Effect of Atmospheric-Pressure Plasmas on Drug Resistant Melanoma: The Challenges of Translating *In vitro* Outcomes into Animal Models

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ABSTRACT: Atmospheric-pressure plasmas (APs) have been identified as a promising cancer therapy that is able to preferentially kill neoplastic cells through apoptosis. *In vitro* studies suggest that AP-generated reactive oxygen species (ROS) are the principal triggers of apoptosis-related signaling cascades via oxidative stress and direct interference with DNA, proteins, and other cellular components. The results of this study corroborate an ROS-mediated mechanism of cancer cell death, with apoptosis in AP-treated Mel-007 melanoma cells inhibited by pretreatment of cells with the ROS scavenger N-acetylcysteine or the caspase inhibitor zVAD-fmk. In an effort to compare apoptosis mechanisms and evaluate the *in vitro–in vivo* correlation for AP-induced apoptosis, Mel-007 cells were injected subcutaneously into mice to form solid tumors and were treated with AP. Histological assessment of tumors from control and AP-treated animals showed no significant difference in tumor volume, mitotic rate, or percentage of necrosis or multinucleate cells. These results vary from those of other studies of AP treatment of xenograft tumors in murine models, in which a decrease in tumor size and tumor volume were observed. These findings focus our attention on challenges associated with translating the *in vitro* results to corresponding *in vivo* outcomes, and highlight concerns about the applicability of mechanisms established *in vitro* to an intrinsically dynamic *in vivo* environment.

KEY WORDS: apoptosis, cell culture, melanoma, plasma treatment, reactive oxygen species

I. INTRODUCTION

Melanoma is the main cause of skin cancer mortality, and Australia has the highest incidence of melanoma in the world.[1] Approximately 50% of melanomas harbor activating (V600E) mutations in the serine-threonine protein kinase B-RAF (BRAF^{V600E}). The *BRAF* gene provides instructions for making a protein involved in transmitting chemical signals from outside a cell to a cell's nucleus as part of the RAS/mitogen-activated protein kinase signaling pathway that regulates cell proliferation, migration,

and apoptosis. Mutations cause the BRAF protein to be constitutively expressed, even in the absence of chemical signals, which may contribute to the growth of cancers by allowing abnormal cells to grow and divide uncontrollably. The most effective chemotherapeutic treatments for BRAF mutant melanomas are BRAF inhibitors (e.g., dabrafenib, trametinib, vemurafenib), but resistance invariably develops through BRAF truncation, upstream mutation in NRAS, or downstream mutations in MEK.[2,3] Similar to *BRAF* mutations, mutations in the *NRAS* gene lead to a continuously active NRAS protein and consequential abnormal cell proliferation and differentiation. Activated *NRAS* mutations are detected in 15−20% of melanomas.[4] Mutations in MEK genes are found in about 8% of melanomas and result in the production of the abnormally active proteins MEK1 and MEK2, which function as part of the RAS/mitogen-activated protein kinase signaling pathway; these mutations lead to faster cell division and may contribute to tumor formation.[5]

An alternative therapeutic approach to the treatment of melanoma is the tumor necrosis factor–related apoptosis-inducing ligand, which specifically induces apoptosis in melanoma; unfortunately, resistance to this ligand may also develop.[6,7] Indeed, resistance to chemotherapeutic drugs is considered the greatest obstacle to the successful clinical management of cancer patients and the main contributor to the failure of chemotherapy.[8] An anticancer treatment should ideally be cytotoxic to cancer cells, have minimal effects on normal cells, and reduce the possibility of resistance. Atmospheric-pressure plasma (AP) has the potential to fill this niche as an anticancer therapy because it can selectively induce apoptosis in neoplastic cells.[9,10] Plasmas used for cancer treatment operate near room temperature in air. They create a mixture of charged and neutral reactive oxygen species (ROS) and reactive nitrogen species (RNS) that may be involved in apoptosis, with specificity for cancer cells.[9–12] Since oncogenesis involves the dysregulation of apoptosis, targeting apoptosis pathways is an attractive therapeutic approach.[13–15]

The effect of AP treatment on the signaling and checkpoint pathways that govern the cell cycle of a wide range of cancer cells has been investigated by many groups, leading to the identification of several molecular mechanisms that potentially drive selective apoptosis in cancer cells.[10,16–22] These researchers generally agree that the specific effects of plasmas depend on the plasma dosage (which is broadly defined by the proximity of plasmas to the treated object and the treatment duration) and the plasma chemical composition, which in turn is determined by the gas composition and the mode and energy used to produce these plasmas.[23] The respective contributions of specific plasma effects—such as ion and neutral species, energetic electrons, photons, electric and magnetic fields, charging of surfaces, current flows, and others—to apoptotic cancer cell death remain a subject of active debate.[10,24,25] Given the reports of selective apoptosis of cancer cells by plasma-activated media *in vitro* and *in vivo*, in the absence of direct contact with the plasma,[26–30] many researchers are of the view that AP-generated ROS and RNS are the primary drivers of plasma-induced apoptosis. However, other investigators failed to obtain a significant apoptotic effect using plasma-activated media alone,[31] warranting further investigations into plasma-induced apoptosis specifically,

and into the nature of plasma–cell/tissue interactions more broadly. Indeed, in addition to the induction of apoptosis, *in vitro* plasma treatments have been shown to promote cancer cell detachment and loss, and impede cell growth, migration and invasiveness, and clonogenicity, leading to cell cycle arrest and cell senescence, mitochondrial dysfunction, downregulation of integrins, DNA damage, and modulation of the ATM/p53 pathway.[20,22,25,32–35]

Translation of the *in vitro* results to corresponding *in vivo* outcomes presents an equally significant challenge because of the highly complex and dynamic nature of the latter. Quantitative and qualitative *in vitro* to *in vivo* extrapolations to predict the efficacy and evaluate the potential side effects of any type of cancer therapy is not trivial; concerns are often raised about the applicability of *in vitro*–derived chemical-specific parameters and mechanisms established *in vitro* to model intrinsically dynamic and complex *in vivo* processes. Direct AP treatment or exposure to AP-treated media *in vivo* have been shown to inhibit tumor growth, hinder cell proliferation, and induce cell death, leading to an overall reduction in tumor size.[30] In this study AP generated with the kINPen Plasma MED (KPM)[36,37] was used to explore the extent to which *in vitro* treatment parameters can be effectively transferred into the *in vivo* environment, and whether the mechanisms involved in the regulation of cellular responses to atmospheric-pressure plasma treatment *in vitro* would be observed *in vivo*.

II. METHODS

A. Atmospheric-Pressure Plasma Jet Device

The KPM (INP Greifswald/Neoplas Tools GmbH, Greifswald, Germany), a European Conformity–certified medical device that meets EU safety, health, and environmental regulations, generates a cold atmospheric-pressure plasma jet. The apparatus consists of a handheld unit (length, 170 mm; diameter, 20 mm), a power supply, and a gas supply unit (Fig. 1A). The handheld unit includes a pin-type electrode (diameter, 1 mm) located in the center of a quartz capillary (inner diameter, 1.6 mm) and a grounded electrode surrounding the dielectric capillary. A high-frequency voltage (1.1 MHz) is coupled to the internal electrode. The device is operated in continuous mode. A plasma plume is generated in the pen and extends to the surrounding air outside the nozzle, and the discharge effluent is directed toward the sample. Argon is used as a working gas at a flow rate of 1−2 standard L/minute.

The parameters of plasmas generated by KPM are well described in the literature.[12,38] A systematic investigation of KPM-generated plasma parameters by Weltmann et al.[38] showed that at an argon gas flow rate of 1 standard L/minute and a distance of 5 mm, the plasma temperature is within the acceptable range. The same study estimated that at this distance and a maximum input power of 6 W, the maximum recorded irradiance in the 260- to 360-nm ultraviolet range is approximately 5 W/cm^2.[38] Fourier transform infrared analysis of biologically and chemically active species by various researchers showed the absorption features of CO_2, CO, nitric oxide (NO), NO_2, N_2O, HNO_2, and HNO_3,

whereas OH and O_3 species, which exist in the small volume of the active plasma, were not detected because of their high reactivity.[39–41] Spatial mapping of the O_3 concentration in the KPM plasma plume by Kelly et al.[42] showed peak values of 238 ppb within the vicinity of the nozzle (<25 mm), with a radius of ~5 mm. The diffusion of ambient air species, such as molecular oxygen and nitrogen, into the effluent of plasma produced by KPM have been discussed by Reuter et al.[43]

For *in vitro* experiments, cells were treated with a KPM plume for 5, 10, 15, 30, and 60 seconds. For *in vivo* experiments, solid subcutaneous tumors were treated with a KPM plume for 8 minutes/day for 7 consecutive days. The KPM plume was positioned 5 mm from the skin (Fig. 1B).
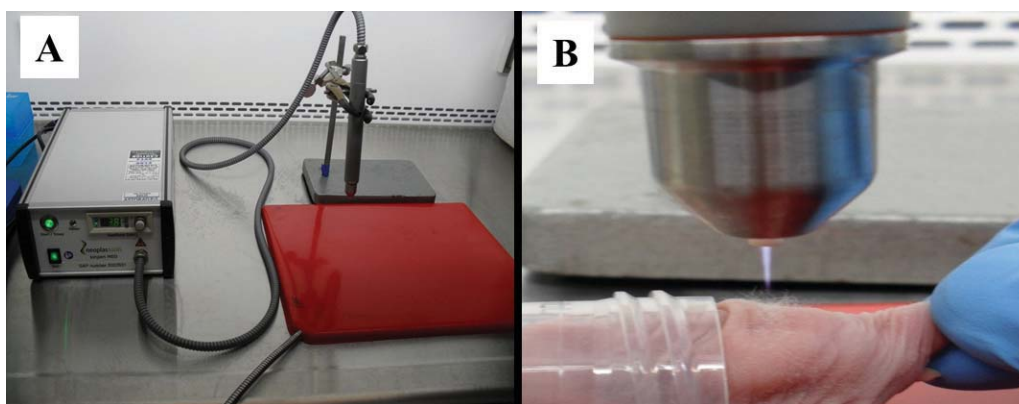

FIG. 1: (A) kINPen Plasma MED (KPM) atmospheric-pressure plasma device. (B) Treatment of a tumor-bearing mouse with KPM.

B. Cell Culture and Reagents

The human melanoma cell lines Mel-RM, Mel-007, and Mel-JD were a gift from Peter Hersey (Melanoma Institute, University of Sydney, Sydney, Australia) and were maintained in Dulbecco's modified Eagle medium (Invitrogen) plus 10% fetal bovine serum. The caspase inhibitor ZVAD-fmk (G7231) and N-acetylcysteine (NAC) (a ROS scavenger; A0737) were purchased from Sigma-Aldrich (St. Louis, MO).

C. Cell Proliferation and Caspase 3/7 Apoptosis Assays

All cells were seeded overnight in 96-well plates at a concentration of 2×10^4 cells/well, treated with KPM for the indicated time periods, and incubated for 18–24 hours. Cell viability was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (G5421; Promega, Madison, WI) following the manufacturer's protocol. In some experiments the caspase inhibitor zVAD-FMK (50 µmol/L) or NAC (3 mmol/L) was added 1–2 hours before KPM treatment. Assays were performed in triplicate. For the

apoptosis assay, cells were seeded in 96-well plates at a concentration of 2×10^4 cells/well, incubated for 24 hours, and then treated with KPM for the time periods indicated. Four to 8 hours after KPM treatment, caspase 3/7 activity was quantified by adding Caspase Glo 3/7 reagent (G8091; Promega). In some experiments the caspase inhibitor zVAD-FMK (50 µmol/L) or NAC (3 mmol/L) was added 1–2 hours before KPM treatment. Triplicate samples were run in standard 96-well plates to quantify the apoptotic response. Luminescence values were then determined using a LUMIstar Omega plate reader.

D. Animal Studies

The use of animals in these experiments was approved by the Commonwealth Scientific and Industrial Research Organisation North Ryde Animal Ethics Committee. Six-week-old BALB/c-Fox1nu/Ausb(BALB/c nude) mice (n = 23) were purchased from Australian BioResources Ltd (Moss Vale, NSW, Australia) and used for 3 experiments. These mice are immunodeficient and were selected to allow the growth of human melanoma cells into tumors; they have the added benefit that they do not need to be shaved or depilated to apply the plasma treatment and evaluate the growth of the subcutaneous tumor. Mice were allowed to acclimatize for 1 week before the experiments. There were 3 experimental lineages (described below) with predetermined end points. Tumors were collected postmortem for histopathology, and these were fixed in 4% neutral buffered formalin and embedded in paraffin. Sections of paraffin-embedded tissue were stained with hematoxylin and eosin, then viewed using a transmitted light microscope and scored by a registered veterinary pathologist (Veterinary Pathology Diagnostic Services, University of Sydney).

Experiment 1 (n = 3) tested the effect of KPM on normal mouse skin (no tumors were induced). A plume of KPM was held 5 mm above the sacral dorsal midline of each mouse for 5 minutes. Mice were killed 0, 24, and 48 hours after treatment. Treated and untreated skin was removed and prepared for histopathology.

Experiment 2 (n = 6) tested the optimal concentration of Mel-007 cells needed to form a tumor in mice (no KPM treatment was applied). Pairs of mice were subcutaneously injected with either 1×10^5, 1×10^6, or 1×10^7 cells in the left flank while under isoflurane general anaesthesia. In each case, 200 µL of cells was mixed with 200 µL Matrigel (BD Matrigel Basement Membrane Matrix, catalog no. 354234; BD Biosciences) for a total injection volume of 400 µL. Tumors were measured with digital calipers and volumes (V) (in millimeters cubed) were calculated according to the formula:

$$V = \frac{\text{length} \times \text{width}^2}{2},$$

where length is the longest axis of the tumor. Tumors were excised at day 28 and prepared for histopathology.

Experiment 3 (n = 14) tested the impact of KPM treatment on tumors induced in mice using Mel-007 cells. Twelve mice were subcutaneously injected in the left flank

with 1×10^7 cells in a total injection volume of 200 μL (100 μL cells + 100 μL Matrigel) while under isoflurane general anaesthesia. Two control mice were injected with 100 μL phosphate-buffered saline (PBS) + 100 μL Matrigel only (no cells). Tumors were allowed to establish for 7 days because this was the time required for the injected cells to adhere and grow into tumors and for tumors to develop vasculature. Then, six mice were treated with KPM for 8 minutes/day for 7 days. The treatment duration was inferred from prior *in vitro* experiments where desired *in vitro* activity, specifically significant apoptotic efficacy, was observed for 5- to 20-minute treatments without deleterious effects to healthy cells. The treatment protocol was similar to that used by Daeschlein et al.[44] for the treatment of subcutaneous melanoma tumors, where tumors were treated through the intact skin with a different version of the KPM device for 5 minutes daily for 5 days. The plasma plume was positioned 5 mm from the skin. A study of the luminous plasma plume produced by the KPM device showed the plasma expanding from the nozzle and reaching/maintaining a maximum length of about 4.5−6 mm (up to 7 mm) until the voltage is terminated.[45] The other 8 mice were controls (6 with tumors and 2 with Matrigel/PBS alone) and received no KPM treatment. All mice were killed on day 24 after tumor inoculation, at which point tumors were removed and prepared for histopathology.

III. RESULTS AND DISCUSSION

Malignant melanoma is resistant to drug therapy,[2,3] and its incidence is steadily increasing at a rate of 2–5% per year.[46] In Australia, between 2006 and 2010, melanoma accounted for 14% and 23% of all cancers diagnosed among 15- to 19- and 20- to 24-year-olds.[47] Because of its superficial location, melanoma is a good candidate for direct AP treatment, and such possibility has been explored by several research groups.[26,27,46,48] The outcomes of these studies and the cell death mechanisms proposed varied with the plasma device used and the treatment protocol, suggesting alterations to the cell cycle, apoptosis, and necrosis.[49] However, the nature of AP selectivity and the specific mechanisms by which AP preferentially induces cell death in tumor cells remain to be fully explored. One potential reason for this is the variability of the experimental procedure (plasma device, cell line, treatment duration). Another significant aspect lies in the nature of most studies, wherein the focus is generally placed on individual effects related to a particular application of interest rather than an attempt to correlate cell responses and intracellular dynamics.[50] Further complexities arise from *in vivo* studies in which the model is both highly complex and dynamic, with a high degree of inherent variability. In practical terms this means that process parameters and mechanisms derived from *in vitro* experimentation have limited relevance and/or require significant optimization to be accurately applied *in vivo*.

    In this article we identified an example where *in vitro*–derived parameters did not translate into expected *in vivo* outcomes, bringing to the attention of the community the potential challenges associated with *in vitro*–to–*in vivo* translation. This is particularly relevant to the field of plasma medicine now, as it is transitioning from cell culture–based to tissue and animal models. First, we examine further the mechanism of cancer

cell death *in vitro*, and then we investigate the extent to which the killing effect, as well as the toxicity of the treatment to healthy tissues, can be replicated *in vivo*.

A. KPM Induces Apoptosis in Melanoma Cells *In vitro*

Direct and indirect exposure to plasma-generated effects has been shown to effectively and selectively kill different melanoma cancer cell lines, and a number of mechanisms have been proposed. Exposure of premetastatic and metastatic melanoma cell lines to plasma generated using surface micro-discharge resulted in irreversible cell inactivation, DNA damage, induction of sub-G_1 phase and proapoptotic events such as p53 and Rad17 phosphorylation, activation of caspase 3, and release of cytochrome c.[46] While not able to induce apoptosis, shorter durations of the same treatment led to senescence in melanoma cells. Apoptosis was also identified as the key cancer cell death mechanism for melanoma cells treated with plasmas generated using other types of devices.[48,51,52] In another study the adhesion molecules of G361 melanoma cells were significantly affected by the exposure to a radiofrequency microplasma jet (e.g., with filamentous actin changing its morphology), leading to the significant detachment of cells from the collagen-coated surfaces and a lack of directionality in the attached cells.[53] The expression of integrin α_2, integrin α_4, and FAK was also inhibited as a result of the plasma treatment. These proteins play notable role in malignant transformation and the attainment of metastatic phenotypes.[54,55] Morphological changes and subsequent cell death were also observed in 1205Lu melanoma cells.[56]

Our previous studies using a custom-made plasma device showed that APs induced apoptosis in cancer cells by activating caspases via JNK/p38 kinases, inducing expression of the proapoptotic phorbol-12-myristate-13-acetate-induced protein (NOXA), and expression of the tumor suppressor protein TP73.[26,27,48] In this study we tested the effect of a different device, the KPM atmospheric-pressure plasma jet, on melanoma cancer cell lines. Three melanoma cell lines (Mel-RM, Mel-007, and Mel-JD) were sensitive to the cytotoxic effects of KPM (Fig. 2). Mel-007 cells also showed a significant increase in caspase 3/7 activity when treated with KPM, but this activity was inhibited by pretreating cells with the ROS inhibitor NAC or the caspase inhibitor zVAD (Fig. 3). These results suggest that the apoptotic pathway induced by KPM is regulated by caspases 3/7 and is dependent on KPM-produced ROS. The susceptibility of neoplastic cells to AP and ROS may be a function of a dysregulated cell cycle and a higher cellular metabolic rate. Hence the effects of ROS could be amplified in neoplastic cells compared with those under normal homeostatic control mechanisms.

B. *In vivo* Effects of KPM on Melanoma

Unlike the ideal *in vitro* (static) conditions in which plasma anticancer efficacy is routinely demonstrated, *in vivo* exposure conditions are systemically interactive and dynamic. Consequently, it is often challenging to predict the required conditions that would generate chemical concentrations and induce biological processes in the target cancer tissue
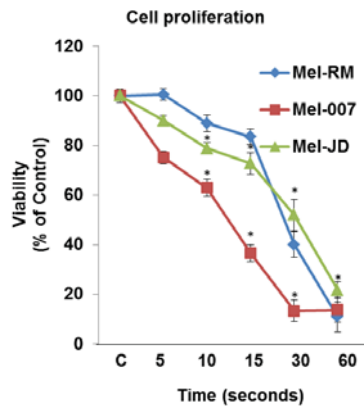
FIG. 2: kINPen Plasma MED (KPM) decreases cancer cell survival. Melanoma (Mel-RM, Mel-007, and Mel-JD) cells were treated with KPM for 5, 10, 15, 30, and 60 seconds. After 24 hours of treatment, cell viability was assessed by the MTS/PMS cell proliferation assay, which measures the activity of live cells. Cell viability is expressed as a percentage of that in control (C) untreated cells.
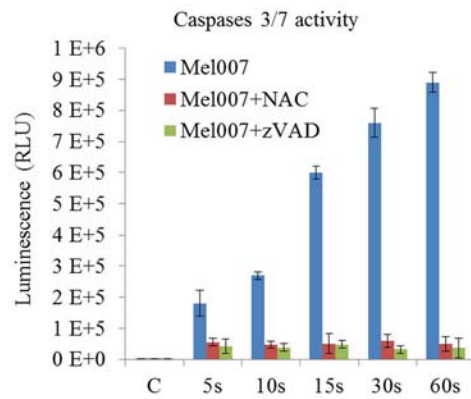
FIG. 3: Caspase 3/7 activity in kINPen Plasma MED (KPM)–treated cells. Mel-007 cells were pretreated with the reactive oxygen species scavenger N-acetylcysteine (NAC) or the caspase inhibitor zVAD for 1–2 hours and treated with KPM for 5, 10, 15, 30, and 60 seconds. After 24 hours of treatment with KPM, the Caspase-Glo 3/7 apoptosis assay was used to determine apoptosis by recording luminescence. Luminescence (as relative light units [RLUs]) is proportional to the relative caspase activity of plasmas for the induction of apoptosis. C, control.

(where cancer cells present as a solid tumor) that are equivalent to those at which effects were observed in an *in vitro* environment (where tumor cells are growing in a monolayer). As such, extensive optimization is often necessary to translate the technology from an *in vitro* to an *in vivo* setting. Equally difficult is the prediction of *in vivo* outcomes based on mechanisms derived from *in vitro* studies and/or modeling efforts. This information transfer is further complicated by the selection of an animal model and the type of tumor cells used to induce a solid tumor, as well as inconsistencies in the methodology and approach used for *in vitro* studies, which is certainly the case for plasma medicine.

In our first experiment we confirmed that treatment with KPM had no deleterious effect on the skin of the mice used for all experiments. Histopathology of skin samples showed no difference between KPM-treated and control skin specimens. Each specimen showed an epidermis that was 3–4 cells thick, with generalized epidermal hyperkeratosis and a moderate stratum granulosum. The superficial dermis consisted of relatively dense connective tissue with a moderate cellularity above the normal subcutis and cutaneous musculature.

In our second experiment we sought to confirm that Mel-007 cells, which have not previously been used in animal studies, would form a tumor at the injection site. Eight

days after inoculation, tumors (125 and 150 mm^3) were present in the pair of mice injected with 1×10^7 cells. No tumors were observable in the other pairs of mice at day 8. By day 28, these tumors were 250 and 400 mm^3, respectively. At this point mice were killed and tumors were harvested for analysis by histopathology. The appearance of the tumors was consistent with amelanotic malignant melanoma, demonstrating successful tumor cell implantation. Tumors of similar sizes were present in both of the mice injected with 1×10^6 cells, but only one of the mice injected with 1×10^5 cells.

Among the numerous species generated during the course of plasma treatment, NO (in RNS) and hydrogen peroxide (H_2O_2) and hydroxyl free radicals (in ROS) are considered as important players in inducing cancer cell apoptosis in the literature.[57–59] Other biologically relevant species include O_2^-, $^1O_2$, NO_2^-, NO_3^-, $ONOO^-$, and, more recently, Cl_2^- and ClO^- that arise from the reaction between oxygen atoms and Cl^- in a solution[60]; however, the specific contributions of these species to the apoptotic effect are yet to be elucidated. Furthermore, since many of the reported apoptosis studies were drawn from the experiments involving direct application of plasmas on cancer cells, it is not yet clear whether these species play an equally important role in inducing apoptosis in cells treated with plasma-activated media.[61] Yan et al.[61] recently investigated the accumulation of RNS and ROS species in plasma-activated media, demonstrating that, with a half-life of only a few microseconds, OH radicals are too reactive to penetrate deep into the media (farther than perhaps several hundred micrometers from the liquid surface). Reactive Cl_2^- and ClO^- species are also relatively short-lived under normal cellular conditions, and as such quickly transform into a less reactive form. On the other hand, H_2O_2 (which to a large degree is formed from OH) and NO are sufficiently stable to penetrate the media and subsequently induce the biological effects in media-treated cells.[40] The interactions of plasma-generated species with components of biological fluid and cellular membranes also influence the kinetics of consumption of plasma-generated ROS; for example, alkyl-peroxide radicals (ROO·) can be generated as a result of interactions between plasma-generated ROS and serum components.[62]

In addition to species stability in media, the speed at which they can diffuse across the cellular membrane plays an important role in determining the amount of intracellular ROS. A degree of similarity between water and H_2O_2 enables the latter to be transported across the cellular membrane by aquaporins (e.g., AQP8).[62] According to Yan et al.,[61] the uptake—and hence the efficacy—of H_2O_2 in inducing apoptosis depends on the properties of the cell membrane and hence varies among different types of cancer cells. Within a cell, in the presence of Fe^{2+}, H_2O_2 can further form reactive OH (via the Fenton reaction), which in turn can damage DNA and other intracellular molecules. It is important to note that although H_2O_2 may indeed be the dominant species involved in inducing apoptosis in the cancer cells subjected to plasma-treated media,[61] it is by no means the only one. Indeed, an equivalent solution of H_2O_2 does not induce the same apoptotic effect as plasma-treated media, suggesting an important role for other plasma-generated species. NO has been shown to affect mitochondrial function through interaction with components of the electron-transport chain, and as such it not only regulates cell respiration but also

increases the generation of ROS by mitochondria, activating mechanisms of cell survival or death.[63] NO can further increase the toxicity of H_2O_2 by inhibiting H_2O_2 degradation[64] and inactivating glutathione peroxidase[65] and other antioxidant molecules.

In the third experiment we sought to test the efficacy of KPM treatment on tumors of melanoma origin induced in mice. Tumors from control and KPM-treated animals were assessed by histopathology, and no significant difference was found in tumor volume, mitotic rate per 10 high-powered fields, percentage of necrosis or multinucleate cells per 10 high-powered fields at day 24 after tumour inoculation (Table 1). Seven of 12 tumors were 10–30% necrotic, with no observed difference between the treated and untreated groups (Table 2). No tissue change at the injection site was observed postmortem in the 2 control mice injected with Matrigel/PBS. Using a smaller volume of injection for experiment 3 (200 μL) resulted in a more localized injection and more uniform tumors than in experiment 2 (400 μL).

Together, these experiments showed that Mel-007 cells form a localized, vascularized melanoma when injected subcutaneously into nude mice (Fig. 4). Injecting 1×10^7 cells mixed with Matrigel (1:1) in a total injection volume of 200 μL was optimal for the concentrations tested. The KPM treatment regime used had no deleterious effect on the skin of the nude mice used in our study. We noted with interest that KPM treatment of the tumors in our study did not reduce tumor volume, and minimal evidence of necrosis was observed when analyzed by histopathology. This was unexpected given published reports by other research groups, where significant differences were observed between tumors harvested from control and plasma-treated mice. For example, in a similar study using melanoma implantation into the flank of C57BL/6N mice, treatment of the tumor

TABLE 1: Characteristics of Tumors from Control and kINPen Plasma MED–Treated Animals as Assessed by Histopathology

	Volume (mm³)	Mitotic Rate (per 10 hpfs)	Multinucleate Cells (per 10 hpfs)
Control (n = 6)	149.3 ± 39.6	25.2 ± 15.8	3.5 ± 1.5
Treatment (n = 6)	166.1 ± 53.7	26.8 ± 14.4	3.8 ± 2.1

Data are the sample means ± standard deviations.
hpf, high-powered field.

TABLE 2: Contingency Table for Necrosis Scoring of Histology Samples

	Necrosis (%)				
	0–10	10–20	20–30	30–40	40–50
Control (n = 6)	1	2	2	0	1
Treatment (n = 6)	1	2	1	1	1
Total	2	4	3	1	2

There is one count for the whole tumor from each animal.
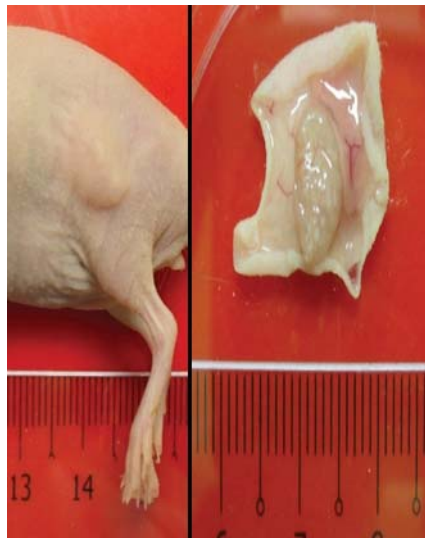
FIG. 4: A tumor-bearing mouse killed on day 24 after tumor inoculation (left), with the excised tumor (right).

with plasmas generated by either an atmospheric-pressure plasma jet or dielectric barrier discharge resulted in a significant delay in tumor growth acceleration.[44] A significant reduction in tumor volume and weight—16 mm^3 and 42 mg for treated mice compared with 80 mm^3 and 96 mg for control mice, respectively—was observed for plasma-treated pancreatic tumors in a murine model; this was largely attributed to a high rate of ROS generation in the vicinity of the cells and ensuing apoptosis.[17] The observed accumulation of cells in the S phase of the cell cycle indicated an arrest of tumor proliferation, whereas a 3-fold increase in caspase-3-positive cells and their homogenous distribution in the treated tumors confirmed the induction of apoptosis in plasma-treated tumors.[19] Reduced tumor volume positively contributed to the animals' life span, increasing it by 60% (from 9.5 to 15.0 days).[18] Although none of the aforementioned *in vivo* studies reported any significant deleterious effects of plasma treatment on healthy tissues, the repeated long plasma treatment (20 min./day) of U87-luc glioma tumors using pulsed dielectric barrier discharge with microsecond pulses resulted in an increase in the subcutaneous temperature and a cutaneous skin pH reduction during the treatment, as well as a superficial burn.[66]

It is possible the KPM plasma plume used in these experiments was too narrow to be effective. In the experiments by Keidar et al.,[10] the plasma treatment was applied when the tumor diameter was <5 mm, corresponding to a volume of ~65 mm^3, which is significantly smaller than that used in this study. Considering that the diameter of KPM plasma plume is ~1 mm, the plume may cover only a very limited surface of the tumor within the treatment time, especially in the case of larger tumors. It is also possible that the intensity of the electric and magnetic fields applied to generate plasma (and hence

the plasma dosage) may have not been sufficient to induce the desired effect *in vivo*.[18,66] Interestingly, with an initial melanoma tumor diameter of ~8 mm, Daeschlein et al.[44] were able to attain some positive antitumor effects using a different version of kINPen; in their experiment, however, the device was brought into direct contact with the surface of the skin. It is also possible that the chemical composition of the generated plasma species differed between our experiment and those of others in which reductions in tumor size and volume were observed."

In addition to using a broader beam to cover the entire tumor, indirect treatments such as the injection of atmospheric gas plasma-treated tissue culture media into tumors may be more effective. The indirect application (by injection) of a plasma-treated medium into xenografted tumors of chronic paclitaxel- or cisplatin-resistant ovarian cancer cells in a mouse model resulted in an average inhibition of 66% ($P <$ 0.05) and 52%, respectively.[30] The use of the ROS scavenger NAC counteracted the growth-inhibitory effects of plasma treatment, suggesting that, similar to our results for melanoma, ROS are the key contributors to plasma-induced apoptosis. It would be intuitive to expect direct plasma treatment to be even more effective because of the added contribution of, for example, an electric field and ultraviolet light. Alternatively, it is possible that, by treating media, a different range of biologically active, long-lived species may be generated (e.g., through chemical reactions between plasma-generated species and amino acids present in the media). This opens an interesting avenue for further research.[67,68]

Better therapeutic outcomes may be attained by first surgically excising the tumor, then using KPM treatment on the tissues in the wound bed to induce apoptosis in any residual cancer cells to potentially avoid tumor regrowth and metastasis. Bringing plasma-generated reactive species and plasma-induced effects (e.g., an electric field) in close proximity to cancer cells may augment the apoptotic effect. Furthermore, a study by Partecke et al.[21] showed that apoptotic and necrotic effects were confined to the top layers of the treated tumor (depth of tissue penetration up to 60 μm), with cells below the top 5 layers of the tumor retaining their usual rate of proliferation. This may provide an explanation regarding the relatively insignificant effect of reported plasma treatments on survival rates.[10,21,44,66] Following the same argument, injection of the plasma-activated media directly inside the tumor may reach the deepest layers of the tumor,[30] and hence may be better suited to the treatment of larger tumors, where higher concentrations of reactive species may need to be delivered with minimal detriment to the surrounding healthy tissues. Removal of the solid tumor before treatment, or treatment of tumors while they are relatively small, may deliver a more permanent resolution of the tumor and positively contribute to patient survival rates.

Other strategies to improve the efficacy of atmospheric-pressure plasma treatment would involve optimization of the treatment protocol—such as specific gas composition and flow rates, treatment time and periodicity, distance between the plasma jet nozzle and the skin surface—as well as synergistic treatments involving anticancer drugs that are currently in use or being developed.[69]

IV. CONCLUSIONS

APs may ultimately provide a specific cancer treatment that overcomes the development of resistance that occurs with many conventional chemotherapy protocols through the activation of apoptotic pathways in neoplastic cells. Although successful in monolayer cell culture experiments, further trials with AP are required to determine how to deliver an effective treatment through multiple layers of different tissues (e.g., skin, fat, and connective tissue) to all cells in the solid, 3-dimensional tumours that occur *in vivo*.

ACKNOWLEDGMENTS

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