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Understanding the Differences Between Antimicrobial and Cytotoxic Properties of Plasma Activated Liquids

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ABSTRACT: The aqueous environment plays an important role in the transmission of cold plasma effects to both prokaryotic and eukaryotic cells. The exposure of liquids to cold atmospheric plasma discharges results in the generation of secondary reactive species; specifically, hydrogen peroxide (H_2O_2) seems to be one of the most important amongst the reactive species contained in plasma activated liquids (PALs) in causing cytotoxicity. Detailed understanding of the effects of PALs on cells is essential to harness this new technology. Liquids acting as models for non-complex solutions were generated using a dielectric barrier discharge atmospheric cold plasma (DBD-ACP) system. The chemical characterization of the PAL included its pH and concentrations of hydrogen peroxide, nitrite, and nitrate. The antimicrobial effects of PALs on Gram-positive and Gram-negative bacteria were examined, and cytotoxicity assays were used to elucidate the cytotoxic properties of PALs. The research outcomes showed acidification of plasma activated nonbuffered solutions and differences in concentrations of hydrogen peroxide, nitrite, and nitrate. PALs with different compositions varied in their antibacterial activity and cytotoxic effects, indicating that different reactive species may be responsible for these inactivation processes. Our results suggest that antimicrobial and cytotoxic effects are distinct from each other, which may offer promising approaches for future targeted applications in medicine.

KEY WORDS: atmospheric cold plasma, ROS, RNS, microbial inactivation, cytotoxicity

I. INTRODUCTION

Cold atmospheric plasma has emerged as a new technology for biomedical applications such as microbial decontamination, anticancer therapy, and dermatological applications.[1–4] Plasma activated liquids (PALs), prepared by cold atmospheric plasma treatment of liquids, are of increasing interest because they offer advantages over direct exposure to plasma. Retention of plasma reactive species to liquids is a promising new technology, an alternative to conventional sterilization techniques. This technique is useful for applications in numerous settings (food industry, agriculture, medicine)[5–11] and also as a new eco-friendly disinfection product. New technologies like this are urgently needed in the biomedical field and agri-food sectors, especially now that there is a trend in eliminating chlorine from disinfection processes, due to health risks associated with chlorine-based byproducts.[12]

Liquid phase processes are the main key to understanding detailed mechanisms of the effects of atmospheric pressure plasma on living systems.[13] Building an understanding of a PAL's mechanisms responsible for inactivation processes, such as microbial inactivation and

cytotoxicity, is essential, but it still remains a field of intensive work due to the complex composition of plasma. Atmospheric cold plasmas contain electrons, positively charged ions, neutral particles, and numerous biologically relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as ozone, atomic oxygen, superoxide anion, hydroxyl radical, hydrogen peroxide, nitric oxide, and peroxynitrite.[14,15] Depending on the plasma system, voltage, working gas, and the chemical composition of the enclosing environment, different chemical reactions can be triggered by plasma exposure, leading to primary and secondary chemical species penetrating or dissolving into the aqueous environment.

Noncomplex liquids such as water have antimicrobial and cytotoxic properties after exposure to plasma. These effects are reported to be caused by a pH reduction, from neutral to an acidic range and the generation of long-lived secondary chemical species such as hydrogen peroxide, nitrite, and nitrate[16] and other short-lived species. ROS, such as hydrogen peroxide, play an important role in the toxic effects of PAL as well and can cause oxidizing reactions in biological cells, including the peroxidation of lipids and induction of DNA damage.[17] The different RNS, such as nitric oxide and its derivatives, including nitrite, nitrate, and peroxynitrite, have been reported to be necessary for antibacterial effects observe in PAL.[18,19] Synergistic effects of some or all of the reactive species are also possibly involved in these inactivation processes. Among them, H_2O_2, NO_2^- and NO_3^- are the central players in the cytotoxic activities of cold atmospheric plasma applications on mammalian cell lines, both normal and cancer cell lines.[11,13,15] Recently some groups have shown that the concentration of nitrate in plasma activated water or phosphate buffered saline (PBS) does not seem to contribute to cell death induced by PAL, indicating that the long-lived chemical species hydrogen peroxide and nitrites in PAL may act synergistically to induce cell death.[20,21] It is important to note that it is difficult to compare concentrations and biological effects of these long-lived species in different experiments because of the variation in discharge parameters and biological models used in each study.

The type of plasma device, the discharge parameters, and also the type of liquid exposed to plasma define the type and concentration of the chemical species in PALs, because different chemical processes occur. Liquids such as plasma treated saline solutions have been reported to have effects similar to those of plasma treated water after exposure to plasma. Oehmigen et al. investigated the bactericidal activity of plasma treated sodium chloride solution using a surface DBD in atmospheric air and reported a 7 log reduction of *Escherichia coli*.[22] Biologically relevant liquids, such as phosphate buffered saline, consist of additional ingredients, which are further sources of radicals. Jablonowski et al. found that sodium chloride solution contained smaller amounts of plasma generated oxygen radicals than Dulbecco's phosphate buffered saline (DPBS) because simple sodium chloride solution does not contain any additional source of oxygen.[23] Differences between the chemistry of more complex solutions, such as cell culture media and other buffered solutions, occur because they contain ingredients (amino acids and vitamins) that act as scavengers and are able to change the concentrations of plasma generated chemical species in liquids.

In order to establish PALs as a technology for medical applications, understanding liquid-mediated effects is required to reveal their full potential. The aim of this

study was to investigate the bactericidal and cytotoxic effects of four different PALs, plasma activated distilled water (PAW), phosphate buffered saline (PAPBS), saline (PAS) and phosphate buffer solution (PAPB). The effects of pH, hydrogen peroxide, and nitrate and nitrite anion concentrations on the inactivation processes and the stability of bactericidal efficiency of PAL were assessed after plasma activation of the liquids. Antimicrobial efficacy of PALs against *Escherichia coli* and *Staphylococcus aureus* was examined by counting colony-forming units (CFU) for up to 2 days postcreation, and the Chinese hamster ovary K1 (CHO-K1) cell line was used for the cytotoxicity assay.

II. MATERIALS AND METHODS

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (Arklow, Ireland).

A. Plasma Settings

A high voltage DBD-ACP system (DIT-120) custom built at Dublin Institute of Technology was used to generate PALs. Details are described in Moiseev et al.[24] The DIT-120 has a maximum voltage output of 120 kV_{rms} at 50 Hz in air. DIT-120 consists of two 15-cm diameter aluminium disk electrodes that are separated by a rigid polypropylene container (310 × 230 × 22 mm), which serves as both a sample holder and a dielectric barrier with a wall thickness of 1.2 mm. Below the top electrode there is a primary dielectric barrier, which is a 10-mm Perspex layer. The distance between the two electrodes was equal to the height of the container and the dielectric barrier. The voltage of the DIT-120 is transformed onto the top electrode, which means that the top electrode is the main output voltage carrier, and the bottom is the ground electrode.

B. Plasma Treatment of Liquids

Liquid samples containing 10 mL of distilled water, phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer solution), saline (137 mM NaCl, 2.7 mM KCl), and 10 mM phosphate buffer (100 mM KH_2PO_4, 100 mM K_2HPO_4), were added separately to a sterile petri dish 90 mm in diameter and placed without the lid at the center of the polypropylene plastic container and then sealed in a high barrier polypropylene bag (Cryovac, B2630, Charlotte, NC, USA). The plastic containers were placed at the center between the electrodes of the DIT-120 and treated with plasma for 0, 1, 5, or 10 min at a voltage of 80 kV_{rms} and 50 Hz using atmospheric air as the inducer gas. To assess the retention effect of plasma generated reactive species over time, samples were stored at room temperature for 24 h posttreatment storage time (PTST) following each plasma treatment time at room temperature. Liquids with no plasma treatment were kept as negative controls (0 min) and stored under the same conditions throughout the study.

C. Measurement of pH and Hydrogen Peroxide, Nitrite, and Nitrate Concentrations

The chemical characterization of PAL was determined with regard to H_2O_2, nitrites and nitrates. The pH of PAL was measured by an Orion pH meter (model 420A, Thermo Electron Corporation, USA). Hydrogen peroxide concentrations in PAL were determined using oxidation of potassium iodide to iodine, which has been described in detail.[25] Concentration of NO_2^- was detected using the Griess reagent based method, and NO_3^- concentrations were determined photometrically with 2,6-dimethylphenol (DMP).[26] Standard curves of known hydrogen peroxide, nitrite, and nitrate concentrations were included on each plate and used to convert absorbance into concentrations. All measurements of H_2O_2, NO_2^- and NO_3^- concentrations and pH changes were measured immediately after the PTST. The error bars show the standard deviation of the measurements.

D. Bacterial Strains and Culture Conditions

Escherichia coli NCTC 12900 and *Staphylococcus aureus* ATCC 1803 were used to investigate the antimicrobial efficacy of PALs. Microbial stock cultures were maintained at −80°C in the form of protective beads (Technical Services Consultants Ltd, UK). One bead of each culture was streaked onto separate tryptic soy agar plates (TSA; Biokar, France). The plates were incubated at 37°C for 24 h and further maintained at 4°C. A single isolated colony of each culture was inoculated in tryptic soy broth (TSB; Biokar, France) and incubated at 37°C for 18 h. The bacterial cells were harvested by centrifugation, and the cells were washed three times with PBS (Sigma, Aldrich, Ireland). Finally, 30 μL of bacterial suspension was diluted in 970 μL of PBS, and this was the bacterial working solution.

For the determination of each plasma activated solution's antimicrobial effect, 10% bacterial suspension was added to 90% PAL and incubated at room temperature for 15, 30, or 60 min (PAL contact time). After each contact time, a concentrated PBS solution (4.5 × PBS concentration) was added to the bacterial solution to neutralize the pH, and cells were diluted in Maximum Recovery Diluent (MRD; Merck, Ireland) and drop-plated on TSA plates. The plates were incubated aerobically at 37°C for 24 h, after which colonies were counted to determine the number of viable cells. To detect any subsequent increase in visible colonies, the plates were incubated for another 48 h. Results are represented as surviving bacterial population in $\log_{10}$ CFU/mL units with error bars representing standard deviation. Antimicrobial testing was performed on days 1 and 2 to determine the stability of the PAL over short storage time.

E. Cytotoxicity Assay

The cytotoxicity of PALs was examined using the CHO-K1 cell line as a biological model. CHO-K1 cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 Nutrient Mixture (DMEM/F12) supplemented with 2 mM L-glutamine and 10% (v/v)

fetal bovine serum (FBS), supplemented with 20% PAL (v/v). Cells were grown at 37°C and 5% carbon dioxide (CO_2) in a humidified incubator. Cell growth was assessed by crystal violet staining of trypsinized cells seeded at 2.5×10^4 cells/mL after 3 days of culture. For the crystal violet staining, the culture supernatant was aspirated and attached cells were fixed with 70% methanol for 1 min. Then, cells were stained with 0.2% crystal violet solution for 10 min. Excess stain was rinsed off with tap water, plates were air-dried, and the dye bound to the adherent cells was resolubilized with 10% acetic acid. The absorbance was measured at a wavelength of 560 nm on a spectrophotometric microplate reader (BioTek, Winooski, VT, USA). Cell growth was expressed as percentage of control cells.

F. Data Analysis

Results are presented as means with standard deviations using GraphPad Prism (GraphPad Software Inc., La Jolla, USA). The microbiological, cytotoxic, and chemical analyses were performed in three independent experiments.

III. RESULTS AND DISCUSSION

A. Chemical Properties of PAL

1. pH Measurements of PAL

Figure 1 shows the pH decrease in PAL as plasma treatment time of the liquids is increased up to 10 min. The pH of the buffered solutions decreased linearly with treatment time, whereas the nonbuffered solutions showed a sharp drop in pH after a 1-min exposure to plasma and were stable thereafter. PAW and PAS, which were the nonbuffered solutions, had lower pH values than the buffered solutions. Specifically, the pH value of the PAW and PAS decreased exponentially from 3.75 ± 0.6 and 4.35 ± 0.1 to 2.73 ± 0.3

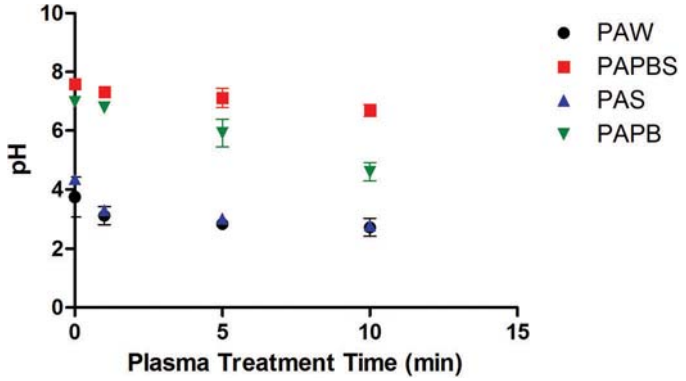

FIG. 1: Dependence of pH on time of activation for PAL (n = 3)

and 2.8 ± 0.13, respectively, after 10 min of plasma activation time. The decrease of pH may be due to the nitrogen oxides produced in the plasma interacting with liquids and producing nitric and nitrous acids.

2. Chemical Composition

Measurement of the ROS and RNS concentrations and an understanding of their role in antimicrobial and cytotoxic activity of PALs are important for any possible application. Slopes of the graphs show a time-dependent H_2O_2 concentration increase as plasma treatment time increases (Fig. 2A). H_2O_2 concentration increased up to 925 μM for PAW after 10 min treatment and 524 μM for PAPBS. The highest concentrations of peroxide were obtained for PAS and PAPB, which were 1300 μM and 2300 μM, after 10 min plasma exposure, respectively. Of note, although concentrations generally ranged from 500 to 1500 μM in all samples after 10 min of treatment, up to 5500 μM were reproducibly measured in set 3 of PAPB, resulting in the much higher average concentration.

Formation of nitrite in PAL was investigated using a colorimetric assay. As shown in Fig. 2B, the concentration of nitrite in PAPBS and PAPB increased with treatment time to around 38 μM and 27 μM, respectively, whereas there was no detectable NO_2^- concentration for PAW and PAS after 10 min of plasma treatment. An increase of nitrate concentration with treatment time was also detected in all PALs after plasma exposure, reaching 0.5 to 1 mM after 10 min. High concentrations of up to 3.7 mM were observed in PAPB treated for 10 min in data set 3, resulting in an average concentration of about 2 mM (see Fig. 2C).

B. Effects of PALs on Prokaryotic Cells

1. Bacterial Inactivation of PALs

Pure microbial strains were incubated with PAL for periods of 15, 30, or 60 min. Compared to the control group sample (0 min PAL treatment time), considerable inactivation was observed for both bacteria. Figure 3 and Tables 1–3 present the microbial inactivation of PAW, PAPBS, PAS, and PAPB obtained on day 1, expressed in $\log_{10}$ CFU/mL. Generally speaking the bactericidal activity of all PALs increased with increasing contact time between bacteria suspension and liquids, with 60 > 30 >15 min obtained in descending order for bacterial inhibition. Specifically, PAW treated for 1, 5, or 10 min caused at least a 5 log reduction to both bacteria after 30 min of contact time (see Fig. 3A,B). A 1-min treated PAS reduced the *E. coli* population to undetectable levels after 15 min contact time (see Fig. 3E). However, for inactivation of *S. aureus*, 10 min of plasma treatment time was needed (see Fig. 3F). A 5 $\log_{10}$ reduction of microbial load is usually required, in order to report that an antiseptic can inactivate microbes,[27] therefore the 6 log reduction caused by PAW and PAS with reasonable contact time is a promising result. With regards to PAPBS, 1 min of plasma treatment of liquid was not sufficient to cause any significant log reduction at any of the contact times for *E. coli*, but 10 min of plasma treatment combined with 30 or 60 min PAL contact time with the target reduced *E. coli*

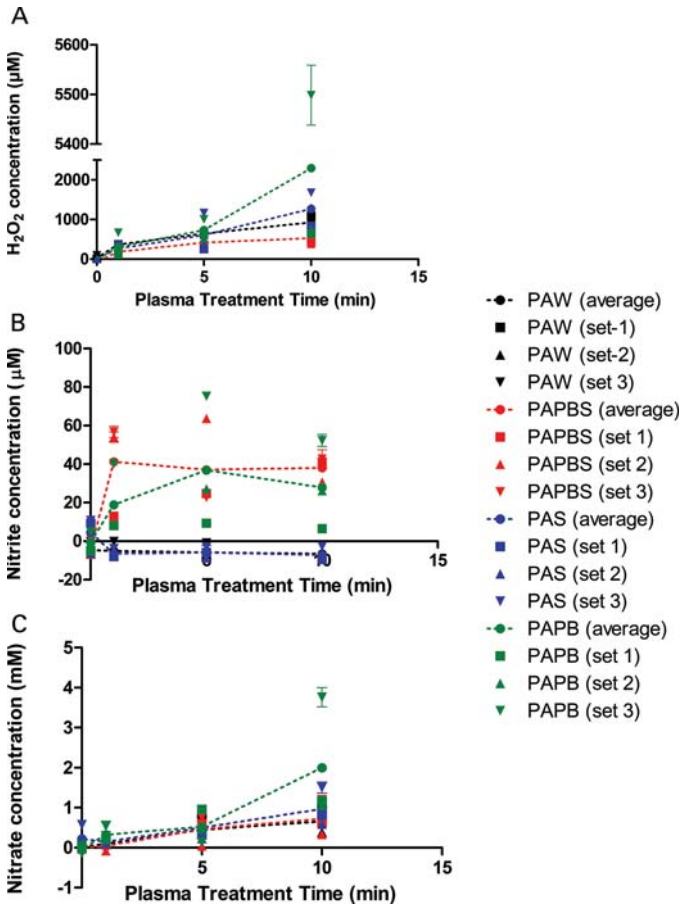

FIG. 2: (A) Generation of hydrogen peroxide, (B) nitrite, and (C) nitrate concentrations in PAL after 1, 5, or 10 min DBD plasma activation time at 80 kV (n = 3). Graphs represent averages of 3 independent plasma treatments (circles + lines), with the individual data sets represented as symbols only.

below the detection limit (see Fig. 3C). Decreasing PAPBS contact time to 15 min was sufficient for inactivation of *S. aureus* (see Fig. 3D). However, PAPB treated with plasma for 10 min was able to reduce *S. aureus* below the detection limit only after 60 min contact time (see Fig. 3H), with no inactivation obtained for *E. coli* (see Fig. 3G). Of note, PAPB set 3, which had displayed exceptionally high concentrations of H_2O_2 and nitrate, only showed slightly higher antimicrobial activity than sets 1 and 2 (Table 4).

2. Antibacterial Stability of PAL

The antimicrobial testing experiments were repeated on day 2 after PTST, to investigate the bactericidal stability of PAL by the same methods (Fig. 4; see Tables 1–4). Over-

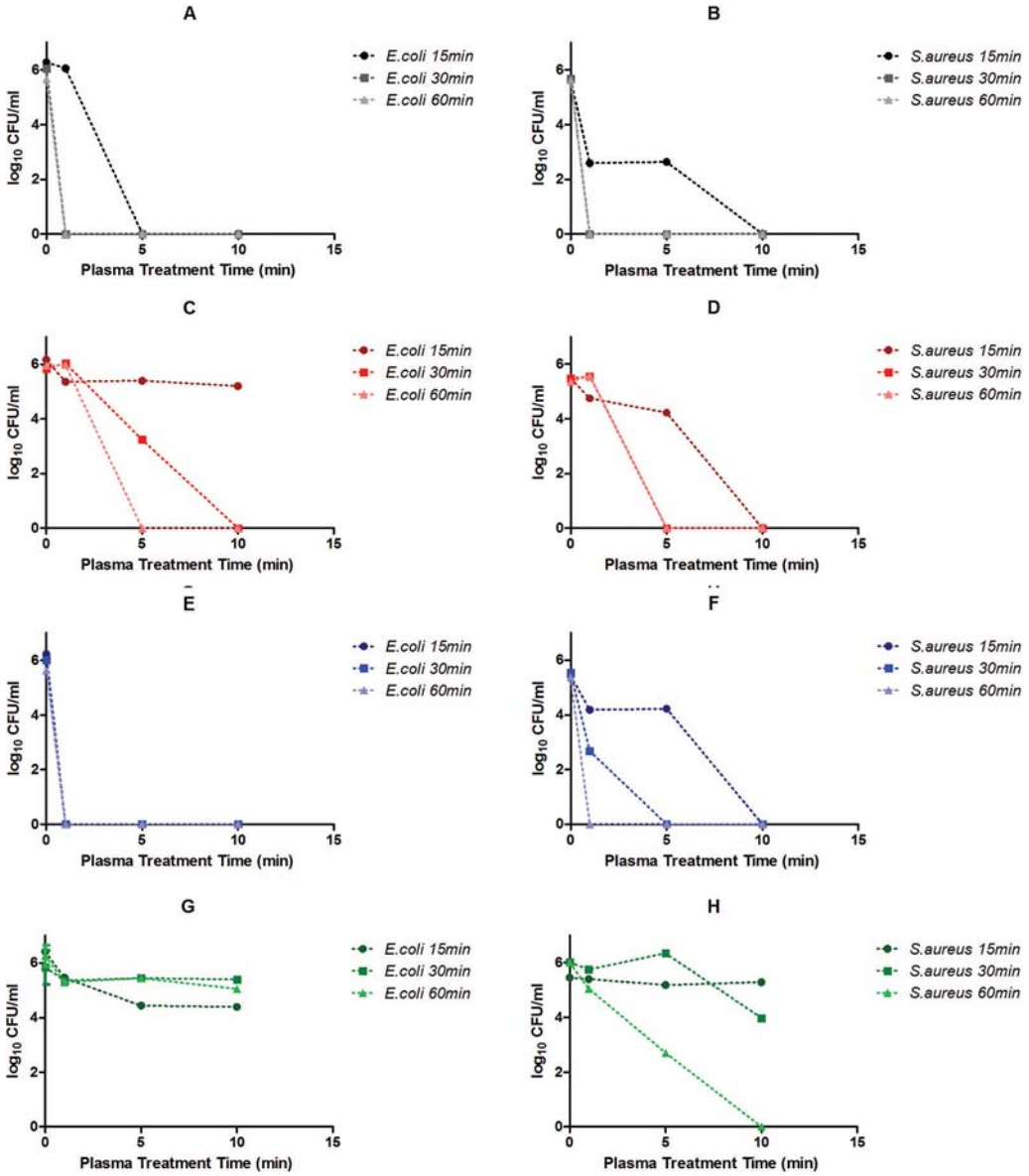

FIG. 3: Surviving bacterial cells in response to PAL after 1, 5, or 10 min plasma treatment. (A) PAW - *E. coli*, (B) PAW - *S. aureus*, (C) PAPBS - *E. coli*, (D) PAPBS - *S. aureus*, (E) PAS - *E. coli*, (F) PAS - *S. aureus*, (G) PAPB - *E. coli*, (H) PAPB - *S. aureus* (set 2).

all, PAL had lower antimicrobial activity after this 1 day of storage at 4°C, and loss of bactericidal activity in PAW and PAS was less compared to PAPBS and PAPB, which retained antimicrobial activity on the first day only. The nonbuffered solutions retained bactericidal activity against *E. coli* and *S. aureus* up to day 2 (see Fig. 4) and day 3 (see

TABLE 1: *E. coli* and *S. aureus* planktonic inactivation efficacy by PAW on days 1–3 for sets 1–3

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
1	15	0	5.39	0.056	6.26	0.045	5.63	0.072	5.06	0.048	5.63	0.072	5.18	0.073
		1	ND	—	6.05	0.029	3.21	1.19	ND	—	2.59	—	2.84	0.153
		5	ND	—	ND	—	ND	—	ND	—	2.63	0.072	4.46	0.048
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
	30	0	5.61	0.041	6.05	0.029	5.21	0.173	4.94	0.128	5.67	0.072	5.15	0.039
		1	ND	—	ND	—	ND	—	ND	—	ND	—	2.95	0.062
		5	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
	60	0	5.70	0.022	5.67	0.072	5.21	0.173	5.24	0.018	5.63	0.072	4.44	0.043
		1	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		5	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
2	15	0	5.65	0.049	6.15	0.039	6.03	0.151	5.52	0.043	6.01	0.054	5.24	0.064
		1	ND	—	ND	—	3.33	0.053	4.16	0.021	5.59	—	4.19	0.036
		5	ND	—	ND	—	3.37	0.083	4.16	0.057	5.47	0.101	2.78	0.055
		10	ND	—	ND	—	1.40	1.220	ND	—	5.57	0.151	ND	—
	30	0	5.27	0.060	5.63	0.072	5.31	0.173	5.35	0.038	6.11	0.043	6.18	0.073
		1	3.57	0.030	ND	—	ND	—	ND	—	4.46	0.061	2.69	0.173
		5	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—

TABLE 1: (*continued*)

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
2	60	0	6.24	0.082	6.12	0.092	6.26	0.065	5.46	0.048	6.53	0.068	5.05	0.029
		1	0.70	1.220	ND	—	1.60	1.394	ND	—	ND	—	4.30	0.076
		5	ND	—	ND	—	2.43	0.275	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
3	15	0	6.64	0.028	6.19	0.036	—	—	5.62	0.047	6.21	0.020	—	—
		1	6.59	0.044	6.05	0.029	—	—	5.50	0.027	5.88	0.073	—	—
		5	5.51	0.034	5.59	—	—	—	5.28	0.029	6.05	0.029	—	—
		10	ND	—	6.25	0.031	—	—	3.35	0.083	6.01	0.054	—	—
	30	0	6.50	0.061	5.59	—	—	—	6.31	0.054	6.28	0.058	—	—
		1	5.83	0.045	5.21	0.173	—	—	5.52	0.033	6.03	0.029	—	—
		5	5.28	0.074	5.83	0.045	—	—	ND	—	4.59	—	—	—
		10	ND	—	ND	—	—	—	ND	—	5.31	0.027	—	—
	60	0	6.23	0.081	6.03	0.029	—	—	6.64	0.044	5.47	0.101	—	—
		1	6.32	0.066	ND	—	—	—	ND	—	2.77	0.101	—	—
		5	ND	—	ND	—	—	—	ND	—	ND	—	—	—
		10	ND	—	ND	—	—	—	ND	—	ND	—	—	—

Cell density = log CFU/ml; ND, Not detected; SD, standard deviation.

TABLE 2: *E. coli* and *S. aureus* planktonic inactivation efficacy by PAPBS on days 1 and 2 for sets 1–3

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
1	15	0	6.36	0.048	6.16	0.021	6.33	0.128	5.56	0.031	5.48	0.028	6.49	0.072
		1	6.12	0.023	5.36	0.027	6.12	0.092	4.47	0.051	4.74	0.020	5.24	0.064
		5	5.88	0.073	5.39	0.033	5.31	0.173	4.16	0.021	4.22	0.033	6.20	0.138
		10	6.17	0.075	5.20	0.053	ND	—	4.08	0.069	ND	—	2.47	0.101
	30	0	6.29	0.061	5.83	0.045	6.38	0.069	5.53	0.025	5.46	0.039	6.24	0.049
		1	6.40	0.079	6.03	0.029	5.53	0.101	5.28	0.029	5.55	0.024	5.97	0.088
		5	4.78	0.055	3.23	0.038	ND	—	4.39	0.033	ND	—	4.37	0.075
		10	5.62	0.047	ND	—	ND	—	3.47	0.037	ND	—	ND	—
	60	0	6.47	0.058	5.95	0.062	5.46	0.048	5.30	0.043	5.35	0.037	5.46	0.039
		1	5.25	0.062	5.97	0.088	ND	—	ND	—	5.56	0.038	4.15	0.117
		5	4.36	0.027	ND	—	ND	—	ND	—	ND	—	ND	—
		10	5.25	0.031	ND	—	ND	—	ND	—	ND	—	0.70	1.22
2	15	0	6.12	0.092	5.34	0.025	4.24	0.048	5.56	0.038	5.34	0.025	4.99	0.090
		1	6.20	0.053	5.46	0.043	4.18	—	5.56	0.045	5.46	0.043	4.20	0.102
		5	6.36	0.014	5.33	0.067	4.34	—	5.55	0.046	5.33	0.067	4.15	0.079
		10	6.25	0.031	5.31	0.027	4.16	—	5.68	0.024	5.31	0.027	2.83	0.045
	30	0	6.30	0.016	6.08	0.069	4.49	0.036	5.56	0.015	6.08	0.069	4.96	0.135
		1	6.56	0.032	5.83	0.045	4.26	0.065	5.57	0.031	5.83	0.045	3.92	0.130
		5	6.35	0.053	6.11	0.043	3.92	0.105	5.59	0.070	6.11	0.043	3.70	0.111
		10	5.97	0.088	5.78	0.055	ND	—	5.43	0.041	5.78	0.055	4.19	0.114

TABLE 2: (*continued*)

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 3)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 20)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
2	60	0	6.63	0.072	5.83	0.045	5.06	0.098	5.44	0.050	5.44	0.031	6.65	0.066
		1	6.63	0.072	5.74	0.055	4.16	0.083	5.66	0.032	5.36	0.014	6.29	0.061
		5	7.15	0.039	5.90	0.102	ND	—	5.48	0.028	5.43	0.056	6.41	0.136
		10	7.05	0.029	5.59	—	ND	—	5.37	0.050	5.49	0.049	5.11	—

Cell density = log CFU/ml; ND, Not detected; SD, standard deviation.

TABLE 3: *E. coli* and *S. aureus* planktonic inactivation efficacy by PAS on days 1–3 for sets 1–3

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
1	15	0	6.20	0.053	6.22	0.033	4.90	0.021	5.48	0.046	5.43	0.020	5.99	0.033
		1	ND	—	ND	—	ND	—	ND	—	4.18	0.092	1.40	1.220
		5	ND	—	ND	—	ND	—	ND	—	4.22	0.033	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
	30	0	6.03	0.029	6.01	0.054	5.83	0.045	5.22	0.067	5.55	0.024	5.06	0.712
		1	ND	—	ND	—	3.31	0.082	ND	—	2.67	0.72	1.40	1.220
		5	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	0.70	1.220
	60	0	5.67	0.072	5.63	0.072	5.67	0.072	5.36	0.027	5.36	0.024	6.09	0.050
		1	ND	—	ND	—	2.11	—	ND	—	ND	—	ND	—
		5	ND	—	ND	—	0.70	1.22	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
2	15	0	5.91	0.038	6.03	0.276	5.47	0.101	5.49	0.028	5.86	0.045	4.95	0.062
		1	ND	—	5.36	0.027	4.88	0.073	5.15	0.039	6.05	0.029	5.25	0.031
		5	ND	—	ND	—	2.37	0.241	4.08	0.069	4.05	0.029	4.47	0.101
		10	ND	—	ND	—	ND	—	4.26	0.045	ND	—	4.41	—
	30	0	5.95	0.062	5.86	0.045	5.45	0.054	5.52	0.045	5.97	0.033	5.31	0.173
		1	ND	—	4.63	0.072	3.03	0.029	ND	—	5.63	0.072	4.63	—
		5	ND	—	ND	—	ND	—	ND	—	5.47	0.101	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	ND	0.013

TABLE 3: (*continued*)

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
3	60	0	6.25	0.079	6.05	0.029	4.97	0.033	5.74	0.055	6.43	0.041	5.31	0.173
		1	ND	—	ND	—	ND	—	ND	—	5.97	0.033	ND	—
		5	ND	—	ND	—	ND	—	ND	—	ND	—	ND	—
		10	ND	—	ND	—	ND	—	ND	—	ND	—	3.18	0.073
	15	0	6.21	0.053	5.67	0.072	—	—	6.35	0.038	6.23	0.049	—	—
		1	5.39	0.045	5.36	0.024	—	—	6.26	0.075	5.48	0.028	—	—
		5	5.60	0.028	5.41	0.085	—	—	6.40	0.034	5.29	0.061	—	—
		10	5.28	0.058	ND	—	—	—	6.08	0.069	5.48	0.046	—	—
	30	0	6.39	0.033	5.53	0.101	—	—	6.21	0.053	6.19	0.036	—	—
		1	6.27	0.060	5.30	0.100	—	—	5.97	0.033	5.47	0.101	—	—
		5	5.63	0.072	5.42	0.174	—	—	5.53	0.101	5.59	—	—	—
		10	5.27	0.270	ND	—	—	—	5.21	0.053	5.23	0.018	—	—
	60	0	6.24	0.064	6.25	0.031	—	—	6.28	0.058	5.97	0.033	—	—
		1	ND	—	ND	—	—	—	ND	—	3.27	0.101	—	—
		5	ND	—	ND	—	—	—	ND	—	2.78	0.055	—	—
		10	ND	—	ND	—	—	—	ND	—	1.40	1.22	—	—

Cell density = log CFU/ml; ND, Not detected; SD, standard deviation.

TABLE 4: *E. coli* and *S. aureus* planktonic inactivation efficacy by PAPB on days 1 and 2 for sets 1–3

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 2)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
1	15	0	6.18	0.102	6.41	0.021	6.10	0.125	6.05	0.029	5.44	0.011	5.26	0.045
		1	6.11	0.043	5.45	0.019	5.27	0.275	6.22	0.033	5.39	0.033	4.61	0.109
		5	6.36	0.024	4.43	0.020	6.24	0.082	5.19	0.114	5.18	0.021	4.41	0.086
		10	6.48	0.028	4.39	0.022	1.40	1.220	4.18	0.102	5.28	0.029	ND	—
	30	0	6.35	0.038	5.83	0.623	5.59	—	6.21	0.053	6.01	0.054	4.53	0.101
		1	6.43	0.041	6.35	0.014	5.31	0.173	5.49	0.049	5.74	0.055	5.52	0.075
		5	6.39	0.033	5.44	0.043	5.57	0.151	5.05	0.029	6.34	0.025	5.51	0.084
		10	5.95	0.062	5.39	0.038	0.70	1.220	3.09	0.050	3.97	0.033	5.23	0.114
	60	0	6.48	0.046	6.26	0.038	5.21	0.173	6.28	0.029	5.99	0.033	4.74	0.055
		1	6.3	0.038	5.29	0.033	5.21	0.173	5.06	0.048	5.05	0.029	4.37	0.241
		5	5.11	—	5.44	0.043	3.37	0.241	4.23	0.070	4.05	0.029	ND	—
		10	ND	—	5.05	0.029	ND	—	ND	—	ND	—	ND	—
2	15	0	6.32	0.066	6.23	0.049	5.51	0.173	6.29	0.061	5.93	0.038	5.53	0.101
		1	6.08	0.066	6.19	0.036	5.51	0.173	6.25	0.052	5.74	0.055	5.47	0.101
		5	6.26	0.045	5.81	—	5.53	0.101	6.14	0.023	5.86	0.045	5.03	0.078
		10	6.14	0.023	6.08	0.026	4.67	0.072	6.08	0.069	6.15	0.039	3.23	0.049
	30	0	6.15	0.039	5.83	0.045	5.03	0.151	6.29	0.036	5.78	0.055	5.70	0.111
		1	6.01	0.054	5.74	0.056	5.47	0.101	6.25	0.054	5.86	0.045	5.80	0.088
		5	6.25	0.031	5.88	0.073	5.21	0.173	6.14	0.069	5.67	0.072	5.11	0.043
		10	5.78	0.055	5.93	0.038	ND	—	6.08	0.050	5.80	0.088	ND	—

TABLE 4: (*continued*)

Day	Contact time (min)	Treatment time (min)	*E. coli* (set 1)		*E. coli* (set 3)		*E. coli* (set 3)		*S. aureus* (set 1)		*S. aureus* (set 2)		*S. aureus* (set 3)	
			Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD	Cell density	SD
2	60	0	6.14	0.023	6.19	0.036	5.61	0.173	6.17	0.143	6.19	0.036	5.04	0.079
		1	6.22	0.033	5.97	0.033	5.27	0.275	6.20	0.041	6.05	0.029	5.24	0.120
		5	6.06	0.098	5.97	0.033	4.74	0.055	6.09	0.050	5.83	0.045	2.51	0.173
		10	5.78	0.055	5.83	0.045	2.07	1.797	6.34	0.051	5.83	0.045	ND	—

Cell density = log CFU/ml; ND, Not detected; SD, standard deviation.

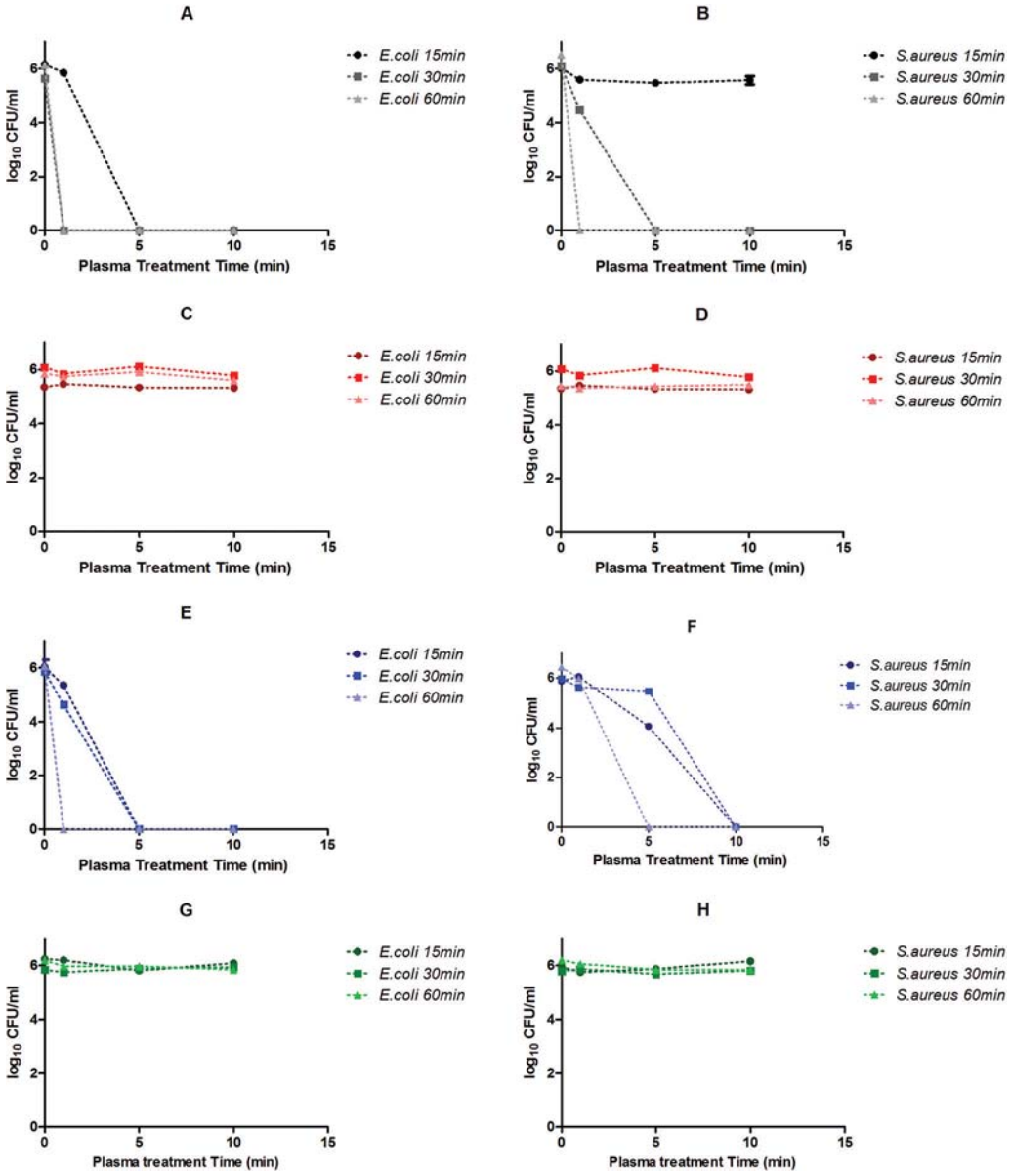

FIG. 4: Antibacterial stability of PAL after 1, 5, or 10 min plasma treatment on day 2. (A) PAW - *E. coli*, (B) PAW - *S. aureus*, (C) PAPBS - *E. coli*, (D) PAPBS - *S. aureus*, (E) PAS - *E. coli*, (F) PAS - *S. aureus*, (G) PAPB - *E. coli* , (H) PAPB - *S. aureus* (set 2).

Tables 1-4) after PTST. It is important to note that PAW and PAS treated with plasma for 5 min were still able to cause 6 log reduction for *E. coli* for all the contact times tested on day 2 (see Fig. 4A, E). The variations between the different PALs might be explained by

differences in the physicochemical properties of PALs in the same conditions. Among all four PALs investigated in this study, the nonbuffered solutions are proposed as the ideal candidates for antimicrobial agents, as they can retain their antimicrobial properties for days. In addition, the best conditions for generating antimicrobial solutions with the DBD-ACP system employed are longer plasma treatment times in conjunction with extended contact times (30 min and 60 min).

A number of studies have reported that Gram-positive bacteria are more resistant than Gram-negative bacteria[28,29] to direct treatment by plasma discharge. Han et. al reported two mechanisms of microbial inactivation between Gram-negative and Gram-positive bacteria. Specifically, the same strain of *E. coli* studied in these experiments was inactivated mainly by cell leakage and low-level DNA damage, whereas *S. aureus* was inactivated by intracellular damage.[30] In this study, PALs were effective against both *E. coli* and *S. aureus*, on the first day of the experiment, but on the second day some of the liquids were less effective against *S. aureus*. This could be explained through oxidative stress exerted by ROS and RNS generated in the liquids by plasma, on bacterial components, attacking the membrane's macromolecules, such as lipopolysaccharides, and thus making Gram-negative bacteria more vulnerable to plasma reactive species overall.

3. Effects of Chemical Composition on Antimicrobial Effects

As antibacterial efficacy is influenced by chemical characteristics of PAL, the influence of the buffering solutions needs to be taken into consideration. A number of studies have shown that microbial inactivation of PAL is related to the pH of the solutions, but the mechanism is still not fully understood.[31–34] Moreover, it is possible that the pH governs the generation of other active compounds. In acidic conditions, nitrites can be converted into nitrous acid. Nitrous acid is an unstable, monobasic acid and disassociates to nitrates and nitric oxide, which exerts broad-spectrum antimicrobial activity.[35]

The antimicrobial effects of hydrogen peroxide have been reported.[36] Hydrogen peroxide in acidic environments can react with nitrites to form peroxynitrite and further byproducts.[22,37] Therefore, higher concentrations of hydrogen peroxide relative to nitrites could enhance the production of peroxynitrite. Hänsch et al. showed that plasma treated saline had strong antibacterial effects against *E. coli* (NCTC 10538) and it was largely due to the interaction between nitrite, nitrate, and hydrogen peroxide generated in liquids.[38] Therefore, the effects on biological systems are more likely caused by different agents with different targets, which possibly lead to synergistic effects.

C. Cytotoxic Evaluation of PALs

In this study, we used the CHO-K1 cells as a well-established mammalian epithelial cell model. Cells cultured in 20% PAL, generated through plasma treatment for 1, 5, or 10 min, demonstrated a treatment time–dependent reduction in cell growth compared to the controls, indicating that PALs have cytotoxic effects. Figure 5 presents the percentages of the cell growth as plasma treatment time of liquids increased. When liquids were

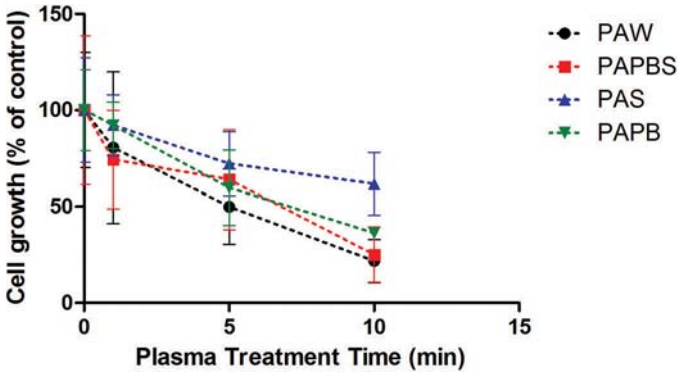

FIG. 5: Growth inhibition of PAL on CHO-K1 cells (n = 3)

prepared with a 10-min plasma treatment, PAW, PAPBS, and PAPB had stronger effects on the cells than PAS, with minimum cell growth of 61% ± 16%.

Air plasma results in a variety of ROS and RNS and both induce oxidative or nitrosative stress in mammalian cells.[16] When the concentration of hydrogen peroxide increased due to plasma treatment, higher cytotoxicity was obtained, which is in accordance with other publications.[7,25] Thus, hydrogen peroxide plays one of the major roles in cell cytotoxicity. It is yet unclear why PAS, which had high concentration of hydrogen peroxide and similar chemical composition as PAW, had the lowest cytotoxic activity. The generation of hypochlorite (OCl^-) might be possible in plasma activated saline solution, but it has not been investigated in this study. Of note, exceptionally high concentrations of H_2O_2 in PAPB set 3 of 5500 µM did not show cytotoxic effects beyond those observed in the other samples.

Nitrites were not generated in detectable concentrations for nonbuffered solutions in contrast to buffered solutions similar to other PALs generated using jet-based systems.[39,40] Boehm et al. previously reported that cell cultures supplemented with high concentrations of nitrite, did not show cytotoxic effects on the CHO-K1 cell line under the same conditions.[11] Concentrations of nitrate were generated in all PALs; however, no cytotoxic effects of nitrate were found in tenfold higher concentrations in the mammalian cells tested according to the same study. In agreement with numerous studies, these results confirm that mammalian cytotoxicity correlates with hydrogen peroxide content in PALs, but that this does not, however, constitute the only cytotoxic factor. Moreover, hydrogen peroxide in liquids can react with oxygen to form hydrogen peroxide radicals (HOO•), which then can form protons and superoxide anion (O_2•). Other ROS may then be generated, which should not be excluded from plasma cytotoxicity.

IV. CONCLUDING REMARKS

The concentrations of some representative long-lived chemical species in PAL increased along with plasma treatment time. Large differences in cytotoxicity and antimicrobial

activity trends were found among the different PALs. We demonstrated that nonbuffered PALs exert strong bactericidal activity that persisted up to 2 days after PAL generation. Of the studied PALs, the antimicrobial activity of PAW and PAS was stable up to the second day, in contrast to PAPBS and PAPB, which lost their antimicrobial activity over the same time frame and storage conditions. Cytotoxicity experiments showed that PAW, PAPBS, and PAPB generated by plasma treatment for 10 min caused cell death in more than 50% of cells. However, PAS was less cytotoxic and showed significant antimicrobial effects on both microbes, so it is potentially a suitable novel candidate for microbial decontamination *in vivo*. These results highlight the complexity of PAL solutions where multiple chemical components exert varying biological effects. As a broad measure of adverse biological responses, cytotoxicity is a useful metric to gauge the health impacts of disinfectant agents. Our results documented that different PALs may carry different concentrations of chemical species, maintain diverse antimicrobial properties and cytotoxic effects, and may offer approaches for future targeted applications in medicine.

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REFERENCES

1. Hertel M, Schwill-Engelhardt J, Gerling T, Weltmann K-D, Imiolczyk SM, Hartwig S, Preissnerb S. Antibacterial efficacy of plasma jet, dielectric barrier discharge, chlorhexidine, and silver diamine fluoride varnishes in caries lesions. Plasma Med. 2018;8(1):73–82.
2. Mine K, Miyamaru Y, Hayashi N, Aijima R, Yamashita Y. Mechanism of inactivation of oral cancer cells irradiated by active oxygen species from DBD. Plasma Med. 2017;7(3):201–13.
3. Ranieri P, Shrivastav R, Wang M, Lin A, Fridman G, A. Fridman A, Han L-H, Miller V. Nanosecond-pulsed dielectric barrier discharge–induced antitumor effects propagate through depth of tissue via intracellular signaling. Plasma Med. 2017;7(3):283–97.
4. Schmidt A, Bekeschus S, Wende K, Vollmar B, von Woedtke T. A cold plasma jet accelerates wound healing in a murine model of full-thickness skin wounds. Exp Dermatol. 2017 Feb 26;(2):156–62.
5. Judée F, Fongia C, Ducommun B, Yousfi M, Lobjois V, Merbahi N. Short and long time effects of low temperature plasma activated media on 3D multicellular tumor spheroids. Sci Rep. 2016 Feb 22;6:21421.
6. Schnabel U, Sydow D, Schlüter O, Andrasch M, Ehlbeck J. Decontamination of fresh-cut iceberg lettuce and fresh mung bean sprouts by non-thermal atmospheric pressure plasma processed water (PPW). Mod Agric Sci Technol. 2015;1(1):23–39.
7. Van Boxem W, Van der Paal J, Gorbanev Y, Vanuytsel S, Smits E, Dewilde S, Bogaerts A. Anti-cancer capacity of plasma-treated PBS: effect of chemical composition on cancer cell cytotoxicity. Sci Rep. 2017;7(1):16478.
8. Yan D, Sherman JH, Keidar M. Cold atmospheric plasma, a novel promising anti-cancer treatment modality. Oncotarget. 2017 Feb 28;8(9):15977–95.
9. Tanaka H, Nakamura K, Mizuno M, Ishikawa K, Takeda K, Kajiyama H, Utsumi F, Kikkawa F, Hori M. Non-thermal atmospheric pressure plasma activates lactate in Ringer's solution for anti-tumor effects. Sci Rep. 2016;8(6):36282.

10. Su X, Tian Y, Zhou H, Li Y, Zhang Z, Jiang B, Yang B, Zhang J, Fang J. Inactivation efficacy of non-thermal plasma-activated solutions against Newcastle disease virus. Appl Environ Microbiol. 2018 Apr 16;84(9):e02836–17.
11. Boehm D, Curtin J, Cullen PJ, Bourke P. Hydrogen peroxide and beyond - the potential of high-voltage plasma-activated liquids against cancerous cells. Anticancer Agents Med Chem. 2017;17:1.
12. Toxicological profile for chlorine. Centers for Disease Control and Prevention; 2010 [cited 2018 Aug 16]. Available from: https://www.atsdr.cdc.gov/ToxProfiles/TP.asp?id=1079&tid=36.
13. Jablonowski H, von Woedtke T. Research on plasma medicine-relevant plasma–liquid interaction: what happened in the past five years? Clin Plasma Med. 2015 Nov;3(2):42–52.
14. Lu X, Naidis GV, Laroussi M, Reuter S, Graves DB, Ostrikov K. Reactive species in non-equilibrium atmospheric-pressure plasmas: generation, transport, and biological effects. Phys Rep. 2016 May 4;630:1–84.
15. Graves D. The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology. J Phys D: Appl Phys. 2012 Jul;45:263001.
16. Machala Z, Tarabová B, Hensel K, Spetklikova 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 Apr 10;10(7)649–59.
17. Yost A, Joshi S. Atmospheric nonthermal plasma-treated PBS inactivates Escherichia coli by oxidative DNA damage. PLoS One. 2015 Oct 13;10(10):e0139903.
18. van Gils C, Hofmann S, Boekema B, Brandenburg R, Bruggeman P. Mechanisms of bacterial inactivation in the liquid phase induced by a remote RF cold atmospheric pressure plasma jet. J Phys D: Appl Phys. 2013 Apr 8;46(17):175203.
19. Lukes P, Dolezalova E, Sisrova I, Clupek M. Aqueous-phase chemistry and bactericidal effects from an air discharge plasma in contact with water: evidence for the formation of peroxynitrite through a pseudo-second-order post-discharge reaction of H_2O_2 and HNO_2. Plasma Sources Sci Technol. 2014 Feb 4;23(1):015019.
20. Girard P-M, Arbabian A, Fleury M, Bauville G, Puech V, Dutreix M, Sousa JS. Synergistic effect of H_2O_2 and NO_2 in cell death induced by cold atmospheric He plasma. Sci Rep. 2016 Jul 1;6:29098.
21. Lu P, Boehm D, Cullen P, Bourke P. Controlled cytotoxicity of plasma treated water formulated by open-air hybrid mode discharge. Appl Phys Lett. 2017 Jun 28;110(26):264102.
22. Oehmigen K, Winter J, Hähnel M, Wilke C, Brandenburg R, Weltmann K-D, von Woedtke T. Estimation of possible mechanisms of Escherichia coli inactivation by plasma treated sodium chloride solution. Plasma Process Polym. 2011 Jun 8;8(10):904–13.
23. Jablonowski H, Hammer M, Weltmann K-D, Reuter S. Effects of atmosphere composition and liquid type on plasma-generated reactive species in biologically relevant solutions. Plasma Med. 2013;3(1-2):45–55.
24. Moiseev T, Misra NN, Patil S, Cullen PJ, Bourke P, Keener K, Mosnier JP. Post-discharge gas composition of a large-gap DBD in humid air by UV-Vis absorption spectroscopy. Plasma Sources Sci Technol. 2014 Oct 20;23(6):065033.
25. Boehm D, Heslin C, Cullen PJ, Bourke P. Cytotoxic and mutagenic potential of solutions exposed to cold atmospheric plasma. Sci Rep. 2016 Feb 24;6:21464.
26. Lu P, Boehm D, Bourke P, Cullen PJ. Achieving reactive species specificity within plasma activated water through selective generation using air spark and glow discharges. Plasma Process Polym. 2017 Jan 23;14(8):1600207.
27. von Woedtke T, Kramer A, Weltmann K-D. Plasma sterilization: what are the conditions to meet this claim? Plasma Process Polym. 2008 Aug 12;5(6):534–9.
28. Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, Moisenovich MM, Romanova JM, Murashev AN, Selezneva II, Shimizu T, Sysolyatina EV, Shaginyan IA, Petrov OF, Mayevsky EI, Fortov VE, Morfill GE, Naroditsky BS, Gintsburg AL. Bactericidal effects

of non-thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011 Jan 1;60:75–83.
29. Lee K, Paek K-H, Ju WT, Lee Y. Sterilization of bacteria, yeast, and bacterial endospores by atmospheric-pressure cold plasma using helium and oxygen. J Microbiol. 2006 Jun;46(3):269–75.
30. Han L, Patil S, Boehm D, Milosavljevic V, Cullen PJ, Bourke P. Mechanism of inactivation by high-voltage atmospheric cold plasma differs between Escherichia coli and Staphylococcus aureus. Applied Environmental Microbiology. 2015 Oct 30;82(2):450–8.
31. Naïtali M, Kamgang-Youbi G, Herry JM, Bellon-Fontaine MN, Brisset JL. Combined effects of long-living chemical species during microbial inactivation using atmospheric plasma-treated water. Appl Environ Microbiol. 2010 Nov;76(22):7662–4.
32. Traylor MJ, Pavlovich MJ, Karim S, Hait P, Sakiyama Y, Clark DS, Graves DB. Long-term antibacterial efficacy of air plasma-activated water. J Phys D: Appl Phys. 2011 Nov 4;44(47):472001.
33. Schnabel U, Niquet R, Schmidt C, Stachowiak J, Schlüter O, Andrasch M, Ehlbeck J. Antimicrobial efficiency of non-thermal atmospheric pressure plasma processed water (PPW) against agricultural relevant bacteria suspensions. Int J Environ Agric Res. 2016 May 31;2(5)212–24.
34. Niquet R, Boehm D, Schnabel U, Cullen P, Bourke P, Ehlbeck J. Characterising the impact of post-treatment storage on chemistry and antimicrobial properties of plasma treated water derived from microwave and DBD sources. Plasma Process Polym. 2017 Nov 7;15(3):e1700127.
35. Fang FC. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest. 1997 Jun 15;99(12):2818–25.
36. Raffellini S, Schenk M, Guerrero S, Alzamora SM. Kinetics of Escherichia coli inactivation employing hydrogen peroxide at varying temperatures, pH and concentrations. Food Control. 2011 Jun;22(6):920–32.
37. Brisset J-L, Pawlat J. Chemical effects of air plasma species on aqueous solutes in direct and delayed exposure modes: discharge, post-discharge and plasma activated water. Plasma Chem Plasma Process. 2016 Mar;36(2):355–81.
38. Haensch M, Mann M, Weltmann K-D, von Woedtke T. Analysis of antibacterial efficacy of plasma-treated sodium chloride solutions. J Phys D: Appl Phys. 2015 Oct 8;48(45):454001.
39. Chauvin J, Judée F, Yousfi M, Vicendo P, Merbahi N. Analysis of reactive oxygen and nitrogen species generated in three liquid media by low temperature helium plasma jet. Sci Rep. 2017 Jul 4;7(1):4562.
40. Reuter S, von Woedtke T, Weltmann K-D. The kINPen—a review on physics and chemistry of the atmospheric pressure plasma jet and its applications. J Phys D: Appl Phys. 2018 May 16;51(23):233001.