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Evaluation of the Effects of a Plasma Activated Medium on Cancer Cells

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Evaluation of the effects of a plasma activated medium on cancer cells

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The interaction of low temperature plasma with liquids is a relevant topic of study to the field of plasma medicine. This is because cells and tissues are normally surrounded or covered by biological fluids. Therefore, the chemistry induced by the plasma in the aqueous state becomes crucial and usually dictates the biological outcomes. This process became even more important after the discovery that plasma activated media can be useful in killing various cancer cell lines. Here, we report on the measurements of concentrations of hydrogen peroxide, a species known to have strong biological effects, produced by application of plasma to a minimum essential culture medium. The activated medium is then used to treat SCABER cancer cells. Results indicate that the plasma activated medium can kill the cancer cells in a dose dependent manner, retain its killing effect for several hours, and is as effective as apoptosis inducing drugs. © 2015 AIP Publishing LLC.

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I. INTRODUCTION

Plasma activated media (PAM) has been shown to be at least as effective in killing cancer cells as direct treatment by low temperature plasma (LTP).^{1,2} Several investigators used PAM successfully and showed that it has killing and anti-proliferative effects against various cancers including ovarian cancer, gastric cancer, glioblastoma, and breast cancer.^{1–5} Therefore, using PAM can be a practical alternative for treating cancerous lesions since PAM has been shown to remain effective after a relatively long time of storage (hours to days depending on storage temperature).

The study of plasma in contact with liquids is an important topic with great relevance to plasma medicine since, in most biomedical applications of LTP, the plasma comes in direct contact with biological fluids. However, even before such interest came to the forefront of plasma science and plasma medicine research, many early studies on plasma-liquid interaction were already carried out (as far back as 1952; see Ref. 6) for various applications. For example, in the quest to generate a non-equilibrium air plasma discharge, several investigators used water or electrolyte electrodes.^{6–9} In the case of water electrode, an intense air discharge was generated and found to be an abundant source of the hydroxyl radical (OH) in the plasma-water boundary layer⁹ and that such discharge causes acidification of the liquid itself.^{10,11} Hydrogen peroxide (H_2O_2) was also found to be one of the main products/species generated when an electrical discharge was ignited on top of inert electrolytes.⁶ More recently and mostly driven by the advent of the many advances in the field of plasma medicine, more detailed experimental/diagnostics, and computational work, was carried out on the interaction of LTP with liquids, resulting in an in-depth understanding of the chemistry in plasma activated liquids. Details can be found in Ref. 12 (and references therein) and in Ref. 13, which discusses the case of wounds covered by a liquid layer.^{12,13}

Low temperature plasma generated in environments containing humid air produces various chemically reactive species such as OH, O_2^- , NO, H_2O_2 , and O that are known to have biological implications.^{14,15} At high concentrations, these reactive oxygen species (ROS) are known to be associated with certain diseases such as cardiovascular diseases and even cancer. But at safe concentration levels, they are also known to play key roles in protective mechanisms, such as in host immune defenses.

Some of the LTP-generated species described above have short lifetimes and do not penetrate deep into the liquid media. On the other hand, they can interact with the liquid to generate relatively stable long lived species in the bulk of the liquid. These include hydrogen peroxide (H_2O_2), nitrites (NO_2^-), nitrates (NO_3^-), and organic peroxides (RO_2). In particular, hydrogen peroxide is known to cause various oxidizing reactions in biological cells, including the peroxidation of lipids and induction of DNA damage. Hydrogen peroxide is also known to play a role in mitogenic stimulation and cell cycle regulation. Recently, Adachi *et al.* reported that hydrogen peroxide and its reaction byproducts in PAM reduced the mitochondrial membrane potential, down-regulated the expression of the anti-apoptotic protein Bcl2, activated poly (ADP-ribose) polymerase-1 (PARP-1), and released apoptosis-inducing factor (AIF) from mitochondria.¹⁶ These authors' results indicate that H_2O_2 and its byproducts in PAM disturbed the mitochondrial-nuclear network in cancer cells through a caspase-independent apoptotic pathway.¹⁶

In this paper, we report on hydrogen peroxide concentration measurements in Minimum Essential Medium that was exposed to LTP generated by the plasma pencil, and on the effects of the produced PAM on a SCABER (ATCC[®] HTB3[™]) cancer cell line originating from a human bladder cancer.

II. THE PLASMA PENCIL

The plasma pencil was used in this study to generate the low temperature plasma that was subsequently applied to a medium to produce PAM. The plasma pencil is made of a

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FIG. 1. Schematic and photograph of the plasma pencil. The photograph of the plasma pencil shows the plasma plume emitted by the device. The plume which appears as a continuous volume of plasma is in fact a train of plasma bullets propagating as an ionization wave guided in the direction of the helium flow.

hollow dielectric tube with a diameter of 2.5 cm.¹⁷ There are two disc electrodes about the same diameter as the tube that are inserted in the tube with a 0.5 cm separation and are each composed of a thin copper ring attached to a centrally perforated dielectric disc. Figure 1 shows schematic of the device and a photograph of the plasma pencil in operation.

To ignite the plasma, a high voltage in the 1–10 kV range was used in the form of repetitive, microseconds-wide pulses and applied between the two electrodes. The operating gas was helium with flow rates in the 4–6 slm range. When the discharge is ignited, it launches a plasma plume that can reach up to 5 cm into the ambient room air. The plasma plume which appears continuous to the naked eye (see Figure 1) was found to be in fact a series of fast moving plasma bullets.^{18,19} These plasma bullets are generated by fast moving guided ionization waves that follow the trajectory of the gas flow.²⁰ The plasma bullets can be looked at as vehicles delivering reactive species to a surface or a medium to achieve a certain function/outcome.

III. MEASUREMENTS OF THE H₂O₂ CONCENTRATION

A. Method and materials

To measure the concentration of hydrogen peroxide generated in a Minimum Essential Medium (MEM) after LTP exposure, an Amplex red hydrogen peroxide assay kit was used (Molecular Probes, Invitrogen, Burlington, Ontario, Canada). This kit contains Amplex red reagent, dimethylsulfoxide (DMSO), 5× reaction buffer, horseradish peroxidase, and hydrogen peroxide. This kit must be stored at −20 °C and protected from light for optimal use. The Amplex red reagent is one of the most commonly used to measure concentrations of hydrogen peroxide. Some other substrates that could be used for similar purposes are homovanillic acid and diacetylchloro-fluorescein.²¹ The fluorescence detected is proportional to the increase in the amount of hydrogen peroxide that react with the fluorescent product to produce an increase in fluorescence. When Amplex red is used, the Amplex red is oxidized by the hydrogen peroxide when horseradish peroxidase (HRP) is also present. This combination creates resorufin, a highly colored compound that can be detected using different methods. One is colorimetric which can detect resorufin using absorption at 570 nm. Another is by fluorescence which can detect resorufin using emission around 585 nm and excitation

around 570 nm. In this work, to record the fluorescent light intensity, a microplate reader (BMG Labtech FLUOstar) was used with an excitation wavelength of 544 nm. The fluorescence was collected at a wavelength of 590 nm. In this assay, the intensity of the fluorescence is typically proportional to the concentration of hydrogen peroxide.

The experimental setup for exposing the media to LTP is shown in Figure 2. For each experimental sample, 500 μl of a liquid MEM was pipetted into a 24 well plate. The bottom of the plate of samples was placed at a constant distance of 2.5 cm from the nozzle of the plasma pencil. To ignite the plasma, high voltage pulses at frequency of 5 kHz and constant pulse width of 1 μs were applied to the device electrodes and helium gas at a constant flow rate of 5 slm was flown. For the experimental results presented in this paper, three pulse amplitudes of 5, 7, 9 kV and five exposure times at 15 s, 30 s, 1 min, 2 min, and 4 min were used.

B. Results and discussion

First, a standard calibration curve was constructed. This standard curve was made up of diluted 3% hydrogen

FIG. 2. Experimental setup for generating the plasma activated media.

FIG. 3. Standard curve relating the measured fluorescence to the absolute H_2O_2 concentration.

peroxide with MEM into concentrations of 20 μM , 15 μM , 10 μM , 5 μM , and 2.5 μM . As a 0 μM , a sample of MEM was used with no hydrogen peroxide added and no exposure to plasma. Figure 3 is a plot of the standard curve.

Figures 4 and 5 show the measured fluorescence and concentrations of H_2O_2 for the three selected voltage amplitudes and at five different plasma exposure times. Based on these results, we can see that at 5 kV, the fluorescence and H_2O_2 concentration increase until around 1 minute exposure time. At 1 min, the fluorescence values and H_2O_2 concentration stop increasing and remain around the same value (28 μM), for the rest of the exposure times. At 7 kV, the fluorescence and H_2O_2 concentration increase only until 0.5 min (30 s) exposure time. After 30 s exposure time, the fluorescence intensity remains around the same value with a slight decrease at 4 min exposure time. Equally, the H_2O_2 concentration stays around a value of 28 μM and then decreases at 4 min. At 9 kV, the fluorescence intensity reaches a saturation value at 0.25 min (15 s) and remains around this level until 4

min exposure time. The corresponding H_2O_2 concentration is around 28 μM , but at 4 min, the concentration seems to drop.

From these results, some observations can be made. At an exposure time of 15 s, the hydrogen peroxide concentration for 5 kV is the smallest while the largest concentration is for a pulse magnitude of 9 kV. This is expected, as with higher applied voltages larger fluxes of species hit the surface of the media causing the hydrogen peroxide concentrations to increase. At an exposure time of 30 s, the concentration for 5 kV is smaller than those at 7 kV and 9 kV, which correlates with our previous statement. Once the hydrogen peroxide concentration levels-off around 28 μM , it appears that it reaches a saturation level indicating that there are no more reactants to bond together and form additional hydrogen peroxide. This saturation point seems to be reached quicker, the higher the voltage applied: For 5 kV, the saturation was reached after 1 min exposure time; at 7 kV, saturation was reached at 30 s; and at 9 kV, saturation was reached

FIG. 4. Fluorescence intensity versus plasma exposure time for three different applied voltages.

FIG. 5. H_2O_2 concentration in micromoles versus plasma exposure time for three different applied voltages.

Cell viability at different time after treatment

FIG. 6. SCaBER Cell viability at different times after PAM treatment showing the number of viable cells counted at 0, 12, 24, and 48 h after PAM incubation. The trend shows that PAM produced at longer LTP exposure time is more efficient in killing cells. However, PAM does not have an immediate effect on the viability of cells. Plasma pencil operating conditions are: Pulse magnitude of 7.00 kV, frequency of 5.00 kHz, 800 ns pulse width, and gas flow rate of 5 slm.

even quicker, at 15 s. The drop of fluorescence and hydrogen peroxide concentration at 4 min could be due to decomposition of hydrogen peroxide molecules by reactions with other species the concentrations of which reach high enough levels for long plasma exposure times.

IV. QUANTIFYING THE EFFECTS OF PAM ON SCaBER CELLS

A. Method and materials

The cancer cells used in this study are of the squamous cell carcinoma type and more specifically SCaBER cells isolated from a human bladder cancer. These cells are adherent with epithelial morphology. The medium was a complete growth medium (Minimum Essential Medium, MEM, with 2 mM L-glutamine, and Earle's Balanced Salt Solution) and was adjusted to contain 10% fetal bovine serum and 1% antibiotics (Penicillin/Streptomycin). The SCaBER cells were grown in the complete MEM growth media in a 75 cm² vented cell culture flask for three days to reach high confluence. One (1) ml of the suspension of SCaBER cells with the concentration of $\sim 10^6$ cells per ml were trypsinized by Trypsin solution (1×), transferred, and seeded into the 24-well plate. The cells were incubated overnight, and adherence was monitored.

The plasma treatment protocol is the following: The complete MEM is first treated by plasma to obtain a Plasma Activated Media (PAM), which is then used to replace the non-treated medium covering the SCaBER cells. So, to obtain the PAM, one milliliter of the fresh MEM growth media (containing 10% FBS, 2 mM L-glutamine and 1% antibiotics) were treated by LTP for 2 and 4 min (method of exposure is similar to the one shown in Figure 3). Immediately after exposure, the treated/activated media were transferred to the plate wells containing the SCaBER cells and incubated overnight at 37 °C and 5% CO₂. The cell viability immediately after PAM application and at various hours post PAM application was determined using trypan blue exclusion assay.

B. Results and discussion

Figure 6 shows the effects of two PAMs created by exposure of the medium to LTP for two different times, 2 min and 4 min. The 0 min represent medium that was not exposed to LTP (control). Cell viability was determined immediately after PAM application to the cells (0 h), and at 12 h, 24 h, and 48 h post-PAM application. First, it is clear from the figure that PAM created by longer exposure to LTP has the strongest effect on the cells (higher kill rate). Second, the data show that PAM had no effect on cell viability immediately after application (0 h). However, the data at 12 h, 24 h, and 48 h post-PAM application show that the longer the cells are exposed to PAM the stronger the effect on cell viability, especially for the 4 min PAM where more than 90% of the cells were killed starting at 12 h post-PAM application. These results indicate that PAM created by longer exposure to LTP contains higher concentrations of reactive species that react with the cells and therefore induce greater cell damage. The results also indicate that cell damage occur only after some time of interaction of the reactive species with the cells since no immediate effect of PAM was observed. It also appears that most of the damage occurs within the first few hours of application of PAM to the cells. Beyond this time, only some residual effect is observed.

To investigate how long the effects of plasma activated media last, PAM was stored/aged at room temperature for different times and then applied to SCaBER cells. The aging (or storage) times were 0 min (immediate application of PAM), 15 min, 1 h, and 8 h. Cells viability was evaluated 10 h post-PAM application. Figure 7 shows the obtained results for PAM created by 2 min and 4 min exposure to LTP. The non-aged PAM (marked immediate in the plot) had the best killing effect on the cells, with the 4 min PAM exhibiting the strongest kill. The 2 min PAM lost most of its effect if not applied immediately. However, the 4 min PAM maintained most of its killing effect even if aged/stored for 1 h before application and still retained some killing effect after 8 h aging/storage time. The above results indicate that PAM is better suited for immediate application after it is produced.

FIG. 7. Efficiency of PAM at 2 and 4 min LTP exposure times. Cell viability was determined 10 h post-PAM application to the cells. Longer time of plasma exposure can generate a critical amount of reactive species in the liquid media required for cell death. PAM efficiency decreases by increasing the aging time indicating that killing factors decayed with time. Plasma pencil operating conditions are: Pulse magnitude of 7.00 kV, frequency of 5.00 kHz, 800 ns pulse width, and gas flow rate of 5 slm.

However, if produced by long exposure to LTP, it can still retain some potency even if used many hours after storage at room temperature. Some investigators have recently reported that if frozen at low temperature (below 0°C), PAM can retain its potency against cancer cells for up to one month.¹⁶

To get a measure of the potency of PAM against cancer cells, a comparison between the efficacy of PAM and that of Staurosporine, an apoptosis-inducing drug, was conducted. Figure 8 shows the results. It is clear that PAM produced by 4 min exposure to LTP is at least as effective as Staurosporine in killing the SCABER cells. In fact, at 10 h post treatment, PAM outperforms Staurosporine by killing an order of magnitude higher number of cells, but for longer times, both PAM and Staurosporine seem to have similar effect.

The efficacy of PAM against cancer cells as described in Section IV is an indication that LTP causes chemical changes to the cell culture liquid medium, which, via various biochemical pathways, affect the cells themselves leading to their eventual death. Although a number of reactive species are produced in the gas-liquid interface when LTP interacts with a liquid medium, only the long-lived solvated species have direct access and impactful reactions with the cells. Measurements of the rate of production of ROS and reactive nitrogen species (RNS) in the liquid and inside the cells have previously been reported.²² Amongst these, hydrogen

FIG. 8. Staurosporine vs. PAM treatment. SCABER cells were incubated by 1 μ M Staurosporine and cell viability counted at 10 h and 24 h after treatment. The number of live cells indicates that PAM produced by 4 min plasma exposure and used for 10 h treatment has an efficiency equivalent to 1 μ M Staurosporine applied for 24 h. Plasma pencil operating conditions are: Pulse magnitude of 7.00 kV, frequency of 5.00 kHz, 800 ns pulse width, and gas flow rate of 5 slm.

peroxide is one of the most stable species with long-lasting biological effects, as has been reported by various investigators. This led us to conduct measurements of the absolute concentrations of H_2O_2 in EMEM which was used to produce PAM. Our measurements show that H_2O_2 was generated abundantly and reached a saturation concentration value rather quickly. We suspect this species, alone or in synergy with others, and its chemical byproducts (when it reacts with biological cells) to play an important role in the observed efficacy of PAM to kill SCABER cancer cells. We also suspect that other solvated species (other than H_2O_2), such as organic radicals, play some biological role and contribute to the killing efficacy of PAM, but we have no reliable experimental data on these that we can report at this time. However, it is important to point out at this juncture that, in general, the observed effects of LTP on cancer cells have been mostly attributed to the ROS and RNS that are produced in the gaseous and liquid phases, and those induced inside the cells. This makes LTP a technology that has the potential to be at the core of a novel cancer therapy.^{23,24}

V. CONCLUSIONS

In this paper, we presented measurements of absolute concentrations of hydrogen peroxide produced in plasma activated Minimum Essential Medium, MEM. We found that the H_2O_2 concentration reaches a saturation value at a time that is a function of the magnitude of the applied voltage: The higher the voltage the shorter the saturation time. The plasma activated medium, PAM, was used to kill SCABER cancer cells. PAM produced by longer exposure time to LTP is most potent, but this potency decreases with time. PAM did not show an immediate effect on cancer cells, but after several hours of exposure to PAM produced by 4 min LTP treatment (generated by the plasma pencil), cell viability of SCABER was reduced by more than 90%. PAM produced in this way can be stored at room temperature for up to 8 h and still be able to kill a substantial number of cells. The efficacy

of PAM compared favorably to that of an apoptosis inducing drug, Staurosporine, indicating that PAM could be an alternative method to kill and/or stop the proliferation of cancerous cells.

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