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Nitrogen Shielding of an Argon Plasma Jet and Its Effects on Human Immune Cells

Sander Bekeschus, Sylvain Iséni, Stephan Reuter, *Member, IEEE*, Kai Masur, and Klaus-Dieter Weltmann, *Member, IEEE*

Abstract—Atmospheric pressure plasmas are widely used in research for biomedical or clinical applications. Reactive oxygen species and reactive nitrogen species (RNS) produced by plasmas are thought to be of major significance in plasma-cell interactions. Different applications, however, may demand for different plasma properties. Tailoring plasma devices by modulating the supply gas or the surrounding is a suitable way to alter reactive species composition, vacuum ultra violet emission, or temperature. Treatment regimens involving availability of oxygen or humidity may lead to increased hydrogen peroxide deposition in liquids and thus will be toxic to cells. Using an atmospheric pressure argon plasma jet, we applied a nitrogen gas curtain to its visible effluent during treatment of human immune cells. The curtain deprived the plasma of molecular oxygen. This excluded gas-phase oxygen plasma chemistry and led to generation of highly energetic metastables. Planar laser-induced fluorescence spectroscopy verified laminar gas flow and complete elimination of surrounding air by the gas curtain. We used human immune cells to monitor cytotoxic effects as they are highly relevant in potential clinical plasma applications, e.g., treatment of chronic wounds. Air curtain plasma treatment led to significantly higher cytotoxicity compared with nitrogen curtain plasma treatment. Scavenging of hydrogen peroxide abrogated cell death in both gas curtain conditions. This indicated a negligible contribution of highly energetic metastables or increased gas temperature to cytotoxicity. Finally, the results suggested an oxygen-independent generation of hydrogen peroxide pointing to an indirect role of UV or RNS in plasma-mediated cytotoxicity.

Index Terms—Atmospheric pressure plasma jet, gas curtain, hydrogen peroxide, immune cells, plasma medicine.

I. INTRODUCTION

IN PLASMA medicine, cold atmospheric pressure plasmas are applied to cells and tissues [1]. Details on plasma-cell interactions, however, still remain to be elucidated. A vital parameter influencing reactive species composition and cellular responses is the feed gas and gas curtain being used [2]. The gas curtain provides a controlled gas area around the plasma effluent, enhancing the reproducibility of the plasma properties [3]. In addition, external parameters,

e.g., humidity can be monitored to tailor the reactive species produced in the effluent and its vicinity [2], [4]. However, various input parameters can be modified, which affects the plasma and its chemistry of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [5]. We recently reported on the laminar or turbulent flow pattern resulting from the feed gas flow rate [6]. Following these observation, this paper focuses on using the same plasma source in a laminar regime. Moreover, we applied a gas curtain cap to fully control the surrounding of the plasma effluent from the jet nozzle to the cell suspension. Here, a laminar argon flow propagated into an ionization front, whereas a pure and dry nitrogen shroud prevented any molecular oxygen or water molecules to interact with the plasma. Consequently, the plasma chemistry in the gas phase was reduced to RNS only. Optical emission spectroscopy revealed the presence and spatial distribution of highly energetic metastables. Liquid chemistry was not investigated in this paper.

To differentiate between air and nitrogen curtain plasma effects, we evaluated their cytotoxicity on human immune cells. Specifically, we referred to three specific populations within those cells. Two of them are lymphocytic origin and their important role in pathological wounds has been implied previously [7]. In addition, they display the highest sensitivity toward plasma compared with various other cell types [8], [9]. This also allows one to distinguish subtle differences in oxidation by plasma [10]. As a third cell type, monocytes were investigated. When differentiated to macrophages, these phagocytes are highly relevant in tissue regeneration [11]. Our previous work had identified significant amounts of plasma-generated hydrogen peroxide (H_2O_2) being deposited in treated liquids [12], [13]. H_2O_2 is thought to be derived from hydroxyl radical ($\cdot HO$) and has a dominant role in cell toxicity [10]. The nitrogen gas curtain shielded the plasma from any oxygen and we hypothesized to find toxic agents other than H_2O_2 in this regime. While cell survival differed between gas curtain conditions, cytotoxicity remained to be dependent on H_2O_2 as results using catalase suggested.

II. PLASMA JET AND GAS CURTAIN

The plasma source used was the *kinpen* (Neoplas, Germany). This jet was operated at atmospheric pressure and argon (99.999% purity, Air Liquide, France) was used as feed gas [14]. It consists of a concentric needle electrode located in a 1.6-mm diameter dielectric capillary and a ground ring

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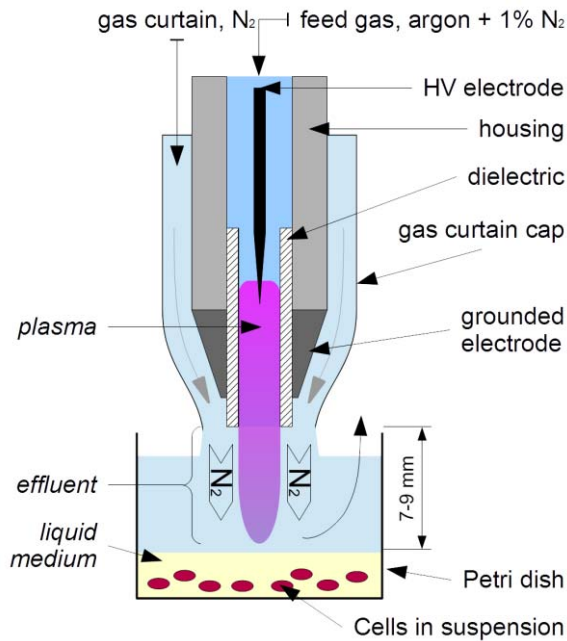


Fig. 1. Schematic of the plasma source and the gas curtain used to treat the human immune cells in suspension.

electrode on the outer diameter. The electrode was powered with an electric excitation of 1 MHz and dissipates between 0.9 and 2.2 W. The device can be operated at various feed gas flow rates ranging from 0.5 to 3 slm and yields a visible effluent length between 8 and 15 mm. At the tip of the effluent (~ 10 mm, 3 slm feed gas flow rate), the average gas temperature does not exceed 350 K.

Fig. 1 shows the geometrical and electrical configuration of the plasma source including the gas curtain cap. The feed gas flow rate was fixed at 1 slm argon to operate the plasma jet in a laminar regime. The plasma was shielded from the capillary nozzle to the liquid surface by 2 slm of nitrogen or dry synthetic air (99.999% purity, Air Liquide, France). By this way, any room air molecules were excluded. Feed gas and gas curtain were run for at least 1 h prior to experiments to eliminate residual humidity (<20 ppm) in tubing. All gases were low on molecular oxygen (<2 ppm) as stated by the supplier. Independent of the gas curtain condition, 1% of N_2 was admixed to the feed gas to increase the production of RNS.

III. MATERIALS AND METHODS

A. Planar Laser-Induced Fluorescence (PLIF)

The optimum shielding conditions were determined experimentally. By injecting an organic compound into the gas curtain, it was possible to visualize the flow pattern using planar laser-induced fluorescence (PLIF). Here, a planar laser sheath excited a fluorescent probe within the feed or curtain gas. Its fluorescent emission can be used as a flow profile visualization tool. Details on this method can be read elsewhere [6].

B. Optical Emission Spectroscopy of $N_2(C)$

To the human eye, the plasma effluent is visible due to radiation in the visible spectra. Radiation is emitted by

molecules and atoms being excited by the plasma. This is a result of either electron impacts or nonelastic collisions between particles. Spatially resolved observations of the spontaneous emission of the second positive system of nitrogen $N_2(C)$ was important to identify the area of potentially high energy within the effluent. The experimental apparatus consisted of an intensified charge-coupled device camera (LaVision GmbH, Germany) with a 100-mm focal length optics mounted on the front. An optical bandpass (bp) filter centered at 335 nm (7-nm bandwidth, Semrock, USA) was inserted to exclusively visualize $N_2(C)$ emission.

C. Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using lymphocyte separation medium (PanBiotect, Germany), as described before [15]. Subsequent counting was done using a hemocytometer. Cell viability after isolation was always greater than 97%, as determined by trypan blue exclusion assay. Cells were suspended at $1 \times 10^6 \text{ ml}^{-1}$ in RPMI1640 without phenol red and supplemented with 1% penicillin/streptomycin, 2% glutamine (all PanBiotect), and 10% fetal bovine serum (Sigma, USA). Upon blood donation at the blood bank (University Medicine of Greifswald, Germany), healthy blood donors gave written and informed consent about anonymous usage of blood for research purposes in the case of the donation is not suitable for clinical application.

D. Plasma Treatment of Cells

The plasma device was oriented vertically at a distance of 8–10 mm from the tip of the capillary to the liquid surface (1 ml of cell suspension). The effluent was close to the surface, but did not connect to it. In this setup, the electrical properties of the plasma, e.g., the current, were identical compared with the jet running in free atmosphere [16]. Cells were plasma-treated in 24 well plates (Sarstedt, Germany) for 1 or 5 min and in duplicates for each condition and experiment. Where indicated, catalase (50 $\mu\text{g/ml}$ in phosphate-buffered saline (PBS); Sigma, USA) was added to each well prior to treatment. The jet was not moved during treatment of one well and immediately and manually transferred to the next well of the plate after treatment was finished. In addition to untreated controls, cytotoxicity of gas but not plasma-treated cells was also evaluated and always resembled untreated controls (data not shown). After exposure, cells were incubated at 37 $^\circ\text{C}$ in a cell culture incubator (Binder, Germany) with humidified air and 5% CO_2 . Twenty-four hours after treatment, the cells were harvested in 12 mm \times 75 mm tubes (Sarstedt, Germany) and washed once with PBS prior to antibody-staining.

E. Flow Cytometry

Cells were investigated using a Gallios flow cytometer (Beckman Coulter, USA) equipped with three solid-state diode lasers (405 nm at 40 mW, 488 nm at 22 mW, and 638 nm at 25 mW). Determination of viability was done as described before [8], [10], [17]. Briefly, 24 h after

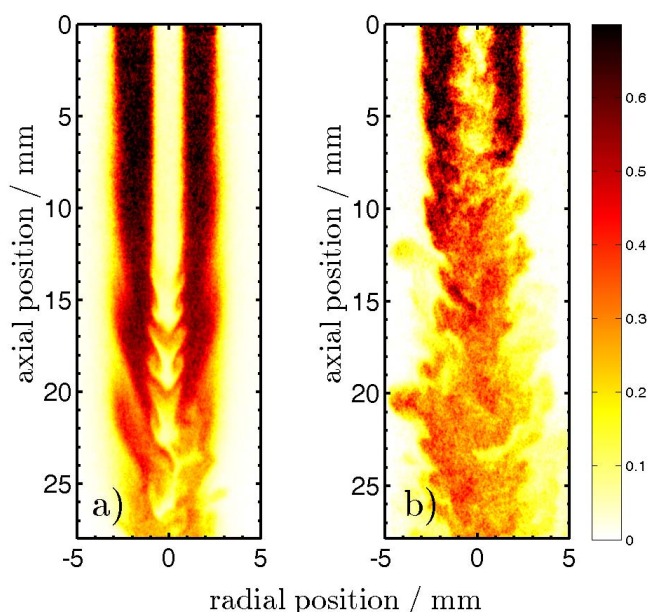


Fig. 2. PLIF image (200-ns exposure time) from an organic compound admixed within the gas curtain (here nitrogen). The plasma is off, but argon feed gas is flowing. Depicted are the flow patterns of the gas curtain at 1.7 slm with 1 slm argon feed gas flow rate (a, laminar), and gas curtain at 5 slm with 3 slm argon flow rate (b, turbulent).

treatment, the cells were stained with annexin V fluorescein isothiocyanate, allophycocyanin-conjugated anti-CD4 (T helper cell marker), phycoerythrin-conjugated anti-CD8 (cytotoxic T cell marker), and phycoerythrin-cyanine-7-conjugated anti-CD14 (monocyte marker) monoclonal antibodies in annexin V binding buffer for 15 min in the dark (all Biolegend, USA). After washing, cells were suspended in binding buffer containing 4', 6-diamidino-2-phenylindole (DAPI; Thermo, USA) to stain deoxyribonucleic acid of cells with compromised membranes. Light was collected using the following filters (center/bandwidth in nanometer; all Semrock, USA): 450/40 (bp, DAPI), 525/40 (bp, Annexin V), 660/20 (bp, CD4), and 755 nm longpass (CD14). Only Annexin V-negative/DAPI-negative cells were counted as viable cells. At least 5×10^4 cells were acquired per sample using *Kaluza Acquisition* 1.0 software (Beckman Coulter). Flow-cytometry-standard 3.0 files (>200 single measurements) were batch-analyzed applying the same gating strategy with *Kaluza* 1.3 software (Beckman Coulter).

IV. RESULTS AND DISCUSSION

A. Gas Curtain Effectively Minimized Ambient Air Influx

The efficiency of the gas curtain has been reported before [2] using pure argon as feed gas and molecular nitrogen or oxygen as curtain gas. This allows to control plasma properties, e.g., the reactive species output [4]. However, the gas flow rate used in these studies resulted in a turbulent effluent [6]. Comparison of gas curtain flow patterns in laminar [Fig. 2(a)] or turbulent conditions [Fig. 2(b)] revealed differences in gas flow structures. The figures can be compared with in [6, Fig. 1(b)]. In case of plasma on, the visible plasma effluent is located along the first 10–15 mm (axial position). Using higher flow rates, turbulences resulted in mixing of

plasma and shielding gas [Fig. 2(b)]. Thus, the plasma was fully shielded only in laminar but not in turbulent conditions. Due to this full shielding, effects of the gas curtain on the plasma chemistry composition reported previously [2]–[4] are very likely to be enhanced in the present conditions. Effective nitrogen shielding also suggested ROS depletion in the plasma gas phase due to exclusion of molecular oxygen. Any remaining ROS may exclusively rely on the impurities remaining from the gas bottles. Concurrently, an enhancement of the RNS in the gas phase was expected, e.g., N_2^+ , $N_2(A)$, and N.

Having a laminar gas flow results in an increase of the gas temperature [18]. The rotational temperature of NO is assimilated to the gas temperature, and thus reflects the temperature on the ionization propagation time scale. The temperature at a 7.5-mm distance from the nozzle was measured to be around 400 K in the same experimental conditions.

B. $N_2(C)$ Emission and Metastables

Preventing influx of oxygen to the plasma and its effluent inhibits the formation of ozone and atomic oxygen. The dissociation energy of molecular oxygen is 6 eV which is close to the $N_2(A)$ energy level requiring 6.2 eV. Thus, molecular oxygen is a serious quencher for the highly energetic metastable state of nitrogen, which has a natural lifetime of ~ 2 s and acts as a significant energy carrier [19]. Nonetheless, its direct detection remains a challenge at atmospheric pressure. Only recently, chemical plasma modeling was compared with experimental data on the production of nitric oxide and atomic oxygen [20], and the plasma jet used in this previous study is similar to the device used in the present work. Interestingly, it was reported that $N_2(A)$ is a very important precursor for the generation of NO and O. Using nitrogen as gas curtain, molecular oxygen was not present. As the ground state of molecular nitrogen cannot quench $N_2(A)$ [21], the latter is a credible energy carrier to be transported from the plasma to the liquid surface, possibly reaching cells. Argon metastables are also important to consider and their effects on plasma chemistry were reported in similar conditions [4].

Fig. 3 shows the spontaneous emission of $N_2(C)$ from plasma. Significant energy is needed to excite the second positive system of nitrogen which can be provided by either electron impacts or can originate from near resonant energy transfer between argon metastables of the ($^3P_{0,2}$) states to excite $N_2(X)$ to $N_2(C)$ [22]. In a similar plasma jet configuration operated with argon, the same mechanism was proposed to explain the excitation of $N_2(C)$ [16]. As discussed in [6], diffusion of nitrogen from the vicinity into the argon channel takes place a few millimeter from the nozzle (on-axis position). In addition, the ionization front propagates along an argon channel bounded by the surrounding atmosphere and follows the higher argon density. If the flow is laminar, the highest argon density remains along the axis of the argon channel. In contrast, the discharge and the highest argon density had identical trajectories in turbulent conditions and the memory effect of the argon metastable was motivated [6]. Subsequently, nitrogen and argon metastables were seriously considered

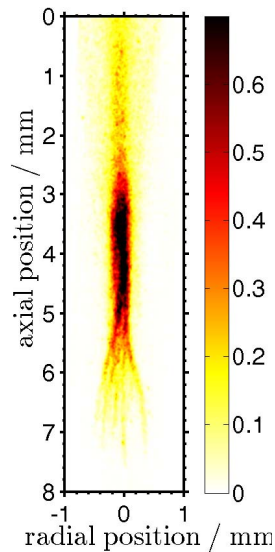


Fig. 3. Optical emission spectroscopy of spontaneous emission of the second positive system of nitrogen, $N_2(C)$, collected via a 7-nm bp filter centered at 335 nm. The plasma was operated using 1 slm argon and 2 slm N_2 gas flow rate for the gas curtain. The image was averaged over 300 single image acquisitions using an exposure time of 200 ns each.

to be transported from the gas phase to the liquid surface. Due to their high energy, a change of the liquid chemistry is anticipated but is out of the scope of this paper.

C. Lymphocyte Viability After Gas Curtain Plasma Exposure

We measured cell viability in primary human immune cells to investigate differences in shielding gas effects. Previously, we have shown that plasma treatment induced apoptosis in human blood cells (PBMC) [17]. Two distinct susceptibility types were: fragile lymphocytes and robust monocyte [8]. In this paper, we found similar differences [Fig. 4(a) and (c) versus (e)]. Compared with the previous setup (3 slm of argon, no gas curtain), cytotoxicity was much lower. A treatment of 1 min resulted in $\sim 75\%$ [10], compared with $\sim 20\%$ [Fig. 4(a) and (c)], nonviable cells 24 h after treatment. This may be due to differences in gas fluxes.

Comparing the effects between air and nitrogen shielding, the latter was significantly less toxic in lymphocytes but not in monocytes. This was in line with our hypothesis in which nitrogen shielded plasma yields lower concentrations of H_2O_2 due to the lack of $\cdot HO$ as discussed above. On the other hand, highly energetic argon and nitrogen metastables may have also led to cytotoxic effects.

To investigate the origin of plasma cytotoxicity in either setup, we added catalase prior to exposure of cells. Independent of the gas curtain used or treatment time applied, the cells remained fully viable as determined by flow cytometry. Generation of H_2O_2 was previously thought to originate from gaseous $\cdot HO$ [12], [13]. Here, results using catalase suggested that in the case of nitrogen shielding, H_2O_2 must be a product of RNS and liquid molecules [18]. The contribution of vacuum ultra violet (V-UV) to H_2O_2 production may also be of significance. It was estimated that one-thirds of $\cdot HO$ radical deposition in cell culture medium may originate from V-UV

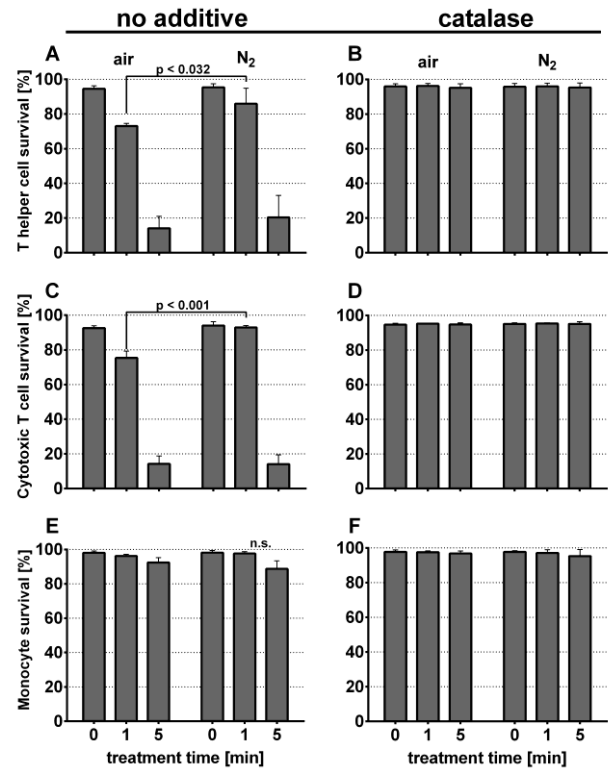


Fig. 4. (a) and (c) Comparison of nitrogen and air gas curtain showed significant differences in viability of T lymphocytes. (e) Monocytes were not strongly affected in either shielding configuration. (b), (d), and (f) Addition of H_2O_2 scavenging enzyme catalase prior to plasma treatment protected each cell type from cytotoxic plasma effects. Data are presented mean + SD of three-independent experiments.

[23]. Finally, temperatures of up to 400 K in the core of the plasma seem to be only local and did not adversely affect viability as our results suggested [Fig. 4(b), (d), and (f)].

V. CONCLUSION

Although no oxygen was present in nitrogen shielded plasma treatment of immune cells, cytotoxicity was still directly or indirectly dependent on the presence of H_2O_2 . We speculate that H_2O_2 originated from the reaction of RNS or V-UV with components of the liquid cell suspension. Effective protection via catalase also suggested that highly energetic plasma argon or nitrogen metastables or increased temperatures in this particular setup were negligible in plasma-mediated cytotoxicity.

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Mr. Bekeschus is a member of the Society for Leukocyte Biology, the German Society for Immunology, the European Society for Free Radical Research, and the International Society for Plasma Medicine.



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Prof. Weltmann is the President of the International Society for Plasma Medicine, and a member of the German Physical Society and several consulting committees in the industry and research.