RESEARCH ARTICLE

Non-Thermal Dielectric Barrier Discharge (DBD) Effects on Proliferation and Differentiation of Human Fibroblasts Are Primary Mediated by Hydrogen Peroxide

Julian Balzer[1☯], Kiara Heuer[1☯], Erhan Demir[2‡], Martin A. Hoffmanns[1‡], Sabrina Baldus[3], Paul C. Fuchs[2], Peter Awakowicz[3], Christoph V. Suschek[1], Christian Oplander[1]*

1 Department of Trauma and Hand Surgery, Medical Faculty of the Heinrich-Heine-University, Düsseldorf, Germany, 2 Department of Plastic Surgery, Hand Surgery, Burn Center, Merheim Hospital Cologne, University of Witten/Herdecke, Köln, Germany, 3 Institute for Electrical Engineering and Plasma Technology, Ruhr University, Bochum, Germany

☯ These authors contributed equally to this work.
‡ These authors also contributed equally to this work.
* christian.oplaender@hhu.de

Abstract

The proliferation of fibroblasts and myofibroblast differentiation are crucial in wound healing and wound closure. Impaired wound healing is often correlated with chronic bacterial contamination of the wound area. A new promising approach to overcome wound contamination, particularly infection with antibiotic-resistant pathogens, is the topical treatment with non-thermal "cold" atmospheric plasma (CAP). Dielectric barrier discharge (DBD) devices generate CAP containing active and reactive species, which have antibacterial effects but also may affect treated tissue/cells. Moreover, DBD treatment acidifies wound fluids and leads to an accumulation of hydrogen peroxide (H_2O_2) and nitric oxide products, such as nitrite and nitrate, in the wound. Thus, in this paper, we addressed the question of whether DBD-induced chemical changes may interfere with wound healing-relevant cell parameters such as viability, proliferation and myofibroblast differentiation of primary human fibroblasts. DBD treatment of 250 µl buffered saline (PBS) led to a treatment time-dependent acidification (pH 6.7; 300 s) and coincidently accumulation of nitrite (~300 µM), nitrate (~1 mM) and H_2O_2 (~200 µM). Fibroblast viability was reduced by single DBD treatments (60–300 s; ~77–66%) or exposure to freshly DBD-treated PBS (60–300 s; ~75–55%), accompanied by prolonged proliferation inhibition of the remaining cells. In addition, the total number of myofibroblasts was reduced, whereas in contrast, the myofibroblast frequency was significantly increased 12 days after DBD treatment or exposure to DBD-treated PBS. Control experiments mimicking DBD treatment indicate that plasma-generated H_2O_2 was mainly responsible for the decreased proliferation and differentiation, but not for DBD-induced toxicity. In conclusion, apart from antibacterial effects, DBD/CAP may mediate biological processes, for example, wound healing by accumulation of H_2O_2. Therefore, a clinical DBD treatment

must be well-balanced in order to avoid possible unwanted side effects such as a delayed healing process.

Introduction

Human skin consists of the two tissue layers, epidermis and dermis. The epidermis is a stratified squamous epithelium composed of proliferating and differentiated keratinocytes.

The underlying thick layer, the dermis, is a collagen-rich connective tissue providing support and nourishment. As the major cell type in the dermis, fibroblasts play a pivotal role in maintaining the skin by synthesis and deposition of various extra cellular matrix (ECM) proteins [1]. Dermal fibroblasts have an important part in the process of wound healing by synthesizing relevant cytokines such as keratinocyte growth factor. In fibroplasia and granulation tissue formation during wound healing, proliferating fibroblasts form a provisional extracellular matrix by generating and depositing collagen and fibronectin [2].

About a week after wounding, a portion of fibroblasts can transform into myofibroblasts, which are primarily known for their key role in wound healing and physiological reconstruction of connective tissue [3]. They express α-smooth muscle actin (α-SMA) and resemble smooth muscle cells in their capacity for generating strong contractile forces [4].

The transformation of fibroblast into myofibroblast begins with the appearance of proto-myofibroblasts expressing beta and gamma cytoplasmic actins containing stress fibers but no alpha-SMA, the marker of differentiated myofibroblasts. This complex process is driven by at least one cytokine, the tissue growth factor-β1 (TGF-β1), extracellular components and mechanical tension [5]. The contractile activity by mature myofibroblasts is beneficial for tissue remodeling and after wound closure, myofibroblasts regularly undergo apoptosis[6]. The persistence of active contractile myofibroblasts can be a detrimental pathological factor in the development of hypertrophic scars, keloids and fibrotic diseases [7,8].

Many factors, such as oxygenation, infection, age, nutrition and diabetes, affect wound healing, leading to improper or impaired tissue repair. The contamination and/or colonization of wounds with bacteria such as *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and β-hemolytic *streptococci*, can cause to prolonged wound inflammation driving the wound to a chronic state [9,10]. Often the bacteria in infected wounds occur in the form of biofilms, which are more resistant to conventional antibiotic treatment [11].

A new promising approach to overcome wound contamination, particularly infection with antibiotic-resistant pathogens, is the topical treatment with non-thermal "cold" atmospheric plasma (CAP). In physics, plasma is a fully or partially ionized gas and has been also called the fourth state of matter, after solid, liquid and gas. Plasma can technically be generated by electric gas discharges. By applying strong electric fields, air or other gases can be transformed into the plasma state with the consequent generation of a complex mixture of inter-reacting and short-living ions, atoms, excited species, photons and free electrons [12]. The properties of plasma depend on the type of gas used, gas pressure and discharge geometry.

In medicine, high-temperature plasmas are already used to sterilize equipment as well as to cut and cauterize tissues during surgery [13]. The development of non-thermal plasma devices operating under atmospheric pressure (CAP) makes it possible to treat living matter, for example, to sterilize human skin without thermal damage or obvious adverse effects. It has been shown that non-thermal plasma effectively kills or inactivates a broad spectrum of bacteria [14,15]. Apart from antimicrobial effects, CAP stimulates microcirculation and reveals some

antipruritic and anti-inflammatory effects, making the use of CAP attractive in the treatment of many skin diseases [13,16,17].

In general, CAP devices can be categorized into two main types that produce either an "indirect" or a "direct" plasma for treatments [18]. By using a dielectric barrier discharge (DBD) device in dermal application, the human skin represents the counter electrode to ignite the plasma straight onto the body to achieve a direct plasma treatment. Indirect plasma treatment can be obtained by igniting the plasma inside a tube within a flowing process gas such as argon, helium or air. The flowing process gas carries the active particles to the treated object. Such devices are already in use—e.g. plasma torch, plasma jet, plasma needle or plasma pencil. Plasma comprises a mixture of different radical species, UV radiation and a significant flux of charges, which are all potentially influencing biological functions when directly applied to tissue or cells. However, indirect plasma avoids direct contact with the conductive plasma, so no electrical current is drawn by the treated tissue [19,20]. Operated with ambient air, plasma generates reactive nitrogen species such as nitric oxide (NO), nitrogen dioxide (NO_2) and reactive oxygen species like ozone (O_3), superoxide (O_2^-) and hydroxyl radicals (^-OH) [21]. In particular, NO is an important molecule that has been shown to regulate many processes in human skin physiology [22,23]. The treatment of liquids and buffers with DBD leads to acidification and accumulation of nitrite and nitrate, which in turn may influence the cell physiology of adult human fibroblasts. In addition, the formation of hydrogen peroxide (H_2O_2) has been identified for various plasma sources and settings [24,25]. Apart from its function as an oxidant with antibacterial and cell toxic effects, H_2O_2 may serve as a signaling agent in signal transduction pathways in multicellular organisms [26].

The aim of the current study is to evaluate the impact of DBD treatment on viability, proliferation and differentiation of human dermal fibroblasts with focus on the role of DBD- induced nitrite/nitrate/H_2O_2 accumulation and acidification.

Material and Methods

Materials

If not indicated chemicals were from Sigma (Deisenhofen, Germany).

Plasma source

The DBD device used has one driven, cylindrical copper electrode covered with aluminum oxide with a total diameter of 10 mm. Plasma was generated by applying voltage pulses with a maximum of ~14 kV and a trigger frequency of 120 Hz.

Plasma characterization

The plasma was characterized by using absolutely calibrated optical emission spectroscopy (OES). Spectra were measured using a spectrometer (Ocean Optics, QE 65000) with a spectral resolution of about 1.3 nm in the 200–800 nm wavelength range. The averaged gas temperature was determined by comparing the measured rotational temperature of nitrogen at 337.1 nm to a simulated spectrum under the same conditions [27]. Gas temperature (T_g) was 390 ± 20 K, UV-A was 7.6 ± 2.3 x 10^{-3} mW/cm^2, UV-B was 1.3 ± 0.4 x10^{-3} mW/cm^2, electron density was 2.6 ± 0.9 x 10^{14} cm^{-3} and ozone 210 ± 10 ppm. The concentrations and total flux of the generated nitric oxide (NO) and nitrogen oxides (NO_x) were measured in the gas phase as described previously [28]. The measured amounts of 0.41 ppm NO and 11.42 ppm NO_2 yields to a flux of Γ_{NONO2} = 5 x 10^{18} cm^{-2} s. At the treatment time of 5 min, the dose of NO/NO_2 was Φ_{NONO2} = 0.75 x 10^{21} cm^{-2}, which equals 1.2 mmol.

Cell culture

Human juvenile foreskin samples were obtained from five patients (5–6 years old), undergoing medically indicated circumcision after written informed consent from parents or legal guardians, with approval of the Ethics Commission of Düsseldorf University (Study No. 3634) and in accordance with the Declaration of Helsinki. Primary cultures of human foreskin fibroblasts were prepared, cultivated and cryoconserved as described elsewhere [29]. For experiments, cryoconserved stocks of fibroblast were thawed and further cultured with cell culture media (DMEM/10% FCS/PEN/STREP) in T 75 flasks (Cellstar, Greiner Bio-One, Frickenhausen, Germany) under normal culture conditions (5% CO_2, 37°C). For seeding, the cells were detached by two rinses with 0.13 M NaCl and 0.01 M sodium phosphate, pH 7.4 (PBS) and incubated with 0.05% trypsin/0.02% EDTA/0.9% NaCl solution for 3 to 5 min. After the cells became detached, the remaining trypsin activity was neutralized by addition of 10 ml culture media and subsequently centrifuged (5 min/400 x g). After centrifugation, cells were resuspended and counted by using a Neubauer counting chamber and seeded at a cell density of 2.5 x 10^4 in 24-well-plates (0.79 cm^2) one day before experiments. All measurements were performed with foreskin fibroblasts in passage 2.

Plasma treatments of HDF

Prior to plasma treatment, cell culture media was carefully replaced with PBS (250 μl) with or without sodium ascorbate (1 mM) after one PBS washing step (500 μl). The cell culture plate was placed on a grounded metal plate under the DBD electrode and the distance between electrode and bottom of the culture plate well was kept as 2 mm (Fig 1A).

For indirect treatments, 250 μl PBS was DBD-treated (0–300 s) and transferred directly after treatment to the cell culture plate with fibroblasts and incubated for 5 min. In addition, for control experiment, fibroblasts were incubated for 5 min with PBS containing varying freshly prepared combinations of H_2O_2 (200 μM), nitrite (300 μM), nitrate (1 mM), hydrochloric acid and sodium ascorbate (1 mM). The range of treatment times (60, 150, 300 s) were chosen in accordance with other studies using DBD as plasma source [30,31] and data (not shown) from preliminary experiments with skin fibroblasts. The change of media temperature by DBD treatment (5 min) was less than 0.1°C.

Determination of pH Values

The pH values of media and buffers were measured 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 determined by titration with HCl. The obtained data were used to calculate the concentration and accumulation rate of oxonium ions in plasma-treated buffer.

Detection of nitrite and nitrate

The concentrations of nitrite were quantified by iodine/iodide-based assay NO-analyzer CLD 88 (Ecophysics, Munich, Germany) as described elsewhere [32,33]. To determine the total nitrite and nitrate, samples were added to 0.1 mol/l vanadium (III) chloride in 1 M hydrochloric acid refluxing at 95°C under nitrogen [34]. The calculated NOD amounts were validated by injecting freshly prepared nitrite/nitrate standards into the respective reaction mixtures. Concentrations of nitrate were calculated by subtraction of nitrite from NOD values.
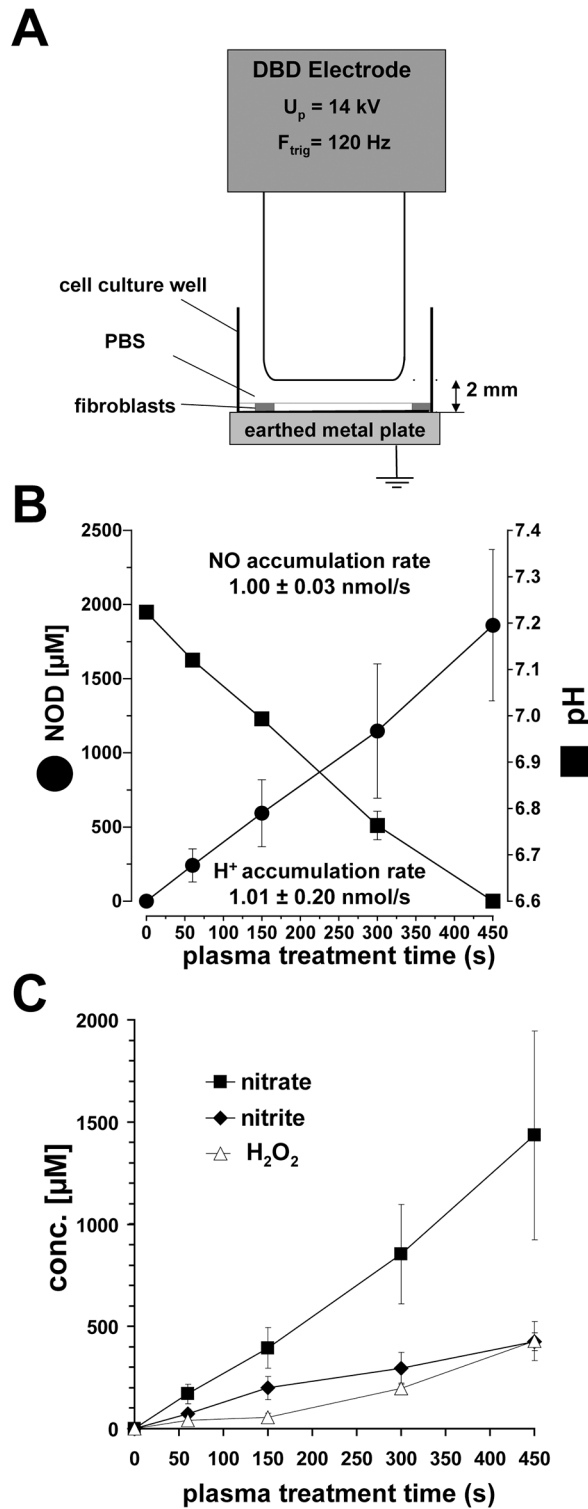
Fig 1. DBD-induced acidification and accumulation of nitrite, nitrate and hydrogen peroxide. A
Experimental set-up for DBD treatments of fibroblasts. B Amounts of NOD (nitrite + nitrate) and pH values
obtained after DBD treatment times as indicated. Given are the mean ± sd values (n = 6).

doi:10.1371/journal.pone.0144968.g001

Toxicity, viability, proliferation and differentiation

For excluding a possible detachment, the cells were observed by a Zeiss light microscope directly after treatment without changing the media in control experiments. Necrotic effects on the cells 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). Living and dead cells were analyzed using a fluorescence microscope (Zeiss, Wetzlar, Germany).

Viability of the cells or relative cell numbers in relation to the untreated control were determined by a resazurin-based assay (Celltiterblue, Promega, Madison, WI, USA) at time points as indicated. Briefly, HDF were incubated for 1 h with alamarBlue™-reagent (1:20 with Medium; 400 µl) and 2 x 100 µl were taken for direct measurement at room temperature using a fluorescence spectrometer (VICTOR II Plate Reader, PerkinElmer, Waltham, MA, USA) at excitation wavelength 540 nm, emission wavelength 590 nm. The linear range of growth curves were used to calculate doubling times.

On day 12 after treatment, the cells were fixed with paraformaldehyde/PBS (4%; 15 min), washed 3x with PBS and permeabilized by 0.2% Triton X-100/PBS. After incubation with blocking buffer (4% BSA/PBS; 12 h; 4°C), the cells were stained with mouse anti—SMA-antibodies (1:400; 60 min; 37°C). Then the cells were washed 3x with PBS and incubated for 60 min with an AlexaFluor488-conjugated goat-anti-mouse antibody (1:1000; Invitrogen, Carlsbad, CA, USA) in blocking buffer. After three washing steps, the cells were incubated with Hoechst 33342 (1 µg/ml) in PBS for 10 min and visualized by using an invert fluorescence microscope. Photos (4x) of each well were taken and the total number of cells, area of single cell nucleus and the area of -SMA$^+$-signal were determined by using Fiji (National Institutes of Health, Bethesda, MD, USA) [35].

Measurement of H$_2$O$_2$

The concentration of H$_2$O$_2$ in DBD-treated PBS was determined by the titanium oxide oxalate method [36]. Briefly, a stock solution of potassium dihydrate (0.1 M) with 2 M sulfuric acid was freshly prepared.

Directly after DBD treatment, the tested solution (50 µl) was taken and diluted 1:4 with PBS, before 200 µl of the titanium oxide oxalate stock solution was added. The reaction of potassium titanium oxide with H$_2$O$_2$ produces a yellowish color. Concentrations of H$_2$O$_2$ were measured by a photometer at an absorbance of 400 nm. Calibration curves with known H$_2$O$_2$ concentrations (0–500 µM in PBS) were used for quantifications. No significant differences were found when the calibration curves were prepared in the presence of high nitrite/nitrate concentrations (both 2 mM) and low pH values (5.5 and 6.4).

Quantification of TGF-β1

The levels of TGF-β1 in culture supernatants were determined on day 7 after treatment by using sandwich ELISA (DuoSet, R&D Systems, Abdingdon, UK) according to the manufacturer's instructions.

Quantitative immunocytochemical detection of gamma-H2AX

Within minutes of the induction of DNA double-strand breaks in somatic cells, histone H2AX becomes phosphorylated at serine 139 and forms γ-H2AX foci at the sites of damage [37]. One our after DBD/H$_2$O$_2$ treatments or UVB irradiation (500 mJ/cm^2) as positive control, the cells were washed 3x with PBS and fixed with paraformaldehyde/PBS (4%; 30 min). After an additional fixation step with methanol (10 min; -20°C), the cells were washed 3x with PBS and

permeabilized by 0.3% Triton X-100/PBS. After incubation with blocking buffer (5% BSA/PBS; 12 h; 4°C), the cells were stained with rabbit γ-H2AX -antibody (1:500; 60 min; 37°C; Abcam, Cambridge, UK), washed 3x with PBS and incubated for 60 min with an AlexaFluor488-conjugated donkey-anti-rabbit antibody (1:500; Invitrogen, Carlsbad, CA, USA) in blocking buffer. After washing steps (3x) and an incubation step with propidium iodide (1 μg/ml, 10 min; 37°C), the cells were visualized by using a fluorescence microscope (Axiovert, Zeiss, Wetzlar, Germany) and photos (2x) of each well were randomly taken. The number of nuclei and γ-H2AX foci were determined by using Fiji (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

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

Results

DBD induces acidification and accumulation of nitrite, nitrite and H_2O_2

It is known that cold plasma can acidify media and increase the levels of nitrite, nitrate and perhaps H_2O_2. Therefore, to elucidate the impact of DBD treatment on cells, we first analyzed the pH and the concentrations of nitrite, nitrate and H_2O_2 in DBD-treated buffers (PBS). Our data revealed that under our experimental conditions, plasma treatment of PBS induced a significant increase in nitrite, nitrate and H_2O_2 concentrations coinciding with a decrease in pH (Fig 1B). For example, after a 300 s treatment of 250 μl PBS we found 1147.05 ± 452.78 μM of total nitric oxide derivatives (nitrate + nitrite). We calculated an accumulation rate of 1.00 ± 0.03 nmol/s. In parallel, the oxonium accumulation rate was 1.01 ± 0.20 nmol/s; thus, the pH value had dropped from 7.2 to 6.7 after 300 s plasma treatment. Coincidently, H_2O_2 and nitrite concentrations increased to 197.19 ± 24.25 μM and 294.09 ± 78.33 μM, respectively (Fig 1B)

DBD treatment reduces the viability of fibroblasts

As many studies have reported plasma-induced toxicity in a number of cell types, we wanted to determine whether DBD treatment can influence the viability of human dermal fibroblasts. Preliminary experiments have shown that under our experimental conditions (Fig 1A), a minimal volume of 215 μl buffer (PBS) is necessary to avoid direct DBD-induced necrotic events. Using 250 μl, we could not observe either necrotic cells or cell detachment after treatment even after longer treatment times of up to 750 s. Analysis of cell viability 24 h after treatment revealed significant decreases (Fig 2A). For example, after direct DBD treatment for 300 s, the cell viability was ~66% in comparison to the untreated controls. The presence of the antioxidant sodium ascorbate could remarkably reverse (~75%) these observed effects, but not significantly. The plasma-induced accumulation of nitrite/nitrate/H_2O_2 in the media with coincident acidification suggests that the biological effects observed after DBD treatment, were not only mediated by the generation of short-living ROS. We therefore examined whether the buffer, which was DBD-treated separately from cells, has similar effects on the viability of fibroblasts. In addition, the buffer was acidified (pH 6.7) and nitrite, nitrate and H_2O_2, equivalent to amounts obtained after a 300 s DBD treatment, were freshly added to the cells and incubated for 5 min. Indeed, we observed a significant decrease in viability when the cells were exposed with DBD-treated buffer (e.g. ~55%, 300 s; Fig 2B). Here, the presence of ascorbate significantly reversed this effect (e.g. ~75%, 300 s). Incubation with acidified buffer containing nitrite/nitrate/H_2O_2 did not show any significant effect on cell viability.

A direct treatment

B indirect treatment
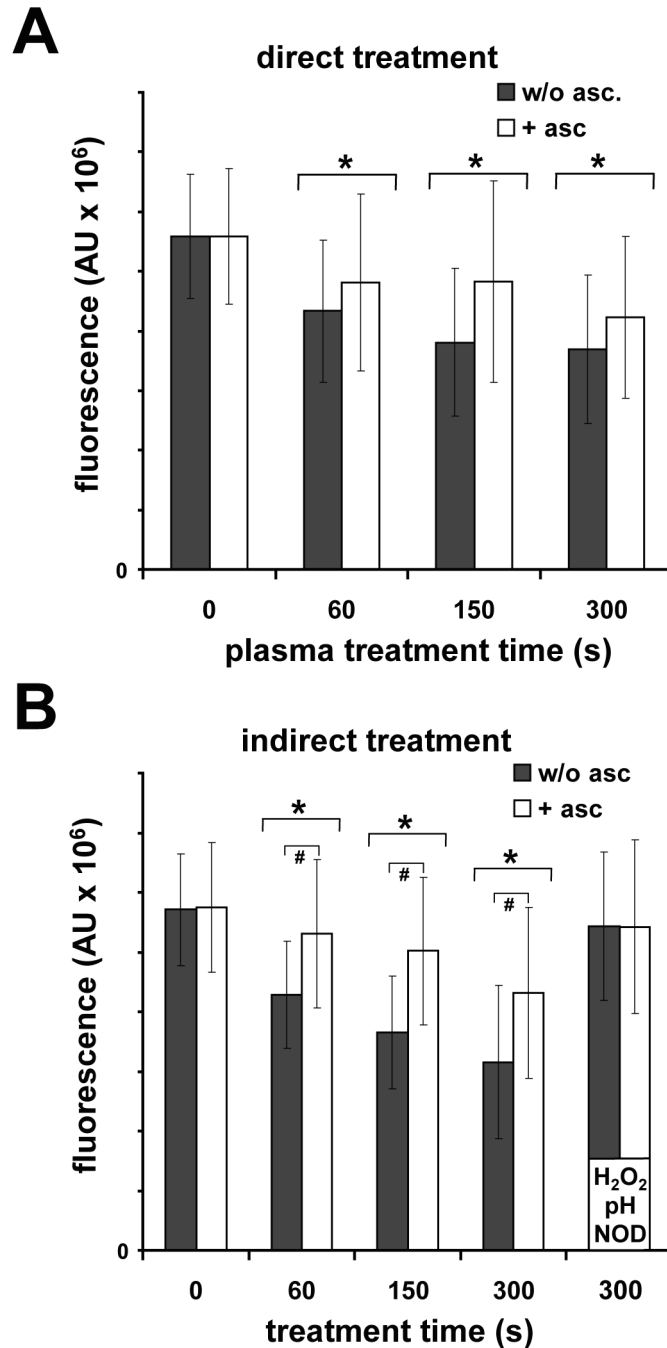
Fig 2. DBD treatment reduces fibroblast viability. A Human dermal fibroblasts were treated with DBD (0–300 s) ± ascorbate (**asc**; 1.0 mM) and cell viability was measured 24 h later by a resazurin-based assay. **B** Cells were exposed for 5 min to buffer ± ascorbate (1.0 mM), which had been DBD-treated (0–300 s) shortly before or exposed to a freshly prepared buffer with a pH of 6.7 (**pH**) containing hydrogen peroxide (200 μM; H2O2), nitrite and nitrate (**NOD**; 300 μM, 1.0 mM). Given are mean ± sd values (n = 5),*P < 0.05 as compared with the control values,#P < 0.05 as compared to respective values without ascorbate.

doi:10.1371/journal.pone.0144968.g002

DBD treatment inhibits proliferation of fibroblasts

The possible toxic effects of cold plasma were observed in many studies. However, few studies have focused on the proliferation of treated cells. To elucidate possible DBD-induced effects, we determined the proliferation rate of the remaining fibroblasts after direct DBD treatment or exposure to DBD-treated buffer. In addition, fibroblasts were incubated for 5 min with freshly prepared acidified buffer containing nitrite (300 µM), nitrate (1.0 mM) and H_2O_2 (200 µM) amounts equivalent to 300 s of DBD treatment. By using a resazurin-based assay, we observed that the proliferation rate was significantly lower after 300 s of direct DBD-treatment, whereas the rates were somewhat lower in shorter treatment times (Fig 3A). In contrast, the exposure of cells to DBD-treated buffer for 5 min results caused a significant breakdown of proliferation (Fig 3B). An analogous result was obtained when acidified nitrite/nitrate/H_2O_2 buffer were used (Fig 3B). The addition of ascorbate during treatment did not have any effect on proliferation (data not shown).

Further analysis revealed that the doubling time of directly DBD-treated cells was significantly prolonged after a 300 s treatment in comparison to the untreated control (4.8 ± 0.6 d vs. 2.8 ± 0.4d; Fig 3C). Incubation of fibroblasts for 5 min with DBD-treated buffer (60–300 s) led to doubling times of 6–8 d. Also the incubation of fibroblasts with acidified buffer containing nitrite/nitrate/H_2O_2 significantly prolonged doubling time (5.8 ± 0.7 d). Since the resazurin-based assay only gives a measure of metabolic active cells, we therefore additionally validated the cell number by manual counting of Hoechst-stained nuclei on day 12 after treatment. Direct and indirect DBD treatment (60–300 s) as well as incubation of cells with acidified nitrite/nitrate/H_2O_2 buffer led to a significant decrease in cell number (Fig 3E and 3G). Here, 150–200 cell nuclei were counted for treatments longer than 60 s, whereas we found three-fold more nuclei (578 ± 42) in the untreated control.

Interestingly, comparing the absolute fluorescence signals obtained from the resazurin assay (Fig 3D) to the number of cell nuclei (Fig 3E) showed some discrepancy on day 12. Here, using resazurin, the values of direct treatments were reduced but not to such an extent as the number of cell nuclei. For example, a direct DBD-treatment (150 s) led to a strong reduction in the number of nuclei (-66%), whereas the resazurin-based assay showed a moderate reduction (-28%), suggesting that not only the number of active cells but also the metabolism and/or phenotype of fibroblasts may be influenced by DBD-treatment. Indeed, an analysis of the mean size of nuclei revealed that the cell nuclei were roughly 1.6-fold larger on 12 d after direct and indirect DBD treatment, suggesting a DBD-induced cell cycle arrest or reduced mitotic activity. As the exposure of fibroblasts to acidified buffer containing nitrite/nitrate/ H_2O_2 showed similar effects, we therefore subsequently examined which of the components may inhibit cell proliferation.

Using various combinations, we found that the presence of H_2O_2 was necessary to significantly inhibit proliferation, to reduce the number of cell nuclei and to prolong doubling time to the same extent as an indirect DBD treatment (300 s), whereas acidification and nitrite/ nitrate alone or combined did not reveal any effects (Fig 4A–4C). Furthermore, after direct and indirect DBD treatment (0–300 s) as well as exposure to H_2O_2 (0–1 mM, 300 s) no significant increase in gamma-H2AX foci as early marker of DNA double-strand breaks [37] could be found. However, an increasing tendency of gamma-H2AX correlating with treatment time could be recognized, particularly after direct treatments (S1 Fig).

DBD treatment affects fibro-/myofibroblast differentiation

Myofibroblast activity during wound healing is crucial and an inhibition of differentiation of fibroblast to myofibroblast may result in problems in wound closure and healing. Thus, we
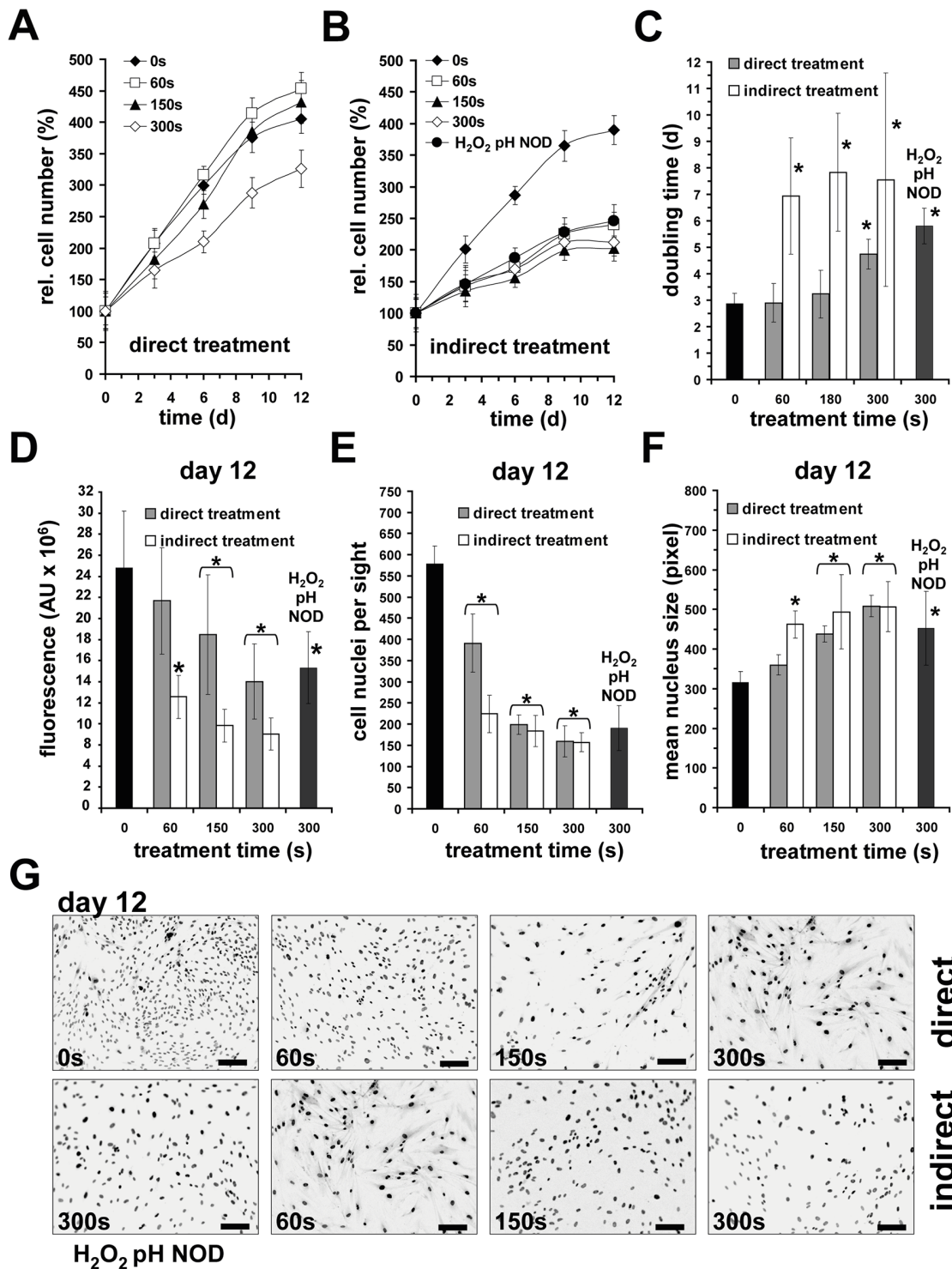
Fig 3. **DBD treatment inhibits proliferation of human dermal fibroblasts. A** Proliferation curve of remaining fibroblasts after direct DBD treatment measured by a resazurin-based assay. **B** Proliferation curve of remaining fibroblasts after exposure for 5 min to buffer, which was DBD-treated as indicated (indirect treatment). Additionally, fibroblasts were exposed to acidified buffer (**pH** 6.7) containing nitrite/nitrate (**NOD;** 300 µM / 1.0 mM) and H_2O_2 (200 µM). **C** The calculated doubling times for proliferation curves A and B. **D** Values obtained by resazurin-based assay on day 12 after treatment. **E** Counted Hoechst 33342-stained nuclei on day 12 after treatment. **F** Quantification of mean fibroblast nucleus size on day 12 after treatment. **G** Representative microphotographs of Hoechst 33342-stained nuclei of fibroblasts on d12 after treatment. Bars = 200µm. Given are the mean ± sd values (n = 5), *$P < 0.05$ as compared with the control values.

doi:10.1371/journal.pone.0144968.g003
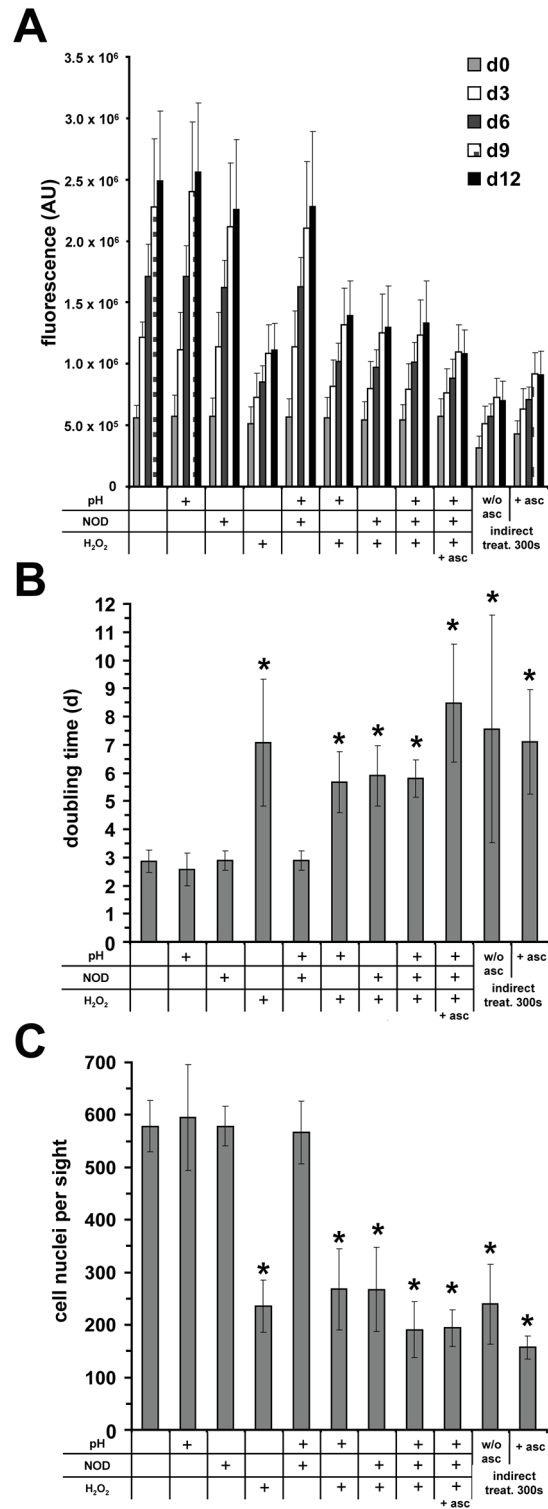
Fig 4. Hydrogen peroxide reduces proliferation of fibroblasts. Fibroblasts were exposed (5 min) to various combinations of hydrogen peroxide (H_2O_2; 200 µM), nitrite/nitrate (NOD; 300 µM/1.0 mM) and acidified PBS (pH 6.7) or normal PBS (pH 7.2) and sodium ascorbate (**asc**; 1 mM).The used concentrations were equivalent to concentrations found after 300 s DBD treatment. Additionally, fibroblasts were exposed for 5 min to DBD-treated PBS (300 s) with or without sodium ascorbate. **A** shows fluorescence values obtained by a resazurin-based assays as the measure for cell viability after treatment as indicated. **B** Calculated

doubling time using obtained resazurin values. **C** Number of Hoechst 33342-stained nuclei on day 12 after treatment. Given are the mean ± sd values (n = 5),*$P < 0.05$ as compared with the control values.

evaluated the amount of myofibroblasts 12 days after DBD treatment by visualization of the myofibroblast marker α-smooth muscle actin (α-SMA). We observed that the α-SMA$^+$ signals were significantly reduced by direct and indirect DBD treatments as compared to the untreated control. Particularly, after longer treatments times (300 s), only 20.2 ± 7.9% (direct) or 22.5 ± 10.1% (indirect) of the cell culture bottom area was positively stained, whereas the control showed 35.2 ± 9.9%. Analogous results could be achieved by the exposure of cells to H_2O_2 (Fig 5A). However, TGF-β1 levels were not significantly changed (Fig 5B).

With regard to the number of cells being coincidently reduced by DBD treatment, we noted a significant increase in α-SMA$^+$ signal normalized to cell number by direct or indirect DBD treatment (Fig 5C and 5D).

Discussion

Since non-thermal plasma has many potential clinical applications, for example, in the treatment of contaminated wounds or impaired wound healing, this study has addressed the interaction of plasma with dermal fibroblasts, which play an important role in wound healing.

Many studies have demonstrated that non-thermal plasma can produce dose-dependent effects ranging from increased cell proliferation to cell death [38–44]. It is hypothesized that cell death in response to non-thermal plasma is primarily through the induction of apoptosis as an effect of the production of reactive oxygen/nitrogen species, which can be scavenged by antioxidants [21].

It has also been demonstrated that *in situ* generation of the hydroxyl radical from superoxide anion and hydrogen peroxide (H_2O_2) plays a critical role in plasma-induced cell death [45].

Using DBD as CAP source, we have observed similar effects on fibroblast viability already after clinically relevant treatment times [46]. Here, the antioxidant ascorbate could attenuate toxic effects. Determining the toxic range in preliminary experiments, we could not find any toxic effects in human foreskin fibroblasts after 15 and 30 s direct DBD treatments (data not shown). However, the exposure of fibroblasts to DBD-treated buffer also reduced cell viability, suggesting that besides high-reactive, short-living radicals (superoxide, singlet oxygen, hydroxyl radical) are also a more stable reactive species such as H_2O_2 within the buffer or other plasma-induced chemical and physical changes (e.g. accumulation of nitrite/nitrate, pH, osmolarity) may be responsible for the observed effects.

Mimicking indirect DBD treatment (300 s) by the acidification of buffer and addition of equivalent amounts of nitrite/nitrate/H_2O_2 has shown slight but not significant effects on viability. In contrast, an indirect DBD treatment reduced cell viability by more than 50%, thereby suggesting that the final products such as H_2O_2, nitrite and nitrate were not the main responsible agents for toxicity. Furthermore, we observed significant inhibition of fibroblast proliferation and nuclei swelling, which was more pronounced by indirect DBD treatments. In preliminary experiments with foreskin fibroblasts, we could not find any effects on proliferation after 15 and 30 s direct DBD treatments (data not shown). Although we observed no effects on viability, acidified buffer containing nitrite/nitrate/H_2O_2 could significantly inhibit cell proliferation to the same degree as indirect treatments. Further experiments revealed that these inhibition effects were solely dependent on the presence of H_2O_2 and not on nitrite/ nitrate and/or acidification. It is well-known that brief exposures to H_2O_2 (50 μM; 1 h) can induce delayed cell death and a prolonged inhibition of cell division in endothelial cells[47].
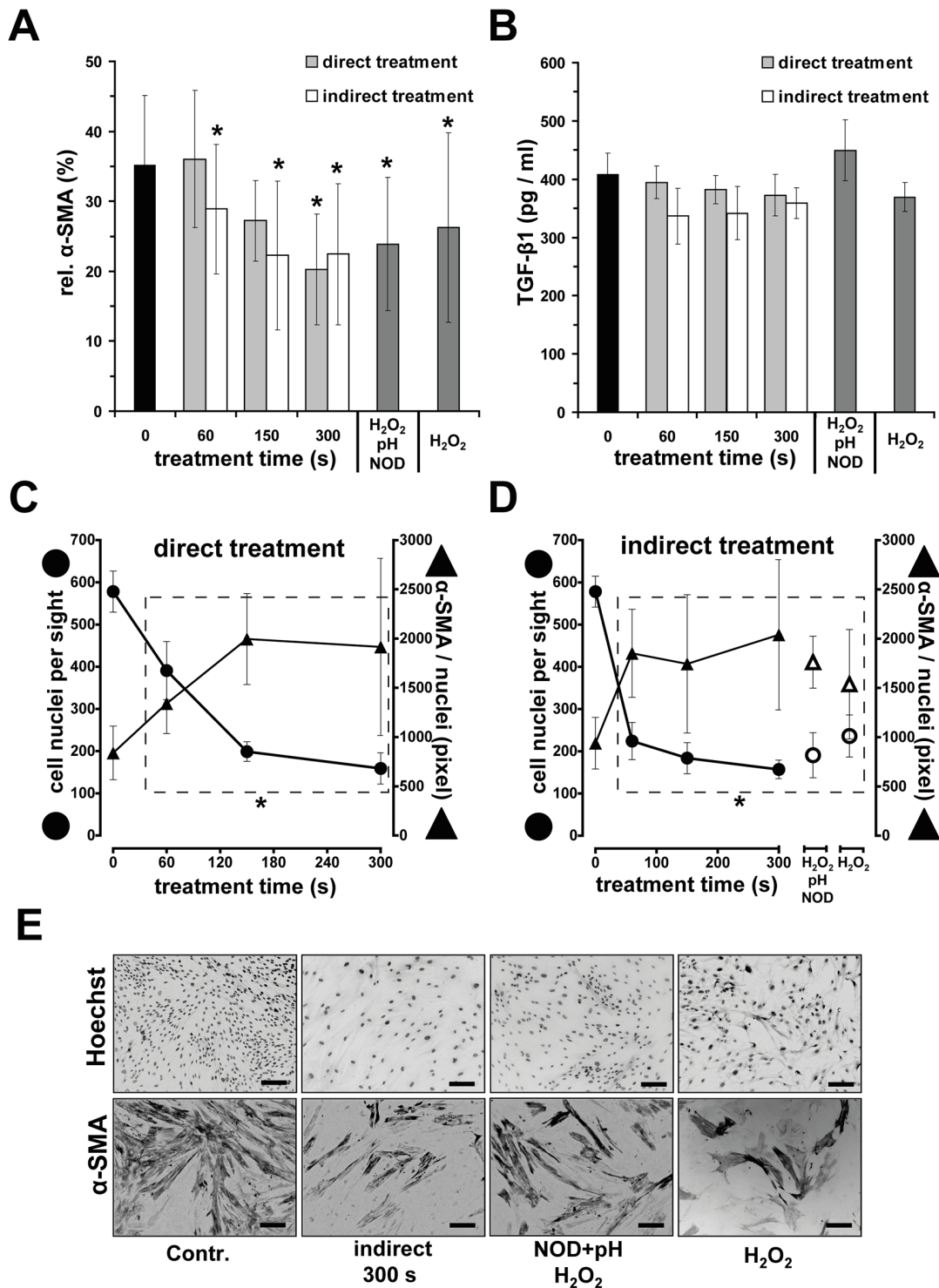
Fig 5. DBD treatment interferes with fibroblast/myofibroblast differentiation. A Quantification of alpha-smooth muscle actin of fibroblasts (α-SMA) 12 days after direct/indirect DBD treatment. B TGF-β1 concentrations in the supernatants seven days after treatment. C and D show number of Hoechst 33342 stained cell nuclei and α-SMA positive-stained area normalized to number of nuclei in 1.4 mm² cell culture plate 12 days after DBD treatment. E Representative microphotographs of Hoechst 33342-/α-SMA-stained fibroblasts.In control experiments, fibroblasts were exposed (5 min) to acidified PBS (pH 6.7) containing concentrations of nitrite/nitrate (NOD; 300 μM/1.0 mM) and hydrogen peroxide (H2O2; 200 μM), which are equivalent to concentrations obtained after a 300 s DBD treatment. Additionally, fibroblasts were exposed to PBS (pH 7.2) containing only H2O2 (200 μM). Given are the mean ± sd values (n = 5),*P < 0.05 as compared with the control values.

doi:10.1371/journal.pone.0144968.g005

Sub-lethal treatment of H_2O_2 (200 μM; 2 h) "stunned" also fibroblast F65 cells and caused them to enter a state resembling senescence [48].

In addition, H_2O_2 can cause DNA damage, which in turn induces cell toxicity and inhibition of proliferation [49]. In our experiments we could not find a significant increase of γ-H2AX as a marker for DNA double-strand breaks after direct or indirect plasma treatment or H_2O_2

However, a treatment time-dependent, but not significant, increase in γ-H2AX levels could be observed after direct DBD treatment. It is important to mention that in contrast to most studies, the cells in our experiments were only exposed for a short time (300 s) to a comparably low concentration of H_2O_2; thus, it seems probable that H_2O_2 -induced DNA damages can occur after longer DBD treatments. In general, the biological impact of H_2O_2 depends on cell type, cell cycle status, cellular antioxidative defense, concentration, exposure time and also the cell environment. Therefore, toxic or antiproliferative doses of H_2O_2 treatment can vary dramatically between studies. The same applies for cold plasma treatments; however, here the use of different plasma sources, operation parameters and therefore many more factors (e.g. radicals, UV, temperature, electric fields etc.) further complicate the matter.

Thus, it is not surprising that, in contrast to other studies [24,50], our data indicate that toxic effects by DBD are not predominantly mediated by H_2O_2.

In our experiments we used human foreskin fibroblasts, which in contrast to adult skin fibroblasts undergo myofibroblast differentiation in cell culture media without addition of inductors such as TGF-β1. Apart from proliferation, the differentiation of fibroblasts to myofibroblasts was affected by DBD treatment (direct/indirect) as well as by H_2O_2-containing buffers. On the one hand, the number of α-SMA$^+$ cells was decreased by treatment, and on the other hand, normalized to cell nuclei, the differentiation frequency was increased. In addition, TGF-β1 levels did not change significantly.

We hypothesize that reactive species such as hydroxyl/superoxide radicals and nitrogen dioxide, which can be scavenged by the antioxidant ascorbate, are partly responsible for loss of cell viability. In addition, we assume that DBD-generated nitrite/nitrate and acidic pH under certain conditions are also involved in toxicity as observed previously in adult skin fibroblasts. However, further unknown mechanisms may take part in the meditation of cell toxicity. Our results indicate that myofibroblast differentiation can be modified by DBD treatment and H_2O_2. In this context, one has to mention again that plasma-mediated effects differ between cell types and cell states. Resting, slow proliferating and/or predifferentiated cells such as proto-myofibroblasts producing TGF-β1 [51], may be less sensitive to DBD/ROS exposure and preferably remain so after treatment. Additionally, generated H_2O_2 inhibits proliferation of the remaining cells. These combined effects could explain that on the one hand, DBD treatment reduces the total number of myofibroblasts, but on the other hand, increases the relative number of myofibroblasts. It is also possible that DBD treatment induces differentiation in fibroblast by further mechanisms, for example, intracellular oxidative stress and/or cell cycle arrest, which were described to promote differentiation processes [52,53].

In conclusion, understanding the mechanisms underlying the different effects of CAP on cell viability, proliferation and differentiation is essential for clinical use. We demonstrated that direct and indirect DBD treatments can reduce fibroblast viability *in vitro*. This reduction is not dominantly mediated by generated H_2O_2, which in turn seems to be responsible for the observed proliferation, inhibition and reduction of myofibroblasts. Due to antibacterial effects, cold plasma treatment represents an effective weapon against resistant bacteria in contaminated wounds. In a recent clinical trial, Brehmer *et al*. have shown the reduction of the bacterial load of chronic wounds by a DBD device (PlasmaDerm; 45 s/cm^2) without harmful side effects [46]. Moreover, by acidification, by generating various biological-active reactive species and by enhancing the local microcirculation cold plasma may positively affect cell proliferation

and healing process, particularly in chronic wounds [28]. In addition, a CAP-induced inhibition of cell proliferation in the context of hyperproliferative skin diseases, such as excessive scarring or psoriasis, may have beneficial therapeutic effects. However, "Right Drug, Right Dose, Right Time" as one basic pharmacological principle also needs to be applied in this matter to avoid disadvantageous effects—for example, delayed wound healing. Therefore, further investigations and clinical trials are necessary to evaluate the possible applications of CAP in clinical use.

Supporting Information

S1 Fig. DBD treatment does not induce significantly DNA double-strand breaks. A Immunocytochemical quantification of gamma-H2AX histone in fibroblasts as marker for DNA double-strand breaks one hour after direct/indirect DBD treatment and **B** 5 min exposure to hydrogen peroxide (0–1000 µM). **C** Representative microphotographs of propidium iodide (**PI**) and gamma-H2AX histone stained fibroblasts treated as indicated. Cells were treated in the presence of ascorbate (**asc**; 1 mM) or irradiated with **UVB** (500 mJ/cm^2) as positive control. Given are the mean $\pm$ sd values (n = 5), $^*P < 0.05$ as compared with the control values. (TIF)

Acknowledgments

Author Contributions

References

1. Pieraggi MT, Bouissou H, Angelier C, Uhart D, Magnol JP, Kokolo J (1985) [The fibroblast]. Ann Pathol 5: 65–76. PMID: 3899128

2. Baum CL, Arpey CJ (2005) Normal cutaneous wound healing: clinical correlation with cellular and molecular events. Dermatologic Surgery: Official Publication for American Society for Dermatologic Surgery [et Al 31: 674–686; discussion 686-674-686; discussion 686.

3. Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200: 500–503. PMID: 12845617

4. Darby IA, Hewitson TD (2007) Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol 257: 143–179. PMID: 17280897

5. Desmouliere A, Chaponnier C, Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. Wound Repair Regen 13: 7–12. PMID: 15659031

6. Desmouliere A (1995) Factors influencing myofibroblast differentiation during wound healing and fibrosis. Cell Biol Int 19: 471–476. PMID: 7640660

7. Shin D, Minn KW (2004) The effect of myofibroblast on contracture of hypertrophic scar. Plast Reconstr Surg 113: 633–640. PMID: 14758226

8. Hinz B (2007) Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 127: 526–537. PMID: 17299435

9. Edwards R, Harding KG (2004) Bacteria and wound healing. Curr Opin Infect Dis 17: 91–96. PMID: 15021046

10. Jones SG, Edwards R, Thomas DW (2004) Inflammation and wound healing: the role of bacteria in the immuno-regulation of wound healing. Int J Low Extrem Wounds 3: 201–208. PMID: 15866816

11. Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM (2008) Microscopic and physio-
     logic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen 16: 23–29. doi:
     10.1111/j.1524-475X.2007.00303.x PMID: 18211576

12. Heinlin J, Isbary G, Stolz W, Morfill G, Landthaler M, Shimizu T, et al. (2011) Plasma applications in
     medicine with a special focus on dermatology. J Eur Acad Dermatol Venereol 25: 1–11.

13. Heinlin J, Morfill G, Landthaler M, Stolz W, Isbary G, Zimmermann JL, et al. (2010) Plasma medicine:
     possible applications in dermatology. J Dtsch Dermatol Ges 8: 968–976. doi: 10.1111/j.1610-0387.
     2010.07495.x PMID: 20718902

14. Isbary G, Shimizu T, Li YF, Stolz W, Thomas HM, Morfill GE, et al. (2013) Cold atmospheric plasma
     devices for medical issues. Expert Rev Med Devices 10: 367–377. doi: 10.1586/erd.13.4 PMID:
     23668708

15. Maisch T, Shimizu T, Li YF, Heinlin J, Karrer S, Morfill G, et al. (2012) Decolonisation of MRSA, S.
     aureus and E. coli by cold-atmospheric plasma using a porcine skin model in vitro. PLoS One 7:
     e34610. doi: 10.1371/journal.pone.0034610 PMID: 22558091

16. Heinlin J, Isbary G, Stolz W, Zeman F, Landthaler M, Morfill G, et al. (2013) A randomized two-sided
     placebo-controlled study on the efficacy and safety of atmospheric non-thermal argon plasma for pruri-
     tus. J Eur Acad Dermatol Venereol 27: 324–331. doi: 10.1111/j.1468-3083.2011.04395.x PMID:
     22188329

17. Heuer K, Hoffmanns MA, Demir E, Baldus S, Volkmar CM, Rohle M, et al. (2015) The topical use of
     non-thermal dielectric barrier discharge (DBD): nitric oxide related effects on human skin. Nitric Oxide
     44: 52–60. doi: 10.1016/j.niox.2014.11.015 PMID: 25435001

18. Kong MG, Kroesen G, Morfill G, Nosenko T, Shimizu T, Dijk Jv, et al. (2009) Plasma medicine: an intro-
     ductory review. New Journal of Physics 11: 115012.

19. Weltmann K-D, Kindel E, Brandenburg R,Meyer C, Bussiahn R, Wilke C, et al. (2009) Atmospheric
     Pressure Plasma Jet for Medical Therapy: Plasma Parameters and Risk Estimation. Contrib Plasma
     Physics, 49: 631–640.

20. Stoffels E, Flikeweert AJ, Stoffels WW, Kroesen GMW (2002) Plasma needle: a non-destructive atmo-
     spheric plasma source for fine surface treatment of (bio)materials,. Plasma Sources Science and Tech-
     nology 11: 383–388.

21. Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, et al. (2011) Effects of non-thermal
     plasma on mammalian cells. PLoS One 6: e16270. doi: 10.1371/journal.pone.0016270 PMID:
     21283714

22. Suschek CV, Schewe T, Sies H, Kroncke KD (2006) Nitrite, a naturally occurring precursor of nitric
     oxide that acts like a 'prodrug'. Biol Chem 387: 499–506. PMID: 16740120

23. Liebmann J, Born M, Kolb-Bachofen V (2010) Blue-light irradiation regulates proliferation and differenti-
     ation in human skin cells. J Invest Dermatol 130: 259–269. doi: 10.1038/jid.2009.194 PMID: 19675580

24. Bekeschus S, Kolata J, Winterbourn C, Kramer A, Turner R, Weltmann KD, et al. (2014) Hydrogen per-
     oxide: A central player in physical plasma-induced oxidative stress in human blood cells. Free Radic
     Res 48: 542–549. doi: 10.3109/10715762.2014.892937 PMID: 24528134

25. Chen LC, Suzuki H, Mori K, Ariyada O, Hiraoka K (2009) Mass Spectrometric Detection of Gaseous
     Hydrogen Peroxide in Ambient Air Using Dielectric Barrier Discharge as an Excitation Source. Chemis-
     try Letters 38: 520–521.

26. Stone JR, Yang S (2006) Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 8: 243–
     270. PMID: 16677071

27. Bibinov N, Knake N, Bahre H, Awakowicz P, Gathen VSVD (2011) Spectroscopic characterization of
     an atmospheric pressure μ-jet plasma source. Journal of Physics D: Applied Physics 44.

28. Heuer K, Hoffmanns MA, Demir E, Baldus S, Volkmar CM, Röhle M, et al. (2015) The topical use of
     non-thermal dielectric barrier discharge (DBD): Nitric oxide related effects on human skin. Nitric Oxide:
     Biology and Chemistry / Official Journal of the Nitric Oxide Society 44: 52–60.

29. Oplander C, Hidding S, Werners FB, Born M, Pallua N, Suschek CV (2011) Effects of blue light irradia-
     tion on human dermal fibroblasts. J Photochem Photobiol B 103: 118–125. doi: 10.1016/j.jphotobiol.
     2011.02.018 PMID: 21421326

30. Blackert S, Haertel B, Wende K, von Woedtke T, Lindequist U (2013) Influence of non-thermal atmo-
     spheric pressure plasma on cellular structures and processes in human keratinocytes (HaCaT). J Der-
     matol Sci 70: 173–181. doi: 10.1016/j.jdermsci.2013.01.012 PMID: 23619096

31. Kaushik NK, Kaushik N, Park D, Choi EH (2014) Altered antioxidant system stimulates dielectric barrier
     discharge plasma-induced cell death for solid tumor cell treatment. PLoS One 9: e103349. doi: 10.
     1371/journal.pone.0103349 PMID: 25068311

32. Feelisch M, Rassaf T, Mnaimneh S, Singh N, Bryan NS, Jourd'Heuil D, et al. (2002) Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. FASEB J 16: 1775–1785. PMID: 12409320

33. Suschek CV, Paunel A, Kolb-Bachofen V (2005) Nonenzymatic nitric oxide formation during UVA irradiation of human skin: experimental setups and ways to measure. Methods Enzymol 396: 568–578. PMID: 16291263

34. Yang F, Troncy E, Francoeur M, Vinet B, Vinay P, Czaika G, et al. (1997) Effects of reducing reagents and temperature on conversion of nitrite and nitrate to nitric oxide and detection of NO by chemiluminescence. Clin Chem 43: 657–662. PMID: 9105269

35. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9: 676–682. doi: 10.1038/nmeth.2019 PMID: 22743772

36. Sellers RM (1980) Spectrophotometric determination of hydrogen peroxide using potassium titanium (IV) oxalate. Analyst 105: 950–954.

37. Kuo LJ, Yang LX (2008) Gamma-H2AX—a novel biomarker for DNA double-strand breaks. In Vivo 22: 305–309. PMID: 18610740

38. Liebmann J, Scherer J, Bibinov N, Rajasekaran P, Kovacs R, Gesche R, et al. (2011) Biological effects of nitric oxide generated by an atmospheric pressure gas-plasma on human skin cells. Nitric Oxide 24: 8–16. doi: 10.1016/j.niox.2010.09.005 PMID: 20883806

39. Kalghatgi S, Friedman G, Fridman A, Clyne AM (2010) Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release. Ann Biomed Eng 38: 748–757. doi: 10.1007/s10439-009-9868-x PMID: 20013154

40. Haertel B, Straßenburg S, Oehmigen K, Wende K, von Woedtke T, Lindequist U (2013) Differential influence of components resulting from atmospheric-pressure plasma on integrin expression of human HaCaT keratinocytes. BioMed research international 2013.

41. Haertel B, Hähnel M, Blackert S, Wende K, von Woedtke T, Lindequist U (2012) Surface molecules on HaCaT keratinocytes after interaction with non-thermal atmospheric pressure plasma. Cell Biol Int 36: 1217–1222. doi: 10.1042/CBI20120139 PMID: 22973947

42. Blackert S, Haertel B, Wende K, Woedtke TV, Lindequist U (2013) Influence of non-thermal atmospheric pressure plasma on cellular structures and processes in human keratinocytes (HaCaT). J Dermatol Sci 70: 173–181. doi: 10.1016/j.jdermsci.2013.01.012 PMID: 23619096

43. Shi XM, Zhang GJ, Yuan YK, Ma Y, Xu GM, Yang Y (2008) Effects of Low-Temperature Atmospheric Air Plasmas on the Activity and Function of Human Lymphocytes. Plasma Process Polym 5: 482–488.

44. Haertel B, Volkmann F, von Woedtke T, Lindequist U (2012) Differential sensitivity of lymphocyte sub-populations to non-thermal atmospheric-pressure plasma. Immunobiology 217: 628–633. doi: 10.1016/j.imbio.2011.10.017 PMID: 22130036

45. Xu D, Liu D, Wang B, Chen C, Chen Z, Li D, et al. (2015) In Situ OH Generation from O2- and H2O2 Plays a Critical Role in Plasma-Induced Cell Death. PLoS One 10: e0128205. doi: 10.1371/journal.pone.0128205 PMID: 26046915

46. Brehmer F, Haenssle HA, Daeschlein G, Ahmed R, Pfeiffer S, Gorlitz A, et al. (2015) Alleviation of chronic venous leg ulcers with a hand-held dielectric barrier discharge plasma generator (PlasmaDerm ((R)) VU-2010): results of a monocentric, two-armed, open, prospective, randomized and controlled trial (NCT01415622). J Eur Acad Dermatol Venereol 29: 148–155. doi: 10.1111/jdv.12490 PMID: 24666170

47. de Bono DP, Yang WD (1995) Exposure to low concentrations of hydrogen peroxide causes delayed endothelial cell death and inhibits proliferation of surviving cells. Atherosclerosis 114: 235–245. PMID: 7605392

48. Chen Q, Ames BN (1994) Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. Proc Natl Acad Sci U S A 91: 4130–4134. PMID: 8183882

49. Driessens N, Versteyhe S, Ghaddhab C, Burniat A, De Deken X, Van Sande J, et al. (2009) Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. Endocr Relat Cancer 16: 845–856. doi: 10.1677/ERC-09-0020 PMID: 19509065

50. Winter J, Tresp H, Hammer MU, Iseni S, Kupsch S, Schmidt-Bleker a, et al. (2014) Tracking plasma generated H 2 O 2 from gas into liquid phase and revealing its dominant impact on human skin cells. Journal of Physics D: Applied Physics 47: 285401–285401.

51. Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. The Journal of pathology 200: 500–503. PMID: 12845617

52. Dimitrijevic-Bussod M, Balzaretti-Maggi VS, Gadbois DM (1999) Extracellular matrix and radiation G1 cell cycle arrest in human fibroblasts. Cancer Research 59: 4843–4847. PMID: 10519394

53. Toullec A, Gerald D, Despouy G, Bourachot B, Cardon M, Lefort S, et al. (2010) Oxidative stress promotes myofibroblast differentiation and tumour spreading. EMBO molecular medicine 2: 211–230. doi: 10.1002/emmm.201000073 PMID: 20535745