Received: 30 June 2016 | Revised: 29 August 2016 | Accepted: 20 September 2016

DOI 10.1002/ppap.201600118

PLASMA PROCESSES AND POLYMERS

FULL PAPER

Temporal evaluation of the anti-tumor efficiency of plasma-activated media

Soheila Mohades[1] | Nazir Barekzi[2] | Hamid Razavi[1] | Venkat Maruthamuthu[3] | Mounir Laroussi[1]*

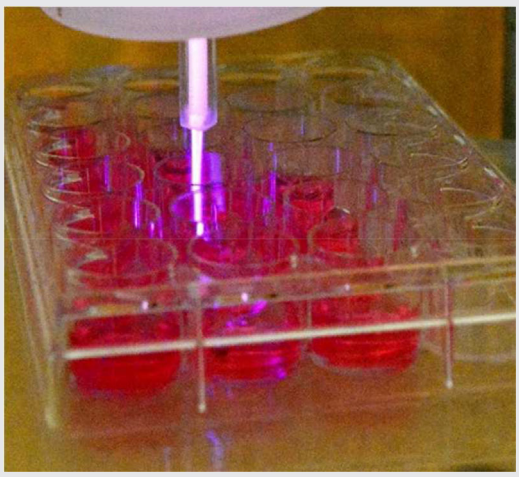
[1] Plasma Engineering and Medicine Institute, Old Dominion University, Norfolk, Virginia 23529, USA

[2] Department of Biological Sciences, Old Dominion University, Norfolk, Virginia 23529-0266, USA

[3] Department of Mechanical and Aerospace Engineering, Old Dominion University, Norfolk, Virginia 23529, USA

***Correspondence**
Mounir Laroussi, Plasma Engineering and Medicine Institute, Old Dominion University, Norfolk, VA 23529, USA.
Email: mlarouss@odu.edu

Plasma-activated media (PAM) can be as effective as direct plasma treatment in killing cancer cells. PAM is produced by exposing liquid cell culture media to low temperature plasma. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the aqueous state play key role in the anti-tumor effects of PAM. The effectiveness of PAM is highly dependent upon the dose of reactive species. The concentrations of reactive species and consequently the effectiveness of PAM decreases over time after plasma exposure. In this paper, the effect of PAM on the viability of SCaBER cells, originally obtained from a bladder squamous cell carcinoma, is shown and its efficiency at different aging times is evaluated. To investigate the selective effect of plasma on normal epithelial cells, MDCK (Madin-Darby Canine Kidney) cells from normal epithelial tissue of a dog kidney were treated by PAM. The concentrations of hydrogen peroxide at different times after plasma exposure were measured. Our findings indicate that there is a correlation between PAM efficiency and H_2O_2 concentration, as both decrease over time.



KEYWORDS
cancer, cold plasma, plasma-activated media, plasma jet, ROS

1 | INTRODUCTION

Different approaches have been taken to treat cancer cells using low temperature plasma.[1–6] In a direct treatment procedure, cancer cells cultured in vitro are exposed to a plasma discharge. Results indicated the capability of plasma treatment in reducing cell viability in a time-dependent manner.[7–12] Several studies reported that in vivo treatment of tumors using low temperature plasma resulted in tumor size reduction or tumor growth inhibition.[13–15] Alternatively, a plasma treated biological medium can be used to treat cancer cells. Recently, several investigators have been studying the biological effects of plasma-activated media (PAM) or plasma activated water (PAW) and the chemistry of liquid-plasma interactions.[16–19] In addition, it has been reported that cancer cells are more susceptible to plasma

treatment than normal cells and reactive oxygen species (ROS) stress induces death mainly through apoptosis.[20–22]

There are several factors in low temperature plasma that are responsible for biomedical impacts on a treated organism such as reactive species, UV radiation, and charged particles; however, it has been shown that ROS and RNS (reactive nitrogen species) perform the major role among other factors in the biomedical effects of LTP.[23–25] Interactions of plasma with liquid media through the process of PAM preparation results in diffusion/dissolution of ROS in the treated medium. The concentrations of reactive species generated in PAM is directly related to the duration of plasma exposure and to the chemical makeup of the medium.[26] However, the lifetime of reactive species is relatively short which makes it an important issue to determine the time span during which PAM remains effective. Most of the reactive species such as nitric oxide (NO), ozone (O_3), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), and superoxide anion (O_2^-) are highly reactive, short-lived, and unstable.[27,28] Chemistry of the treated media is a factor that affects the stability of ROS in PAM as well as pH[27] and temperature. Yan et al.[29] reported on the influence of regulating the composition of PAM on the stability of hydrogen peroxide (H_2O_2) and indicated that a tyrosine derivative amino acid added in DMEM (Dulbecco's Modified Eagle Medium) PAM attenuated H_2O_2 at 8 °C. Besides, it was shown that keeping PAM at room temperature[30] or frozen[17] has a significant effect on the degradation rate of reactive species. Therefore, identifying the concentrations of ROS in PAM as well as their lifetime are important for a better understanding of the anti-cancer effects of PAM.

H_2O_2 with an oxidation potential of 1.8 V has a relatively higher stability than other ROS species such as superoxide anion and hydroxyl radical.[27] It is well-known that H_2O_2 is toxic to cells and causes macromolecular damage such as peroxidation of lipids and induction of DNA damage.[31,32] Considering the important biological impact and long lifetime of H_2O_2, we measured its concentration in PAM immediately and at a specific time interval after PAM preparation.

One of the most common reactions of H_2O_2 is known as Fenton chemistry where H_2O_2 reduces into $OH^{\bullet}$ and $HO_2^{\bullet}$ (hydroperoxyl radical) in the presence of Cu^{2+} or Fe^{2+} ions. Serum in cell culture media contains proteins and metallic compounds such as ferrous ion which can destroy H_2O_2 in PAM.[33] Therefore, we evaluated the H_2O_2 concentration in a serum-free PAM as well as a serum supplemented PAM.

2 | EXPERIMENTAL SECTION

2.1 | Cell culture

Two adherent epithelial cell lines were used in this study: SCaBER (ATCC® HTB3™) cell line from a urinary bladder tissue with the squamous cell carcinoma disease was purchased from ATCC (Manassas, VA). This cell line was cultured in MEM (Minimum Essential Medium) with 10% Bovine Calf Serum and 1% Penicillin/Streptomycin/Glutamine all purchased from HyClone (Logan, UT). MDCK (ATCC® CCL-34™) cell line derived from normal epithelial tissue of kidney of a dog. MDCK was grown in EMEM media supplemented with 10% Bovine Calf Serum and 1% Penicillin/Streptomycin/Glutamine. Both cell lines were seeded in a 75 cm^2 vented cell culture flask and maintained at 37 °C in a humidified incubator with 5% CO_2. Once cells had reached a high confluence, a cell suspension with a density of around 400 000 cells ml^{-1} was prepared and 100 µl $well^{-1}$ of cell suspension was seeded into a 96-well plate and were incubated overnight before treating with PAM.

2.2 | Treatment by plasma-activated media

The plasma source in this research is the plasma pencil which generates low temperature plasma plumes at atmospheric pressure. Figure 1 shows a schematic of the plasma pencil and more details are described in.[4] The plasma pencil is a DBD-based plasma source with two ring shape electrodes. One electrode is connected to a pulsed high voltage and the second one is grounded. Plasma is ignited by applying a high voltage and introducing a flow of working gas which was 99.99% pure helium in the current experiment. For all experiments, plasma was ignited with the following conditions unless otherwise mentioned: the voltage of 7 kV, frequency of 5.00 kHz, and pulse width of 800 ns. The flow rate of He was 5.0 ± 0.1 slm (the gas velocity was around 3 m s^{-1}). The distance between the nozzle tip and media surface was ~22 mm while the depth of media was ~3 to 4 mm.

The same cell culture media that was employed to grow each cell line was used to make PAM as described in Section 2.1; that is, MEM (Minimum Essential Media) for SCaBER and EMEM (Eagle's Minimum Essential Media) for MDCK, both supplemented with serum and antibiotics. To address the

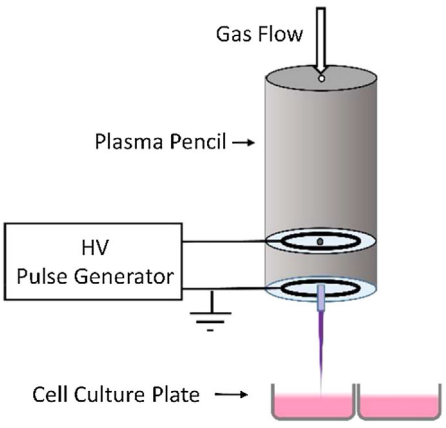


FIGURE 1 Experimental setup for generating the PAM

media used in making PAM we call them MEM PAM or EMEM PAM in the rest of this paper.

It was observed that MDCK cells grow better in EMEM complete media. Therefore, EMEM PAM was used to treat normal cells to provide the best-growing condition for the control sample. The chemical composition of MEM and EMEM is slightly different. EMEM contains more amino acid compounds and 1 mM Sodium Pyruvate while MEM contains 2.2 g L^{-1} Sodium Bicarbonate according to the manufacturer data sheet. We assumed that the difference in the composition does not have a significant effect on the final results and to validate our assumption the H_2O_2 was measured in both plasma treated media. In addition, Kaushik et al.[34] reported that addition of 10 mM Sodium Pyruvate in their investigated media resulted in 20% reduction in the effectiveness of the PAM. However, 1 mM sodium pyruvate in the EMEM media is 10 times lower than what was used in Kaushik et al.[34] experiment and accordingly its scavenging effect is negligible.

To make PAM, 1 ml of fresh complete cell culture media was added to each well of a 24-well plate. Each well was exposed to the plasma pencil for a designated exposure time. Right after exposure, the media on top of cells grown in a 96-well plate was replaced by 100 µl of PAM. After PAM treatment, cells were stored in an incubator for monitoring viability at post-treatment times. Non-plasma-treated media was used to treat the control sample.

To evaluate the stability of ROS and effectiveness of PAM through aging time, the treated media was stored in the dark at room temperature for the specific time of 1, 8, and 12 h to make what is called aged-PAM. Later, the "aged-PAM" was used to treat SCaBER cells. The viability of SCaBER cells was measured at post-aged-PAM treatment times.

The pH of PAM was measured before and after plasma exposure. The result indicated that plasma exposure does not decrease/increase the pH of the cell culture media and media remained at stable pH even after 10 min plasma exposure (data not shown).

2.3 | Cell viability assay

Cell viability was quantified using The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, MI). The solution reagent contains a tetrazolium compound [3-(4, 5-imethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]. Metabolically active cells reduce the MTS tetrazolium compound into a colored formazan product that is soluble in cell culture medium. At post-treatment time, 20 µl of the MTS solution was added to 100 µl of cell culture media in each sample well of the 96-well plate and incubated for 1 to 2 h in an incubator. Absorbance was recorded at 490 nm using a microplate reader (AgileReader, ACTGene Inc.). All experiments were conducted in at least three sets of independent experiments and results are expressed as a percentage of cell viability normalized to the control at each post-assay time.

Trypan blue exclusion assay was used to count the number of live cells in a control sample in order to quantify results of MTS assay. In this dye exclusion method, non-viable cells take the dye in and turn blue in color while viable cells remain unstained since the intact membrane of live cells prevents penetration of the dye.

2.4 | Hydrogen peroxide detection assay

The concentration of H_2O_2 generated by the plasma in PAM and aged-PAM was measured using Amplex red H_2O_2 assay kit (Invitrogen, Burlington, Ontario, Canada). The Amplex red assay is sensitive and one of the most commonly used molecular probes to measure the concentration of H_2O_2. The reaction of Amplex red reagent with H_2O_2 in the presence of horseradish peroxide (HRP) – which plays the role of a catalyzer in this reaction – results in the production of resorufin. Resorufin is a highly colored compound that can be detected fluorometrically or spectrophotometrically.

The H_2O_2 concentration was measured in MEM PAM immediately after plasma exposure and after 1, 8, and 12 h aging. To evaluate the effect of serum on H_2O_2 concentration, a serum-free PAM made by MEM and 1% antibiotics was used and the H_2O_2 concentration was measured after 8 h aging. A standard curve of known H_2O_2 concentration (in MEM complete media) within the range of 0–50 µM was prepared to quantify the acquired absorbance. Correspondingly, the H_2O_2 concentration was analyzed in EMEM PAM immediately after plasma exposure.

In all experiments, each sample was diluted 1:10 with a fresh complete media to be within the acceptable range of the assay. The control sample was complete media with no plasma treatment. Fifty microliters of the standard curve solutions, PAM, and the control sample were transferred into individual wells of a 96-well plate. Fifty microliters of the working solution composed of 100 µM Amplex red reagent and 0.2 U ml^{-1} HRP was loaded in each well. Then, the 96-well plate was incubated in a dark place at room temperature for 30 min. The absorption of samples was measured at 570 nm using the microplate reader.

3 | RESULTS AND DISCUSSION

3.1 | Effects of PAM and aged-PAM on cell viability

Figure 2 shows the effect of PAM (immediate PAM) on SCaBER cell viability when it was applied right after plasma exposure. Plasma exposure time was 2–4 min and 0 min represents the control sample. Cell viability was analyzed at

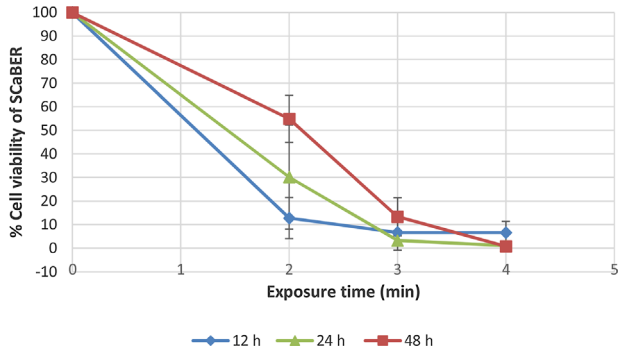


FIGURE 2 Effect of PAM produced by different exposure times on the viability of SCaBER cells using MTS assay. Metabolic activity of cells was measured at 12, 24, and 48 h post-PAM treatment. Results from three independent experiments with two replications are shown as means ± SD (standard deviation)

12, 24, and 48 h post-PAM treatment. The percentage of metabolically active cells decreases with increasing exposure time and as can be seen in Figure 2, 3 and 4 min PAM were toxic enough to induce more than 80% cell death. There is not a significant change in viability of cells as a delayed effect at post-treatment times except the 2 min PAM that indicates gradual cell growth in survived cells.

Figure 3 indicates the result of aged-PAM on SCaBER cell viability at 12 h post-PAM treatment. PAM was stored at room temperature for 1, 8, and 12 h before the application. No significant change in the cell viability was observed at 24 and 48 h after PAM treatment (data not shown). Comparing the cell viability outcome of SCaBER treated with aged-PAM indicates that PAM efficiency decreases with increasing aging time, depending on the duration of plasma exposure. Reduction in the

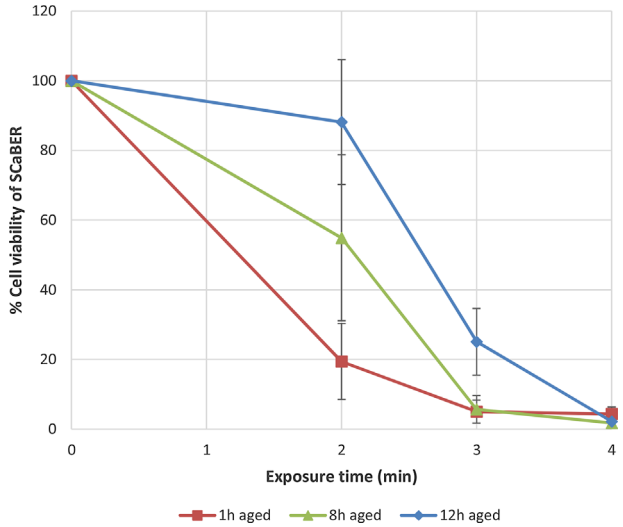


FIGURE 3 Effectiveness of aged-PAM to induce cell death in SCaBER cells when stored for 1, 8, and 12 h before utilizing. Metabolic activity of cells was measured at 12 h post-aged-PAM treatment. Results are shown as means ± SD from three independent experiments with two samples replications

efficiency of PAM is more remarkable at shorter exposure times such as 2 min. For instance, aging the 2 min PAM for 8 and 12 h leads to 50% and 80% reduction, respectively in the PAM efficiency in comparison with immediate application (see Figure 2 at 12 h). However, we did not observe a significant reduction in the efficiency of longer exposure time PAM with aging. This outcome suggests that the concentration of reactive species is higher than a tolerable level for cells and PAM remained toxic to SCaBER cells.

In addition to checking the effects of PAM treatment on a cancer cell line, the effect of PAM on normal epithelial cells was analyzed, finding no significant changes in metabolic viability of MDCK cells up to 3 min PAM (Figure 4A). Using 4 min PAM induces around 10% reduction in metabolic activity of normal cells which is much lower than the 90% reduction in the viability of SCaBER cells treated with same exposure time PAM, as shown in Figure 2. In fact, using a longer exposure time PAM (10 min) caused only one log reduction in the number of viable normal cells. This clearly reveals that, unlike the case of SCaBER, PAM treatment does not have severe damaging effects on normal MDCK cells at exposure time below 4 min.

For a better interpretation of MDCK cells response to PAM treatment, the raw data of cell viability (cell ml^{-1}) is shown in Figure 4B. The number of viable cells was quantified at 12 and 24 h post-PAM treatment using MTS and trypan blue exclusion assays. The black column (initial seeding) represents the number of cells at the time of PAM treatment. Figure 4B reveals that PAM with up to 6 min plasma exposure arrests cell proliferation in normal cells; however, 10 min PAM induces death in treated cells.

3.2 | Hydrogen peroxide concentration in PAM and aged-PAM

H_2O_2 is an important ROS by being able to penetrate the cell membrane. It is not a radical species but it intermediates in many reactions and results in more ROS production such as $OH^{\bullet}$.[35] The concentration of H_2O_2 was measured in MEM PAM immediately after plasma exposure and after aging (Figure 5A). The Amplex red measurements indicate the presence of H_2O_2 in PAM in an exposure time-dependent manner. As it can be seen in Figure 5A, H_2O_2 has been degraded in PAM over time. The longer the aging time is the lower the concentration of H_2O_2 will be. For instance, H_2O_2 concentration in 2 min PAM reduces 18, 45, and 80% after 1, 8, and 12 h aging, respectively. These results are in agreement with the efficiency reduction of 2 min PAM as shown in Figure 3. The correlation between the efficiency of PAM and the H_2O_2 level at different aging time manifests the important role of H_2O_2 in anti-cancer effects of PAM.

Figure 5B presents H_2O_2 concentration in the serum-free PAM after 8 h aging. The level of H_2O_2 is approximately two times higher in the serum-free PAM than serum-included

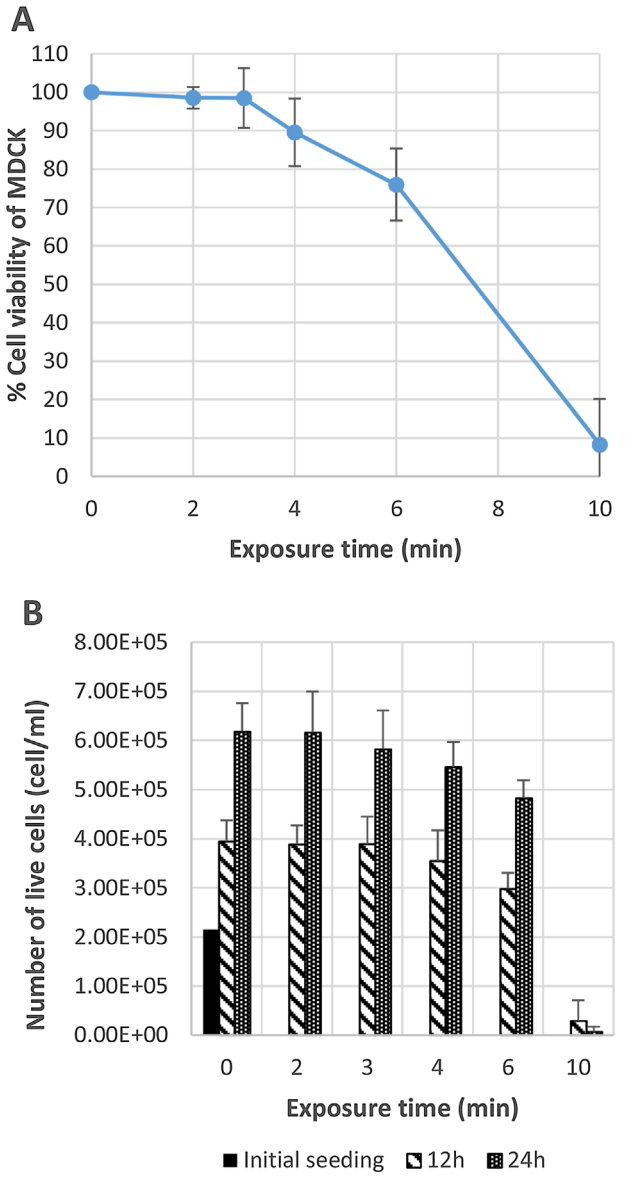


FIGURE 4 Effect of PAM produced by different exposure times on the viability of MDCK normal cells using MTS assay and trypan blue exclusion assay. A) Percent of cell viability at 12 h after PAM treatment. B) Number of live cell (cell ml^{-1}) at 12 and 24 post-PAM treatment. Initial seeding represents the number of cells at the time of PAM treatment. Results from three independent experiments with two replications are shown as means ± SD

PAM (compare with 8 h aged-PAM in Figure 5A). As it is expected the iron supplemented serum scavenges H_2O_2 generated in PAM via Fenton reaction.

EMEM PAM was used to treat normal cells and the H_2O_2 concentration in it was measured immediately after plasma exposure as shown in Figure 6. Comparing results of Figure 6 with immediate measurement presented in Figure 5A reveals that the H_2O_2 concentration (μM) induced in two PAM types is in the same order of magnitude. This outcome supports our assumption that the minor differences in EMEM and MEM chemical formula have a negligible impact on the cell viability results.

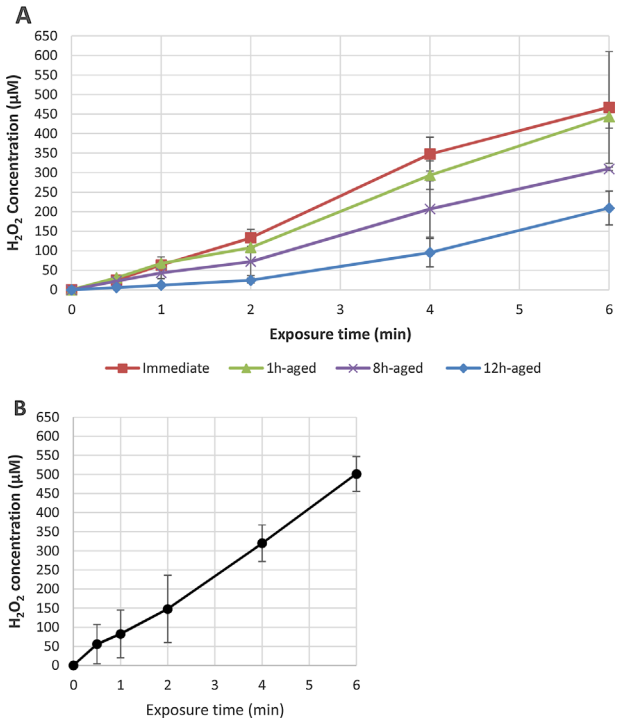


FIGURE 5 A) Concentration of H_2O_2 versus plasma exposure time measured in MEM at immediately after plasma exposure and 1, 8, and 12 h after aging. B) H_2O_2 concentration in a serum-free PAM was measured after 8 h aging. Data represents the mean ± SD of three independent experiments

4 | CONCLUSION

One of the most feasible ways to transport the reactive species in a gas phase to a target is via direct exposure of a sample to the effluent of the plasma. However, plasma treatment of a

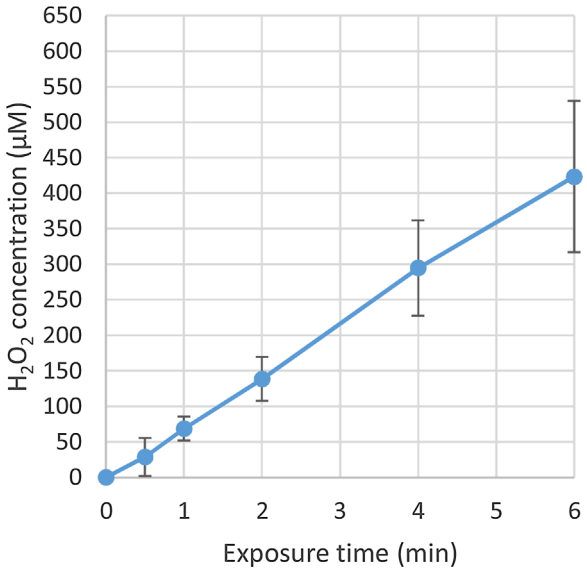


FIGURE 6 H_2O_2 concentration was measured in EMEM PAM immediately after plasma exposure

liquid sample results in the activation of the treated media by generating/dissolving reactive species in the liquid. Indeed, PAM allows for storage of long-lived reactive species for a specific time and for later application. This procedure provides an indirect treatment of a sample. Hence, the stability of the reactive species in the aqueous state is an important issue to determine the shelf life of PAM. For instance, it was shown here that added serum in media reduces the level of H_2O_2 by 50% in 8 h aged-PAM. Plasma exposure time, storage temperature, and pH of the media are parameters that can impact the stability of reactive species induced in PAM. In this paper, it was shown that the efficiency of PAM to destroy cancer cells attenuates with increasing aging time. On the other hand, the measurement of H_2O_2 induced in the treated media revealed a relatively large amount of this reactive molecule in EMEM and MEM PAM. The reduction in the level of H_2O_2 after aging indicated degradation of H_2O_2 over the time which correlates well with the reduction in the efficiency of PAM. Despite the severe killing impact of PAM on SCaBER cells, no significant reduction in the viability of normal MDCK cells was observed. Rather, our findings suggest that it is likely that PAM with shorter exposure time inhibits proliferation in normal cells which requires further investigation.

REFERENCES

[1] H. Tanaka, M. Mizuno, K. Ishikawa, K. Takeda, K. Nakamura, F. Utsumi, H. Kajiyama, H. Kano, Y. Okazaki, S. Toyokuni, S. Maruyama, F. Kikkawa, M. Hori, *IEEE T Plasma Sci*. **2014**, *42*, 3760.

[2] S. Mohades, N. Barekzi, M. Laroussi, *Plasma Process. Polym*. **2014**, *11*, 1150.

[3] C. H. Kim, S. Kwon, J. H. Bahn, K. Lee, S. I. Jun, P. D. Rack, S. J. Baek, *Appl. Phys. Lett*. **2010**, *96*, 243701.

[4] M. Laroussi, *IEEE T Plasma Sci*. **2015**, *43*, 703.

[5] A. R. Gibson, H. O. McCarthy, A. A. Ali, D. O'Connell, W. G. Graham, *Plasma Process. Polym*. **2014**, *11*, 1142.

[6] M. Laroussi, S. Mohades, N. Barekzi, *Biointerphases* **2015**, *10*, 029401.

[7] S. Kalghatgi, C. M. Kelly, E. Cerchar, B. Torabi, O. Alekseev, A. Fridman, G. Friedman, J. Azizkhan-Clifford, *PLoS ONE* **2011**, *6*, e16270.

[8] N. Barekzi, M. Laroussi, *J. Phys. D: Appl. Phys*. **2012**, *45*, 422002.

[9] S. Mirpour, H. Ghomi, S. Piroozmand, M. Nikkhah, S. H. Tavassoli, S. Z. Azad, *IEEE T Plasma Sci*. **2014**, *42*, 315.

[10] N. K. Kaushik, H. Uhm, E. H. Choi, *Appl. Phys. Lett*. **2012**, *100*, 084102.

[11] X. Zhang, M. Li, R. Zhou, K. Feng, S. Yang, *Appl. Phys. Lett*. **2008**, *93*, 021502.

[12] S. J. Kim, T. H. Chung, S. H. Bae, S. H. Leem, *Appl. Phys. Lett*. **2010**, *97*, 023702.

[13] M. Vandamme, E. Robert, S. Dozias, J. Sobilo, S. Lerondel, A. Le Pape, J. M. Pouvesle, *Plasma Med*. **2011**, *1*, 27.

[14] M. Vandamme, E. Robert, S. Pesnel, E. Barbosa, S. Dozias, J. Sobilo, S. Lerondel, A. Le Pape, J. M. Pouvesle, *Plasma Process Polym*. **2010**, *7*, 264.

[15] S. P. Kuo, C. Y. Chen, C. S. Lin, S. H. Chiang, *IEEE T Plasma Sci*. **2010**, *38*, 1908.

[16] M. J. Traylor, M. J. Pavlovich, S. Karim, P. Hait, Y. Sakiyama, D. S. Clark, D. B. Graves, *J. Phys. D: Appl. Phys*. **2011**, *44*, 472001.

[17] T. Adachi, H. Tanaka, S. Nonomura, H. Hara, S. Kondo, M. Hori, *Free Radic. Biol. Med*. **2015**, *79*, 28.

[18] R. Laurita, D. Barbieri, M. Gherardi, V. Colombo, P. Lukes, *Clin Plasma Med*. **2015**, *3*, 53.

[19] K. Wende, P. Williams, J. Dalluge, W. Van Gaens, H. Aboubakr, J. Bischof, T. von Woedtke, S. M. Goyal, K. D. Weltmann, A. Bogaerts, K. Masur, P. J. Bruggeman, *Biointerphases* **2015**, *10*, 029518.

[20] M. Wang, B. Holmes, X. Cheng, W. Zhu, M. Keidar, L. G. Zhang, *PLoS ONE* **2013**, *8*, e73741.

[21] S. Iseki, K. Nakamura, M. Hayashi, H. Tanaka, H. Kondo, H. Kajiyama, H. Kano, F. Kikkawa, M. Hori, *Appl. Phys. Lett*. **2012**, *100*, 113702.

[22] S. J. Kim, T. H. Chung, *Sci. Rep*. **2016**, *6*, 20332.

[23] D. B. Graves, *J. Phys. D: Appl. Phys*. **2012**, *45*, 263001.

[24] X. Lu, G. V. Naidis, M. Laroussi, S. Reuter, D. B. Graves, K. Ostrikov, *Phys. Rep*. **2016**, *630*, 1.

[25] Y. F. Yue, S. Mohades, M. Laroussi, X. Lu, *IEEE T Plasma Sci*. **2016**, *44*, 2550805. DOI: 10.1109/TPS

[26] S. Mohades, M. Laroussi, J. Sears, N. Barekzi, H. Razavi, *Phys. Plasmas*. **2015**, *22*, 122001.

[27] F. A. Villamena, *Chemistry of Reactive Species*, John Wiley & Sons, Inc., Hoboken, NJ, USA **2013**.

[28] D. B. Graves, *Clin. Plasma Med*. **2014**, *2*, 38.

[29] D. Yan, N. Nourmohammadi, K. Bian, F. Murad, J. H. Sherman, M. Keidar, *Sci. Rep*. **2016**, *6*, 26016.

[30] D. Yan, J. H. Sherman, X. Cheng, E. Ratovitski, J. Canady, M. Keidar, *Appl. Phys. Lett*. **2014**, *105*, 224101.

[31] X. Yan, Z. Xiong, F. Zou, S. Zhao, X. Lu, G. Yang, G. He, K. Ostrikov, *Plasma Process. Polym*. **2012**, *9*, 59.

[32] J. Köritzer, V. Boxhammer, A. Schäfer, T. Shimizu, T. G. Klämpfl, Y. F. Li, C. Welz, S. Schwenk-Zieger, G. E. Morfill, J. L. Zimmermann, J. Schlegel, *PLoS ONE* **2013**, *8*, 64498.

[33] S. Gebicki, J. M. Gebicki, *Biochem. J*. **1993**, *289*, 743.

[34] N. Kaushik, N. Uddin, G. B. Sim, Y. J. Hong, K. Y. Baik, C. H. Kim, S. J. Lee, N. K. Kaushik, E. H. Choi, *Sci. Rep*. **2015**, *5*, 8587.

[35] J. Nordberg, E. S. Arner, *Free Radic. Biol. Med*. **2001**, *31*, 1287.