On Plasma Fractionation Treatment and Its Implications in Cells

Nishtha Gaur, Robert D. Short, and Sarah L. Allinson

***Abstract*—Here, we present a novel plasma treatment regime—plasma fractionation, analogous to the concept of dose fractionation in radiotherapy, which could see the application in plasma-based cancer treatment. In plasma fractionation, a single acute dose of plasma is divided into multiple small dosages (fractionated dosages) and administered to the cells *in vitro* at 24-h intervals. We utilized a helium plasma jet and studied the effects of plasma fractionation in an immortalized keratinocyte line (HaCaT) and a squamous cell carcinoma line (A431). The effects were assessed over three cell seeding densities—8000, 3500, and 1000 cells/well. Our results show that at all seeding densities, plasma fractionation produced lower levels of cell death in both cell types compared to the same dose administered as a single plasma treatment. This highlights the potential of plasma fractionation as a potentially safer method to conduct plasma treatments in the future. We also show that A431 cells were more sensitive to a single acute plasma treatment than HaCaT cells, at cell densities that are subconfluent (1000 cells/well). A similar difference in sensitivity between HaCaT cells and A431 cells was not observed on exogenous treatment with hydrogen peroxide, pointing to the importance of other shorter lived plasma components.**

***Index Terms*—Cold atmospheric plasma, keratinocytes, plasma fractionation, plasma jet, plasma oncology.**

I. Introduction

COLD ATMOSPHERIC plasma, or plasma hereon, has gained significant attention as a possible new medical therapy for the treatment of different types of cancers, including glioblastoma, osteosarcoma, lung cancer, breast cancer, and melanoma [1]. In this direction of plasma-based oncology, various avenues, including plasma device designs, plasma operational parameters, and treatment regimes, are being researched. Two common approaches being investigated for the plasma-based treatment of tumors are—1) direct method wherein the tumor is directly irradiated with a plasma treatment device and 2) indirect method wherein the target is injected with a liquid (such as cell-culture media or buffered solution) pretreated with plasma [2], each posing its own advantages and disadvantages. For instance, shorter lived and highly reactive plasma-generated reactive species are delivered into the tumor in case of direct treatment, thus making it perhaps more effective than indirect treatment, however, it may induce cytotoxic and genotoxic effects in noncancerous cells as well [3], [4]. This highlights the significance of optimizing the operational parameters of plasma devices to achieve the therapeutic benefits safely.

In this study, we investigate the method of "plasma fractionation" wherein a single acute dose of directly applied plasma is divided into multiple small doses and administered over a period of a few days. The concept of dose fractionation has proven critical to the effective use of radiotherapy in cancer treatment, where it is essential that tumor killing is maximized while minimizing side effects due to damage to adjacent normal cells. Dose fractionation aims to address one or more of the classic five Rs—1) repair; 2) repopulation; 3) reoxygenation; 4) redistribution; and 5) radiosensitivity [5]. For instance, applying small dosages over a prolonged period of time can allow time for normal cells to "repair" their DNA from sublethal damage [6], with their damage response pathways, typically being more effective than those in cancer cells, thus reducing the side effects. Fractionation can also be beneficial for "reoxygenation" wherein hypoxic tumors can progressively recover their level of oxygen tension during the gaps between various dosages. Dose per fraction also impacts the sensitivity of cells to radiation ("radiosensitivity"), which can also depend on the "redistribution" of cells in the cell cycle. For instance, cells in the late-S or early-G1 phase show most radioresistance, while cells in G2/M transition are most radiosensitive [7].

The biological effects of fractionation have, therefore, been extensively explored in the field of radiobiology [8], however, never in the field of plasma medicine. Dose in plasma medicine has proven contentious to define, given the multiple components delivered by the plasma; and we do not attempt to quantify herein what is the total dose. Considering, for this study, the dose is equivalent to the plasma exposure time, then the approach of fractionation does not change the total plasma dose given in each experiment, merely it temporally divides this dose into different quantities. However, we acknowledge that, for example, four short fractionated doses may not induce exactly the same chemistries in the target as one single dose, as the plasma-induced chemistry during solution treatment may reach a different equilibrium for a short versus long plasma

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This work did not involve human subjects or animals in its research.

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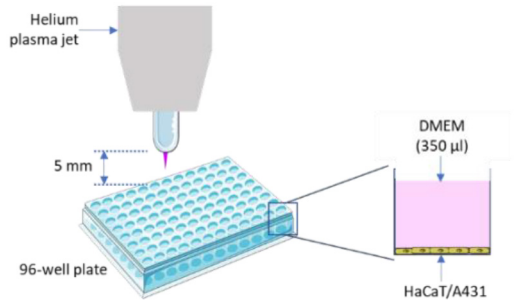

Fig. 1. Schematic of plasma treatment methodology used in this study.

delivery duration. We investigated the cytotoxicity of a fixed plasma dose (1 min) fractionated versus the equivalent single plasma dose in an *in vitro* cell-culture system using immortalized human keratinocytes (HaCaT) and a human squamous cell carcinoma (A431) cell line. Both cell lines are well established and extensively cited epidermal models, see, for example, [9] and [10]. Moreover, proteomic analysis supports their suitability as models for normal human epidermal keratinocytes and malignantly transformed keratinocytes, respectively [11]. Accordingly, both have been employed in the field of plasma medicine to evaluate the sensitivity of cancer cells to plasma compared to normal cells (see, for example, [12]–[14]).

II. Methodology

A. Cell Culture

HaCaT and A431 cell lines were both cultured in DMEM (Lonza) supplemented with 10% fetal calf serum (Labtech) and 100 U/ml penicillin and 100 μg/ml streptomycin (Lonza) at 37 °C in a humidified incubator with 5% carbon dioxide. Cells were seeded at three cell densities—1000, 3500, and 8000 cells/well before culturing overnight in a 96-well multiwell plate.

B. Plasma Treatment

A helium (He) plasma jet (previously described in our work [4] was operated at a peak-to-peak voltage of 10 kV, a frequency of 30 kHz, and a gas flow rate of 0.500 standard liters per minute (slpm).

Cells seeded at indicated densities were then exposed to plasma at a fixed distance of 5 mm (between the top of the plate and the end of the plasma glass tube nozzle) as shown in Fig. 1. Old DMEM was replaced with 350 μl of fresh DMEM, once, before the first plasma treatment.

As described in Table I, fractionated plasma treatments involved 15 and 30 s per treatment over four and two days, respectively, and a single acute plasma dose of 60 s for one day. The volume prior/post plasma treatment was measured showing no significant evaporation. The pH of plasma-treated DMEM remained unchanged under these treatment parameters due to its buffered nature.

C. Trypan Blue Staining

The cells were washed with 1× phosphate buffer saline (PBS) and then fixed with 70% ethanol for 5 min, followed by three washes with PBS. 100 μl of 0.2% v/v trypan blue (TB) solution (prepared in PBS) was added to the wells for 5 min, followed by three washes with PBS. The plates were left to dry in air and observed under a brightfield microscope at 40× objective magnification. The images were captured with a digital camera with appropriate calibrations to assess the scale bar using ImageJ software.

TABLE I
Treatment Methodology for Plasma Fractionation. Three Different Types of Treatments Were Conducted: (I) 15 s on Days 1–4, (II) 30 s on Days 1 and 2, and (III) 60 s on Day 1

	Day 1	Day 2	Day 3	Day 4	Total treatment time
(I)	15 s	15 s	15 s	15 s	15 s x4 = 60 s
(II)	30 s	30 s			30 s x2 = 60 s
(III)	60 s				60 s x1 = 60 s

D. XTT Cell Viability Assay

After the plasma treatment, on the fifth day, XTT cell assay (TACS, R&D Systems, U.K.) was performed as per the manufacturer's instructions. The absorbance was measured at 490 nm (reference data at 630 nm) using a plate reader. The percentage of cell viability was calculated by a formula described as follows:

$$\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\%. \quad (1)$$

E. Hydrogen Peroxide Concentration

The concentration of hydrogen peroxide (H_2O_2) produced in 350 μl of DMEM in a 96-well microwell plate after 15, 30, and 60 s of plasma treatment was measured using an amperometric sensor (ISO-HPO-2, World Precision Instruments).

III. Results and Discussion

To assess the effect of cell density on the extent of damage after fractionated plasma dosages in cancer (A431) and non-cancerous (HaCaT) cells, we studied fractionation at three cell densities—1000, 3500, and 8000 cells/well. After four days (day 5), the cells exhibited different extents of confluency as discussed as follows.

First, a qualitative assessment of cell growth of untreated HaCaT and A431 cells on days 1 and 5 at different seeding densities was determined using a TB staining method. As shown in Fig. 2, in the absence of treatment, both cell lines seeded at 8000 cells/well were fully confluent and, therefore, largely quiescent by day 5, while the cells seeded at 3500 and 1000 cells/well remained subconfluent and were still actively dividing.

Fig. 3 shows the changes in cell viability after fractionated plasma treatments at different seeding densities. For both the cell types (HaCaT and A431), fractionation into multiple short plasma exposures proved less toxic than a single "long" exposure (i.e., cell death: 1×60 s $> 2 \times 30$ s and 4×15 s). In the case of 8000 and 3500 cells/well, cell viability after

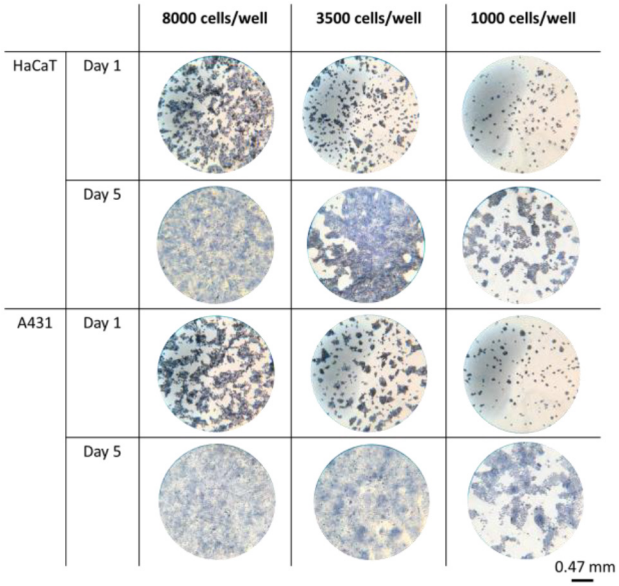

Fig. 2. Panel showing TB-stained HaCaT and A431 cells (untreated), grown overnight, on days 1 and 5 at seeding densities of 8000, 3500, and 1000 cells/well.

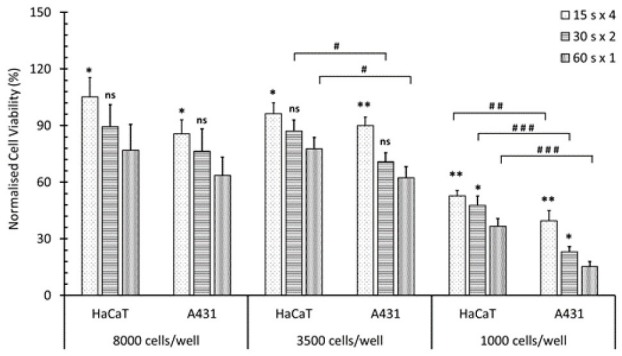

Fig. 3. Cell viability of HaCaT and A431 cells seeded at 8000, 3500, and 1000 cells/well after plasma treatment. The plasma jet was operated for 15, 30, and 60 s for 4, 2, and 1 day(s), respectively, and viability was measured on the fifth day. Statistical analysis was performed using a student t-test. Symbols * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) indicate a statistically significant difference (in HaCaT and A431) between fractionated dosages (15 s $\times$ 4 and 30 s $\times$ 2) and single dose (60 s $\times$ 1), and "ns" implies the difference was insignificant ($p > 0.05$). Symbols # ($p < 0.05$), ## ($p < 0.01$), and #### ($p < 0.0001$) show the statistically significant difference between HaCaT and A431.

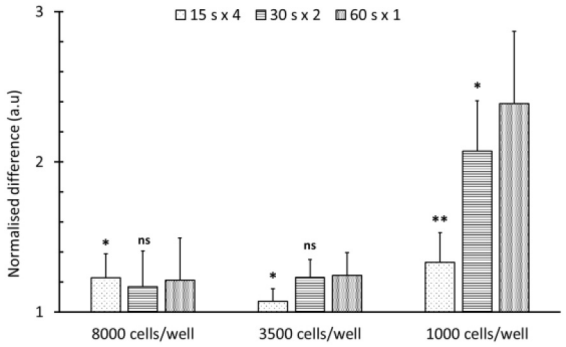

Fig. 4. Percentage viability of HaCaT cells normalized against the percentage viability of A431 cells at 8000, 3500, and 1000 cells/well after plasma treatment. The plasma jet was operated for 15, 30, and 60 s for 4, 2, and 1 day(s), respectively, and viability was measured on the fifth day. Statistical analysis was performed using a student t-test. Symbols * ($p < 0.05$) and ** ($p < 0.01$) indicate a statistically significant difference between fractionated dosages (15 s $\times$ 4 and 30 s $\times$ 2) and single dose (60 s $\times$ 1), and ns implies the difference was insignificant ($p > 0.05$).

one 60 s plasma treatment was found to be significantly lower than after four 15 s plasma treatments, but the difference was insignificant when a single high dose is compared to two 30 s treatments. However, at 1000 cells/well, the extent of cell damage in both the four 15 s and two 30 s doses was significantly lower than one 60 s. All of these data are consistent with the view that fractionation reduces the extent of cell damage.

We also wanted to compare the effects of fractionation between A431 (cancer cell line) and HaCaT (noncancerous) cells. From Fig. 3, it appears that for all the seeding densities, A431 cells were more sensitive to plasma treatment than HaCaT cells. However, we can observe that at high cell density (8000 cells/well), neither single nor fractionated plasma treatments resulted in statistically significant differences in viability ($p > 0.05$) between A431 and HaCaT cells. However, as the cell density was reduced to 3500 and 1000 cells/well, the difference between cell viabilities of A431 and HaCaT cells became more prominent. For example, at 3500 cells/well, the viability of A431 cells was significantly lower after 2 $\times$ 30 s and 1 $\times$ 60 s plasma treatments than that of HaCaT cells under the same treatment conditions. At the lowest cell density of 1000 cells/well, HaCaT cells were significantly less sensitive to plasma than A431 cells under any given treatment condition. To compare more clearly the effects of plasma fractionation in HaCaT and A431 cells, we normalized the cell viability of HaCaT cells against the viability of A431 cells for each plasma treatment condition, as shown in Fig. 4. A normalized value of 1 indicates no preferential impact of the plasma on the viability of either cell type (for clarity, the y-axis of Fig. 4 starts at 1). A normalized value >1 indicates a preferential (negative) impact on A431 and < 1 a greater impact on HaCaT. Fig. 4 shows that at lower cell density (e.g., 1000 cells/well), there may well be a preferential impact on A431 and moreover that this effect is greater in a single plasma dose than in fractionated doses. However, based upon a single study in cell culture, we need to be wary of concluding "selectivity" toward A431. Nevertheless, these results do reveal that various factors, such as the rate of cell division in a tumor, will affect whether a single dose (or indeed a fractionated dose) will be the more effective choice for cancer treatment. This may mean considering different treatment modalities for aggressive versus indolent tumors. And these results highlight the need for greater experimentation and the adoption of more realistic models of cancer.

Figs. 3 and 4 highlight that cell seeding density can be an important variable in determining the sensitivity of different cell types to any plasma treatment modality and the need for careful assessment of results when using *in vitro* assays. The phenomena of contact inhibition, leading to exit of the cell cycle (i.e., quiescence) in confluent cultures and also of quiescent cells being less sensitive to DNA damage-induced cell death are both well-established concepts in cell

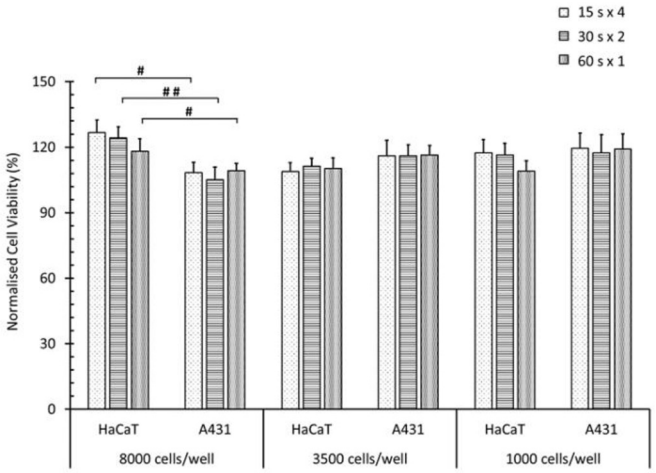

Fig. 5. Cell viability of HaCaT and A431 cells seeded at 8000, 3500, and 1000 cells/well after helium gas (no plasma) treatment for 15, 30, and 60 s for 4, 2, and 1 day(s), respectively, and viability was measured on the fifth day. Statistical analysis was performed using a student *t*-test. The symbols # ($p < 0.05$) and ## ($p < 0.01$) show the statistically significant difference between HaCaT and A431.

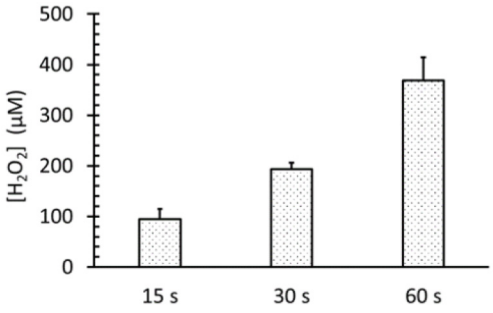

Fig. 6. Concentrations of H_2O_2 delivered by the He plasma jet in DMEM after 15, 30, and 60 s of treatment. The measurements were conducted using an amperometric sensor.

biology. An additional mechanism has also been proposed for the reduced sensitivity of high-density cultures to oxidative stress based on their having a greater antioxidant capacity per well, which results in greater quenching of extracellular reactive oxygen species [15]. Consistent with these phenomena and our observations, it has been previously reported that confluent HaCaT cells are more resistant to oxidative stress than their subconfluent counterparts [16].

Furthermore, studies have shown that other intrinsic properties of the cell type can manifest within the plasma-induced effects [17], [18]. For instance, the metabolic activity of A431 was significantly reduced upon exposure to plasma cf. HaCaT [12]–[14]. The increased inhibitory action of plasma in A431 seemed to be associated with elevated apoptosis in A431 via increase of intracellular reactive oxygen species (ROS) and/or increased production of pro-inflammatory molecules [13], [14]. Moreover, the strength of the surface attachment of A431 cells is lower than that of HaCaT cells [19]. This highlights the potential role that mechanical forces arising from the He gas flow may play during the plasma treatment, which could impact the results. To examine this, we conducted a He gas treatment, i.e., without plasma for the same conditions as previously described in Table I. This also served as a negative control for our experiment.

The results in Fig. 5 show that for all the seeding densities, i.e., 8000, 3500, and 1000 cells/well, He gas treatment (fractionated and single dose) does not affect the viability of A431 and HaCaT cells. When comparing the viabilities of A431 cells at 3500 and 1000 cells/well with HaCaT cells, we do not observe any statistically significant difference. However, at the highest density of 8000 cells/well, cell viability relative to untreated cells was higher for HaCaT cells than that for A431 cells under the same He gas treatment conditions. This result is repeatable and significant. We would like to point to the fact that one of the known effects of He gas is to "sparge" the culture media [20], [21], and the reduced oxygen tension may favor keratinocytes [22] at the higher seeding density. This result may warrant further consideration from the perspective of understanding how the artificial environment of cell culture (at high oxygen tension relative to physiological levels) perturbs cell metabolism. From our perspective, the data in Fig. 5 confirm that the changes in cell viability after the plasma treatment (Fig. 3) are due to plasma-produced ROS, and not the gas flow.

A further effect of gas flow we noticed, most marked at the lowest cell density, was the distribution of the A431 cells toward the edges of the well (data not shown), an observation also made by other researchers [23], [24]. We checked our plasma-treated cells (Fig. 3) and also observed a similar migration of cells to the edges of the well at 1000 cells/well. We do not think this is a direct effect of the gas flow (i.e., blowing cells off the bottom of the culture well), as the gas flow hardly perturbed the top surface of the culture media. However, we note this observation for other researchers to check and acknowledge that this effect is a subject of further investigation.

To further understand the role of plasma-delivered ROS, we measured the production of H_2O_2 in cell-culture media after each treatment condition and administered the same concentrations of H_2O_2 solution in a "fractionated" fashion to the cells. H_2O_2 is regarded as the main species which contributes to plasma-induced damage in cancer cells [25]–[27]. Exogenously generated H_2O_2 enters through aquaporins in the cell membrane and reacts with lipids, proteins, and DNA. Intracellularly, it undergoes Haber–Weiss reaction with superoxide radicals ($O_2^{-\bullet}$) or Fenton reaction with metal ions, such as cellular iron, to form the highly reactive hydroxyl radicals (•OH), which can directly attack DNA. Considering the long duration (five days) of our study, we focused only on investigating this longer-lived ROS. Furthermore, other previous studies have shown that >95% of ROS produced by plasma in tissue fluid model are H_2O_2 [28]–[30].

As shown in Fig. 6, the production of H_2O_2 increases with plasma treatment time, such that 15, 30, and 60 s of plasma treatment produced 95, 193.5, and 368.7 μM of H_2O_2, respectively. To replicate the plasma fractionation, 20 μl of DMEM was replaced with 20 μl of H_2O_2 (95 μM added on days 1–4, 193.5 μM on days 1 and 2, and 368.7 μM on day 1), as shown in Table II.

TABLE II
TREATMENT METHODOLOGY FOR "H_2O_2 FRACTIONATION." THREE DIFFERENT TYPES OF TREATMENTS WERE CONDUCTED: (I) 95 μM ON DAYS 1–4, (II) 193.5 μM ON DAYS 1 AND 2, AND (III) 368.7 μM ON DAY 1

	Day 1	Day 2	Day 3	Day 4
(I)	95 µM	95 µM	95 µM	95 µM
(II)	193.5 µM	193.5 µM		
(III)	368.7 µM			

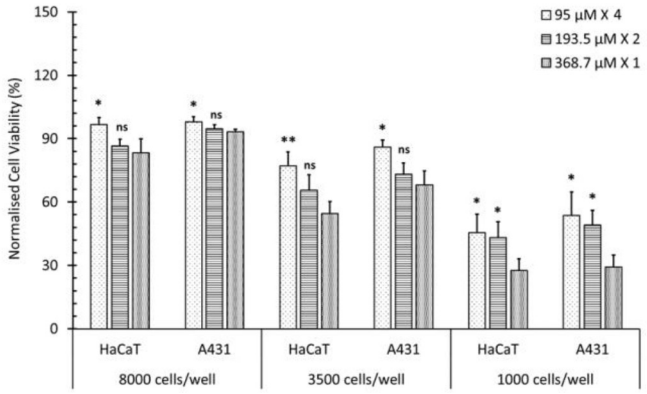

Fig. 7. Cell viability of HaCaT and A431 cells seeded at 8000, 3500, and 1000 cells/well after H_2O_2 treatment. 95, 193.5, and 368.7 μM of H_2O_2 were added to the cells for 4, 2, and 1 day(s), respectively, and viability was measured on the fifth day. Statistical analysis was performed using a student t-test. Symbols * ($p < 0.05$) and ** ($p < 0.01$) indicate statistically significant difference (in HaCaT and A431) between fractionated (95 μM $\times$ 4 and 193.5 μM $\times$ 2) and single dose (368.7 μM $\times$ 1), and ns implies the difference was insignificant ($p > 0.05$).

Fig. 7 shows that at cell densities of 8000 and 3500 cells/well, for A431, four doses of 95 μM H_2O_2 induced a significantly lower extent of cell damage than the single acute dose of 368.7 μM. But the difference between cell viability after two doses of 193.5 μM and a single dose of 368.7 μM was not significant. At the lowest cell density of 1000 cells/well, fractionated doses of two 193.5 μM and four 95 μM were both significantly less harmful to A431 than a single dose of 368.7 μM. The same trend was also observed in HaCaT cells. Overall, Fig. 7 indicates that similar to plasma fractionation, fractionated doses of H_2O_2 are significantly less harmful to both the cell types than a single dose. However, unlike plasma treatment (Fig. 3), the data in Fig. 7 show that following H_2O_2 treatment, the viability of cancer cells (A431) was not significantly different from that of noncancerous cells (HaCaT), irrespective of the cell number.

Taken together, the results (Figs. 3 and 7) highlight that the effects induced by plasma treatment may arise from an interplay between a wide range of plasma-generated species other than H_2O_2,, such as $O_2^{-\bullet}$, $\bullet$OH, peroxynitrite ($ONOO^{--}$), and singlet oxygen ($^1O_2$). These species may also undergo reactions with H_2O_2 to induce cell-damaging signaling mechanisms. For example, Girard *et al.* [31] demonstrated the synergistic effect of H_2O_2 and nitrites in plasma-induced cell death. It has further been shown that plasma-induced H_2O_2 and $ONOO^-$ undergo reactions in the vicinity of cells to form $^1O_2$, which in turn can inactivate membrane-bound catalases, increasing local concentrations of H_2O_2, depleting intracellular glutathione, and ultimately inducing apoptotic pathways in nearby cancer cells [32], [33]. This $^1O_2$-induced catalase inactivation and associated bystander effects are not observed in nonmalignant cells after plasma treatment [34]. It is important to note that fractionation does not change the total dose of any of these species delivered over the course of the entire experiment, merely how they are delivered—in several smaller quantities, or in one larger amount.

The phenomenon of enhanced sensitivity of cancer cells toward plasma treatment cf. nonmalignant cells has been widely reported [35]–[37], but the exact underlying mechanism(s) remain unanswered. It has been suggested that unlike normal cells, cancer cells are more vulnerable to oxidative stress due to their higher intrinsic ROS levels [38], [39]. As a result, they are unable to protect themselves and cannot recover from oxidative stresses. It has also been proposed that differences in membrane composition between cancer and normal cells contribute to the difference in toxicity of plasma [40]. For instance, low levels of cholesterol molecules in the cancer cell membrane reduced the membrane integrity making them more susceptible to damage. Studies also suggest that an increased number of aquaporins in the cancer cell membrane contributes to an enhanced transport of ROS into the cell and, thus, a higher oxidative stress in cancer cells [25], [41].

However, as shown here, caution must be taken when studying the specificity of plasma treatment toward cancer cells due to the limitations of an *in vitro* model and other treatment parameters such as seeding density. Conclusions drawn from tissue-culture experiments conducted in a 96-well plate cannot be considered wholly representative of the real cell environment. Therefore, as for other studies involving monolayer culture, the observations and outcomes of this study offer some insight into the biological mechanism but cannot tell the whole story from the perspective of real tissue/tumor. Future studies need to be conducted to study the effects of plasma in a 3-D tumor model.

IV. CONCLUSION

The primary aim of our study was to introduce the concept of plasma fractionation which remains an unexplored area in the plasma medicine community. Our findings show that plasma fractionation induces less damage in both cancer (A431) and noncancerous (HaCaT) cells cf. a single acute dose of plasma, which is more damaging. The smaller the dose fractions, the lower the damage in cells. The results with normal cells are similar to what has been observed in radiotherapy (x-ray) fractionation, implying that plasma and x-ray induce similar effects in cells and that plasma fractionation may similarly serve to protect normal cells from damage.

We also show that the sensitivity to plasma in cancer and noncancerous cells is subject to seeding density. For instance, in the case of subconfluent cell populations (3500

and 1000 cells/well), where more cells are likely to be actively cycling, there is a greater extent of plasma-induced damage in the cancer cells cf. noncancerous cells. The sensitivity of cancer cells to plasma (either single or fractionated) is however decreased in fully confluent cells (8000 cells/well). This could imply that while actively dividing, cancer cells are more susceptible to plasma treatment, with potential parallels to the concept of redistribution within the "five Rs" of radiotherapy. Consistent with these observations, other studies have also shown that plasma induces cell cycle arrest at the G1/S and G2/M stages [42]–[44].

We finally show that while subconfluent cancer cells (A431) are more sensitive to plasma treatment than noncancerous cells (HaCaT). This is not the case when H_2O_2 is applied to these cell types. This highlights the potential role of other ROS and other constituents of plasma, which may be involved in invoking plasma sensitivity specifically in the cancer cells.

There are multiple next steps required in developing plasma fractionation, which include understanding how it affects DNA damaging mechanisms and apoptotic pathways, as well as understanding the role of oxygen and bystander effects. In context to plasma treatment, reoxygenation may be of key significance. Reoxygenation can be achieved by single as well as repeated successive plasma treatments as studies suggest plasma can improve oxygen tension within a tissue [45], [46], but how plasma fractionation changes oxygen levels still remains a largely unexplored field. Previously, Kisch *et al.* [47] did report a study to show that repetitive and successive plasma exposure can improve the microcirculatory effects in wounded tissue by successive tissue oxygenation *in vivo*. However, this study did not include a comparison where the same overall dose was delivered in a single fraction. If developed as a therapy, plasma can improve oxygen levels and enhance tumor killing by delivering damaging oxygen radicals into the tumor [48], thus reducing "repopulation" and improving "sensitivity."

In longer term we envisage that plasma fractionation may have the potential in the field of adjuvant therapy [49], [50]. If demonstrated to be safe, one can imagine a treatment regime with post- or preconditioning of tumors with fractions of short plasma treatments coupled with fractions of small doses of radiotherapy/chemotherapy, with maximal anticancer effects and minimal side effects. Plasma fractionation may also be a method to induce adaptive immunity in healthy cells against cancer, as previously trailed in x-ray fractionation [51]–[53]. Overall, plasma fractionation could be an exciting strategy to make plasma oncology more effective and, maybe, safer.

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