We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 6

Physicochemical Analysis of Argon Plasma-Treated Cell Culture Medium

Claudia Bergemann, Cornelia Hoppe, Maryna Karmazyna, Maxi Höntsch, Martin Eggert, Torsten Gerling and Barbara Nebe

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61980

Abstract

The effects of cold plasma under atmospheric pressure are being explored for medical applications. It was found that plasma effects on cells correspond to a plasma–medium interaction; thus, plasma-treated cell culture medium alone is able to influence the cell behavior. Here, we discovered that the liquid-mediated effect of atmospheric-pressure argon plasma on mouse liver epithelial cells persists up to 21 days of storage; i.e., the liquid preserves the characteristics once induced by the argon plasma. Earlier investigations of our group revealed that temperature and pH, hydrogen peroxide production and oxygen content can be excluded as initiators of the detrimental biological changes. As we found here, the increased osmolality in the media caused by plasma treatment can also be excluded as a reason for the observed cell effects. Conversely, we found changes in the components of cell culture medium by fast protein liquid chromatography (FPLC) and decreased cell viability in plasma-treated media independent of the presence of fetal calf serum (FCS) during plasma treatment. The persistent biological effect on plasma-treated liquids observed here could open up new medical applications. Stable plasma-treated liquids could find application for dermatological, dental, or orthopedic therapy.

Keywords: Non-thermal atmospheric-pressure plasma jet, Plasma–liquid interaction, Osmolality, Size-exclusion chromatography, Cell viability

1. Introduction

Plasma medicine is an emerging field of interdisciplinary research combining physics, biology, and clinical medicine [1,2]. In the first applications, gas plasma was used in the form of hot plasma for cauterization [1,3]. Currently, the effects of cold plasma under atmospheric pressure are being explored for medical applications. Several experiments showed the achievement of

INTECH
open science | open minds

© 2016 The Author(s). Licensee InTech. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CC BY

blood coagulation and bacterial decontamination, with little effect on the surrounding tissue [4–9], as well as the promotion of wound healing and tissue regeneration [10–13]. This proliferative reaction was correlated with the secretion of growth factors induced by plasma treatment [14]. Conversely, adverse effects such as anti-proliferative and anti-tumorigenic effects were reported [15–18]. Also, the detachment of cells from the substrate, an effect that accompanies the change to a rounded cell morphology, and apoptosis were reported for animal cell lines like CHO-K1 (Chinese hamster ovary epithelial cells), 3T3 (mouse fibroblasts), BAEC (bovine aorta endothelial cells), H5V (mouse endothelial cells), and RASMC (rat aorta smooth muscle cells) [1,19,20]. This is in line with the observation that the expression of cell adhesion molecules is changed upon plasma treatment [21]. Detrimental cell effects like DNA damage were shown by Kalghatgi et al. [22]. Plasma treatment of epithelial cells led to cell detachment and additional DNA damage, which was proven by the phosphorylation of the histone protein H2AX [22].

It was discovered that these effects can be mediated by the use of plasma-treated liquids alone [23]. Plasma treatment of cell culture medium resulted in DNA damage to the subsequently incubated cells [22,24]. Similar to the direct treatment, liver epithelial cells lost their substrate attachment when incubated with argon plasma-treated medium alone [25]. In addition, cell–cell contact proteins, e.g., the tight junction protein zonula occludens (ZO-1), as well as the cell membrane morphology were shown to be impaired [25,26]. Based on these observations, it could be concluded that plasma effects are not only solely based on the direct plasma–cell interaction but also on the interaction between plasma and medium, and these plasma-induced changes in the complex Dulbecco's modified Eagle's medium (DMEM) were long lasting: up to 7 days and more [26].

Further analysis revealed that neither lipid oxidation [24] nor the generation of ozone [27] was responsible for these effects. Also, changes in temperature, pH-value, or the concentration of hydrogen peroxide could be excluded as a reason [25,26]. In addition, it was shown that decreasing the oxygen concentration of the medium as a result of plasma treatment was not the reason for the respective cell defects [26]. Several publications speculated that the generation of reactive oxygen species (ROS) through plasma treatment would be an explanation for DNA damage [20,28,29]. However, the cell-damaging effect of plasma-treated medium could be detected after 1 h as well as up to 7 days after treatment [22,25,26], indicating that the component(s) remained stable for a long time. This result excludes the short-lived ROS as an inducing agent.

The present work aimed to examine the contribution of different medium components, e.g., fetal calf serum and gentamicin, to the detrimental cell effects resulting from plasma treatment. Furthermore, physicochemical parameters other than temperature, pH, oxygen concentration, or the presence of hydrogen peroxide, which had already been excluded as a reason [25, 26], were investigated. Taking into consideration that the plasma effect is mediated only by liquids [22,23,25,26,29], the cell culture medium was treated with argon plasma and cells were incubated afterward with the medium. The plasma treatment was performed using an atmospheric-pressure argon plasma jet.

http://dx.doi.org/10.5772/61980

2. Material and methods

2.1. Argon plasma source

The atmospheric-pressure plasma jet kINPen®09 (neoplas tools GmbH, Greifswald) was used for all experiments. This plasma source consists of a quartz capillary (inner diameter of 1.6 mm) with a needle electrode (diameter of 1 mm). A high-frequency voltage of 1.1 MHz/2–6 kV is applied at this electrode. Argon gas (purity 99.996%) is used as a feed gas with a gas flow of 1.9 slm. A gas discharge is ignited at the tip of the high-voltage needle exciting the argon gas. In this way, low-temperature plasma is generated and blown out of the capillary. The so-called plasma jet outside the nozzle has a length of 12–14 mm and is about 1 mm wide. The temperature at the visible tip of the plasma jet does not exceed 50°C. Details for the characterization of the plasma source are given by Weltmann *et al.* [12,26].

2.2. Argon plasma treatment of cell culture media

One-hundred microliters of the defined cell culture medium (see below) was supplied per well in a 96-well plate (Greiner Bio-One). The kINPen®09 was mounted vertically and the quartz capillary was positioned at the top edge of each well in the 96-well plate. The distance between the tip of the quartz capillary and the 96-well plate does not exceed 1 mm. In this way, the argon plasma had an immediate contact with the cell culture medium (Figure 1).

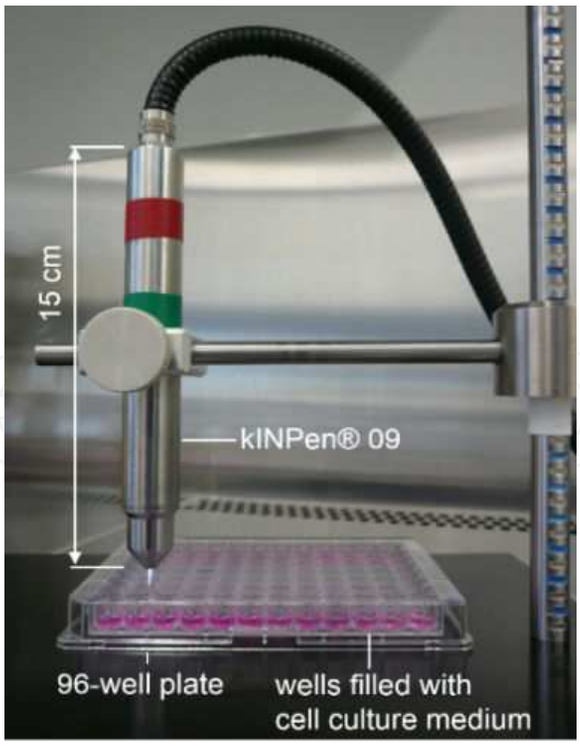


Figure 1. Experimental setup for argon plasma treatment of cell culture media. The kINPen®09 was mounted vertically. The distance between the tip of the quartz capillary and the 96-well plate does not exceed 1 mm.

Dulbecco's modified Eagle's medium (DMEM; Invitrogen) was used for all experiments. For cell culture experiments, DMEM is usually supplemented with 10% fetal calf serum (PAA Gold, PAA Laboratories) and 1% gentamicin (Ratiopharm). To explore the effect of the supplements on plasma treatment results, pure DMEM without supplements or with one supplement was treated as well. We refer to the different media as follows: pure DMEM = DMEM; DMEM with 10% FCS and 1% gentamicin = DMEM+FCS+Genta; DMEM with 10% FCS = DMEM+FCS; DMEM with 1% gentamicin = DMEM+Genta. The different media underwent argon plasma treatment for 60 or 120 s as indicated for the specific experiment. Argon gas treatment using the kINPen®09 without igniting the plasma was used as a control. Untreated medium was included as a further control. After plasma treatment, the different samples and controls were pooled in separate tubes (1.5 ml, Eppendorf) and analyzed for their physicochemical parameters. For subsequent cell experiments, the medium was stored at 37°C and 5% CO_2 for at least 24 h to ensure that the cell effects observed were neither caused by the changed oxygen concentrations nor by the persistence of hydrogen peroxide; these were shown to be balanced over 24 h after plasma treatment [26]. For investigations regarding the persistent effect of plasma-treated medium on cells, the medium was stored at 37°C and 5% CO_2 for 7 or 21 days. For all experiments, with the exception of the size-exclusion chromatography, the supplements omitted during plasma treatment were added afterward to ensure analogous conditions.

2.3. Physicochemical analysis of argon plasma-treated cell culture media

2.3.1. Osmolality

To acquire the loss of solvent during argon plasma treatment, the osmotic strength was utilized by the measurement of the freezing point depression. The respective medium (50 μl) was applied to an Osmomat 030 (Gonotec) and analyzed in triplicate. The determination of osmolality was performed in three independent experiments for DMEM+FCS+Genta, which had been argon plasma-treated for 60 or 120 s, and for the same medium after argon flow for 60, 120, or 180 s versus the untreated control.

2.3.2. Size-exclusion chromatography

Size-exclusion chromatography was performed by gel filtration on a fast performance liquid chromatography (FPLC) system equipped with a UV detector (ÄKTA purifier, GE Healthcare). After argon plasma treatment, samples were pooled and the protein concentration was measured. The samples were diluted with distilled water to a concentration of 4 mg/ml protein, sterile-filtrated (0.2 μm), applied to a size-exclusion column (Superdex 200, 10/300 GL, GE Healthcare) and separated with a constant flow of 0.5 ml/min for 60 min, using a phosphate buffer (34.3 mM Na_2HPO_4, 14.5 mM NaH_2PO_4, 200 mM NaCl, pH 7.14). The samples were monitored by the UV detector (280 nm) and collected in fractions of 500 μl. Chromatograms were conducted for DMEM, DMEM+FCS, and DMEM+Genta, which were argon plasma-treated for 120 s, for the untreated control and for the same medium after argon flow for 120 s.

2.4. Epithelial cell experiments

2.4.1. Cell culture of mHepR1

The epithelial cell line mHepR1 (murine hepatocytes) [30] was used throughout the experiments. These immortalized mHepR1 cells represent a clone of the HepSV40 line derived from transgenic mice [31] and exhibit characteristic markers for epithelial cells like the tight-junction-associated protein ZO-1. The epithelial cells were cultured in DMEM supplemented with 10% FCS and 1% gentamicin at 37°C and 5% CO_2. Near confluence, cells were detached with 0.25% trypsin/0.38% EDTA (Invitrogen) for 10 min. After stopping trypsinization by the addition of cell culture medium, an aliquot of 100 μL was put into 10 mL of CASY® ton buffer solution (Roche Innovatis) and the cell number was measured in the counter CASY® Model DT (Schärfe System).

2.4.2. Immunofluorescence staining of ZO-1

Into a 24-well plate (Greiner), which was provided with a round coverslip (diameter of 12 mm, MENZEL) and 1 ml DMEM+FCS+Genta, 5×10^4 cells/well were seeded. The mHepR1 cells were incubated for 2 days at 37°C and 5% CO_2 to achieve a confluent cell layer for ZO-1 observation. Then the culture medium was replaced by plasma-treated or control DMEM+FCS+Genta, prepared as described in paragraph 2.2. and stored for 21 days at 37°C and 5% CO_2. The plate was incubated for another 24 h at 37°C and 5% CO_2 followed by immunofluorescence staining of the cells for ZO-1 protein. The cells were permeabilized with ice-cold acetone (−20°C, 200 ml, Lab-Scan) for 5 min and, after washing three times with phosphate buffer solution (PBS; PAA Laboratories), the cells were incubated with rabbit anti-ZO-1 antibody (diluted 1:100, Invitrogen) for 30 min at room temperature. After washing again with PBS, the cells were incubated with fluorescein-conjugated Alexa Fluor 488 secondary antibody goat anti-rabbit (1:100, Invitrogen) for 30 min at room temperature in the dark to avoid fading of the fluorescent dye. Finally, the cells were embedded in mounting medium (FluroShield, Sigma Aldrich) with a coverslip. The ZO-1 protein was analyzed by the inverted confocal laser scanning microscope LSM 780 (Carl Zeiss, Oberkochen, Germany), equipped with a 63× oil-immersion differential interference contrast (DIC) objective.

2.4.3. Cell viability

To monitor the viability of the cells in plasma-treated medium, the CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used. For each medium sample and the control (prepared as described in paragraph 2.2.), three wells containing 100 μl of medium were prepared in a 96-well plate and 2×10^4 mHepR1 cells were added to each well. After an incubation time of 24 h (37°C and 5% CO_2), 20 μl of the MTS solution (Promega) was added to each well and incubated for 2 h (37°C and 5% CO_2) with manual shaking for every 30 min. Afterward, 60 μl of each well was transferred into a fresh 96-well plate and the absorption was determined at 492 nm (reference 620 nm) using a microplate reader (Anthos). The appropriate medium without cells served as a blank. The viability of cells was calculated corresponding to cells in the untreated control medium (100%). The determination of cell viability was

performed in three independent experiments for DMEM+FCS+Genta, which had been argon plasma-treated for 60 or 120 s, for the untreated control and for the same medium after argon flow for 60, 120, or 180 s. Three additional experiments were performed for DMEM and DMEM +FCS, which were argon plasma-treated for 120 s, for the untreated control and for the same media after argon flow for 120 s. The supplements omitted during plasma treatment were added before cell culture to ensure analogous conditions.

2.4.4. Scanning Electron Microscopy (SEM)

Into a 24-well plate (Greiner), which was provided with a round coverslip and 1 ml DMEM +FCS+Genta, 5×10^4 cells/well were seeded. The mHepR1 cells were incubated for 2 days at 37°C and 5% CO_2 to achieve a confluent cell layer. Then the culture medium was replaced by plasma-treated DMEM+FCS+Genta (60 s) or control medium, prepared as described in paragraph 2.2. and stored for 7 days at 37°C and 5% CO_2. After 24 h of incubation, the mHepR1 monolayer was washed three times with PBS and subsequently fixed in glutardialdehyde (GDA, Sigma Aldrich, 0.5% in PBS) and stored for at least 24 h at 4°C. Afterward, the mHepR1 monolayer was critical point-dried. For this purpose, the GDA PBS solution was first gradually removed by drainage using acetone with increasing concentrations of 30, 50, 70, 90, and 100% for 10 s, 5, 10, and 15 min and twice for 10 min, respectively. Subsequently, the acetone was substituted by critical point drying (K 850 EMITECH). Then samples were sputter-coated with gold particles for 100°s (layer thickness approximately 20 nm), achieving a conductive dissipation for SEM. The cell surface structure was analyzed with a DSM 960 A (Carl Zeiss), operated at 10 kV at a magnification of 5,000× and a tilt angle of 60°.

3. Results and discussion

The atmospheric-pressure plasma jet kINPen09 [12] was applied throughout the experiments. The same plasma jet was used in our recent work [25], where we found that plasma effects on cells correspond to a plasma–medium interaction, and thus plasma-treated DMEM+FCS +Genta alone is able to open cell–cell contacts in a confluent cell monolayer of mHepR1 cells. Immunostaining of the zonula occludens tight junction protein (ZO-1) showed large openings between cells, which led to the complete degradation of the tight junction proteins. Normally, untreated cells represent clear, continuous ZO-1 bands between adjoining cells. In contrast, large intercellular openings were observed using plasma-treated DMEM+FCS+Genta. This effect was verified also for the medium that was stored up to 7 days after plasma treatment showing a persistent effect of plasma-treated medium on cells [26].

As we discovered here, this long-lasting effect is persistent for up to 21 days after argon plasma treatment. The tight junction protein ZO-1 of murine hepatocytes mHepR1 is shown in Figure 2. After incubation of cells with DMEM+FCS+Genta, which was plasma-treated for 120 s and stored for 21 days, the expression of ZO-1 in the cell contact zone was either reduced in different areas or disappeared due to the retraction in a centripetal direction. In consequence, the cell

http://dx.doi.org/10.5772/61980

size increased due to the loss of cell–cell contacts in the monolayer. In normal controls, tight cell–cell contacts could be found between neighboring cells.

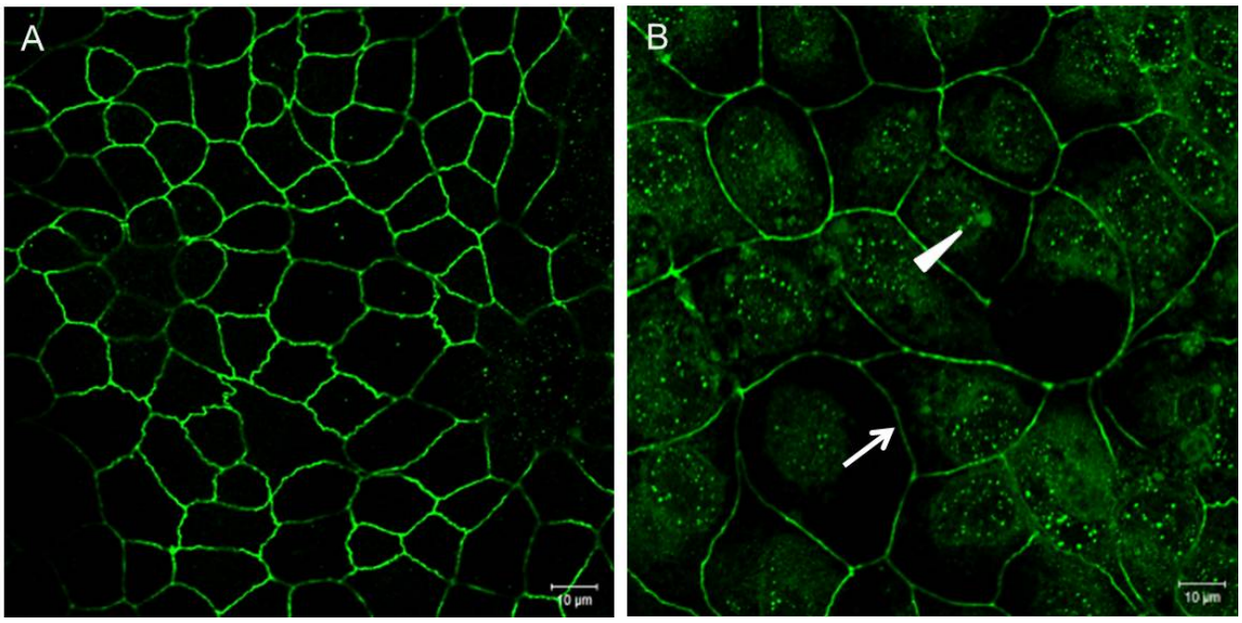


Figure 2. ZO-1 of mHepR1 cells after 21 days. (A) In control cells, the tight cell–cell contacts between neighboring cells are clearly visible. (B) After incubation of cells with plasma-treated DMEM+FCS (120 s) – which was stored for 21 days – either the expression of ZO-1 in the cell contact zone was reduced (arrow) or ZO-1 disappeared due to the retraction in a centripetal direction (arrowhead). Confocal microscopy (LSM 780, Carl Zeiss, bars 10 µm).

Accompanied with the long-lasting effect of plasma-treated medium on the distribution of ZO-1 in mHepR1 cells, we found changes in the cell surface morphology by SEM. The mHepR1 cells exhibited shortened microvilli with a lower density resulting from the plasma-treated cell culture medium [26]. These findings on microvilli shortening were revealed after a 1-day as well as a 7-day storage time of plasma-treated medium (60 s; complete DMEM). SEM images of mHepR1 cells (Figure 3), incubated for 24 h with the plasma-treated (60 s), 7-day stored DMEM+FCS+Genta illustrate this effect. Untreated and even argon-treated mHepR1 cells present elongated microvilli covering the cell surface at a high density. In contrast, the plasma-treated mHepR1 cells showed greatly shortened microvilli and the density over the entire cell surface seems to be reduced.

The formation of cell surface structures, e.g., microvilli, is essential for characteristic functions of specialized cells in tissues. Microvilli increase the cell surface and play an important role in metabolic processes: they regulate cellular functions by external signals as well as Ca^{2+} signaling [32]. Effects similar to those we observed in our work were detected by Pfister and Burstein after the treatment with ophthalmic drugs [33]. They observed the loss of surface epithelial microvilli as well as rupture of the tight junctions on corneal epithelial cells.

As a consequence, the question arises of which parameter that can persist as long as 21 days is changed in cell culture medium due to plasma treatment. As recently shown, plasma effects due to thermal damage of differential proteins in cell culture medium could be excluded for

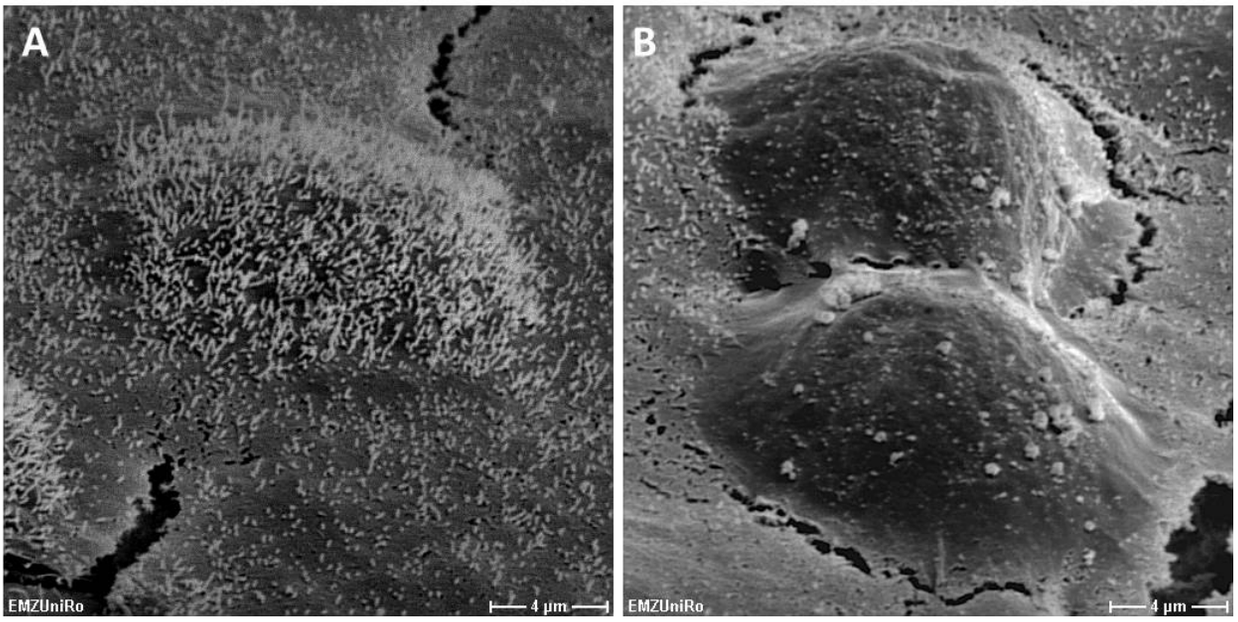


Figure 3. Microvilli of mHepR1 epithelial cells (A) in DMEM+FCS+Genta as control and (B) in the same medium, which was argon plasma-treated for 60 s and stored for 7 days (culture time 24 h). Scanning electron microscopy (SEM, DSM 910, magnification 5,000×, tilt angle 60°, and bar 4 μm).

treatment times below 180 s for the atmospheric-pressure plasma jet kINPen09 [26]. The temperature in the cell culture medium did not exceed 25°C. Plasma–liquid interactions were studied by Oehmigen *et al.* [34] and von Woedtke *et al.* [35] relating to the effective disinfection of liquids. It was shown that the inactivation of bacteria is strongly dependent on the acidification of aqueous liquids (pH decrease). In our work, we could show that the pH of the complete cell culture medium DMEM remained constant. The buffering capacity of sodium hydrogen carbonate in the medium was sufficient to maintain the pH even after a plasma treatment. Furthermore, the redox amphoteric hydrogen peroxide was found in liquids after plasma treatment in various studies [10,34]. As hydrogen peroxide also occurs in the cell metabolism, it can be degraded by various repair and protection mechanisms in the cell. Antioxidants inside the cell are able to detoxify "natural" amounts of ROS. In the presence of an overabundance of ROS, these mechanisms fail. Thus, the concentration of hydrogen peroxide and oxygen in the medium after plasma treatment was investigated earlier. Our investigations revealed that hydrogen peroxide and oxygen concentrations were balanced over the time period of up to 1 day, but the cell behavior was affected by argon plasma-treated medium stored for 1 or 7 days [26] or as described here for 21 days.

These detrimental effects on mHepR1 cells due to argon plasma-treated cell culture medium lead us to investigate other physicochemical parameters, which could change during the plasma treatment of a medium. One parameter to which cells react sensitively is the osmolality [36]. Due to evaporation processes induced by the argon gas flow, the medium volume in the well could be reduced during plasma treatment. Accordingly, the osmotic strength in the medium would increase. To examine this, we utilized the osmolality by measuring the freezing point depression for argon plasma-treated DMEM+FCS+Genta. The results in Figure 4 show

http://dx.doi.org/10.5772/61980

an increasing osmolality for the medium after argon plasma treatment for 60 or 120 s. Interestingly, argon gas flow alone did not influence osmolality as high as argon plasma did. Thus, osmolality in the medium after 60 s of plasma treatment is higher (444.11 mOsmol/kg) than in the argon gas-treated control (384.67 mOsmol/kg). To reach the same osmolality level as after 60 s of plasma treatment, the argon gas flow needs to impact on the medium for 180 s.

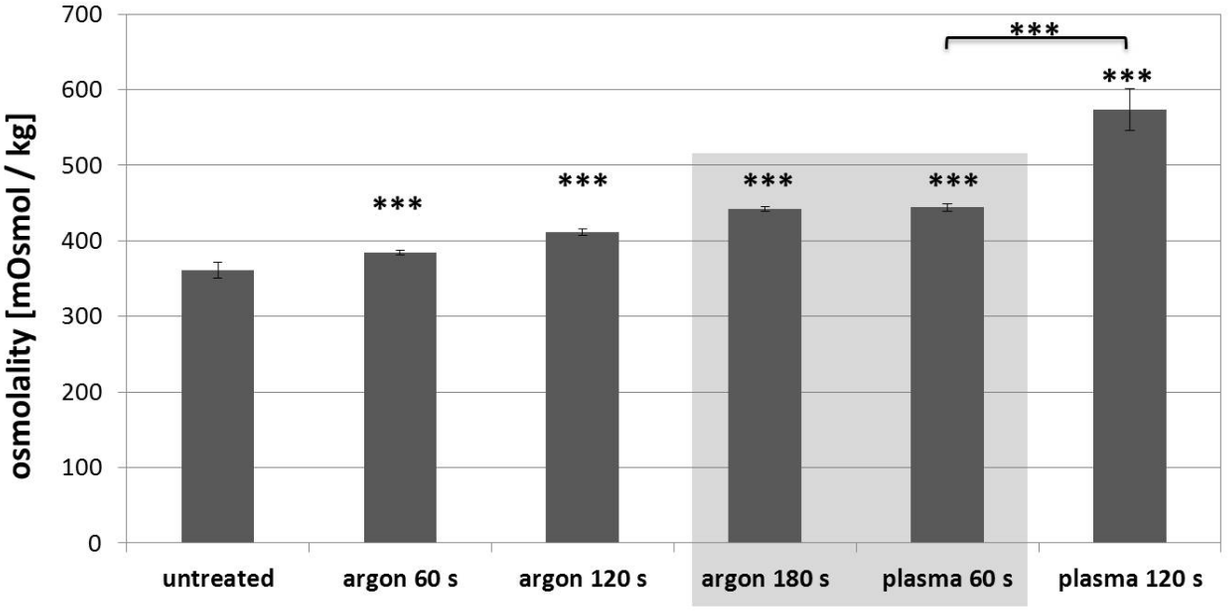


Figure 4. The osmolality of treated DMEM+FCS+Genta increased significantly with longer exposition times. Note that argon plasma treatment (plasma) over 60 s and argon gas treatment (argon) over 180 s result in similar values. (mean ± SD, *** $p < 0.001$, *t*-test, $n = 3$).

To determine the effect of increasing osmolality on the cell behavior, we studied the viability of mHepR1 cells cultured for 24 h in DMEM+FCS+Genta, which was treated similarly to the samples used for osmolality measurements. As can be seen in Figure 5, the viability of the cells was massively reduced due to the 60 s plasma-treated medium. Interestingly, cells in 180 s argon gas-treated medium kept a viability of around 80%, although the osmolality values of 60 s argon plasma versus 180 s argon gas were in the same range.

Thus, increasing osmolality during argon plasma treatment is not the reason for the detrimental cell effects we observed, neither the changing of the pH nor the oxygen concentration nor the persistence of hydrogen peroxide after plasma treatments, which were precluded earlier.

Besides physical parameters, which could be changed due to plasma treatment, alterations in the chemical composition of the cell culture medium were investigated. In a recent study, Kalghatgi *et al.* [24] demonstrated an induction of DNA damage in mammalian breast epithelial cells by plasma-treated cell culture medium. This effect was not reduced if the medium had been stored up to 1 h prior to addition to the cells. The authors hypothesized that this remaining biological effect of plasma-treated cell culture medium is caused by stable organic peroxides

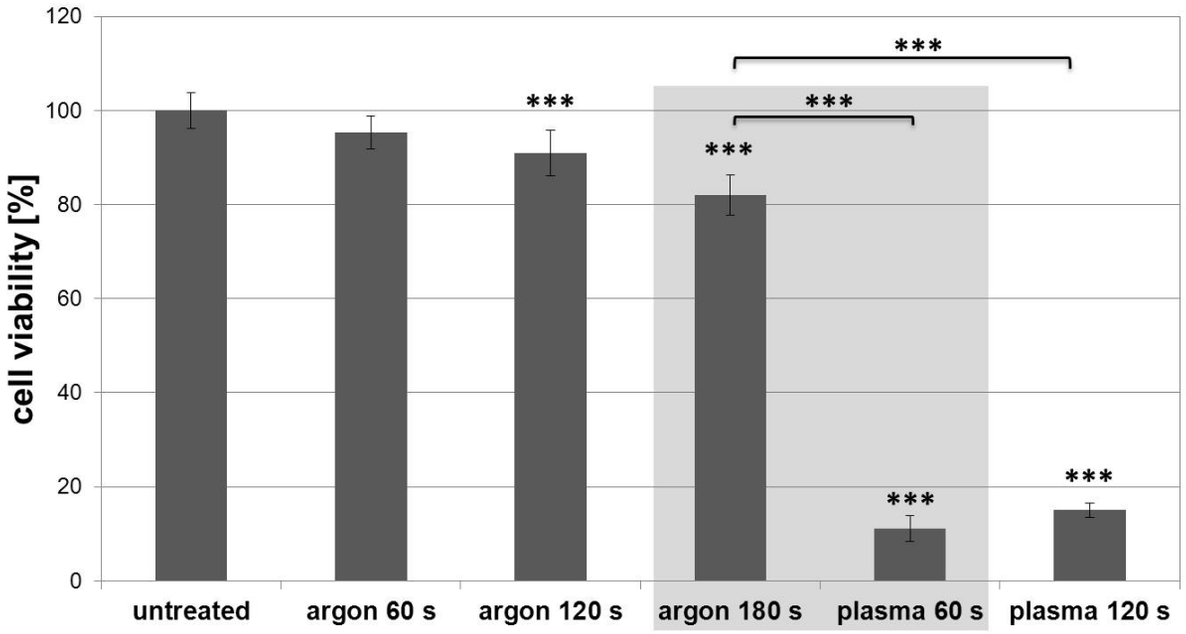


Figure 5. Difference in the viability of cells cultured in argon plasma- and argon gas-treated DMEM+FCS+Genta. Note that argon gas treatment over 180 s did not decrease cell viability as much as the argon plasma treatment did. (mean ± SD, *** $p < 0.001$, *t*-test, $n = 3$).

made up of medium compounds like amino acids [24]. The formation of stable peroxides from amino acids and proteins by reactive oxygen species like an OH radical is a well-known process [37]. In recent years, an active discussion has begun about the very complex ROS chemistry in plasma-treated liquids and its biological effects, including reactive species like OH radical, hyperoxide anion, and also the relatively stable hydrogen peroxide [1,38,39].

As we showed recently by size-exclusion chromatography on an FPLC analysis system, medium components are modified by argon plasma treatment [26]. We detected an additional peak upon plasma treatment of DMEM+FCS+Genta compared with the argon gas-treated control. In these experiments, we separated the components after plasma treatment by gel filtration. Interestingly, the peak height increased depending on the treatment time of the medium. In the present experiments, we examined the contribution of different medium components, FCS and gentamicin, to this additional peak and to the effects on cells. For this purpose, we investigated plasma treatment for the medium with (DMEM+FCS) and without supplements (DMEM). The different medium samples were analyzed after argon gas flow for 120 s and after argon plasma for 120 s by gel filtration with an FPLC system and compared with untreated controls (Figure 6).

All chromatograms of the analyzed media show peaks at a retention volume of about 22 ml (21.67–22.10 ml) and 26 ml (25.41–25.77 ml). The characteristic peak for albumin at about 15 ml (14.38–14.70 ml) was found in DMEM supplemented with FCS. An additional peak was found for the samples, which were treated for 120 s with argon plasma (see arrow) in basic DMEM, in DMEM+FCS (see Figure 6) and in DMEM+Genta (data not shown). No signal around the retention volume of 20 ml could be detected in untreated media and media that

http://dx.doi.org/10.5772/61980

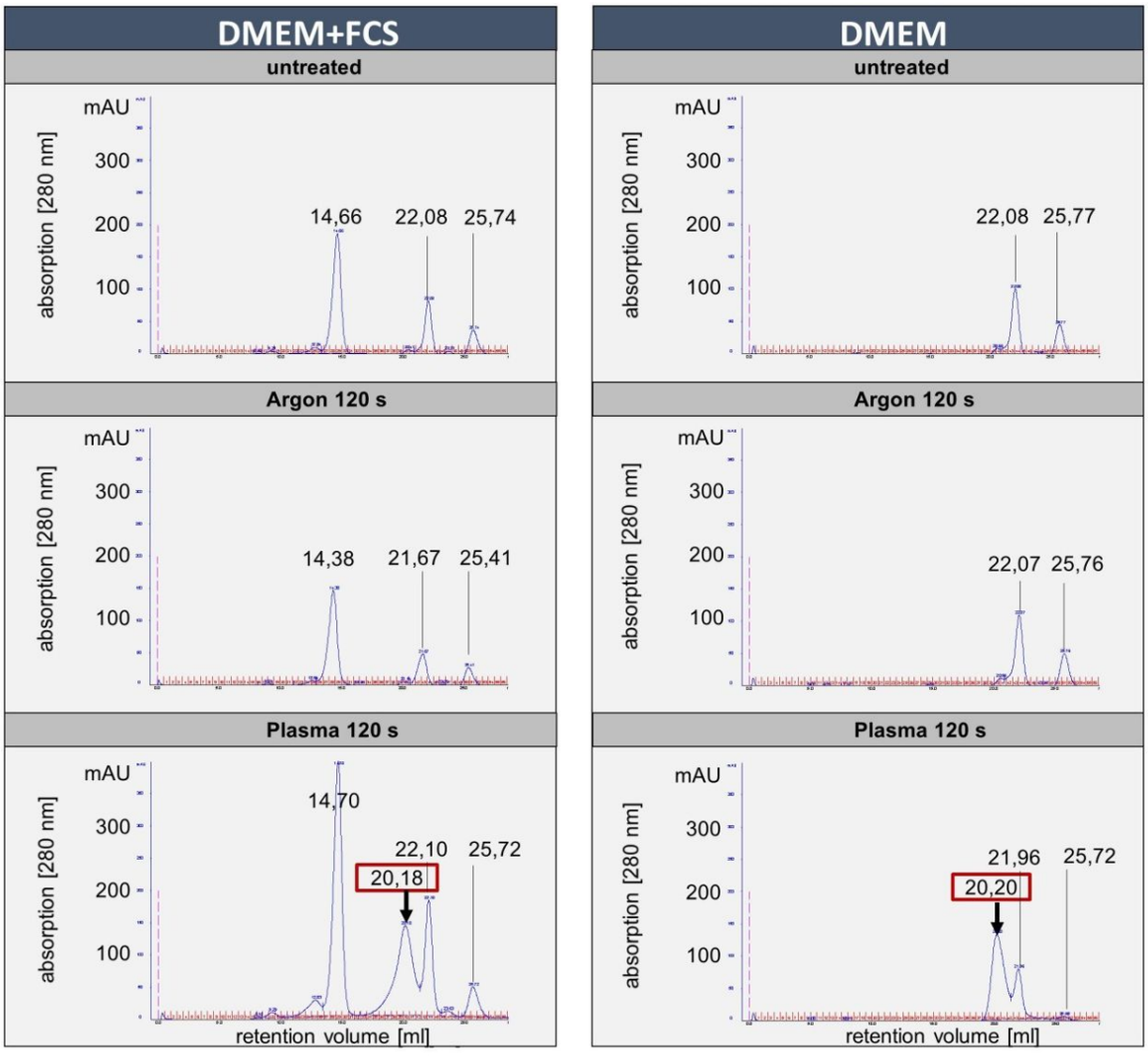


Figure 6. An additional peak at a retention volume of about 20 ml could be detected in the plasma-treated samples using size-exclusion chromatography. Note that cell culture medium DMEM without FCS also showed this signal, indicating that the source of this signal was not the interaction of argon plasma with FCS.

were only exposed to argon gas flow. This result gives rise to the idea that the visible changes in the chromatogram were caused by a change in the basic media (DMEM) independent of the supplements (FCS and gentamicin). Argon plasma seems to initiate the formation of components with higher molecular weight in the basic DMEM that appear at a lower retention time in the chromatogram.

Investigators hypothesized that ROS induces the formation of organic peroxides, which could be responsible for the damaging effects on cells in culture [21,22,37]. This conclusion is mainly based on the comparison of the incubation of cells with plasma-treated PBS, FCS, bovine serum albumin (BSA) or single amino acids [22]. DNA damage could not be shown after incubation of cells with plasma-treated PBS. However, incubation with plasma-treated BSA (in PBS) or FCS (in PBS) resulted in DNA damage of subsequently added cells. Even plasma treatment of PBS containing single amino acids resulted in adverse cell effects. Further analysis revealed a

correlation between the peroxidation efficiency of single amino acids and their potential to induce DNA damage in cells [22]. These findings supported the thesis of amino acid peroxide formation and its potential contribution to DNA damage generation. The damage could be mediated either by single amino acids or by FCS, both being usual components of cell culture media.

As shown in Figure 5 and also observed in an earlier work of our group, DMEM+FCS+Genta impaired cell vitality after argon plasma treatment [25]. Based on the results deduced from size-exclusion chromatography (see Figure 6), it was deemed important to analyze the viability of mHepR1 cells dependent on the medium supplements during plasma treatment. For this purpose, DMEM and DMEM+FCS were argon plasma-treated for 60 and 120 s. Controls without treatment or only argon gas flow were also investigated. It was found that the mHepR1 cell viability decreased significantly in plasma-treated media after 24-h incubation for both approaches (Figure 7). In particular, in the presence of FCS during plasma treatment, the inhibitory effect is more obvious.

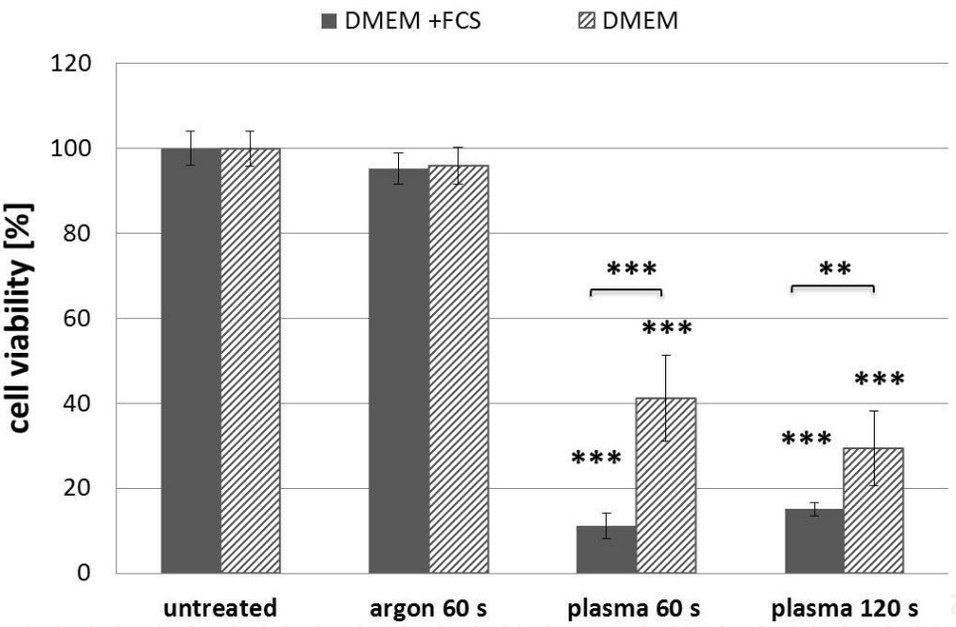


Figure 7. Cell viability after incubation of mHepR1 cells in plasma-treated DMEM and DMEM+FCS. (mean ± SD, ** $p < 0.005$, *** $p < 0.001$, *t*-test, $n = 3$).

Therefore, it can be assumed that components in the basic DMEM without supplements were changed due to the impact of argon plasma and influenced cell viability.

Based on the hypothesis of Kalghatgi *et al.,* for the generation of stable organic peroxides from amino acids during plasma treatment [22] and our cell viability results in mHepR1 cells in plasma-treated DMEM versus DMEM+FCS, it can be concluded that peroxides formed from single amino acids by plasma treatment could be the reason for the detrimental cell effects we found. However, peroxidated amino acids are semi-reactive compounds and it seems possible that they generate compounds with higher molecular weight during storage, e.g., by poly-

merization. The identification of the newly generated substances in the cell culture medium awaits further analysis.

There is increasing evidence that plasma treatment promotes the healing process of tissue and accelerates wound healing [13,40–42]. The skin is a complex architectural multilayer cell system and processes concerning wound healing can be examined on cell monolayers as an *in vitro* model system. However, it is important to keep in sight that a cell monolayer *in vitro* is much more sensitive to agents because there are no cells in the "second row" to protect or replace cells in the apical row [43]. Here, we described effects of argon plasma-treated liquids (e.g., cell culture medium) as an indirect approach for plasma application in medicine. Transferred to *in vivo* systems, the opening of cell–cell contacts we observed by plasma-treated liquids could have a positive effect on the penetration of conventional therapeutics (antibiotics and disinfectants) on skin. Often, the application of conventional liquid antiseptics is not sufficient and sustainable as the borders and the surrounding of chronic wounds frequently consist of sclerotic skin, impeding an effectual penetration of these products [44]. With regard to disinfection, direct plasma treatment of living intact and wounded skin was found to be safe for doses even higher than required for inactivation of bacteria [45,46]. Impaired cell adhesion and reduced cell viability due to plasma-treated liquids are important starting points for further investigations concerning cancer therapy. The work presented here, focused on the basic mechanisms in the interaction of plasma-treated cell culture medium with cells and on the components in the treated medium, which are responsible for the persistent biological effect.

The vision could be the establishment of local plasma centers to prepare relatively stable plasma liquids for dermatological (chronic wounds and tumors), dental (peri-implantitis), or orthopedic (joint rinsing) applications to support or replace the conventional therapy.

4. Summary and conclusions

This study focused on the physicochemical analysis of argon plasma-treated cell culture medium DMEM with the additives FCS and gentamicin. In addition, the efficacy of plasma-treated complete cell culture medium DMEM upon storage and its impact on the cell physiology of epithelial mHepR1 cells were ascertained. We discovered that the liquid-mediated effect of atmospheric-pressure argon plasma on mouse liver epithelial cell–cell contacts and cell membrane microvilli persists up to even 21 days or 7 days of storage, respectively. Earlier investigations of our group revealed that temperature and pH (both were constant) as well as hydrogen peroxide production and oxygen content (both decreased within 1 day) can be excluded as initiators of the detrimental biological changes. As we found here, increased osmolality in the media caused by plasma treatment can also be excluded as a reason for the observed cell effects. On the other hand, we found an additional peak in size-exclusion chromatography analysis in basic DMEM after plasma treatment and significantly decreased cell viability in plasma-treated media independent of the presence of FCS during plasma treatment. High molecular compounds generated during plasma treatment of DMEM without

FCS give an impulse for further investigations on the formation, stability, and reaction of amino acid peroxides in this medium. The persistent biological effect on plasma-treated liquids observed here could open up new medical applications. Stable plasma-treated liquids could find applications for dermatological, dental, or orthopedic therapy.

Acknowledgements

The authors acknowledge the financial support of the University Medical Center Rostock, Germany (FORUN 889061, PlasmaBiomedicine) for C.H., M.K., and C.B.. M.H. is grateful for the doctoral stipendium of the state Mecklenburg-Vorpommern (Germany) and of the University of Rostock, Interdisciplinary Faculty, Department of Life, Light and Matter. The authors appreciate the BMBF Germany Pilot Program Campus PlasmaMed (13N11183). They would also like to express their thanks to the Electron Microscopic Center (EMZ) of the University Medical Center Rostock (M. Frank, G. Fulda) for qualified technical assistance.

Author details

Claudia Bergemann[1], Cornelia Hoppe[1,2], Maryna Karmazyna[1,3], Maxi Höntsch[1,4], Martin Eggert[5], Torsten Gerling[6] and Barbara Nebe[1*]

*Address all correspondence to: barbara.nebe@med.uni-rostock.de

1 University Medical Center Rostock, Department of Cell Biology, Schillingallee, Rostock, Germany

2 Technical University Dresden, DFG-Center for Regenerative Therapies, Dresden, Germany

3 Seracell Stem Cell Technology GmbH, Schillingallee, Rostock, Germany

4 Sarstedt AG and Co, Quality Management/EWZ, Nümbrecht, Germany

5 University Medical Center Rostock, Department of Internal Medicine, Center for Extracorporeal Organ Support, Schillingallee, Rostock, Germany

6 Leibniz-Institute for Plasma Science and Technology e.V., Greifswald, Germany

References

[1] von Woedtke T, Reuter S, Masur K, Weltmann KD. Plasmas for medicine. Phys Rep. 2013;530:291–320.

[2] von Woedtke T, Metelmann HR, Weltmann KD. Clinical plasma medicine: State and perspectives of in vivo application of cold atmospheric plasma. Contrib Plasma Phys. 2014;54:104–17.

[3] Fridman G, Friedman G, Gutsol A, Shekhter AB, Vasilets VN, Fridman A. Applied plasma medicine. Plasma Process Polym. 2008;5:503–33.

[4] Fridman G, Peddinghaus M, Ayan H, Fridman A, Balasubramanian M, Gutsol A, et al. Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air. Plasma Chem Plasma P. 2006;26:425–42.

[5] Kalghatgi SU, Fridman G, Cooper M, Nagaraj G, Peddinghaus M, Balasubramanian M, et al. Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma. IEEE T Plasma Sci. 2007;35:1559–66.

[6] Daeschlein G, von Woedtke T, Kindel E, Brandenburg R, Weltmann KD, Junger M. Antibacterial activity of an atmospheric pressure plasma jet against relevant wound pathogens in vitro on a simulated wound environment. Plasma Process Polym. 2010;7:224–30.

[7] Daeschlein G, Scholz S, Ahmed R, von Woedtke T, Haase H, Niggemeier M, et al. Skin decontamination by low-temperature atmospheric pressure plasma jet and dielectric barrier discharge plasma. J Hosp Infect. 2012;81:177–83.

[8] Daeschlein G, Scholz S, Ahmed R, Majumdar A, von Woedtke T, Haase H, et al. Cold plasma is well-tolerated and does not disturb skin barrier or reduce skin moisture. J Dtsch Dermatol Ges. 2012;10:509–15.

[9] Daeschlein G, Scholz S, Arnold A, von Podewils S, Haase H, Emmert S, et al. In vitro susceptibility of important skin and wound pathogens against low temperature atmospheric pressure plasma jet (APPJ) and dielectric barrier discharge plasma (DBD). Plasma Process Polym. 2012;9:380–89.

[10] Nosenko T, Shimizu T, Morfill GE. Designing plasmas for chronic wound disinfection. New J Phys. 2009;11:115013.

[11] Nastuta AV, Topala I, Grigoras C, Pohoata V, Popa G. Stimulation of wound healing by helium atmospheric pressure plasma treatment. J Phys D Appl Phys. 2011;44:105204-0.

[12] Weltmann KD, Kindel E, Brandenburg R, Meyer C, Bussiahn R, Wilke C, et al. Atmospheric pressure plasma jet for medical therapy: Plasma parameters and risk estimation. Contrib Plasma Phys. 2009;49:631–40.

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

[14] Kalghatgi S, Friedman G, Fridman A, Clyne AM. Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release. Ann Biomed Eng. 2010;38:748–57.

[15] Vandamme M, Robert E, Lerondel S, Sarron V, Ries D, Dozias S, et al. ROS implication in a new antitumor strategy based on non-thermal plasma. Int J Cancer. 2012;130:2185–94.

[16] Plewa JM, Yousfi M, Frongia C, Eichwald O, Ducommun B, Merbahi N, et al. Low-temperature plasma-induced antiproliferative effects on multi-cellular tumor spheroids. New J Phys. 2014;16:043027.

[17] Kalghatgi S, Kelly C, Cerchar E, Azizkhan-Clifford J. Selectivity of non-thermal atmospheric-pressure microsecond-pulsed dielectric barrier discharge plasma induced apoptosis in tumor cells over healthy cells. Plasma Med. 2011;1:249–63.

[18] Kaushik NK, Kaushik N, Attri P, Kumar N, Kim CH, Verma AK, et al. Biomedical importance of indoles. Molecules. 2013;18:6620–62.

[19] Stoffels E, Sakiyama Y, Graves DB. Cold atmospheric plasma: Charged species and their interactions with cells and tissues. IEEE T Plasma Sci. 2008;36:1441–57.

[20] Kieft IE, Broers JLV, Caubet-Hilloutou V, Slaaf DW, Ramaekers FCS, Stoffels E. Electric discharge plasmas influence attachment of cultured CHO k1 cells. Bioelectromagnetics. 2004;25:362–68.

[21] Haertel B, Wende K, von Woedtke T, Weltmann KD, Lindequist U. Non-thermal atmospheric-pressure plasma can influence cell adhesion molecules on HaCaT-keratinocytes. Exp Dermatol. 2011;20:282–84.

[22] Kalghatgi S, Kelly CM, Cerchar E, Torabi B, Alekseev O, Fridman A, et al. Effects of non-thermal plasma on mammalian cells. Plos One. 2011;6:16270.

[23] Haertel B, Hahnel M, Blackert S, Wende K, von Woedtke T, Lindequist U. Surface molecules on HaCaT keratinocytes after interaction with non-thermal atmospheric pressure plasma. Cell Biol Int. 2012;36:1217–22.

[24] Kalghatgi S, Azizkhan-Clifford J. DNA damage in mammalian cells by atmospheric pressure microsecond-pulsed dielectric barrier discharge plasma is not mediated via lipid peroxidation. Plasma Med. 2011;1:167–77.

[25] Hoentsch M, von Woedtke T, Weltmann KD, Nebe JB. Time-dependent effects of low-temperature atmospheric-pressure argon plasma on epithelial cell attachment, viability and tight junction formation in vitro. J Phys D Appl Phys. 2012;45:025206.

[26] Hoentsch M, Bussiahn R, Rebl H, Bergemann C, Eggert M, Frank M, et al. Persistent effectivity of gas plasma-treated, long time-stored liquid on epithelial cell adhesion capacity and membrane morphology. Plos One. 2014;9:e104559.

[27] Kalghatgi S, Fridman A, Azizkhan-Clifford J, Friedman G. DNA damage in mammalian cells by non-thermal atmospheric pressure microsecond pulsed dielectric barrier discharge plasma is not mediated by ozone. Plasma Process Polym. 2012;9:726–32.

[28] Stoffels E, Kieft IE, Sladek REJ, van den Bedem LJM, van der Laan EP, Steinbuch M. Plasma needle for in vivo medical treatment: Recent developments and perspectives. Plasma Sources Sci Technol. 2006;15:S169–S80.

[29] Dobrynin D, Fridman G, Friedman G, Fridman A. Physical and biological mechanisms of direct plasma interaction with living tissue. New J Phys. 2009;11:115020.

[30] Nebe B, Rychly J, Knopp A, Bohn W. Mechanical induction of beta-1-integrin-mediated calcium signaling in a hepatocyte cell-line. Exp Cell Res. 1995;218:479–84.

[31] Paul D, Hohne M, Pinkert C, Piasecki A, Ummelmann E, Brinster RL. Immortalized differentiated hepatocyte lines derived from transgenic mice harboring Sv40 T-antigen genes. Exp Cell Res. 1988;175:354–62.

[32] Lange K. Role of microvillar cell surfaces in the regulation of glucose uptake and organization of energy metabolism. Am J Physiol-Cell Physiol. 2002;282:C1–C26.

[33] Pfister RR, Burstein N. Effects of ophthalmic drugs, vehicles, and preservatives on corneal epithelium – scanning electron-microscope study. Invest Ophth Visual. 1976;15:246–59.

[34] Oehmigen K, Winter J, Hahnel M, Wilke C, Brandenburg R, Weltmann KD, et al. Estimation of possible mechanisms of *Escherichia coli* inactivation by plasma treated sodium chloride solution. Plasma Process Polym. 2011;8:904–13.

[35] von Woedtke T, Oehmigen K, Brandenburg R, Hoder T, Wilke C, Hähnel M, et al. Plasma-liquid interactions: Chemistry and antimicrobial effects. In: Machala Z, Hensel K, Akishev Y, editors. Plasma for bio-decontamination, medicine and food security: Springer, Netherlands; 2012. pp. 67–78.

[36] Waymouth C. Osmolality of mammalian blood and of media for culture of mammalian cells. In Vitro Cell Dev Biol Plant. 1970;6:109–27.

[37] Gebicki S, Gebicki JM. Formation of peroxides in amino-acids and proteins exposed to oxygen free-radicals. Biochem J. 1993;289:743–49.

[38] van Gils CAJ, Hofmann S, Boekema BKHL, Brandenburg R, Bruggeman PJ. Mechanisms of bacterial inactivation in the liquid phase induced by a remote RF cold atmospheric pressure plasma jet. J Phys D Appl Phys. 2013;46:175203.

[39] Machala Z, Tarabova B, Hensel K, Spetlikova E, Sikurova L, Lukes P. Formation of ROS and RNS in water electro-sprayed through transient spark discharge in air and their bactericidal effects. Plasma Process Polym. 2013;10:649–59.

[40] Barekzi N, Laroussi M. Dose-dependent killing of leukemia cells by low-temperature plasma. J Phys D Appl Phys. 2012;45:422002.

[41] Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, et al. Bactericidal effects of non-thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011;60:75–83.

[42] Emmert S, Brehmer F, Hänßle H, Helmke A, Mertens N, Ahmed R, et al. Atmospheric pressure plasma in dermatology: Ulcus treatment and much more. Clin Plasma Med. 2013;1:24–29.

[43] Hoene A, Prinz C, Walschus U, Lucke S, Patrzyk M, Wilhelm L, et al. In vivo evaluation of copper release and acute local tissue reactions after implantation of copper-coated titanium implants in rats. Biomed Mater. 2013;8:035009.

[44] Lademann O, Kramer A, Richter H, Patzelt A, Meinke MC, Czaika V, et al. Skin disinfection by plasma-tissue interaction: Comparison of the effectivity of tissue-tolerable plasma and a standard antiseptic. Skin Pharmacol Physiol. 2011;24:284–88.

[45] Dobrynin D, Wu A, Kalghatgi S, Park S, Shainsky N, Wasko K, et al. Live pig skin tissue and wound toxicity of cold plasma treatment. Plasma Med. 2011;1:93–108.

[46] Fluhr JW, Sassning S, Lademann O, Darvin ME, Schanzer S, Kramer A, et al. In vivo skin treatment with tissue-tolerable plasma influences skin physiology and antioxidant profile in human stratum corneum. Exp Dermatol. 2012;21:130–34.

IntechOpen