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Cold atmospheric plasma (CAP) differently affects migration and differentiation of keratinocytes via hydrogen peroxide and nitric oxide-related products

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ABSTRACT

Background: A promising approach to treat infected chronic wounds is the treatment with "cold" atmospheric plasma (CAP) that has a broad antibacterial spectrum and can enhance microcirculation. Dielectric barrier discharge (DBD) devices generate CAP containing reactive species, leading to acidification and the accumulation of hydrogen peroxide (H_2O_2), nitrite and nitrate within the treated tissue/liquids.
Objective: Since CAP produced species may affect wound healing and cell behavior, we investigated the possible DBD/CAP-induced effects on human keratinocytes.
Methods: Primary keratinocytes were treated by a DBD device (13.5 kV, 300 Hz; 0–300 s). DBD-induced changes (pH; nitrite, nitrate; H_2O_2) in treated media were evaluated. As control and to investigate the impact of the CAP-produced species, equivalents amounts of H_2O_2, HCL, nitrite and nitrate as obtained by CAP treatments (0, 60, 300 s) were added separately or combined to keratinocytes. Cell viability and proliferation were determined by live cell imaging and a resazurin-based assay. Gap closure rates were assessed by migration assays. Differentiation/proliferation states were determined by qRT-PCR analysis of KI67 and involucrin.
Results: We found that even longer CAP-treatment times (300 s) did not reduce cell viability. However, migration/proliferation was affected by longer treatments resulting in a delay of gap closure in migration assays. The mRNA expression of involucrin and KI67 showed a pro-differentiation effect induced by longer CAP treatment. Similar effects could be induced by adding H_2O_2 in amounts found after a 300 s CAP treatment. The effects were reversed by catalase. Shorter CAP treatment (60 s) did not reveal pro-differentiation effects, but significantly accelerated gap closure. Lower H_2O_2 concentrations, equivalent to a 60 s CAP treatment, induced also upregulation of involucrin, which in turn could be diminished by low concentrations of nitrite/nitrate, indicating a potential mediation of H_2O_2-induced effects by parallel CAP-induced accumulation of these nitric oxide derivatives.
Conclusion: CAP treatment theoretically could kill several birds with one stone—overcome bacterial contamination, improve microcirculation and additionally compensate missing H_2O_2 and nitric oxide— facilitating wound healing. However, clinical CAP treatment must be well balanced to avoid possible unwanted side effects, such as a delayed healing process and tissue damage.

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1. Introduction

1.1. Skin and wound healing

The epidermis is a stratified squamous epithelium on the skin's outer surface and acts as the major barrier to protect the body against pathogens and excessive water loss.

The major cellular components of epidermis are proliferating basal and differentiated keratinocytes, which are crucial in the process of wound healing; thus they are involved in the initiation, maintenance, and completion of wound healing [1,2]. Since the epidermis provides a barrier against infection and maintains homeostasis, improving the re-epithelialization process, particularly in impaired healing situations, has attracted a lot of attention in dermatology and surgery. Multiple local and systemic factors can lead to impaired wound healing. In particular, patients with diabetes mellitus have impaired dermal microcirculation resulting in chronic wounds by chronic hypoxia, e.g. the diabetic foot syndrome [3,4]. It is assumed that reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), can act as cellular messengers in normal wound healing, stimulating cell motility, cytokine action, and angiogenesis. Hypoxia as well as hyperoxia cause excessive ROS production, which in turn eliminates the beneficial effects and causes additional tissue damage [5,6]. The contamination and/or colonization of wounds with bacteria can also cause impaired healing. In particular, Pseudomona aeruginosa, β-hemolytic streptococci and Staphylococcus aureus are common bacteria in infected and clinically non-infected wounds [7,8]. For example, many chronic ulcers may not heal by the formation of P. aeruginosa containing biofilms, which shield the bacteria from the phagocytic activity of invading polymorphonuclear neutrophils [9].

1.2. Cold atmospheric plasma for wound treatment

A new promising approach to overcome wound contamination is the topical treatment with non-thermal “cold” atmospheric plasma (CAP) that can easily be generated by electric gas discharges. CAP contains a complex mixture of inter-reacting and short-living ions, atoms, excited species, photons and free electrons [10], which can effectively kill or inactivate a broad spectrum of bacteria [11,12] without known resistance formation. In addition, CAP can enhance microcirculation and reveal some antipruritic and anti-inflammatory effects, making the use of CAP attractive for the treatment of many skin diseases [13-15]. In a Phase I clinical trial, Brehmer et al. have shown the reduction of the bacterial load of chronic wounds by CAP treatment without direct harmful side effects [16]. The CAP device used (PlasmaDerm, Cinogy) is a dielectric barrier discharge (DBD); thus, the plasma is ignited directly between the electrode of the device and the human skin (wound) that acts as a counter electrode.

Operated with ambient air, DBD-derived CAP contains reactive nitrogen/oxygen species such as nitric oxide (NO), nitrogen dioxide (NO_2), ozone (O_3), superoxide (O_2^-) and hydroxyl radicals (^-OH) [17]. In particular, NO is an important molecule in human skin physiology and wound healing [18,19]. In addition, the formation of hydrogen peroxide (H_2O_2) has been identified for many CAP sources and settings [20,21]. Apart from its function as an oxidant with antibacterial and cell toxic effects, H_2O_2 in lower concentrations serves as a signaling agent in signal transduction pathways [22]. Studies have demonstrated the induction of cell death in many cell types, e.g. HaCaT by treatment with other CAP devices [23-29]; thus, DBD-derived CAP may exert toxic effects on keratinocytes, which in turn could interfere with the wound healing process.

1.3. Study objective

Regarding the possible use of CAP for improved wound healing, in this study we evaluated possible CAP-induced toxicity in human primary keratinocytes. As plasma source a CAP device similar to the clinically approved PlasmaDerm system was used. The CAP-induced effects on wound healing parameters, such as proliferation, differentiation, and migration of keratinocytes, was determined in vitro. Since CAP treatment can induce acidification and the accumulation of NO-related products and H_2O_2 in skin tissue [15,30,31], we have investigated whether these physical/chemical changes play a role in the underlying mechanism of the CAP-induced effects.

2. Material/Methods

2.1. Cell isolation and culture

Primary human keratinocytes were isolated from dermal explants of abdominalplasty obtained from plastic surgery, as described in more detail elsewhere [32], with the approval of the Ethics Commission of Düsseldorf University (Study No. 3634) and in accordance with the Declaration of Helsinki. After isolation, the keratinocytes were cultured in Keratinocytes SFM medium (Gibco, Frankfurt, Germany), supplemented with Epidermal Growth Factor 1–53 (EGF 1–53), Bovine Pituitary Extract (BPE), penicillin and streptomycin at 37 °C and 5% CO_2. For the experiments, keratinocytes cells were detached by two rinses with PBS (pH 7.4) and incubated with 0.05% trypsin/0.02% EDTA/0.9% NaCl solution for 3 to 5 min. After the cells were detached, the remaining trypsin activity was neutralized by the addition of 1 ml of soybean trypsin inhibitor (Gibco, Frankfurt, Germany). After centrifugation (5 min/400 x g), the cells were resuspended and counted by using a Neubauer counting chamber and seeded at a cell density of 2.5×10^4 in 24-well-plates (Cellstar, Greiner, Frickenhausen, Germany) (0.79 cm^2) three days before the experiments. Cells from different donors in low passage number (maximum passage 3) were used for each experiment.

2.2. Generation and characterization of cold atmospheric plasma (CAP)

The Dielectric Barrier Discharge (DBD) device for generating CAP has one driven, cylindrical copper electrode covered with aluminum oxide with a total diameter of 10 mm. The device was operated by voltage pulses with an amplitude of ~13.5 kV and a trigger frequency of 300 Hz. The plasma was investigated spectroscopically by means of absolutely calibrated optical emission spectroscopy (OES) using two different spectrometer (Ocean Optics, QE 65,000, Ostfildern, Germany and LLA Instruments, ESA 3000, Berlin, Germany). For the determination of the plasma parameter a spectrometer with a spectral range of 330–400 nm and a spectral resolution of 0.15 nm was used, whereas a spectrometer in the range of 200–800 nm and a spectral resolution of 1.3 nm was used for the ozone and UV irradiance measurements. As the device is operated in air, the emissions of the second positive system of nitrogen N2 (C-B, 0–0) at λ = 337.1 nm and emissions of the first negative system N2 (B-X, 0–0) at λ = 390.6 nm were used for the determination of gas temperature and plasma parameters. Fundamental plasma parameters can be analysed using absolutely calibrated spectrometer in combination with a collisional-radiative model and numerical simulation. The diagnostics used in this study are described in more detail in Rajasekaran *et al.* [33], Bibinov *et al.* [34] and Offerhaus *et al.* [35]. Optical absorption spectroscopy (OAS) in the range of λ = 200–320 nm was applied to investigate the ozone production of the DBD as described in Baldus *et.al.* [36]. The UV irradiances were calculated from the absolutely calibrated spectra using a volume to surface ratio according to the geometry of the DBD. The presented UV irradiances are time averaged values.

2.3. Treatment of keratinocytes with cold atmospheric plasma (CAP)

Prior to CAP treatment, the cell culture media was carefully replaced and the cells were washed with PBS (500 µl). CAP treatment was

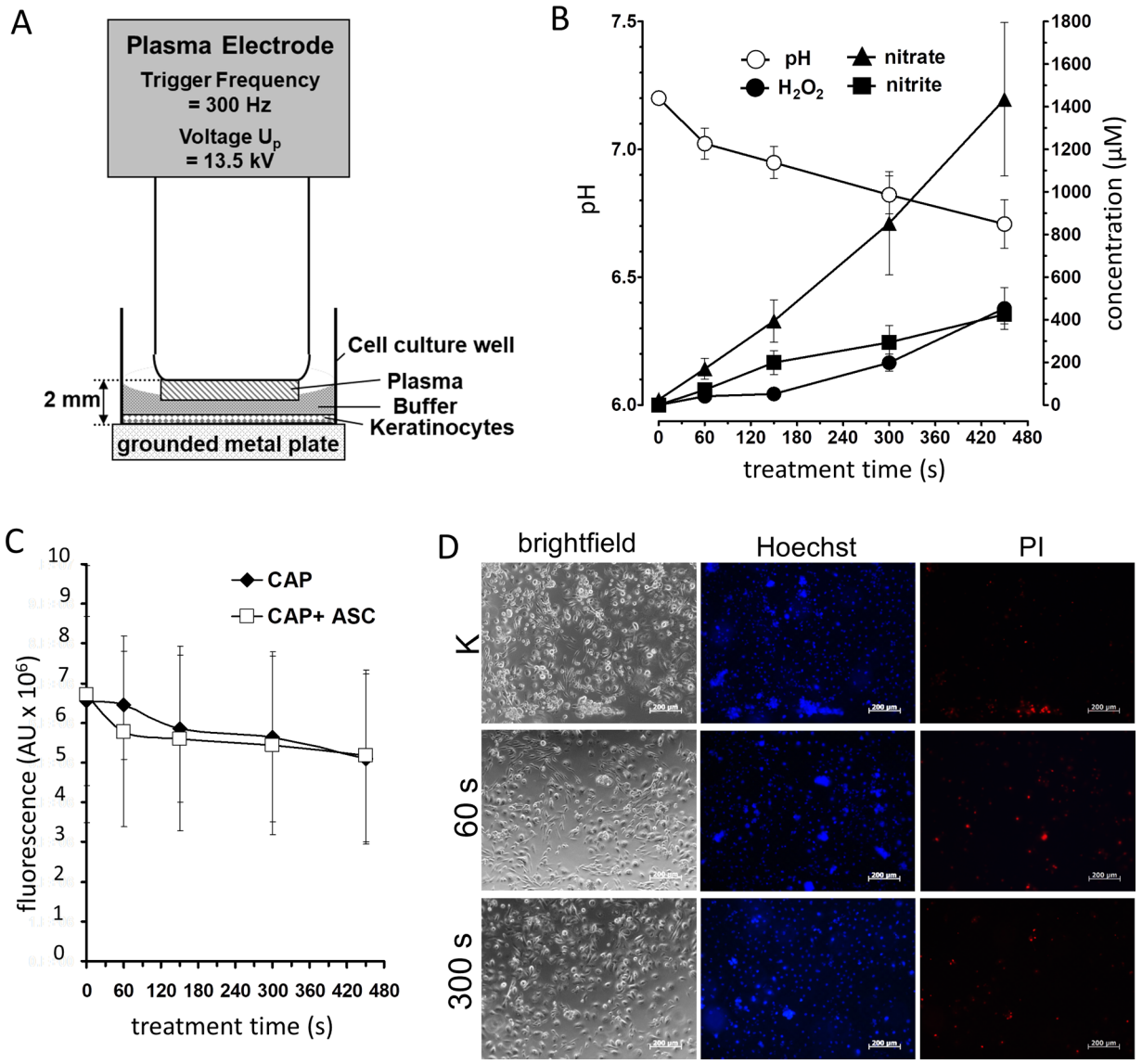

Fig. 1. Effects of cold atmospheric plasma on buffer and keratinocytes
A Experimental set up for cold atmospheric plasma (CAP) treatment of keratinocytes. **B** CAP-induced acidification (pH), accumulation of hydrogen peroxide (H_2O_2) and nitrite/nitrate in 500 µl buffer. **C** Viability of CAP-treated keratinocytes w/wo the antioxidant ascorbate (ASC) measured by a resazurin-based assay. No statistical significance could be found (ANOVA). The mean ± sd of 5 independent experiments are shown.
D Microscopic control staining with the fluorescent dyes Hoechst 33342 and propidium iodide (PI). Cell nuclei of dead cells in the cell layer were visualised (red).

conducted as illustrated in Fig. 1A in PBS (250 µl) with or without sodium ascorbate (1 mM). The cell culture plate was placed on a grounded metal plate under the electrode. The distance between the electrode and the bottom of the culture plate well was kept at 2 mm. For control experiments, the keratinocytes were incubated with PBS containing freshly prepared combinations of H_2O_2, nitrite, nitrate, and hydrochloric acid in comparable concentrations as obtained with CAP treatments (60 s: H_2O_2 = 41.4 µM, sodium nitrite = 55 µM, sodium nitrate = 154 µM, hydrochloric acid = 440 µM; 300s: H_2O_2 = 198 µM, sodium nitrite 217 µM, sodium nitrate = 917 µM, hydrochloric acid = 1 mM).

Moreover, 1000 U of bovine catalase or 1 mM of sodium ascorbate (both Sigma Aldrich, St. Louis, USA) was added as indicated.

2.4. Evaluation of CAP-induced chemical and physical changes of treatment buffer

In preliminary experiments, the plasma-induced pH changes of buffer were measured by using a pH meter (calimatic 766, Knick, Berlin, Germany) and a pH electrode (InLab-micro, Mettler-Toledo, Giessen, Germany). The buffering capacity of PBS was experimentally determined by titration with HCl and the data obtained were used to calculate the concentration and accumulation rate of oxonium ions in the CAP-treated buffer. In addition, the concentrations of nitrite/nitrate of the CAP-treated buffer were quantified by combined iodine/iodide/- and vanadium (III) chloride-based assays using NO-analyzers (CLD 88, Ecophysics, Munich, Germany), as described previously [30]. Alongside this, the H_2O_2 concentration was determined by the titanium oxide oxalate method using a photometer (Specord 205, Analytikjena, Jena, Germany) as described elsewhere [37]. The evaporation rate was determined by measuring the liquid volumes (250 µl) in the cell culture plates (24-well) before and after CAP treatments by a pipette (Eppendorf, Wesseling, Germany).

2.5. Determination of toxicity, cell viability and proliferation

For excluding a possible detachment, the keratinocytes were observed by a Zeiss light microscope directly after CAP treatment without changing the media in the control experiments. Necrotic effects were investigated by live cell imaging directly after treatment using the fluorescence dyes fluorescein diacetate, Hoechst 33342 and propidium iodide (each dye 0.5 μg/ml). The living and dead cells were analyzed using a fluorescence microscope (Zeiss, Wetzlar, Germany).

The viability of keratinocytes and relative cell numbers in relation to the untreated control were determined by a resazurin-based assay (Celltiterblue, Promega, Madison, WI, USA) according to manufacturer's protocols at time points after CAP treatment as indicated. Briefly, the keratinocytes were incubated for 1 h with Celltiterblue reagent (1:20 with medium; 400 μl) and 2 × 100 μl were taken for direct measurement at room temperature by using a fluorescence spectrometer (VICTOR II Plate Reader, PerkinElmer, Waltham, MA, USA) at an excitation wavelength of 540 nm, and an emission wavelength of 590 nm.

2.6. Migration assays

Migration assays were performed by using culture-inserts (ibidi GmbH, Martinsried, Germany) in 24 well plates in accordance with the manufacturer's instructions. Briefly, 70,000 human keratinocytes were seeded into the inserts and cultivated for 1 day at 37 °C 5% CO_2. The insert's removal resulted in a defined gap (500 μm) in the monolayer. The closure of the gap and the cell migration were observed by time-lapse phase-contrast microscopy after CAP treatment. The resulting images were used for the analyses of gap closure rates by Fiji (National Institutes of Health, Bethesda, MD, USA) [38].

2.7. Real-time quantitative-PCR (qRT-PCR)

For investigating the differentiation and proliferation of keratinocytes, we used qRT-PCR analysis of mRNA expression of the proliferation marker KI67 and the differentiation marker involucrin [39]. The total RNA of keratinocytes (4 wells) was extracted by using Qiagen RNeasy (Qiagen, Hilden, Germany) spin columns, and reversed transcripted with Omniscript RT Kit 200 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real time PCR was carried out by using the SYBR Green I kit on 7300 Real Time PCR System, (both Applied Biosystems, Warrington, UK) in a total volume of 20 μl using 9 μl of the diluted cDNA, 1 μl of PCR primer set (300 nM final concentration each), and 10 μl of Power SYBR Green PCR Master Mix according to the manufacturer's instructions. The primers for amplification were as follows: KI67, forward 50- AATTGAAATCCATGAG CAGG −30 and reverse: 50- AACAGACCCATTTACTTGTG −30; involucrin, forward: 50- TTACTGTGAGTCTGGTTGAC −30 and reverse: 50- TGTTTCATTTGCTCCTGATG −30; Cyclophilin b, forward: 50- CCCAAAGTCACCGTCAAGGT −30 and reverse: 50- CACCCGGCCTAC ATCTTCAT −30 (Sigma Aldrich, St. Louis, USA). The PCR conditions consisted of 10 min at 95 °C to activate the enzyme, followed by 40 cycles of denaturation at 95 °C for15 s and annealing/extension at 55 °C for 1 min. The samples were run in triplicates and normalized to levels of the housekeeping gene Cyclophilin b mRNA for each reaction. The CT values were normalized to the housekeeping gene and reported according to the $\Delta\Delta C_T$ method as RNA fold increase: $2^{\Delta\Delta CT} = 2^{\Delta CT\ \text{sample} - \Delta CT\ \text{reference}}$.

2.8. Statistical analysis

For statistical analysis we used GraphPad Prism V 5.01 (San Diego, USA). Significant differences were evaluated using 1-way ANOVA followed by an appropriate post-hoc multiple comparison test (Tukey method). A p-value < 0.05 was considered significant.

3. Results

3.1. Plasma characterization

Operating the plasma device with 300 Hz and 13.5 kV, the gas temperature of the generated CAP was 360 ± 20 K. A time- and space-averaged electron density of 1.33×10^{11} cm^{-3} and a reduced electric field of 330 Td were measured. UV-A irradiance of $3.32 \pm 0.3 \times 10^{-2}$ mW/cm^2 and UV-B irradiance of $3.09 \pm 0.9 \times 10^{-3}$ mW/cm^2 were detected, which are several orders of magnitude smaller in comparison to the amount of UV reaching the Earth's surface (~5 mW/cm^2). The averaged ozone density in the discharge volume amounted to $n_{O3} = 2.7 \times 10^{16}$ cm^{-3}. As the determined ozone density does not exceed the dosage applied to human skin during medical ozone therapy (10–100 μg/ml) the DBD can be assumed to be safe with regard to ozone production [40].

3.2. CAP-induced physical/chemical changes of treatment buffer

By applying CAP on the buffer, we observed a linear treatment time-dependent increase in nitrite (0.9 μM/s; $R^2 = 0.99$) and nitrate (3.1 μM/s; $R^2 = 0.98$) concentrations (Fig. 1B). After 60 s treatment time, the obtained nitrite and nitrate concentrations were 71.5 ± 24.3 μM and 170.2 ± 48.2 μM, respectively. After 300 s, the obtained nitrite concentration increased to 294.1 ± 78.3 μM and the nitrate concentration to 852.9 ± 242.7 μM. The CAP treatment slightly decreased the pH value of the buffer, for example, the pH was 6.82 ± 0.07 after 300 s (Fig. 1B). We also found a considerable accumulation of hydrogen peroxide, for example 41.4 ± 3.0 μM after 60 s and 198.5 ± 39.8 μM after 300 s of plasma treatments (Fig. 1B). Evaporation was negligible (< 10%, 5 min) and did not show significant effects on cells.

3.3. CAP treatment effects on viability of keratinocytes

In some cases CAP treatment induced a direct detachment of keratinocytes only after 450 s, which could be avoided in the presence of ascorbate (SFig.1).

Shorter treatment times showed a slight but not significant reduction of cell viability (Fig. 1C). Without any obvious detachment of the cells, a single 300 s CAP treatment reduced the viability signal by ~18%. By life cell imaging, no noteworthy increase in cell toxic events could be observed (Fig. 1D).

3.4. Migration assays

The CAP treatment in a migration assay model revealed a dual effect on the keratinocytes' migration/proliferation or gap closure. Shorter treatment times (< 60 s) did not show a significant effect on gap closure, whereas moderate treatment times (60 s, 120 s) accelerated the closure process. In contrast, 300 s of CAP treatments led to a significant delay (see Fig. 2A, B, SFig. 2). After 12 h, 50% of the wound area was still open when the cells were CAP-treated for 300 s (Fig. 2C). Here, the onset of closure was delayed and did not start until after 4–6 h; however, the speed of closure seemed unaffected.

In contrast, after 60 s CAP treatments the open gap area was approximately 10% 12 h after wounding, which was significantly smaller compared to the untreated control (23%). After 24 h, all gaps were closed.

3.5. CAP effects on differentiation and proliferation of keratinocytes

On proliferating cultures of keratinocytes, a 300 s CAP treatment did not affect cell proliferation. In contrast, short treatment (60 s) seemed to have a pro-proliferating effect; however, the obtained results here had no statistical significance (Fig. 2D). Only a longer treatment time (450 s) revealed a slight delay on day 2 (not statistically significant).

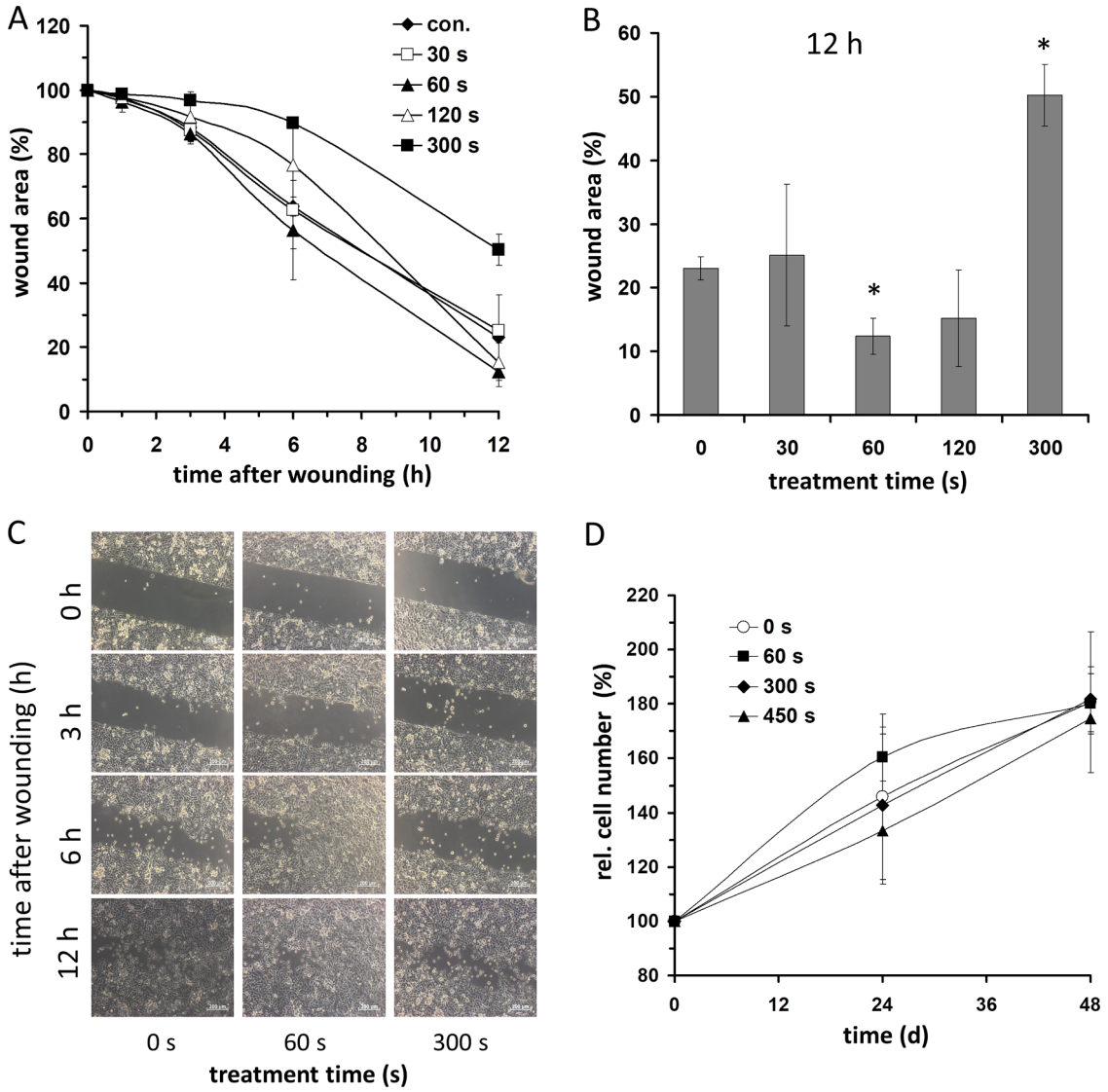

Fig. 2. Effects of cold atmospheric plasma (CAP) on keratinocyte migration
A Quantitative results of migration assays with CAP-treated keratinocytes. The mean ± sd of values obtained by digital image analysis of open gap areas are shown. **B** shows the results 12 h after treatment (n = 5;*p = 0.05, ANOVA). **C** Exemplary microphotographs of gap areas. **D** Results of proliferation assays of subconfluent keratinocytes treated with CAP as indicated (n = 5, ANOVA).

Nevertheless, regarding the mRNA-expression levels of the differentiation and proliferation markers involucrin/KI67, longer treatments (300 s) raised the differentiation level as shown by the two-fold increase in the involucrin/KI67 ratio (Fig. 3). This pro-differentiation effect could be completely reversed by the addition of catalase. Short CAP treatment (60 s) did not affect the differentiation/proliferation status. The addition of H_2O_2, in amounts equivalent to 60 s or 300 s CAP treatment, increased the ratio nearly two-fold as well. No effects were observed after the addition of nitrite/nitrate and acidification of the treatment buffer, equivalent to a 60 s or a 300 s CAP treatment. Interestingly, mimicking the 60 s CAP treatment, the pro-differentiation effects of H_2O_2 were inhibited in the presence of nitrite/nitrate and acidification. In comparison, for mimicking a 300 s CAP treatment, nitrite/nitrate and acidification further enhanced the pro-differentiation effects of H_2O_2.

4. Discussion

Reactive oxygen species (ROS) have been considered to be solely damaging to cells and tissues, promoting pathologies and aging. Tissue injury and inflammation are accompanied by an increase of ROS and H_2O_2 levels produced by phagocytic and inflammatory cells, widely recognized to play an important role in antimicrobial host defense [41,42].

The increased production of ROS can be observed after acute wounding as well as in chronic regenerating wounds [43,44]. Excessive or chronic ROS production is considered to actively contribute to chronic tissue injury or inflammation, due to their ability to oxidize various biomolecules resulting in, for example, protein dysfunction or cell death [45,46].

However, apart from their injurious effects, ROS can also control many biological processes through redox signaling mechanisms. In animal models, the rapid production of H_2O_2 in response to wounding minimizes infection risk, and activates the epithelial signaling pathways involved in epidermal regeneration. Furthermore, neutrophils and other leukocytes are recruited to the wound site by early H_2O_2 [44,47,48]. In mammalian models of wound healing, H_2O_2 production in dermal wounds was found to contribute to wound angiogenesis and closure indicating an essential role of H_2O_2 as an early damage signal in the wound healing response [43]. Hence, during cutaneous wound

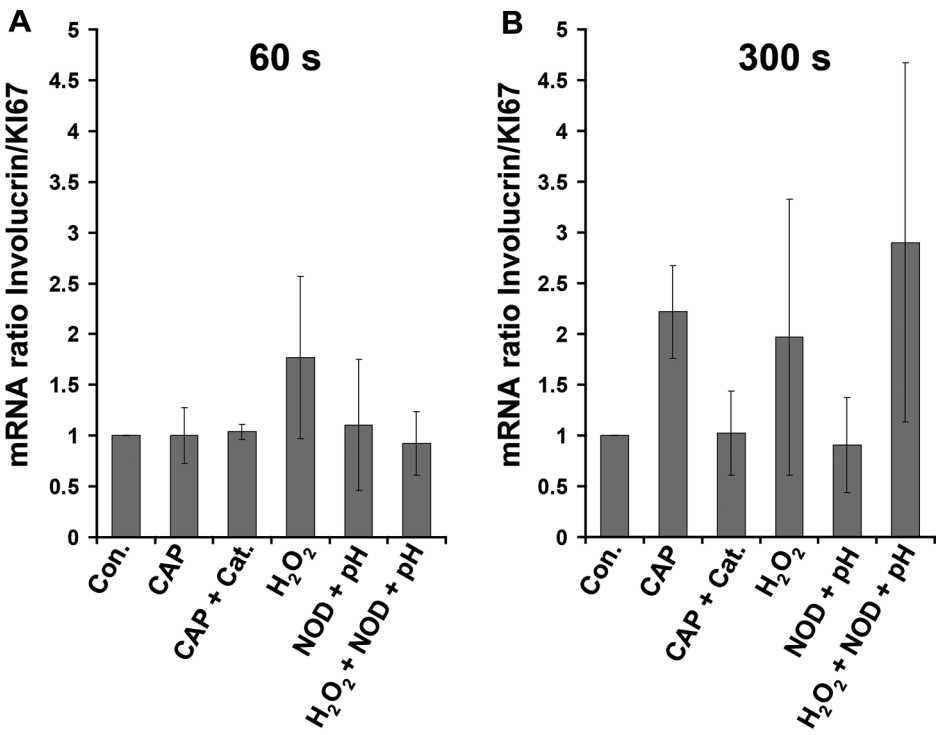

Fig. 3. Effects of cold atmospheric plasma and H2O2 on keratinocyte differentiation
Relative mRNA expression of the keratinocyte differentiation marker involucrin and proliferation marker KI67 for **A** 60 s and **B** 300 s treatments with cold atmospheric plasma (CAP). As indicated catalase (Cat.) was added or instead of CAP treatment, equivalents amounts of hydrogen peroxide (H_2O_2; 41 μM ~60 s; 198 μM ~300 s), hydrochloric acid (pH; 440 μM ~60 s; 1 mM ~300 s) and/or NOD (nitrite 55 μM ~ 60 s; 217 μM ~300 s and nitrate 154 μM ~60 s; 917 μM ~300 s). The means and standard deviation of 5 experiments are given.

healing many growth factors/cytokines i.e. VEGF, IL-l, TGFβ and IGF-1 appear to be produced as a result of oxidant activity. Here, a VEGF gene is known to respond to H_2O_2 [49].

Using a mice model, Hamanake et al. could show that mitochondrial reactive oxygen species—in particular, H_2O_2—promote epidermal differentiation and hair follicle development [50]. In another study that used a keratinocyte/fibroblast co-culture model, a positive effect on migration and proliferation of keratinocytes was observed with the addition of H_2O_2 (250, 500 μM), leading to a faster re-epithelialization in an in vitro wound model [51].

CAP is known to produce various reactive species, which may interfere with the media and/or the cells. We found that under our conditions, CAP induces the accumulation of nitrite, nitrate and H_2O_2 in the treated fluids. We observed that CAP treatment affects human primary keratinocytes in different ways. Longer treatments > = 300 s decrease cell proliferation and migration leading to a retarded gap closure and an upregulation of the differentiation marker involucrin, whereas shorter treatments (60 s), i.e. comparable to the treatment times used for wound healing, accelerated gap closure without an upregulation of involucrin (Fig. 3). With regard to the described H_2O_2-dependent effects on cell differentiation stated above, in particular, the observed concentrations of H_2O_2 (41 μM ~60 s; 198 μM ~300), could possibly be responsible for the observed effects.

It was shown that keratinocytes treated with H_2O_2 at low concentrations have enhanced epidermal growth factor receptor activation and ERK1/2 phosphorylation, which may explain the observed higher potential of migration [51,52].

The addition of H_2O_2 to keratinocytes in equivalent amounts as produced by CAP during treatment (60 s, 300 s) also induced an upregulation of the involucrin level; whereas the presence of catalase during CAP treatments completely inhibited this effect, pointing to a crucial role for the CAP-produced H_2O_2. The parallel accumulation of nitrite and nitrate seems to mediate the H_2O_2-induced effects. The pro-differentiation effect of lower H_2O_2 concentrations is diminished in the presence of low concentrations of nitrite/nitrate (equivalent to 60 s CAP). In contrast, the pro-differentiation effects of higher H_2O_2 concentrations (equivalent to 300 s CAP) are further enhanced by the parallel produced amounts of nitrite/nitrate (Fig 3). There are several concerns that the use of H_2O_2 (3%) to disinfect wounds may hurt nascent regenerating tissues [53]; however, it has been shown that topical application of low concentrations (0.03–0.15%) of H_2O_2 favorably influenced wound angiogenesis [54,55].

Owing to impaired microcirculation, chronic wounds are typically hypoxic, and thus limited in their ability to generate endogenous H_2O_2, which in turn may result in bacterial colonization and impaired wound angiogenesis [56]. In conclusion, we demonstrate for the first time that CAP produced by a DBD device can promote keratinocyte migration at shorter treatment times, while inhibiting migration at longer treatment times. These slowing down effects are linked to the CAP-induced production and accumulation of higher H_2O_2 amounts, which in turn can induce keratinocyte differentiation and therefore hamper the potential of migration. In the best case scenario, a therapeutic approach to chronic wounds with CAP overcomes bacterial contamination, improves microcirculation and additionally compensates the missing necessary H_2O_2, which together promote keratinocyte migration and proliferation, initiate angiogenesis, accelerating tissue regeneration and wound closure. In addition, regarding hyperproliferative skin diseases, such as excessive scarring or psoriasis, an induction of differentiation and inhibition of proliferation by longer CAP-treatments may also have beneficial therapeutic effects.

In contrast to a mere topical H_2O_2 application, CAP treatment offers possible synergistic effects of the various produced plasma components. At the push of the button these components can be “freshly produced” and precisely delivered to the desired wound areas by the practitioner at the bed-side.

Therefore, the basic pharmacological principle of “Right Drug, Right Dose, Right Time” needs to be applied for CAP treatment as well to avoid disadvantageous effects—for example, necrosis or delayed wound healing. Owing to the complexity of CAP itself—there are many different CAP devices, which can be operated with different parameters—and the complexity of possible interactions with biomolecules, cells and living tissues, further investigations and clinical trials are crucial to establish CAP in clinical use.

Conflict of interest

The authors state no conflict of interest.

Ethical Statement

Cell isolation from human skin were performed with the approval of the Ethics Commission of Düsseldorf University (Study No. 3634).

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2018.11.001.

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