

# Comparison of Biological Effects on Human Keratinocytes Using Different Plasma Treatment Regimes

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**ABSTRACT:** This study investigated the influence of the plasma treatment regime on human keratinocytes (HaCaT cells). HaCaT cells were plasma treated with a volume dielectric barrier discharge plasma source in three different ways: directly, directly with culture medium exchange, and indirectly (in which only cell culture medium was exposed to plasma). The influence of dielectric barrier discharge plasma on viability, DNA, cell cycle, and intracellular concentration of reactive oxygen species in HaCaT cells was investigated. Direct and indirect plasma treatment caused a treatment time–dependent decrease of viable cells. An increase in DNA damage was observed immediately after plasma treatment, which was diminished after 24 h. Intracellular reactive oxygen species increased with longer plasma treatment times. The cell cycle analysis showed an accumulation of cells in the G2/M phase at the expense of cells in the G1 phase. An immediate exchange of culture medium after plasma treatment attenuated the described effects. Direct and indirect plasma treatment of adherent HaCaT cells resulted in comparable effects that depend on the plasma treatment time. Physical plasma seems to generate long-living reactive species or to modify organic components of the cell culture medium. Both mechanisms can initiate oxidative stress in human keratinocytes, which is responsible for the observed effects.

**KEY WORDS:** keratinocytes, DBD plasma, DNA damage, cell cycle, ROS, plasma treatment regime

## I. INTRODUCTION

The field of plasma medicine has grown rapidly in recent years and many practical applications in medicine and biology seem to be promising. The antimicrobial properties of physical plasma are well known. These properties can be used to inactivate microorganisms and to sterilize heat-labile and moisture-sensitive material.<sup>1–4</sup> The use of cold argon plasma for blood coagulation after surgical interventions is also well established.<sup>5,6</sup> In dentistry, plasma can be used in the treatment of periodontitis, caused by bacteria.<sup>7</sup> In dermatology, various applications of plasma are conceivable. The strong antibacterial properties of plasma offer a use in the treatment of chronic wounds. Chronic wounds are

often colonized with bacteria, and healing is inhibited if the wound remains infected.<sup>8,9</sup> Moderate amounts of reactive oxygen species (ROS), generated by physical plasma, can improve wound healing.<sup>10</sup> It was previously shown that wound-relevant microorganisms were killed with plasma, and the first clinical trials showed a reduction of bacteria in infected wounds and enhanced healing of CO<sub>2</sub>-laser skin lesions.<sup>11–13</sup> The selective killing of malignant cells could possibly be a tool in skin cancer treatment.<sup>14</sup> Nevertheless, each kind of cell may react in a different way to physical plasma; therefore, it is necessary to investigate the individual behavior of the cell type of the desired application. In addition to the cell type, many other factors are involved in the plasma cell interaction. Plasma itself, with its complex composition (ions, electrons, radicals, electric and magnetic fields, and ultraviolet [UV] radiation), can affect cellular structures and processes. The effects of ROS and UV radiation on DNA, proteins, and other cell components are well known. However, the surrounding liquids (e.g., buffered salt solutions or cell culture medium) of the cells during plasma treatment also influence the degree of cellular reaction.<sup>15–18</sup>

This study aimed to investigate the dimension of the biological effects of physical plasma, generated by a volume dielectric barrier discharge (VDBD), on human keratinocytes depending on the plasma treatment regime (direct, direct with exchange, and indirect). HaCaT cells, a well-established *in vitro* model for human keratinocytes, were used and effects on viability, DNA, cell cycle, and induction of ROS were analyzed.

## II. MATERIALS AND METHODS

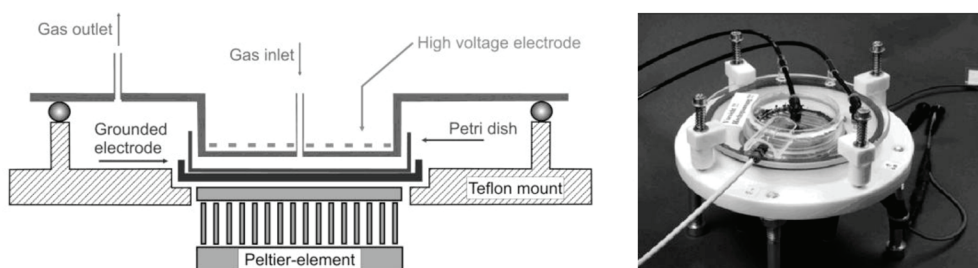
### A. Cell Culture

Adherent HaCaT human keratinocytes, kindly provided by Professor Fusenig (German Cancer Research Center, Heidelberg, Germany), were cultured in an incubator (37°C, 5% CO<sub>2</sub>, 90% humidity) using RPMI 1640 medium supplemented with 1% Penicillin–Streptomycin (PS) and 8% fetal calf serum (FCS). HaCaT cells were subcultivated twice a week and regularly tested for possible mycoplasma contamination.

Twenty-four hours before the experiment was started,  $1 \times 10^6$  HaCaT cells were seeded in 60-mm cell culture dishes and the cell culture medium (3 mL) was exchanged directly before plasma treatment.

### B. Plasma Treatment

A VDBD (Leibniz Institute for Plasma Sciences and Technology e.V., Greifswald, Germany) was used as the plasma source.<sup>19,20</sup> The working gas was argon. Fig. 1 shows a schematic of the source, which consists of two flat round copper electrodes ( $A = 16.6 \text{ cm}^2$ ). The perforated high-voltage electrode is integrated in the chamber lid. The lid is made of 2.4-mm-thick acryl glass and acts as a dielectric barrier. It has two openings for gas exchange and is sealed against the Teflon base plate by a rubber ring. Therefore, a gas gap of 5.6 mm between the lid and grounded electrode is realized. The grounded electrode is



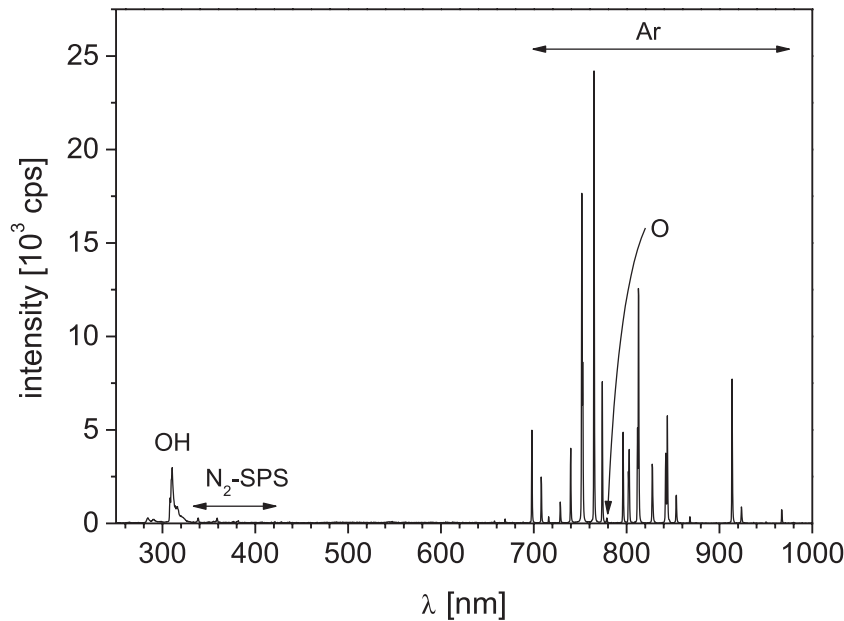
**FIG. 1.** Schematic setup and photography of the VDBD plasma source. The culture dish with adherent HaCaT cells or cell culture medium was located between both electrodes. Working gas was argon and cells were incubated for 1 min with argon before plasma treatment.

cooled by means of a Peltier element. Electrical power is supplied through a homemade transformer, an amplifier (AG1021; T&C Power Conversion Inc., Rochester, NY), and a function generator (AFG 3101; Tektronix, Beaverton, OR). Frequency matching of the setup was done by inserting an empty petri dish. The material of the cell culture dish on the surface of the grounded electrode then acts as an additional dielectric barrier in the setup. Filling the dish with cell culture medium led to further changes in capacitive properties and to humidification of the working gas. With liquid in the dish, a significant decrease of the plasma emission was seen. Furthermore, a shift in emission line ratios was noted. Nevertheless, the frequency matching of the setup with an empty dish inside was used for all treatments. During treatment of liquid-containing petri dishes, plasma filled the whole gap between the chamber lid and liquid surface. Broad, pale, slowly moving plasma filaments were observed. The plasma emission was mainly governed by argon lines in the near infrared region (Fig. 2). Less intensive emission was measured for the OH radical (UVB spectral range), and very low emission of the second positive system of nitrogen (UVA) and atomic oxygen (777 nm) was observed. Table 1 lists the physical parameters of the plasma source obtained during treatment of the cell culture medium. The power rating was obtained by measuring the dissipated electrical energy by the Lissajou method.

To accomplish an air-free atmosphere, all samples (adherent HaCaT cells or culture medium) were locked air-tight and incubated for 1 minute with argon at a flow rate of 0.5 slm before plasma treatment.

HaCaT cells were directly and indirectly treated with dielectric barrier discharge (DBD) plasma. Half of the directly treated samples received fresh culture medium immediately after plasma exposition; the other samples were retained in the plasma-treated medium. For indirect plasma treatment, only cell culture medium was exposed to plasma and was added to adherent HaCaT cells immediately after plasma treatment. The positive control comprised 100  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) (with the same incubation periods as the plasma samples).

The control cells were not exposed to plasma but were otherwise treated identically. At 1 h, 4 h, and 24 h after plasma treatment, HaCaT cells were detached by treatment with phosphate-buffered saline (PBS)/EDTA and trypsin/EDTA (in PBS without



**FIG. 2.** Optical emission of the plasma during treatment of 2-ml cell culture medium with emission bands of the OH radical (OH) and the second positive system of nitrogen (N<sub>2</sub>-SPS) as well as emission lines of argon (Ar) and atomic oxygen (O).

**TABLE 1.** Technical parameters of VDBD (energy and power for treatment of culture dish, filled with 3 mL culture medium)

Parameter	Value
Voltage	9–10 kV
Frequency	33 kHz
Gas flow	0.5 slm
Power	1.8 W
Energy	55 μJ
Area of electrode	16.6 cm <sup>2</sup>
Energy density	3.3 μJ/cm <sup>2</sup>

Ca<sup>2+</sup>/Mg<sup>2+</sup>, 0.05%/0.2%). After centrifugation (3 min/130×g), the cells were resuspended in RPMI 1640 and a Neubauer chamber was used to estimate the cell number. Trypan blue (0.04%) was used to distinguish between living and dead cells.

**C. Single-Cell Gel Electrophoresis**

The alkaline version of single-cell gel electrophoresis (SCGE) developed by Singh *et al.*<sup>21</sup> was used to detect DNA damage 1 h and 24 h after plasma treatment. The ex-

perimental procedure was previously described by Blackert *et al.*<sup>16</sup> Briefly, HaCaT cells were detached by trypsin 1 h and 24 h after plasma treatment and were embedded in agarose on a microscope slide. Cells were lysed in a detergent- and salt-containing buffer and electrophoresis was performed under alkaline conditions.

Fifty nuclei per slide were randomly selected and analyzed using a fluorescence microscope (Olympus CK 40; Olympus, Hamburg, Germany) connected to a camera (Infinity 3; Lumenera, Ottawa, Canada). Nucleoids were analyzed with Comet Assay IV software (Perceptive Instruments, Suffolk, UK). The tail intensity (percentage of DNA in the tail) is the parameter used to indicate DNA damage.

#### **D. Cell Cycle Analysis**

Twenty-four hours after plasma treatment, distribution of HaCaT cells in different cycle stages was analyzed using flow cytometry. Cells were detached and counted as described above. Then,  $5 \times 10^5$  cells were incubated in 70% ethanol for at least 1 h and washed twice with PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ). The cells were incubated for 30 min in RNase (50  $\mu\text{g/mL}$  in PBS) at 37°C.

After washing with FACS buffer (1% FCS, 8  $\mu\text{g/mL}$  sodium azide in PBS), cells were resuspended in FACS buffer and stained with propidium iodide (50  $\mu\text{g/mL}$ ). Then,  $5 \times 10^3$  cells per sample were analyzed with a MACSQuant Analyzer (Miltenyi Biotech, Bergisch Gladbach, Germany). Kaluza software (Beckman Coulter, Krefeld, Germany) was used to evaluate the distribution of HaCaT cells in each cell cycle phase.

#### **E. Detection of Intracellular ROS**

Intracellular ROS were measured using 2',7'-dichlorodihydrofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ ).<sup>22,23</sup> The intracellular ROS were determined at 1 h, 4 h, and 24 h after plasma treatment. HaCaT cells were detached and counted as described above. Then,  $1.5 \times 10^5$  cells were suspended in RPMI culture medium and incubated with 2.5  $\mu\text{M}$   $\text{H}_2\text{DCFDA}$  (30 min, 37°C). After washing with culture medium, HaCaT cells were resuspended in RPMI culture medium and analyzed using flow cytometry (MACSQuant Analyzer). Kaluza software was used for determination of the proportion of cells that were positive for green fluorescence.

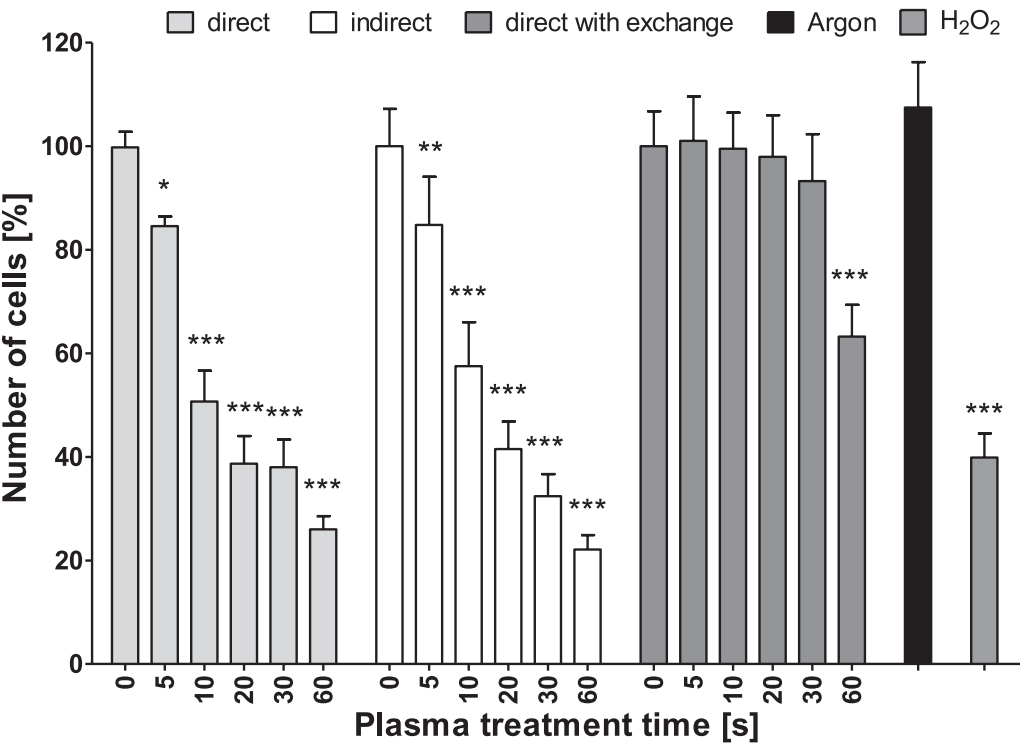
#### **F. Statistical Analysis**

GraphPad Prism software (GraphPad Inc., La Jolla, CA) was used to test result significance (Tukey's honestly significant difference test and the Student's *t* test).

### **III. RESULTS AND DISCUSSION**

This study focused on the comparison of the dimension of the cellular reactions of human keratinocytes toward a plasma treatment regimen. HaCaT cells were treated directly and indirectly with a VDBD.

The number of viable cells was determined 1 hour and 24 hours after plasma treatment. The viability of cells was unaffected 1 h after plasma treatment (data not shown). Twenty-four hours after plasma treatment, a plasma dose-dependent reduction of viability was observed after direct and indirect plasma treatment (Fig. 3), even after the shortest plasma treatment time (5 s). When cell culture medium was exchanged subsequently after plasma exposition, only the longest treatment time (60 s) caused a significant loss of viability. Flushing with argon, without igniting the plasma, had no influence on the viability. HaCaT cells, incubated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (used as a positive control), also showed a significant loss of viable cells corresponding with a plasma treatment time of 20 s (direct and indirect treatment). The viability reduction of indirectly treated cells occurred in the same dimension as the directly treated cells, although the indirectly treated cells never came in direct contact with the plasma. UV radiation and electric and magnetic fields do not seem to be primarily responsible for the described effect, because these plasma components can

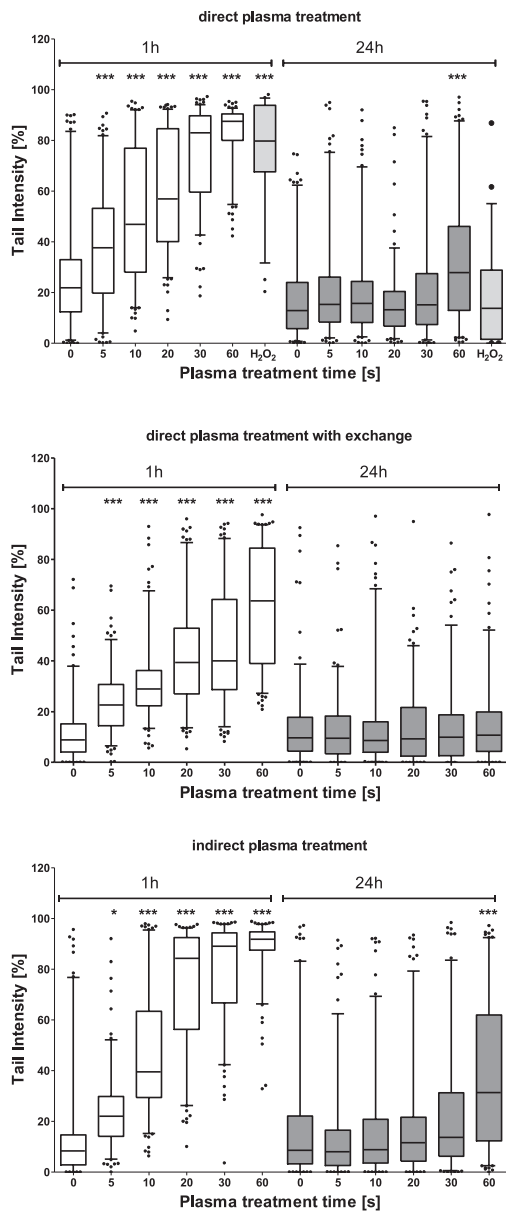


**FIG. 3.** Influence of VDBD plasma on viability of HaCaT cells. The number of viable cells was estimated 24 h after plasma treatment (5–60 s) using a Neubauer chamber and trypan blue to exclude dead cells. A treatment time–dependent loss of viable cells was detected after direct and indirect treatment procedures. Exchanging the culture medium subsequently after plasma treatment showed no effect on viability, with the exception of the longest treatment time. Results are expressed as the mean  $\pm$  SEM ( $n = 4–11$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (versus untreated controls;  $t$  test).

only interact with the cells during direct plasma treatment. The generation of long-living radicals or modification of cell culture medium ingredients by plasma may affect cell viability. Plasma treatment time-dependent loss of HaCaT cells and attenuation of this effect after subsequent medium exchange were also described with a surface DBD in air.<sup>16,17</sup>

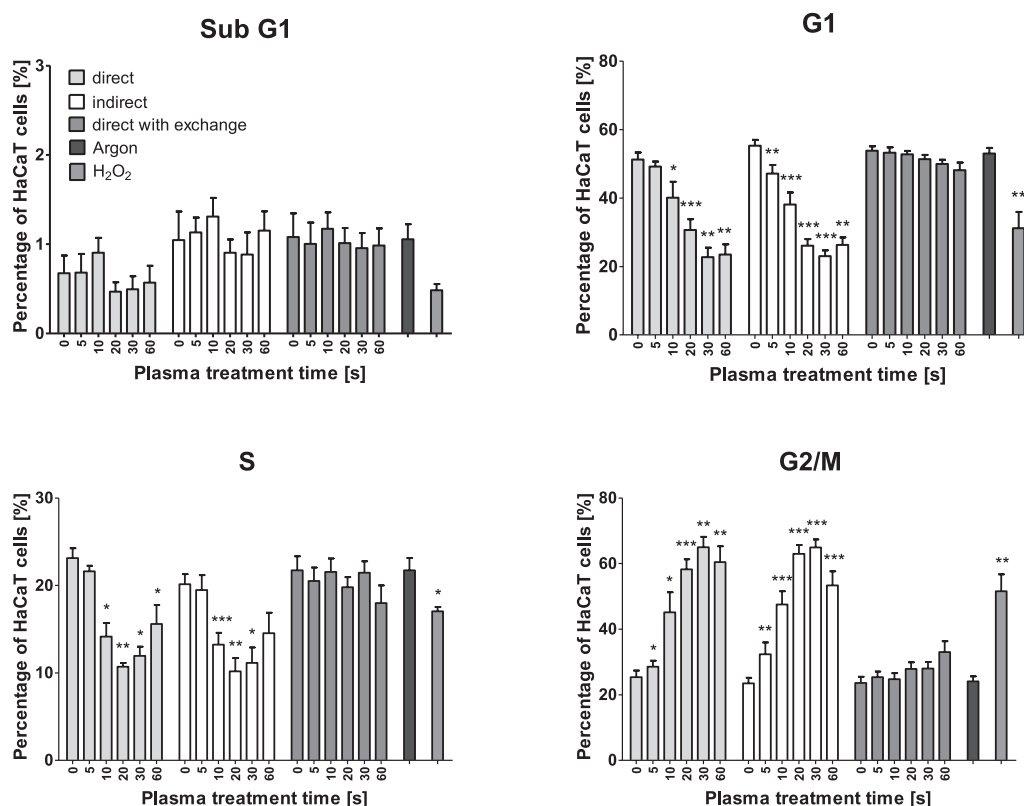
To detect the influence of the volume DBD on DNA of HaCaT cells, the alkaline version of SCGE was used 1 h and 24 h after plasma treatment. This assay detects various kinds of DNA damage such as single and double strand breaks, alkali labile sites, and oxidative base damage in single cells. Tail intensity (percentage of DNA in the tail) was the parameter analyzed. Direct and indirect treatment showed a dose-dependent increase of DNA damage 1 h after plasma exposition (Fig. 4), even after the shortest treatment time. Twenty-four hours after treatment, DNA damage was no longer evident, with the exception of the longest plasma treatment time (60 s). The exchange of the culture medium subsequently to plasma treatment resulted in DNA damage as well, but to a less severe degree (Fig. 4) compared with the direct treatment regime. After 24 h, values of tail intensity showed the same level as untreated control cells. Bringing the data of viability and DNA damage in context, it seems that HaCaT cells are able to repair moderate DNA damage. Plasma treatment with culture medium exchange induces DNA damage but a decrease of viable cells was not detectable up to a plasma treatment time of 30 s (Figs. 3 and 4). Direct and indirect plasma-treated cells also showed diminished values of tail intensity 24 h after treatment, but there was also a loss of viable cells. It cannot be distinguished whether cells are able to repair DNA damage or whether it comes to a selection process of the damaged cells. Similar effects on DNA were shown using a surface DBD.<sup>16</sup> Welz *et al.*<sup>24</sup> investigated the impact of physical plasma on DNA of mucosal tissue with alkaline SCGE. Treatment times up to 120 s did not cause DNA damage, but mucosal tissue was plasma treated without surrounding liquids (cell culture medium). Kalghatgi *et al.*<sup>15</sup> analyzed DNA damage after plasma treatment detecting phosphorylated  $\gamma$ -H2AX (an indicator for DNA double strand breaks) and also found a plasma dose-dependent increase of DNA damage, irrespective of direct or separate plasma treatment.<sup>25</sup> The authors suggest that plasma can modify organic culture medium components (e.g., amino acids) into stable peroxides. If plasma-treated culture medium will not be changed subsequently after plasma treatment (direct and indirect treatment), these stable peroxides can interact with cellular structures during the incubation period (up to 24 h). In addition, the augmented generation of long-living reactive species (e.g.,  $H_2O_2$ ) can disturb the integrity of the genetic information. An intact DNA structure and the unimpeded sequence of the cell cycle are required for the correct transmission of the genetic information. The analysis of the cell cycle did not show any significant changes in the distribution of cells in each cell cycle phase 24 h after direct plasma treatment with medium exchange or after incubation with argon (Fig. 5). By contrast, direct and indirect treatment caused significant changes in cell cycle analysis. The number of HaCaT cells in the G1 and S phases decreased in a plasma treatment time-dependent manner and the percentage of keratinocytes increased significantly in the G2/M phase (Fig. 5). Percentages of cells in the sub-G1 phase were not affected. Increased numbers of cells in the sub-G1 phase would indicate DNA fragmentation, characteristic of apoptotic processes. Using a surface DBD in air for plasma





**FIG. 4.** Detection of DNA damage with alkaline SCGE after plasma treatment (1 h, 24 h). Tail intensity (percentage of DNA in the comet tail) was used to quantify DNA damage. One hour after plasma treatment, a dose-dependent increase of DNA damage was detected (direct and indirect) and effects were diminished with an additional medium exchange. DNA damage was not evident 24 h after incubation with plasma, except for the longest treatment time.  $H_2O_2$  (100  $\mu M$ ) was used as the positive control. Results are expressed in box plots (5%–95% percentiles;  $n = 3$ ). \* $P < 0.05$ ; \*\*\* $P < 0.001$  (versus untreated control; Tukey's honestly significant difference).





**FIG. 5.** Cell cycle analysis of HaCaT cells 24 h after plasma treatment. Percentages of cells in cell cycle phases (subG1, G1, S, G2/M) were analyzed using propidium iodide and flow cytometry. Direct and indirect plasma treatment caused a significant decrease of keratinocytes in G1 and S and an increase of cells in the G2/M phase. No changes in the distribution of cells were detected when HaCaT cells received fresh culture medium immediately after plasma treatment. Results are expressed as the mean  $\pm$  SEM ( $n = 3-5$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (versus untreated control;  $t$  test).

treatment induced comparable effects in human keratinocytes.<sup>16</sup> In mouse skin cancer cells, physical plasma also induced a remarkable increase of cells in the G2/M phase, whereas normal mouse keratinocytes showed only a moderate increase.<sup>26</sup> Arndt *et al.*<sup>27</sup> detected a significant increase of melanoma cells in the sub-G1 phase 24 h after plasma treatment. In ocular cells, elevated level of cells in the sub-G1 phase were detectable within 12 h after plasma exposure.<sup>28</sup> Summing up, the results of cell cycle analyses it becomes obvious that depending on plasma source and cell line different effects on cell cycle were detected.

Physical plasma induces oxidative stress to cells. Many articles report elevated levels of intracellular ROS in several cell lines after treatment with different plasma sources.<sup>16,28-31</sup> Very high amounts of ROS can disturb the homeostatic balance of the cell and lead to oxidative stress responses. Via oxidation, ROS can affect the nucleobases and the sugar

backbone of the DNA.<sup>32</sup> Therefore, the number of ROS-positive cells was determined. Incubation with argon gas only or with direct plasma treatment with a subsequent exchange of the culture medium after plasma treatment caused no increased levels of ROS independently from the plasma dose and incubation period used (Fig. 6). By contrast, incubation with H<sub>2</sub>O<sub>2</sub>, a well-known ROS inducer, resulted in a significant increase in ROS-positive cells. After direct plasma treatment (1 h), a significant increase of ROS-positive cells was also observed at a plasma treatment time of 20 s ( $60.44\% \pm 9.53\%$  versus  $9.24\% \pm 0.72\%$ ; Fig. 6). Values of ROS-positive cells at 4 h and 24 h were still elevated but at lower levels compared with values at 1 h.

Brun *et al.*<sup>28</sup> detected a maximum of intracellular ROS in human keratocytes 5 min after plasma treatment and elevated levels of glutathione peroxidase 1 (GPX-1) 6 h after plasma treatment. Similar results were also found on the gene level by Schmidt *et al.*,<sup>33</sup> who found an increased gene expression of oxidative stress-relevant enzymes (e.g., GPX-1, GPX-3, and GPX-5) in plasma-treated HaCaT cells. This confirms that cells are able to compensate oxidative stress using antioxidative enzymes (e.g., catalase and peroxidase).

#### IV. SUMMARY AND CONCLUSIONS

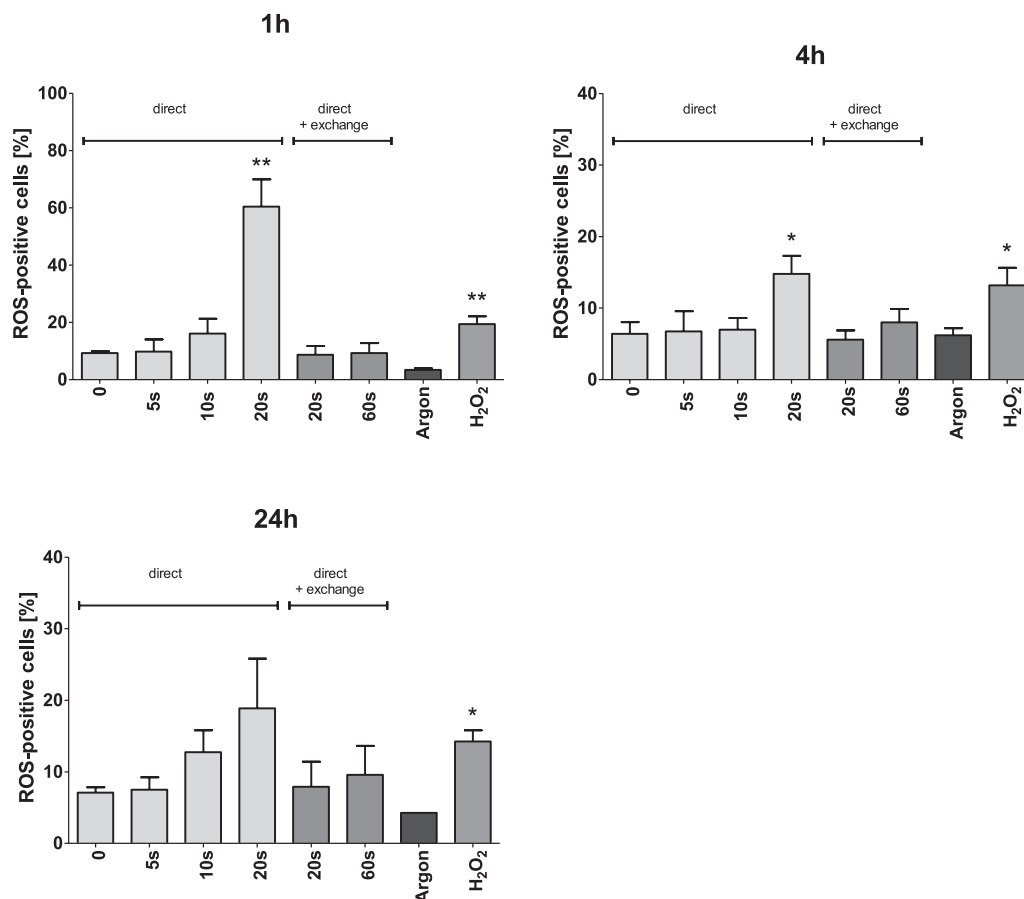
This study describes effects of VDBD plasma on basic cellular processes and structures in human keratinocytes using different plasma treatment regimes. HaCaT cells were treated directly with and without exchange of the cell culture medium and indirectly (in which only culture medium was exposed to physical plasma). The influence on cell viability, intracellular ROS, DNA, and cell cycle was investigated. Direct and indirect plasma treatment showed comparable effects. A plasma dose-dependent loss of cell viability and an induction of DNA damage with a probable repair after shorter treatment times were observed. A cell cycle arrest in the G2/M phase was detected in response to DNA damage, giving cells time for DNA repair.

A maximum of ROS-positive cells was detected 1 h after plasma treatment. Long-living reactive species and modified culture medium components seem to be responsible for the described effects in human keratinocytes. A subsequent cell culture medium exchange attenuated all of the described observations. The interaction of physical plasma with the cell-surrounding liquids, plasma treatment time, and incubation periods (1–24 h) of the experiments ascertain the degree of effects on human keratinocytes.

A comparison of the impact of physical plasma on human keratinocytes with the present plasma source and a surface DBD<sup>16</sup> shows that similar cellular effects occur (e.g., cell viability, DNA damage, intracellular ROS concentration, and cell cycle analysis). Treatment times vary due to different technical parameters of the plasma source (e.g., process gas, energy density, and area of the electrodes).

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**FIG. 6.** Detection of ROS-positive HaCaT cells after plasma exposure using H<sub>2</sub>DCFDA (1 h, 4 h, 24 h). Direct plasma treatment with a dose of 20 s caused 60% ROS-positive cells (1 h). The 4-h and 24-h levels of ROS-positive cells were still elevated but on a lower dimension. An additional medium exchange prevents building of intracellular ROS. Results are expressed as the mean  $\pm$  SEM ( $n = 3-5$ ), except for argon (24 h). \* $P < 0.05$ ; \*\* $P < 0.01$  (versus untreated control;  $t$  test).

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