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Killing adherent and nonadherent cancer cells with the plasma pencil

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The application of low temperature plasmas in biology and medicine may lead to a paradigm shift in the way various diseases can be treated without serious side effects. Low temperature plasmas generated in gas mixtures that contain oxygen or air produce several chemically reactive species that have important biological implications when they interact with eukaryotic or prokaryotic cells. Here, a review of the effects of low temperature plasma generated by the plasma pencil on different cancerous cells is presented. Results indicate that plasma consistently shows a delayed killing effect that is dose dependent. In addition, there is some evidence that apoptosis is one of the pathways that leads to the death of the cells, indicating that plasma initiates cell signaling pathways. © *2015 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4905666]

I. INTRODUCTION

Plasma Medicine is a field of research that deals with the biomedical applications of low temperature plasmas (LTPs) and most specifically plasmas generated at atmospheric pressure and at biologically tolerable temperatures.[1,2] These types of plasmas can be safely applied to biological systems (cells, tissues, organs, etc.) without causing thermal damage. The plasma can be tuned to achieve various effects ranging from killing pathogens, to stimulating the proliferation of healthy cells, to destroying cancer cells.[1–3] Tuning the plasma to achieve desired effects involves judicious selection of the operating gas mixture, the type of waveform of the applied voltage (DC, pulsed DC, RF, pulsed RF, microwave, etc.), and careful adjustment of the amount of applied power to influence the electrons energy distribution function and to control the fluxes of the reactive species. There is a general agreement, based on overwhelming experimental evidence, that the plasma-generated reactive oxygen species (ROS) and reactive nitrogen species (RNS) play significant roles in most of the observed effects of plasma on biological cells and biological systems. This comes as no surprise as it is well known from cell biology investigations that ROS and RNS are involved in various biochemical events that occur on the cellular and subcellular levels. Some of these include lipids peroxidation, DNA damage, and the triggering of cellular signals.[1–4]

Of all the possible applications of LTP in medical therapy none is expected to have as much of an important scientific and societal impact as cancer treatment. Today, the work on cancer is becoming the most exciting topic in plasma medicine research. Here, first what is known about the mechanisms of action of LTP against cancer cells is briefly reviewed, then the results of the application of a particular LTP source termed the "plasma pencil" to kill cancer cells are presented.

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II. MECHANISMS OF ACTION OF LTP AGAINST CANCER CELLS

In general, LTP is applied to cells that are either submerged in a liquid medium or covered by a thin layer of a liquid/fluid. Therefore, the reactive species generated by the plasma in the gaseous state have to go through a gas–liquid interface and then diffuse into the bulk of the liquid. Since most plasma-generated reactive species are short-lived whereas the plasma-induced biological effects on cells have been observed only a relatively long time later, we hypothesize that the short lived species first react with the medium to create reaction by-products that are long-lived and it is these species that subsequently interact with the cells. These longer-lived reaction byproducts would include H_2O_2, NO_2^-, NO_3^-, RO_2 (organic peroxide), etc. Our work showed that if a medium is treated by LTP, then cells are seeded into this plasma activated medium (PAM), the effects on the cells greatly depends on the "age" of the PAM.[5] By age it is meant how long a time PAM is stored before applied on the cells. The older the PAM, the lower the killing effect on cells. Fresh PAM (not aged/applied right after plasma activation) has the strongest killing effect. Hence, we concluded that plasma reaction byproducts in the medium are responsible for killing the cells, but as their concentrations decrease with PAM storage/aging time (hours) most cells would be able to cope and survive. It is important to note that the killing of cells by PAM occurs in a delayed manner, the same as for the case when cells are directly exposed to LTP.

Based on experimental evidence, several investigators reported that cancer cells appear to be more vulnerable to LTP than their healthy counterparts.[6,7] Keidar and coworkers reported that LTP causes an increase in the expression of the oxidative stress reporter γH2A.X (pSer 139) and a decrease in DNA replication in the S-phase of the cell cycle.[8] The S-phase is the phase in the cell cycle when DNA replication occurs. These effects were much less pronounced in the healthy cells. Since LTP appears to interfere with the DNA replication phase and since cancer cells multiply more rapidly than their healthy counterparts, they are much more

susceptible to LTP-induced damage. In general, cancer cells have much higher metabolic rate than healthy cells and therefore exhibit higher levels of intracellular concentrations of ROS and RNS. Hence, exposure of cancer cells to LTP leads to an even higher intracellular oxidative stress, therefore causing their death.

Other investigators have reported that cell signaling plays an important role in the interaction of LTP with cancer cells. One such signaling pathway is the activation of caspases. Caspases are proteins that play a crucial role in apoptosis or programmed cell death. The lack of initiation of apoptosis leads to uncontrolled cell multiplication and tumor development.

Laroussi and co-workers studied caspase-3 activation in the case of squamous cell carcinoma and found higher level of caspase-3 activation in cells treated by LTP than in control samples (untreated cells), indicating that LTP induces apoptosis in these cells.[9] Ishaq *et al*. suggested that because tumor cells are defective in several regulatory signaling pathways they exhibit metabolic imbalance, which leads to a lack of cell growth regulation.[10] According to these authors, LTP-generated ROS affect the metabolism of the cancer cells by impairing redox balance, which leads to slowing down or arresting the proliferation of the cells. These investigators also reported that LTP up-regulates intracellular ROS levels and induce apoptosis in melanoma.[11] They identified that LTP exposure causes a differential expression of tumor necrosis factor (TNF) family members in the cancerous cells but not in the normal cells. They found that apoptosis in the cancer cells was induced by apoptosis signal kinase 1 (ASK1), which is activated by TNF signaling.

III. PLASMA PENCIL

The application of LTP in biology and medicine relies on the availability of various sources that can produce plasmas with gas temperatures less than about 40 °C, and at atmospheric pressure (~760 Torr). This is crucial since if LTP is to be used as a basis for a medical therapy it has to be generated at surrounding room conditions such as in a hospital or clinic setting. Among the various LTP sources, low temperature, atmospheric pressure plasma jets are most practical for biomedical applications because they readily provide plasmas to a target at room conditions.[12–14] These jets can be powered by sources covering a wide frequency spectrum that ranges from DC to microwaves. Various types of plasma jets have therefore been developed with designs tailored for specific needs. One such device, the plasma pencil, a handheld plasma jet generator developed in our laboratory, is presented here in some detail.

The electrodes of the plasma pencil are disk-shaped and spaced by a gap that can be varied from 0.5 to1 cm (see Fig. 1).[15] Each of the two electrodes is made of a thin copper ring attached to the surface of a centrally perforated dielectric disk. To ignite the plasma, high voltage (few kV) pulses at repetition rates in the 1–10 kHz range are applied between the two electrodes and a gas mixture (such as helium and oxygen) is flown through the holes of the electrodes (flow rates in the 1–10 l/min range). Plasma plumes reaching lengths up to 5 cm can be launched through the hole of the outer electrode and in the surrounding room air.[15] The length of the plume depends on the gas flow rate, the magnitude of the applied voltage pulses, their widths, and the frequency. The plasma plume exhibits low temperature and can be touched by bare hands without causing any harm. A photograph of the plume emitted by the plasma pencil and touching the author's hand is shown in Fig. 2. Since the plasma plume does not cause thermal damage even to delicate tissue, it can be applied to disinfect skin wounds resulting from cuts or burns and to destroy cancer cells without damaging healthy cells.

Using a combination of diagnostics involving intensified charge coupled device imaging, optical emission spectroscopy, and electrical probes it was discovered that the plasma plume is in fact not a continuous volume of plasma, as they appear to the naked eye, but discrete small volumes of plasma referred to as "plasma bullets," which travel at very high velocities.[16,17] Unlike regular plasma streamers, which appear at random times and spaces, these plasma bullets exhibit behavior that is highly repeatable and predictable. Lu

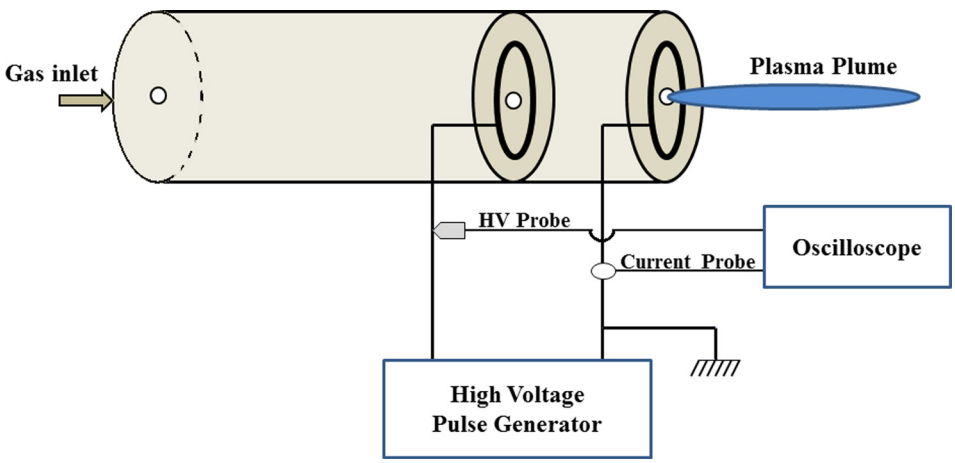

FIG. 1. Schematic of the plasma pencil.

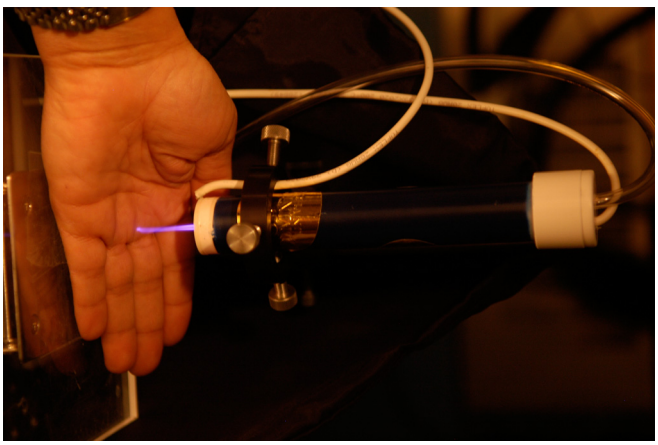

FIG. 2. Cold plasma plume touching the author's hand.

and Laroussi first proposed photoionization as a necessary mechanism to explain the propagation of the plasma bullets;[17] however, today there is consensus that plasma bullets are "guided" ionization waves where the electric field at the head of the plasma bullet plays an important role in its propagation characteristics.[14]

Because plasma bullets are launched in air, they produce very interesting reactive chemistry that can be exploited in biological and medical applications. Reactive oxygen species, such as O, OH, O_2^-, and reactive nitrogen species, such as NO, NO_2, which are known to have biological implications, are abundantly generated by the plasma bullets.

IV. EFFECTS OF THE PLASMA PENCIL ON CANCER CELLS

To investigate the efficacy of the plasma pencil against cancer both adherent and nonadherent cancerous cells were tested. As a representative of nonadherent cell lines leukemia cells were selected. As adherent cells, squamous cell carcinoma and prostate cancer cells were used. The following is a review of the results obtained. The experimental setup used in these studies is shown in Fig. 3. The reader is hereby notified that the following is not a review of the field but rather a review of our own results. These results were originally published elsewhere, in various scientific journals. References to the original papers are provided, where more details of the experiments can be found. The remainder of this paper mainly summarizes our work of the last few years on cancer application using our low temperature plasma generator, the plasma pencil.

A. Case of nonadherent cells

In this study, T-cell line (ATCC No. CCL-119; aka CCRF-CEM) originally isolated from the blood of a patient with acute lymphoblastic leukemia was used. The CCRF-CEM cells are nonadherent leukemia cancer cells, obtained from the American Type Culture Collection. They were

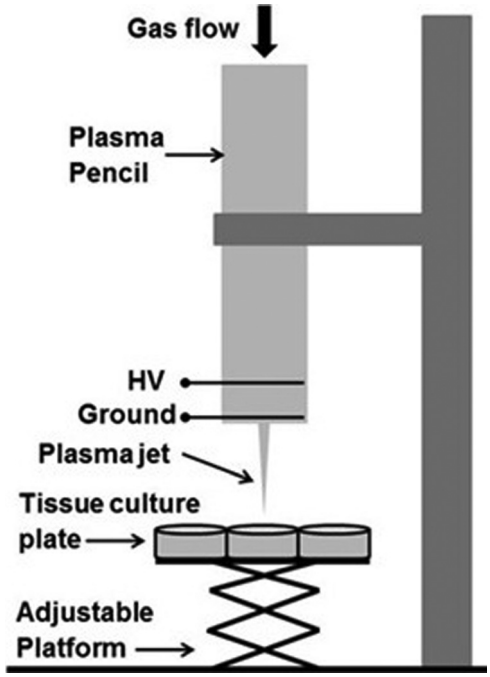

FIG. 3. Experimental setup for the exposure of samples to the plasma pencil.

grown in complete growth media in 75 cm^2 vented sterile polystyrene tissue culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2. The growth media consisted of buffered RPMI-1640 media supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin/streptomycin), and 1% glutamine.

Initial experiments revealed that plasma kills the leukemia cells and that the effects of a single dose of plasma continue for up to few days. Figures 4(a) and 4(b) illustrate results using CCRF-CEM leukemia cells, *in vitro*, treated at various doses and evaluated at 12 and 36 h post-treatment, respectively.[18] Figure 5 shows images that illustrate the difference in color between cells that took up the trypan blue dye (dead) and those that did not (live).

The results revealed that the cell viability did not differ dramatically at time 0 h postplasma treatment even at the highest dose of treatment which was a plasma exposure of 10 min. However, when post-treatment analysis of cell viability was determined at 12, 36, or 60 h, statistically significant killing was observed even at the lower plasma doses. Cell survivability data analyzed at 12, 36, or 60 h postplasma treatment revealed that there was a threshold treatment time of 3 min beyond which a highly statistically significant increase in leukemia cell death was obtained.[18] The results from this study indicate that there is a dose dependent response in the induction of cell death of leukemia cells and single doses of plasma treatment continue killing cells up to 2.5 days post treatment. The delayed response of cell death may be attributed to cell signaling cascades that may result in apoptosis. However, for higher doses, such as for 10 min exposure, necrotic cells were observed immediately after plasma exposure.

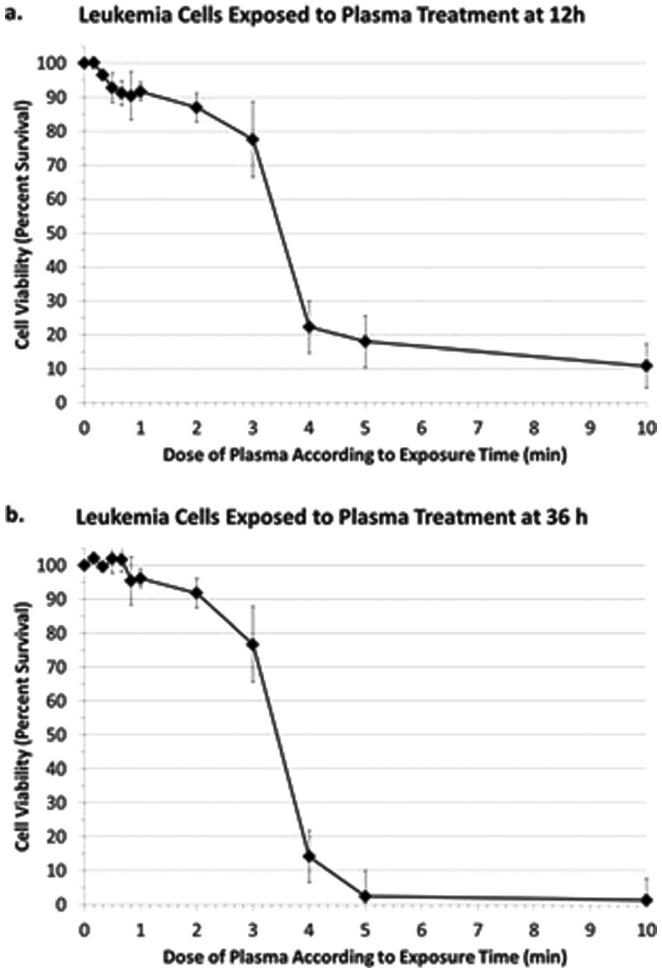

FIG. 4. Effect of varying exposure time of plasma treatment on the viability of CCRF-CEM cells. The cell viability was determined using trypan blue exclusion assays, and the values are expressed as the mean percentage of total viable cells ± standard deviation of three separate experiments. (a) Cell viability determined at 12 h postplasma treatment and (b) cell viability determined at 36 h postplasma treatment.

B. Case of adherent cells

1. Prostate cancer

The prostate cancer cells (DU 145) are metastatic cells isolated from a patient's brain, obtained from ATCC. They have been conditioned to Roswell Park Memorial Institute-1640 media supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin/streptomycin), and 1% glutamine.

Cells were exposed to the plasma pencil in the same manner as described above (see Fig. 3). At 0 h post-treatment, plasma exposure times up to 10 min seemed not to have an effect on the prostate cancer cells (DU 145). However, starting with 24 h post-treatment, a slow but steady trend of cell death for plasma exposure times longer than 5 min was observed. Figure 6 shows cell viability in percent versus plasma exposure time, 48 h post-treatment.[19] The figure shows that plasma exposure times less than 5 min do not induce cell death at all. However,

FIG. 5. Photograph of the trypan blue exclusion assay showing the live vs dead cells after 3 min of exposure to plasma treatment sampled at 12 h post-treatment. A ratio of 1:1 of the trypan blue dye (0.4% concentration) to 10^6 cells in media was used and the mixture was loaded on a hemocytometer. The cells that are dead absorb the trypan blue dye while live cells do not. The difference is clearly observed using a bright field microscope at a magnification of 25×.

starting around 5 min a slow decrease in viable cells is observed as the exposure time increases. At a plasma exposure time of 10 min, more than 20% of the cancer cells are destroyed. Figure 7 shows images of the control DU 145 cells and that of cells treated for 10 min. The cells sample exposed to plasma for 10 min shows extensive cell damage.[19]

The delayed killing of cells (24 h and longer post-treatment) indicates that the destruction of the cells is not achieved by brute force at the time of plasma exposure. It rather seems to indicate that plasma triggers biochemical reactions that require some time to induce internal cellular effects that can lead to cell death. If this hypothesis is correct it would be consistent with cell signaling mechanisms associated with apoptosis.

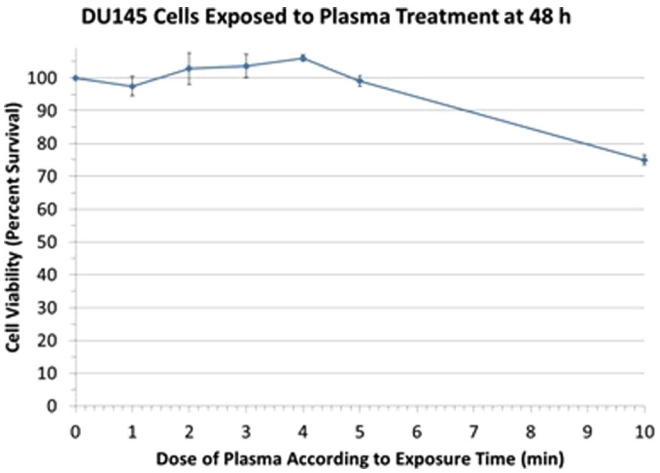

FIG. 6. Percent viability vs exposure time for cancer cells. Data taken 48 h postexposure.

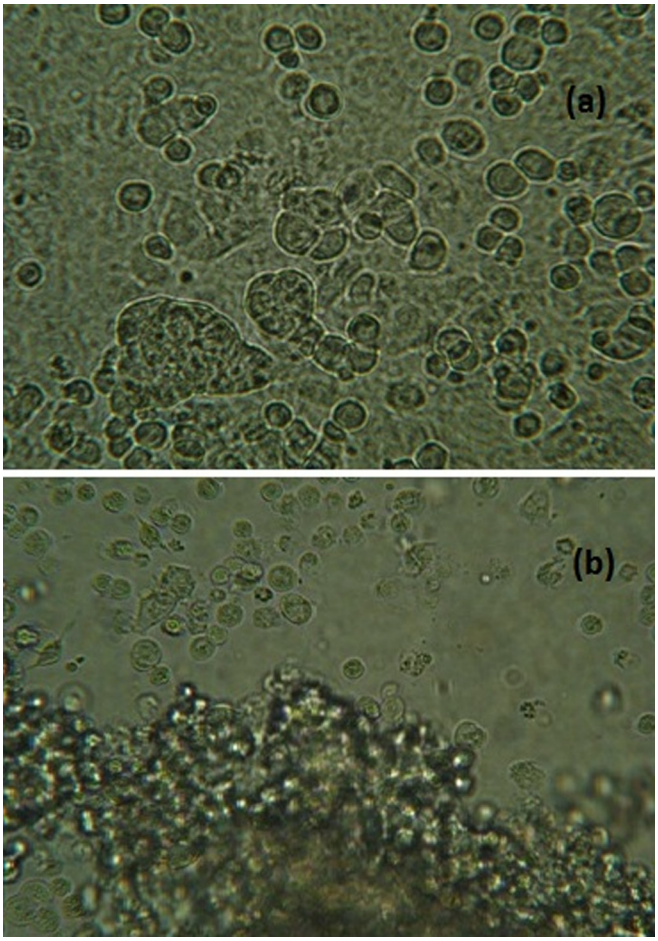

FIG. 7. Morphology of DU 145 cancer cells, control (a) and plasma treated for 10 min (b).

2. Squamous cell carcinoma

In this study, the human bladder cancer cell line SCaBER (ATCC® HTB3™), originating from a patient with squamous cell carcinoma, was used (see image in Fig. 8). These type of cells are adherent with epithelial morphology. They were grown in complete growth media in a vented tissue culture flask and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The complete growth minimum essential medium (MEM with 2 mM L-glutamine and Earle's balanced salt solution) was adjusted to contain 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin).

After adding 1 ml trypsin to the suspension of cells with a concentration of $\sim 10^6$ cells/ml, the suspension was transferred and seeded into the 24-well plate. The cells were incubated overnight and adherence was monitored. Each sample in the 24-well plate was treated by the plasma pencil at different exposure time. The distance from the tip of the nozzle of the plasma pencil and the surface of the media was 2 cm. The plasma exposure times were 2, 3.5, and 5 min, and the untreated cells were kept as control for the cell viability assays.

Trypan blue exclusion assay was used to determine the viability of SCaBER cells. A ratio of 1:1 of the trypan blue dye (0.4% trypan blue solution) to cells in MEM solution was used. Cell counting (live versus dead) was carried out by the use of a hemocytometer counting chamber under a phase-contrast bright field microscope. The image of cells after the trypan blue exclusion assay is shown in Fig. 9. The dead cells retain the blue dye and are indicated by an arrow, while the clear cells demonstrate live cells and are indicated by a circle. The numbers of live and dead cells were counted at 0, 12, 24, and 48 h postplasma exposure. Subsequently, the total numbers of viable cells were calculated.[7]

The results of cells viability are shown in Fig. 10.[9] The counts immediately after plasma pencil treatment at time 0 h

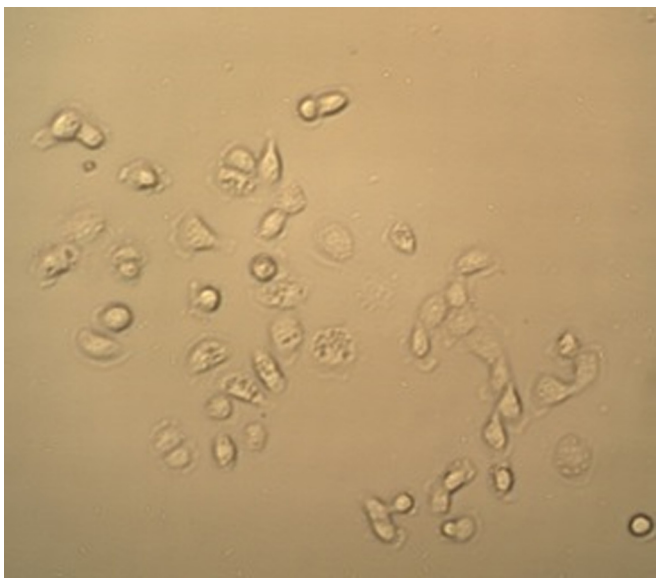

FIG. 8. Image of untreated SCaBER cancer cells grown in MEM complete growth media at a magnification of 10× using an inverted bright field microscope.

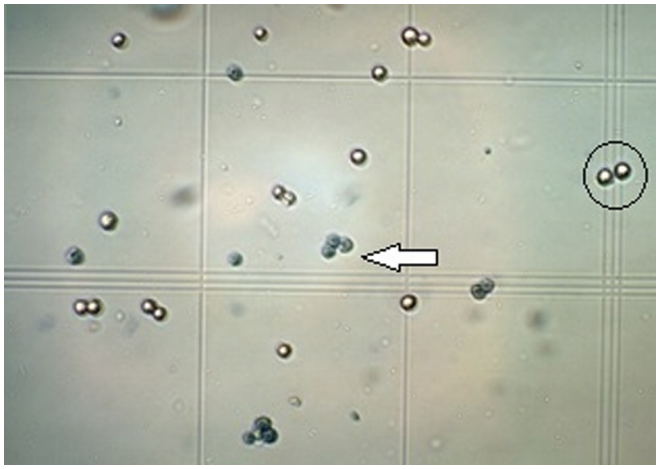

FIG. 9. Image of SCaBER cells after the trypan blue exclusion assay showing cells treated by LTP for 2 min at 12 h postplasma exposure. Live cells are bright/whitish (indicated by the circle) as the dye cannot penetrate into the intact cell membrane while dead cells are stained blue/dark (indicated by the arrow). The image is at 10× magnification using a bright field microscope.

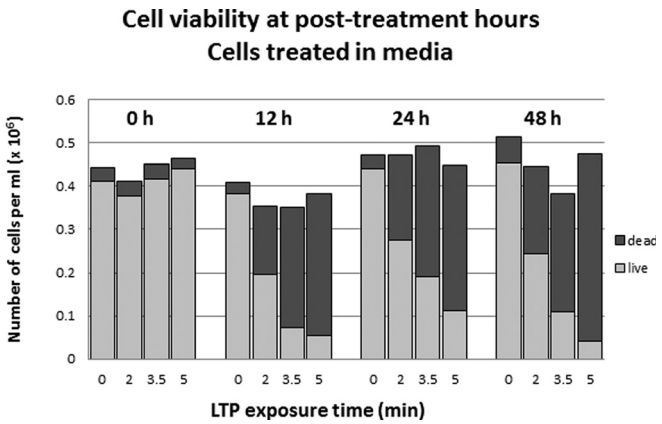

FIG. 10. Cell viability of SCaBER cells treated in media reveal dead (dark bars on top) and live (gray bars below). The viability was monitored for 0, 12, 24, and 48 h post-LTP exposure.

reveal no dead cells, suggesting that there were no immediate physical effects. However, the viability of cells at 24 h after 2 min plasma treatment was reduced to 50%. For 5 min plasma treatment, only about 25% of cells remained viable at 24 h postplasma exposure, indicating that the higher the dose of the plasma the lower the number of the cells that survived. The late effects of plasma at 48 h postplasma exposure revealed higher efficacy of killing where, 5 min plasma treatment diminished the viability of SCaBER cells to approximately 10%.

Experiments were also conducted where the medium was first treated by the plasma pencil and then the PAM was added to the cells. The results were similar to the ones described above when the cells were already in the medium under plasma treatment.[9] They indicate that the ROS, RNS, and their derivatives/byproducts generated in the medium by the plasma play a major role in the killing of the cells.

To test if cell signaling plays a role in leading to apoptosis caspase-3 activation assay was used. Caspase-3 is a protein involved in the apoptotic pathway that interacts with caspase-8 and caspase-9 through a sequential process that activates cell apoptosis. Monitoring the levels of caspase-3 is a good indicator of the apoptotic pathway activity. Results from the caspase-3 activation assay suggested that the onset of apoptosis occurs on or before 12 h postplasma exposure and that 1 min of plasma treatment was the optimal dose for eliciting the highest apoptotic activity based on the caspase-3 levels in this study.[9]

The cells detachment/reattachment function was also examined. Figure 11 shows images that provide a timeline of cell detachment and reattachment after plasma exposure.[9] From these images it is apparent that it takes longer time for the cells to reattach to the surface of the flask as the plasma

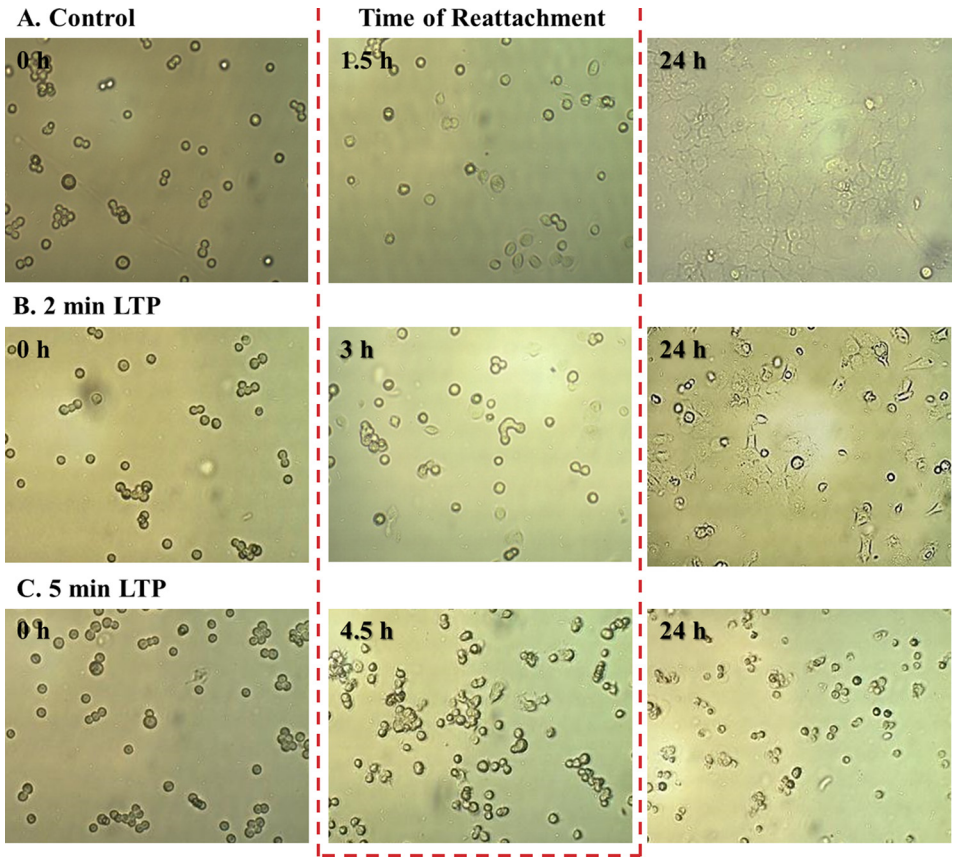

FIG. 11. Images of SCaBER cells reattaching to the surface of the culture plate at different times after reseeding. Photos were taken at specific times as noted on each image. Panel A is the control SCaBER cells that were not treated. The untreated cells adhere and are responsive at 1.5 h post-reseeding. Panel B is a 2 min LTP treatment and panel C is a 5 min LTP treatment. The earliest reattachment time post-reseeding for the 5 min LTP treatment was at 4.5 h as indicated by a few cells beginning to adhere to the surface.

treatment time increases. In this case, as seen in Fig. 11, the onset of reattachment of suspended SCaBER cells takes place after 1.5, 3, and 4.5 h for LTP treatment times corresponding to 0 (control), 2, and 5 min, respectively. This result is an indication that LTP exposure affects the normal function of the cell membrane ligands that allows it to attach and adhere to surfaces.

V. CONCLUSIONS

This paper reviewed our experiments which used one of our plasma jet generators, the plasma pencil, to evaluate the effects of LTPs on various cancer cell lines. This line of research has recently become of great interest to the plasma medicine community since LTP appears to have most of the desirable attributes that could lead to a new therapy. These include selectivity, works as well *in vivo*, minimal side effects, etc.

In our experiments, we tested, *in vitro*, both nonadherent and adherent cells. LTP worked well for both types. All experiments shared some consistent outcomes: (1) dose dependency; (2) delayed effects by hours and days; and (3) single exposures have measurable effects.

Since the cells were always in a suspension within a growth medium, it is reasonable to assume that the plasma interacts more directly with the medium than the cells themselves. Most of the reactive species generated by the plasma are very short-lived and probably do not reach the actual cells. Therefore, we think that these short-lived reactive species react with the media, generating long-lived species that cause the observed effects. These include H_2O_2, NO_2^-, NO_3^-, RO_2, etc. Since the observed effects only appear several hours/days later, we hypothesize that these species trigger biochemical events within the cells (intracellular) that follow slow progressing cascades in cell signaling pathways. As possible evidence, caspase activation, which can lead to apoptosis, was measured: For the case of SCaBER cells, we were able to verify that under some conditions caspase-3 was activated by LTP.

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