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How plasma induced oxidation, oxygenation, and de-oxygenation influences viability of skin cells

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The effect of oxidation, oxygenation, and de-oxygenation arising from He gas jet and He plasma jet treatments on the viability of skin cells cultured *in vitro* has been investigated. He gas jet treatment de-oxygenated cell culture medium in a process referred to as "sparging." He plasma jet treatments oxidized, as well as oxygenated or de-oxygenated cell culture medium depending on the dissolved oxygen concentration at the time of treatment. He gas and plasma jets were shown to have beneficial or deleterious effects on skin cells depending on the concentration of dissolved oxygen and other oxidative molecules at the time of treatment. Different combinations of treatments with He gas and plasma jets can be used to modulate the concentrations of dissolved oxygen and other oxidative molecules to influence cell viability. This study highlights the importance of *a priori* knowledge of the concentration of dissolved oxygen at the time of plasma jet treatment, given the potential for significant impact on the biological or medical outcome. Monitoring and controlling the dynamic changes in dissolved oxygen is essential in order to develop effective strategies for the use of cold atmospheric plasma jets in biology and medicine. *Published by AIP Publishing.* [http://dx.doi.org/10.1063/1.4967880]

There is significant optimism that cold atmospheric plasmas might one day be effectively and routinely utilized for targeted cancer therapy,[1–3] wound decontamination[4–8] and wound healing.[9–11] The action of plasma jets, usually operated with helium (He) and argon (Ar), is linked to reactive oxygen and nitrogen species (RONS).[12–14] RONS are generated upon interaction of the plasma jet with ambient air. The gas flow directs the delivery of RONS to the target tissue fluid or tissue.[15,16] RONS were initially thought to be the underlying cause of free radical ageing.[17] However, it is now recognized that controlled doses of exogenous RONS could potentially be beneficial for disease treatment.[18,19] Numerous studies have shown a possible link between plasma-generated RONS in cell culture media to apoptosis, cell proliferation, migration, and angiogenesis.[20–24]

A UV-Visible absorption spectroscopy (UVAS) procedure was developed to monitor the real-time changes in concentrations of RONS and aqueous oxygen [O_2(aq)] in deionized (DI) water during plasma jet treatment.[25] This and follow-up studies have shown that although He and Ar plasma jets efficiently deliver RONS into DI water, at the same time these plasma jets also de-oxygenate DI water.[26–28] Given that plasma jets deliver RONS, it would have been reasonable to think that water is also oxygenated by plasma jets. But, the de-oxygenation of water by inert gas plasma jets is perhaps not so surprising in the context that inert gases have been utilized to de-oxygenate liquids for many decades in a process referred to as "sparging."[29] And it is important to consider that an almost negligible percentage of the inert gas, estimated 10^{-4}%–10^{-7}%,[30] is ionized in cold atmospheric plasma jets. He is more effective at sparging than other inert gases including Ar, as previously discussed by Rollie *et al.*,[31] and more recently observed for He and Ar plasma jets.[26–28] So what is perhaps surprising is that the effect of sparging of tissue fluids with inert gas plasma jets has not been commented upon before. This phenomenon is important in the context that sparging is likely to produce undesirable hypoxia. Hypoxia impairs healthy cell and tissue function and inhibits tissue regeneration,[32,33] and is likely to reduce the sensitivity of cancer cells to ionizing radiation therapy.[34,35] Sparging might also change pH, with the removal of CO_2 out of the fluid.[36]

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Consequently, as a starting point, the influence of sparging on viability of cells treated with He gas and plasma jets was examined in this study. HaCaT and HFF-1 cells were used for keratinocytes and fibroblasts, respectively, which are the major cells of skin. Skin cells were chosen because of the intense interest in the plasma stimulation of skin cells to aid in the healing of chronic wounds.[23,37–41] Cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. A total of 5000 cells suspended in 200 μl of cell culture medium was added to 96-well tissue culture polystyrene plates and incubated for 24 h prior to treatments. The depth of the cell culture medium in the 96-well was 6 mm. A resazurin-based assay was performed to assess changes in cell viability at 24 h following the He gas and plasma jet cell treatments. A full description of the He plasma jet has been provided in previous studies.[26,28] The 4 mm inner diameter He gas and plasma jet tube was tapered to 650 μm at the nozzle. The distance between the nozzle and top of the 96-well plate was 2 mm. The He plasma jet is a capillary dielectric discharge operated with an applied voltage of 10 kV (peak-to-peak) at 30 kHz and He flow rate of 0.5 or 0.05 standard litres per minute (slpm), with ca. He ionization of $\approx 10^{-7}$%. The flow rate of the He gas jet was fixed at 0.5 slpm. We note that both He gas and plasma jets were operated in open air environments, so there is a possibility that O_2 from air is entrained in the flowing He as it interacts with water. UVAS of DI water was used to monitor dynamic changes in the concentrations of H_2O_2, NO_2^- and NO_3^-, the main longer-lived RONS generated by cold atmospheric plasmas,[42–46] and O_2(aq). Water is the main constituent (>98%) of DMEM. Liquid volumes used in cell treatments were 200 μl, but in order to use UVAS, it was necessary to increase the volume of DI water by 20× to 4 ml. As a consequence, the He gas and plasma jet treatment times of DI water were extended to minutes in order to illustrate the effects on RONS delivery and oxygenation/de-oxygenation. This was considered acceptable because, in the context of this study, the emphasis is on general trends rather than absolute values.

Figs. 1 and 2 show changes in HaCaT cell and HFF-1 cell viability, respectively, following He gas and plasma jet treatments. Positive and negative values in Figs. 1 and 2 refer to an increase and decrease in cell viability, respectively; both are relative to untreated cells.[47] In Fig. 1(a), it can be seen that HaCaT cell viability decreased as a function of the He gas jet treatment time. After 300 s of He gas jet treatment, cell viability was reduced by ≈11.5%. HFF-1s were less sensitive and a short He gas jet treatment of 60 s produced a modest increase in cell viability, whereas longer treatments of 180 and 300 s resulted in a modest decrease in cell viability (Fig. 2(a)).

The cell viability data was compared to dynamic changes in RONS and O_2(aq) in DI water treated with He gas and plasma jets (Fig. 3). From Fig. 3(a), a significant effect of the He gas jet can be seen by a reduction in O_2(aq) of $>4\,mgl^{-1}$ in DI water after 5 min of treatment. The decrease in cell viability, as seen in Fig. 1(a) and to a lesser degree in Fig. 2(a), is attributed to sparging by the He gas

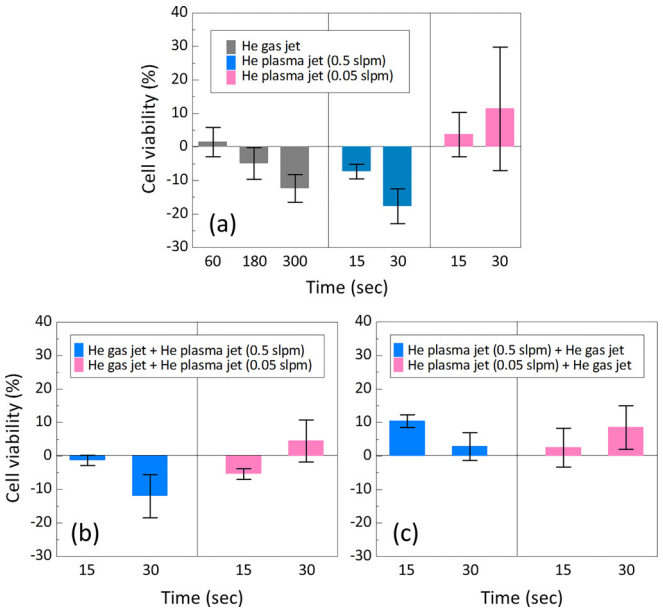

FIG. 1. The effect of He gas and plasma jet treatments on HaCaT cell viability. Cell viability was measured after treatment with the: (a) 0.5 slpm He gas jet and 0.5 or 0.05 slpm He plasma jet; (b) 300 s of 0.5 slpm He jet followed immediately after with the 0.5 or 0.05 slpm He plasma jet; (c) 0.5 or 0.05 slpm He plasma jet followed immediately after with the 0.5 slpm He gas jet for 300 s. He gas and plasma jet treatment times were varied in (a) and the He plasma jet treatment time was varied in (b) and (c).

flow. It is unlikely that the inert He gas could by any other means physically or chemically interact with the cell culture medium. The sparging results show that the He gas jet negatively impacts on the viability of HaCaTs more than HFF-1s, presumably due to the greater sensitivity of keratinocytes to oxygen tension.[48] However, the decrease in cell viability

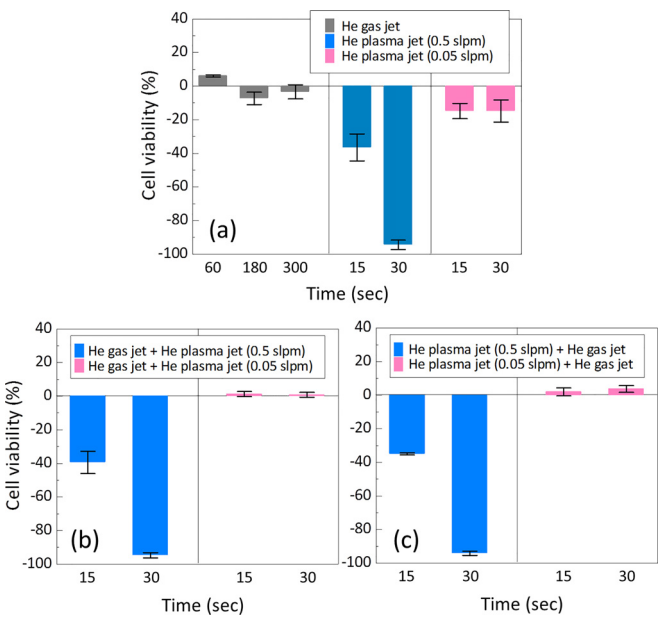

FIG. 2. The effect of He gas jet and He plasma jet treatments on HFF-1 cell viability. Cell viability was measured after treatment with the: (a) 0.5 slpm He gas jet and 0.5 or 0.05 slpm He plasma jet; (b) 300 s of 0.5 slpm He jet followed immediately after with the 0.5 or 0.05 slpm He plasma jet; (c) 0.5 or 0.05 slpm He plasma jet followed immediately after with the 0.5 slpm He gas jet for 300 s. He gas and plasma jet treatment times were varied in (a) and the He plasma jet treatment time was varied in (b) and (c).

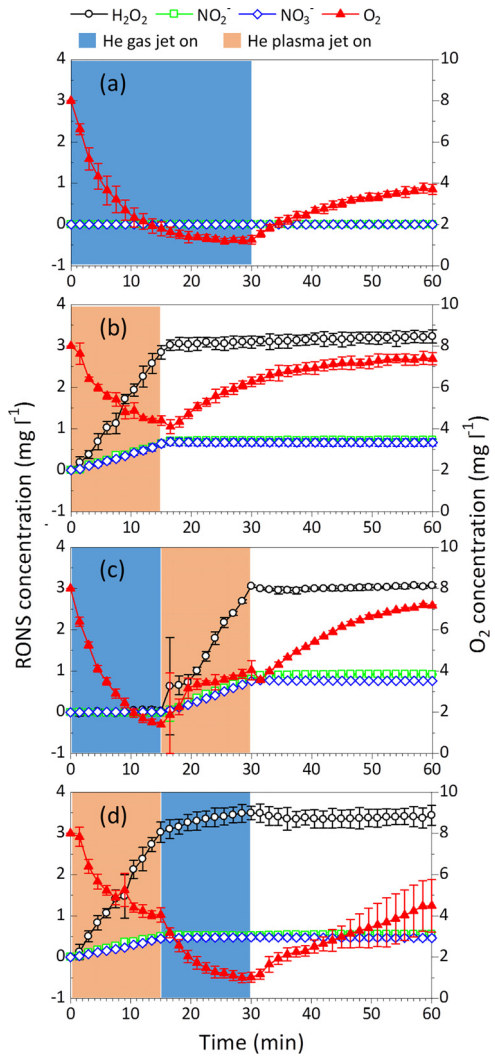

FIG. 3. Dynamic changes in the concentration of H_2O_2, NO_2^-, NO_3^- and O_2(aq) in DI water during and post He gas and plasma jet treatments. DI water was treated with the: (a) 0.5 slpm He gas jet for 15 min; (b) 0.5 slpm He plasma jet for 15 min; (c) 0.5 slpm He gas jet for 5 min followed with the 0.5 slpm He plasma jet for 15 min; and (d) 0.5 slpm He plasma jet for 15 min followed with the 0.5 slpm He gas jet for 15 min.

from sparging was temporary; cell viability fully recovered at 72 h post He gas jet treatment (data not shown). Therefore, the decrease in cell viability from sparging may be due to low O_2(aq), temporarily decreasing cellular metabolism. Cellular metabolic activity can recover after O_2 from the ambient air solvates back into solution after the He gas flow is extinguished, as seen in Fig. 3(a). In contrast to chronic hypoxia, we note an acute hypoxia could benefit wound healing by accelerating keratinocyte migration in response to oxygen gradients in the wound.[48,49]

As shown in Fig. 3(b), concomitant to sparging, He plasma jets also deliver oxidizing species. The combinations of these two effects, as shown below, are interesting and potentially exploitable. A series of experiments were conducted to explore different orders of He gas and plasma jet cell treatments.

The effect of He plasma jet treatment on cell viability is discussed first. He plasma jet treatment was performed at two different He flow rates of 0.5 and 0.05 slpm. Fig. 1(a) shows that at 0.5 slpm, He plasma jet treatment decreased HaCaT cell viability; however, employing the lower flow rate of 0.05 slpm did not impact negatively on cell viability, even at the longer treatment time. A larger decrease in HFF-1 cell viability was observed after 0.5 slpm He plasma jet treatment (Fig. 2(a)). There was an almost 100% reduction in cell viability at t = 30 s, and this cannot be attributed to a sparging effect, as it was not seen in the He gas jet treatment alone. Consequently, it is evident that HFF-1 cells are more sensitive to the oxidizing species delivered by the 0.5 slpm He plasma jet. This effect was still seen for the He plasma jet treatment at the lower He flow rate of 0.05 slpm, but was much less marked, ca. 15% at t = 30 s from Fig. 2(a). These results support the previously reported observation that fibroblasts are more sensitive to oxidative stress compared to keratinocytes.[50] In Fig. 3(b), the DI water was treated with the He plasma jet for 15 min before extinguishing the plasma and He gas flow, and monitoring for a further 45 min. As shown in Fig. 3(b), the He plasma jet generated a higher concentration of H_2O_2 compared to NO_2^- and NO_3^-, which is important because fibroblasts are particularly sensitive to exogenous H_2O_2.[51] During the He plasma jet treatment, the RONS concentration immediately increased in the DI water, but at the same time, the DI water was de-oxygenated. The RONS monitored in this study, H_2O_2, NO_2^- and NO_3^-, are all known to create oxidative stress, which can have stimulatory or inhibitory effects on cells depending on their dosage.[51–53] Therefore, He plasma jets can potentially increase or decrease cell viability through the extent of oxidative stress versus de-oxygenation. The result depends upon the relative contributions of each effect, determined by the treatment parameters such as He gas flow rate and treatment time.

Next examined was how 0.5 slpm He gas jet treatment, followed immediately by He plasma jet treatment, might affect cell viability. Having established the He gas jet alone decreases HaCaT cell viability, it was now seen that cell viability was unaffected by 300 s of He gas jet treatment, if followed immediately for 15 s with the 0.5 slpm He plasma jet (Fig. 1(b)). This is suggestive of a "rescuing" effect from the He plasma jet. But, following the He gas jet treatment with a longer 0.5 slpm He plasma jet treatment of 30 s, decreased cell viability (Fig. 1(b)). With 0.05 slpm He plasma jet treatment for t = 15 s, whilst cell viability remained below untreated cells, cell viability was still higher for these cells compared to cells treated with the He gas jet alone (in Fig. 1(a)); and moreover, cell viability increased after the 0.05 slpm He gas jet treatment was extended to 30 s (Fig. 1(b)). In contrast, with HFF-1s, the He gas jet preceding He plasma jet treatment at 0.5 slpm had no positive effect on cell viability, i.e., HFF-1 cell viability remained highly susceptible to the plasma jet (Fig. 2(b)). At t = 30 s of He plasma jet treatment, cell viability was reduced by almost 100%. A small rescuing effect (from the He gas jet pre-treatment) was seen with the He plasma jet operated at 0.05 slpm. Fig. 3(c) shows the UVAS data of DI water first treated for 15 min with the He gas jet, followed with 15 min of He plasma jet treatment. In this scenario, when the DI water was first de-oxygenated by the He gas jet, the He plasma jet now oxygenates the DI water. Therefore, He plasma jet treatment might help improve cell viability under hypoxic conditions by oxygenating the

solution, provided the flux of RONS is kept low enough to not induce excessive oxidative stress.

In the next experiments, the treatment order was reversed: i.e., cells were first treated with the He plasma jets followed by the He gas jet. Surprisingly, in this order HaCaT cell viability had increased when the 0.5 slpm He plasma jet was first applied for 15 s to cells (Fig. 1(c)). Even at the longer treatment time of 30 s, whilst cell viability was reduced, it was still higher on average than untreated cells (Fig. 1(c)). This result indicates that applying the He gas jet directly after He plasma jet treatment at the higher flow rate (0.5 slpm), somehow (more than) "rescued" cells compared to 0.5 slpm He plasma jet treatment alone. This order produced an overall increase in cell viability. When the He plasma jet was operated at the lower flow rate of 0.05 slpm, changing the order of treatment had only a minor effect compared to treatment with only the 0.05 slpm He plasma jet. In contrast, with HFF-1s no rescuing effect was observed by following the higher flow rate He plasma jet treatment with the He gas jet. A larger decrease in cell viability was observed for treatment with the 0.5 slpm He plasma jet followed with He gas jet treatment (Fig. 2(c)). This effect is almost identical to that seen for HFF-1s treated with the 0.5 slpm He plasma jet (in Fig. 2(a)). At this flow rate, HFF-1s are vulnerable to the oxidative species delivered by the He plasma jet. However, HFF-1s do marginally better when subjected to treatment with the lower flow rate (0.05 slpm) He plasma jet followed by the He gas jet, compared to treatment with the 0.05 slpm He plasma jet alone (Fig. 2(c)). In Fig. 3(d), when the DI water was treated with the He plasma jet for 15 min followed with 15 min of He gas jet treatment, the He gas flow does not affect the RONS concentrations in solution, but it keeps the O_2(aq) concentration lower than in the scenario where no He gas jet treatment was applied (in Fig. 3(b)). O_2(aq) is also a reactive free radical, in that it has two unpaired electrons, and it can itself participate in intracellular RONS generation.[18,54,55] Therefore, keeping the O_2(aq) concentration low, when cells are experiencing excessive oxidative stress, possibly helps maintain cell viability.

H_2O_2 and O_2(aq) levels were measured directly in the cell culture medium immediately after He gas and plasma jet treatments (Table I). These measurements were taken using a "Free Radical Analyzer" (WPI Instruments), as recommended by Taniguchi and Gutteridge.[56] The percentage (tension) of O_2(aq) was 21% for cell medium that was allowed to equilibrate with ambient air; 0% when purged with N_2 and 100% when purged with O_2. O_2(aq) remained unchanged for up to 30 s of 0.05 slpm He gas jet treatment. But the O_2(aq) decreased to 17.5% and 15.3% after 0.5 slpm He gas treatment for 15 and 30 s, respectively. Approximately 75% of O_2(aq) was purged from the cell medium after 300 s of 0.5 slpm He gas jet treatment. Similarly, after 15 and 30 s He plasma jet treatments, the O_2(aq) tension did not change at 0.05 slpm, but decreased at 0.5 slpm; however, not to the same degree as seen with the He gas jet alone; attributed to the plasma jet also delivering oxidative species into the cell medium. As expected, the 0.05 slpm He plasma jet treatments of 15 and 30 s produced lower concentrations of H_2O_2 (2.8 and 5.5 μM) than at the higher flow rate (0.5 slpm) He plasma jet treatments (17.6 and 73.8 μM for t = 15 and 30 s, respectively). Concentrations of H_2O_2 above 10 μM can be cytotoxic,[18] as observed in this study. The pH of the cell medium remained constant at 8.5 ± 0.06 for all treatments.

TABLE I. Level of O_2(aq) and H_2O_2 in cell culture medium after He gas and plasma jet treatments. … Did not measure.

		O_2 (%)		H_2O_2 (μM)	
He flow rate (slpm)		0.05	0.5	0.05	0.5
He gas jet treatment time (s)	15	20.0 ± 0.88	17.5 ± 1.20	…	…
	30	21.1 ± 0.11	15.3 ± 0.52	…	…
	60	…	13.0 ± 0.35	…	…
	180	…	7.52 ± 0.12	…	…
	300	…	5.29 ± 0.09	…	…
He plasma jet treatment time (s)	15	21.1 ± 0.42	19.6 ± 0.26	2.8[a]	17.6 ± 1.33
	30	21.5 ± 0.64	18.3 ± 0.68	5.5[a]	73.8 ± 0.70

[a]Due to low signal obtained with these treatments, values were extrapolated from a calibration curve using longer treatment times of minutes.

These results show that sparging can significantly impact on *in vitro* cell culture experiments where inert gas plasma jets are used to treat cells in small culture wells with small volumes of less than 1 ml. In extrapolating this effect to the treatment of real tissue, it should be considered that the situation is no longer static and that the blood flow might to some extent mitigate de-oxygenation. Also, recent reports on the effects of plasma-skin treatment have shown that plasma treatment increases sub-cutaneous blood flow and blood O_2(aq) content;[57–59] so it should be considered that plasma might also increase local tissue O_2(aq) content. It is also worth noting that plasma-induced oxygenation/de-oxygenation in open wounds could be very different to covered wounds.[26,60]

In conclusion, although the role and effects of RONS such as H_2O_2, NO_2^- and NO_3^-, have attracted a significant amount of attention in plasma medicine studies, particularly in explaining *in vitro* culture studies, potential changes in the concentration of O_2(aq) have largely been overlooked. But the O_2(aq) concentration will affect the biological outcome of the plasma treatment of cells in culture. Monitoring the O_2(aq) concentration is relatively straightforward and could help in the interpretation of *in vitro* studies and therefore in the development of more effective strategies in the application of cold atmospheric plasma in biology and medicine.

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