/ml. The collagen-fibroblast solution was pipetted into 12-well inserts (Greiner Bio-One, Germany) and incubated for 15 min at 37°C and in a 5% CO2 atmosphere to ensure the curing of the matrix. This artificial dermis was incubated at 37°C for 24 h in a submersed medium consisting of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum and 1% gentamycin. The next day, keratinocytes were added on top of the dermis giving a concentration of 1 × 106 cells/ml and incubated with submersed medium consisting of keratinocyte basal medium 2 (Promocell), insulin (5 μg/ml), transferrin (10 μg/ml), bovine pituitary extract (0.004 ml/ml), epidermal growth factor (0.125 ng/ml), hydrocortisone (0.33 μg/ml), epinephrine (0.39 μg/ml), CaCl2 (0.06 mmol/l), 5% fetal calf serum and 1% gentamycin. For 7 days of submersed cultivation, the culture was raised to the medium-air interface while replacing the inserts in 12-well ThinCert™ cell culture inserts (Greiner Bio-One). The defined medium was composed of Dulbecco’s modified Eagle’s medium + Ham’s F12 (ratio 1:1), 5% fetal calf serum, 1% gentamycin, 10 ng/ml human epidermal growth factor, 0.33 μg/ml hydrocortisone, 10−4 M adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 2 × 10−7 M tri-iodothyronine, and 1.88 mM CaCl2. After 12 days of incubation, the models were fully differentiated.

Plasma Treatment

A plasma single jet (Plasma MEF, Tigres, Germany) was used for experiments. This plasma jet consists of a concentric inner electrode and flows out of an opened nozzle. The plasma is ignited between both electrodes and flows out of an opened nozzle. For treatment, 3D skin models were fixed onto a movable x-y-stage aligned with a distance of 10 mm to the burner. The plasma treatment was performed using the plasma MEF system (fig. 1) with either the process gas air or nitrogen (gas pressure: 4.5 bar). A punctual treatment of 5 s was chosen while varying the input power between 80 and 300 W. Thereafter, skin models were incubated for 24 h at 37°C and in a 5% CO2 atmosphere. The supernatant of each sample was collected and frozen at −20°C until analysis, and 3D skin models were transferred to 4% formalin solution (Dr. K. Hollborn & Sohne, Germany) for histology or referred to RNA isolation for gene expression analysis. Untreated 3D skin models were used as controls.
Histopathological Examination

Constructs were prepared using standard histology protocols. Formalin-fixed samples were put through a series of dehydration processes and finally embedded in paraffin (Merck, Germany). Then, cross-sections of 4 μm thickness were cut and mounted on glass slides. Sections were rehydrated and stained with haematoxylin and eosin (Merck) using an automated slide stainer (Leica, Germany). Slides were studied for histological changes, microphotographs were taken using the Axio Scope A.1 (Carl Zeiss, Germany), and images were obtained with the digital camera ColorView II (Soft Imaging Systems).

Gene Expression Analysis

3D skin models were removed from the inserts, and RNA was isolated using a Qiagen RNeasy Mini purification kit (Qiagen, USA). The isolated RNA was converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) using 10 ng RNA. Real-time PCR was carried out using the SensiFAST™ SYBR No-ROX Kit (Bioline, Germany) for interleukin 8 (forward: 5′-ATG ACT TCC AAG CTG GCC GT-3′, reverse: 5′-TCC TTG GCA AAA CTG CAC GT-3′), interleukin 6 (forward: 5′-CCA CCG GGA ACG AAA GAG AA-3′, reverse: 5′-GAG AAG GCA ACT GGA CCG AA-3′), and interleukin 1α (forward: 5′-CGC CAA TGA TGA CTC AGA GGA AGA-3′, reverse: 5′-AGG GCG TCA TTC AGG ATG AA-3′). β-Actin was included as housekeeping gene (forward: 5′-CCA ACC GCG AGA AGA TGA-3′, reverse: 5′-CCA GAG GCC CAC AGG GAT AG-3′). For this, 400 nM of each primer and 2× SYBR Green-Mix at a total sample volume of 20 μl were run on the Rotor-Gene-Q (Qiagen, USA). PCR products were amplified over 40 cycles (95 °C for 120 s, 60 °C for 10 s, 72 °C for 20 s). The relative level of gene expression was calculated using the 2−ΔΔCt method [21].

Detection of Interleukins by ELISA Assay

For this, the supernatants of the skin models were thawed immediately. For quantification of interleukins the IL-6 ELISA kit (Mabtech, Sweden), IL-8 ELISA kit and IL-1α ELISA kit (R & D Systems, USA) were used. The tests were performed according to the manufacturers’ protocols. Optical density was measured at 450 nm with a reference measurement at 620 nm. Interleukin concentrations were calculated according to a 4-parameter fit with lin-log coordinates for optical density and concentration. For evaluation, concentrations of interleukins were normalized to the protein content of the supernatant.

Determination of Protein Content

For protein quantification, the supernatant of the skin models was used. The test was performed using the protein quantification kit (Interchim, France) according to the manufacturer’s protocol. Optical density was measured at 580 nm. Protein concentrations were calculated based on a bovine serum albumin standard curve included in the test.

Statistical Analysis

All tests were run in duplicate. One-way analysis of variance was carried out to determine statistical significances (Microsoft® Excel 2000). Differences were considered statistically significant at a level of p < 0.05. Asterisks indicate significant deviations from the control at the respective incubation time (* p < 0.05; ** p < 0.01; *** p < 0.001).

Results

Dose-Dependent Effects of CAP on 3D Skin Models – Histological Changes

The generated plasmas demonstrated an effect that strongly depended on input power. Treatment at 300 W for 5 s led to damage in the epidermal layer and reached as far as the dermal layer regardless of whether air or nitrogen was used (fig. 2). The epidermis of the 3D skin model shows an almost burned surface, and injured fibroblasts in the dermis are recognizable by their roundish appearance and dark colour. With decreasing power, the damage to the cell layers is distinctly reduced. At 200 and 150 W, extension of harm is limited to upper dermal levels. Differences between results for using air or nitrogen as process gas were observed. While CAP based on nitrogen did not longer induce damage at an input power of 100 W, air-based CAP still evoked injury to the epidermal layers. Application of CAP produced at 80 W was well tolerated, and histological sections looked comparable to untreated controls.

Dose-Dependent Effects of CAP on 3D Skin Models – Gene Expression and IL Release

Relative expression of inflammatory cytokines IL-1α, IL-6, and IL-8 by CAP-treated 3D skin models was analysed after 24 h (fig. 3). IL-1α did not show a significant...
alteration in expression after this time. However, in accordance with the harmful effects of the CAP using air as process gas, an increase in the expression of IL-6 and IL-8 was observed with increasing input power. Similarly, IL-6 and IL-8 expression increased with treatment of nitrogen-based CAP while intensifying input power from 100 to 200 W. Yet, the expression levels dropped at 300 W. The cytokine release measured after 24 h (fig. 4) mirrored the gene expression results. A distinct increase in IL-6 and IL-8 release with enhancing input power was noted. Moreover, IL-1α levels after treatment with CAPs were found to be augmented compared to the untreated control independently if air or nitrogen was used during the plasma treatment. In most cases, slightly lower levels of all 3 cytokines were noted with nitrogen-based CAP compared to CAP using air as process gas. In all experiments cytokine concentrations at maximum input power of 300 W again decreased in keeping with the damage observed in the responding histological sections.

Time-Dependent Effects of CAP on 3D Skin Models – Histological Changes

3D skin models were treated with CAP at an input power of 100 W using air or nitrogen as process gas over an increasing treatment time (fig. 5). Again, it could be shown that nitrogen-based CAP was better tolerated than air-based CAP. However, both working gases evoked cellular damage at prolonged time intervals. First signs of injury were noted at as short as 5 s for CAP generated with air as process gas, moving farther into the cell layers with treatment time. After 25 s, most of the fibroblasts in the lower dermal regions were harmed (depth of 250 μm). Application of nitrogen-based CAP was tolerated up to 10 s; at this point, damage of keratinocytes in the epidermal layer was noted. Prolonging treatment times further, led to spread of cellular injury into the dermal level at a depth of 150 μm.

Time-Dependent Effects of CAP on 3D Skin Models – Gene Expression and IL Release

Time-dependent effects of CAP treatment on expression of the inflammatory cytokines IL-1α, IL-6, and IL-8 by 3D skin models were observed after 24 h (fig. 6). Again, IL-1α demonstrated distinctly slighter changes in expression after this time than IL-6 and IL-8. The relative expression levels of the latter increased distinctly with augmentation of treatment time from 5 to 20 s. Similarly to the drop noted in expression from 200 to 300 W, there
was a decline in expression in 3D skin models treated for 20 compared to 25 s echoing the damage observed by histological methods. Release of IL-6 and IL-8 (fig. 7) represented gene expression results. IL-1α levels were found to be negligible. IL-6 and IL-8 liberation by treatment with nitrogen-based CAP increased in a time-dependent manner. 3D skin models treated with air-based CAP showed an enhancement in IL-6 and IL-8 release from 5 to 15 s while after further prolongation of the treatment lower cytokine levels were found in accordance with the damage observed in the responding histological sections. Again, results reflect a better tolerability of CAP from nitrogen compared to CAP using air as process gas.

**Discussion**

CAP represents an alternative physical procedure for medical antimicrobial treatment. Positive effects of plasma applications have been shown in various diseases including chronic ulcer wounds, bacterial dermatitis, eyelid infections, and pulmonary tuberculosis [22, 23]. Several recent studies have looked at the antimicrobial effects of CAPs. It was demonstrated that bactericidal activity is mediated by reactive radical species, positive and negative ions as well as UV radiation [17, 24–28]. The composition of active constituents varies with the plasma sources and process gases used. With the plasma MEF, for instance, antimicrobial efficacy was found to be higher using nitro-
gen compared to air as process gas, to increase with input power, and to be more pronounced with extended treatment cycles [17]. However, plasma-produced active species may not solely interact with the micro-organisms present but are likely to influence body cells as well. UV radiation has been shown to induce DNA damage leading to cell death [10, 11]. Moreover, reactive oxygen species (e.g. O, OH, O₂⁻) have been considered to prompt apoptosis at certain concentrations [12, 13]. Optimization of treatment protocols for future clinical uses requires the determination of the effects of bactericidal active plasma sources on human cells and tissues.

Hence, this study analyses the influence of CAPs based on nitrogen or air as process gas and generated by the plasma MEF technology on 3D skin models in vitro. Low plasma doses did not affect cells negatively. Good cell compatibility was also observed by other groups [23, 29]. Here, it is thought that the amount of active species that eventually reaches the cell lies in the physiological range and is comparable to the radical concentrations produced by the organism itself during an increased activity such as tissue repair processes [23, 30]. However, it was found that CAPs demonstrate a pronounced effect on 3D skin model morphology as well as on release of inflammatory cytokines at high input power or long treatment intervals, corresponding to administration of higher plasma doses. In addition, it was observed that air as process gas had a more detrimental effect on the 3D skin models compared to nitrogen. These results are in accordance with a study published by Lin et al. [9], who showed a dose-dependent decrease in cell viability. They further signify the importance of plasma-generated charges and reactive oxygen species as contributors for plasma-induced cell death through cell lysis. Moreover, the study showed that the global electric field, generated from plasma, by itself does not play a role in cell killing and that effects of UV in cell killing are negligible and may only play an ancillary role [9]. They also observed a higher cell compatibility following discharge of nitrogen gas. In conformity, our findings demonstrate that the oxygen species produced by air plasma are the major contributors to plasma-induced cell damage in 3D skin models. In previous investigations we could show that the major difference between the use of nitrogen and air as process gases is associated with the formation of different reactive species. Thus, in corresponding optical emission spectroscopy measurements we found for the Tigres MEF system strong atomic oxygen emission lines in the case of using air as process gas.
whereas in the case of using nitrogen we could not find such lines. Furthermore, the formation of NO molecules seems to be stronger when using air as process gas [17]. Concluding, the observed higher plasma-induced cell damage in the 3D skin models by using air as plasma process gas can be associated especially with the interaction of atomic oxygen with the cell matrix. Immediate cellular damage by CAPs at high input power in the 3D skin models through cell lysis would further account for the decreased gene expression rates and reduced release of the pro-inflammatory cytokines IL-1α, IL-6 and IL-8.

**Conclusion**

Generally, the plasma impact may induce cell proliferation, differentiation, and apoptosis [9, 14–16, 30, 31]. These pleiotropic effects on cells reported might be explained by differences between devices, settings, or cellular models [32]. A study by Pai et al. [33] showed different outcomes during plasma treatment of HUVEC-2, neuroblastoma, and HePG2 cells at the same plasma doses, with the most prominent effects on HePG2 while HUVEC cells were more resilient and neuroblastoma cells showed intermediate behaviour. Diversities in cellular behaviour might be attributed to distinctive membrane
compositions and physicochemical properties. It was recently shown that tuning negative charging and membrane strength of liposomal membranes using cholesterol accelerates the plasma membrane reactions and introduces posttreatment phenomena similar to that of plasma-treated cells [34]. Moreover, use of serum-containing or serum-free media affects experimental outcomes when using the same plasma device and settings [32]. In addition, cells might react differently while kept in monolayers or cultured in a more in vivo-like setting using 3D models. Here, we report the treatment of 3D skin models with the plasma MEF nozzle using air or nitrogen as process gas with previously tested settings showing antibacterial activity [17]. A clearly dose- and time-dependent effect of CAPs could be observed in which the CAP based on nitrogen exhibited higher cell compatibility than CAP generated from air. These settings might therefore be recommended for future in vivo applications such as wound decontamination. In contrast, air as process gas might be deemed too toxic when repeated or longer treatments are necessary. Furthermore, it might be of interest to correlate the levels of pro-inflammatory cytokines, which act as alert signals, to anabolic cues, such as transforming growth factor or fibroblast growth factor, to evaluate not only the cellular compatibility of CAPs, but also the interaction with the cells treated. This shall be further explored in adjacent studies.

**Statement of Ethics**

The research did not involve human participants and/or animals. All experimental procedures followed GLP requirements.

**Disclosure Statement**

The authors report no conflicts of interest.

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**References**