

Optimization of a Cold Atmospheric Plasma Treatment to Selectively Affect the Viability of Skin Cancer Cells

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Abstract: Cold atmospheric plasmas (CAPs) are a specific type of non-thermal plasmas mainly composed by reactive oxygen and nitrogen species (ROS and RNS, respectively), UV radiation and charged particles. In the last years, liquids treated by CAPs (indirect CAPs treatments) have attracted a significant interest in oncology due to its ability to kill cancer cells with an effectiveness similar to direct irradiation of the cells by cold plasmas. It is important to point out that indirect treatments have the advantage of avoiding the effects of UV radiation and the electrical fields present in plasmas being their effects mainly dependent on the ROS and RNS produced in the liquid phase. To better understand the mechanisms behind the interaction between CAPs, treated liquids and cells, it was engineered a plasma jet device and studied the vulnerability of different cell lines to the culture medium previously exposed to CAPs. For that, it was analysed the concentration of H₂O₂ produced during the treatments by means of colorimetric assays and evaluated the influence of using different working parameters such as volume of medium and gap. According to the obtained results it could be observed that the cancer cell line (Met-1) in study is more sensitive to the liquids treated by CAPs than the non-cancer one (HGF-1), which in the particular case of this jet, seems to be mainly related with the concentration of RNS species produced in the liquids during the plasma exposure since the concentration of the H₂O₂ produced is very low.

1 INTRODUCTION

Cold atmospheric plasmas (CAPs) are a near-room temperature plasma, generally produced in laboratory conditions, by applying an external source of energy to a neutral gas up to a critical point, at which electrons dissociate from atoms (Fridman, Chirokov, and Gutsol 2005; Weltmann and Von Woedtke 2017). The resulting ionized gas will be mainly composed by a mixture of reactive oxygen and nitrogen species (ROS and RNS), UV, visible and infrared light, electromagnetic fields, electrons and ions (Pipa et al. 2012; Reuter et al. 2009, 2012). It is important to note that, since these plasmas are laboratory-generated, their properties, such as their energy and charged particles density, will be dependent on the features of the used set-up: applied power and its type, and also of the feeding gas (Bekeschus et al. 2013; Weltmann and Von Woedtke 2017).

Since, under *in vivo* conditions, cells and tissues are surrounded by a liquid environment, during the past decade, liquids treated by CAPs (indirect plasma treatments) have attracted attention in clinical plasma medicine (Jablonowski and von Woedtke 2015). So far, a significant number of studies, have shown similar effectiveness between treated liquids and direct irradiation of cells (Keidar et al. 2013; Liedtke et al. 2017; Nakamura et al. 2017; Tanaka et al. 2011; Wende et al. 2014; Yan et al. 2014, 2017), over a wide range of cancer cell lines, including melanomas and carcinomas (Pereira et al. 2019; Yan et al. 2015). Additionally, indirect treatments have the advantage of avoiding the effects of UV radiation and of the electromagnetic fields present in plasmas, being their effects mainly related with the ROS and RNS species produced in the liquid phase. Although, the explanation in detail of which species are active in plasma-treated liquids remains a challenge. Some general

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conclusions have been taken about the anti-cancer mechanisms of CAPs. For example, it was shown that the increase in ROS species will cause damages to the antioxidant system and consequently lead to DNA double-strand breaks (Adachi et al. 2015; Sauer, Wartenberg, and Hescheler 2001). Another conclusion is that the CAPs effects will result in cellular apoptosis or necrosis in a dose-dependent way. Moreover, H_2O_2 and NO produced in treated liquids are proposed to be key molecules to preferably kill cancer cells instead of non-cancerous ones (Bekeschus et al. 2014; Jablonowski and von Woedtke 2015).

In the present work, the indirect treatment of Met-1 cells (Squamous Cell Carcinoma keratinocytes), which represents 24% of all skin cancers, was performed. Squamous Cell Carcinoma represents 20-30% of the reported cases of non-melanoma skin cancers and its incidence is increasing over the world (Graham and Tuchayi 2016; Waldman and Schmults 2019). To understand the mechanisms behind the interaction between CAPs, treated liquids, and eukaryotic cells, it was engineered a plasma jet device, which will be described in this paper, and study the vulnerability of Met-1 and HGF-1 cells (human fibroblasts) to indirect CAPs treatment. It was also investigated if CAPs anti-cancer capacity was dependent on the parameters that were chosen to perform the plasma treatments. For that, different volumes of the medium, distances from the jet to the liquid to be treated and times of plasma exposure were tested. The vulnerability of Met-1 cells to CAPs treatment was then compared to one of the HGF-1 cells (human fibroblasts). In addition, the concentration of H_2O_2 produced in the treated liquid was measured and the vulnerability of Met-1 and HGF-1 cell lines to H_2O_2 rich DMEM w/o sodium pyruvate was studied, to determine if H_2O_2 is the only reactive species responsible for the CAPs effects.

2 MATERIALS AND METHODS

2.1 Experimental Set-up

The CAP jet device used in this research was designed and constructed in our laboratory (Plasmas and Applications laboratory, CEFITEC, Physics Department, FCT/UNL, Portugal). It consists of a hand-held principal unit composed by a borosilicate capillary with an outer diameter of 6.93 mm and an inner diameter of 3.76 mm, with two metal electrodes, a custom made DC power supply (2.5 mA, 20 kV), and a gas supply unit (Pereira et al 2019). The

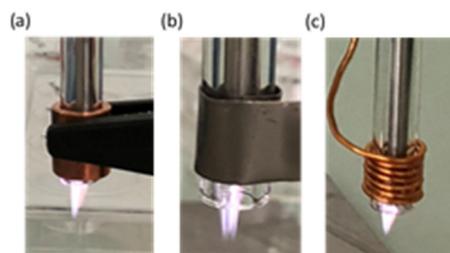


Figure 1: Tested electrodes: (a) copper ring, (b) titanium ring, and (c) copper wire.

electrode on the inside of the capillary is a stainless-steel needle with a diameter of 2 mm. For the outer electrode (connected to the high voltage) three different hypotheses were tested: a copper ring, a titanium ring and one made from an enameled copper wire, Figures 1 (a), (b) and (c), respectively. In order to choose the electrode that allowed a more stable plasma, spectroscopic analysis of the plasma plume obtained using each one of the referred electrodes was performed. According to the obtained results, it was decided to develop the jet using the third configuration (Figure 1 (c)). The copper wire coil used has a 1 mm of thickness and a height of 7 mm, corresponding to seven turns. Once the optimal configuration in terms of stability was determined, the device was coated in order to facilitate safe handling and system automation.

2.2 Optical Emission Spectroscopy

OES was performed using an optic fiber (FC-UV600-2, Avantes) coupled to a spectrometer (SPEC STD, Sarspec's). The size of the spectrometric quartz glass lens covered the whole length of the effluent and was kept at approximately 5 mm perpendicular to the plasma plume. The instrument has a resolution of 1.7 nm and emission intensities on the range 180-1100 nm were recorded.

2.3 Cell Lines and Cell Culture

Human Squamous Cell Carcinoma (Met-1) cells were purchased from Ximbio (153539, London) while human fibroblasts obtained from a gingival biopsy (HGF-1) were purchased from American Type Culture Collection (ATCC® CRL-2014™, Barcelona). Both cell lines were cultured in 75 cm² flasks (Corning®, 4314640) with complete DMEM [(Dulbecco's Modified Eagle's Medium, Sigma, D5030), supplemented with 1.0 g/L D-glucose (Gibco, 15023-021), 3.7 g/L sodium bicarbonate (Sigma-Aldrich, S5761), 1% GlutaMAX™ (L-

alanyl-L-glutamine dipeptide, Life Technologies, 35050-038), 1% sodium pyruvate (Gibco, 11360039), penicillin (100U/ml) and streptomycin (100 µg/mL) (Invitrogen, 15140122), 10% FBS (Fetal Bovine Serum, Invitrogen, 10270106)]. When reaching confluence, the cells had to be transferred into new flasks by an enzymatic method. Met-1 and HGF-1 cells are adherent cell lines that have to be maintained under 37°C in a humidified atmosphere of 5% CO₂ (standard conditions).

2.4 Plasma Treatment Conditions

The protocol used for the both cell lines in study was identical. Met-1 and HGF-1 cells were seeded in a 96-well plate (83.3924, Sarstedt) with a confluence of 3.5×10^4 cells/mL and incubated overnight under standard conditions. A specific volume of complete DMEM without sodium pyruvate were plasma-treated in wells of a 12-well plate (83.3921.005, Sarstedt), for different times. To perform the treatments, the plasma jet was placed above the upper edge of the well containing the liquid to be treated and it rested at the chosen position until the end of the treatment. The treated liquid was mixed by an argon flux of 3 standard liters per minute (slm) controlled by a flowmeter (Dynamal Argon 0-15 L/min, Air Liquid). 100 or 150 µL of the treated culture medium were then immediately transferred to the previously cultured cells in sextuplicate. The same volume of untreated medium was used as a positive control in sextuplicate, in all the performed experiments. Before this, the medium used to culture the cells overnight was discarded and the cells were washed with Phosphate Buffer Saline (PBS). The cells were incubated for 48 hours under standard conditions and only then the cellular viability was assessed by the resazurin assay. On the present study, it was decided to use DMEM without sodium pyruvate, since the last could act as a scavenger for hydrogen peroxide (Pereira et al. 2019; Wende et al. 2015).

2.5 pH and Temperature Measurements

2 mL of cell culture medium were treated as previously described. Immediately after the treatment, the pH of the treated solution was measured using a ph-meter. The temperature of the treated solution was determined using a multimeter associated with a k type thermocouple (RS, Portugal).

2.6 Hydrogen Peroxide Determination in Complete DMEM

The H₂O₂ concentration of the treated liquid was analyzed using a Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, MAK165). After the plasma treatment, 50 µL/well of the treated liquid were transferred in triplicate to different wells of a black 96 well flat-bottom plate. As control, 50 µL of DMEM without sodium pyruvate were also transferred to a well on the same plate in triplicate. 50 µL of a previously prepared master mix (assay reaction solution) was added to all the wells in study (samples, standards, and controls). The plate was then incubated at room temperature for 30 minutes protected from light and the fluorescence intensity was measured at $\lambda_{\text{excitation}}=540$ nm and $\lambda_{\text{emission}}=590$ nm using a fluorescence plate reader (Tecan infinite 200). Final concentrations were calculated using a H₂O₂ standard curve.

2.7 Effects of H₂O₂ Rich Medium

Met-1 and HGF-1 cells were seeded in a 96-well plate with a cell confluence of 3.5×10^4 cells/mL and cultured in an incubator overnight under standard conditions. Then, different solutions of H₂O₂ rich medium were prepared. The H₂O₂ solutions were prepared by mixing a 30% w/w H₂O₂ solution (Sigma) into the complete DMEM without sodium pyruvate. The prepared H₂O₂ rich medium was then transferred to the previously cultured cells in sextuplicate. Before this step, the medium that has been used to culture the cells overnight was discarded and the cells were washed with PBS. After that, the cells were incubated for 48 hours under standard conditions, and only then the cellular viability was assessed by the resazurin assay. Positive control of cells in untreated DMEM without sodium pyruvate and without H₂O₂ was used in all the realized experiments.

2.8 Cell Viability: Resazurin Assay

The resazurin assay is based on the ability of the dehydrogenase enzyme, present in metabolically active cells, to reduce the resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) blue dye into a pink colored and highly red fluorescent resorufin (3H-phenoxazin-3-one) product. The amount of resorufin produced is directly proportional to the mitochondrial enzyme activity, i.e. to the number of viable present cells (Anoopkumar-Dukie et al. 2005; Riss et al. 2004), which can be easily quantified using a

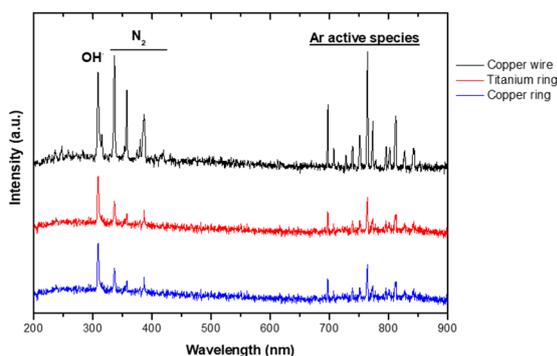


Figure 2: Optical emission spectrum of the plasma plume for three different electrodes, obtained using an Argon flow of 3 slm, where the black spectrum represents the electrode of copper wire, the red the titanium ring and the blue the copper ring.

microplate reader. To assess cell viability, the culture medium in the wells was discarded and 100 μ L of resazurin solution (0.04% resazurin, Life Technologies, USA) were added to all the wells in study. After an adequate time of incubation in the dark at standard conditions, the absorbance was measured in a microplate reader, using a wavelength of 570 nm and a reference standard of 600 nm.

2.9 Statistical Analysis

All data are expressed as mean \pm standard deviation (SD) of at least three independent experiments with six replications. The statistical significance of the differences was evaluated using the Student t-test and it was recognized as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.005$.

3 RESULTS

3.1 OES Obtained for the Different Tested Electrodes

To verify if the plasma produced using the three different electrodes (copper ring, titanium ring, and copper wire) have the same composition, the optical emission spectrum of the discharge was acquired for all of them. Observing Figure 2, despite some small differences in terms of relative intensities, the produced species seems not be dependent on the outer electrode used. The obtained emission spectra show an emission peak belonging to OH radical in the UV-B region at 308 nm and some peaks between 330 and 400 nm representing the nitrogen (N_2) emission in the UV-A range. Since neither oxygen nor nitrogen are

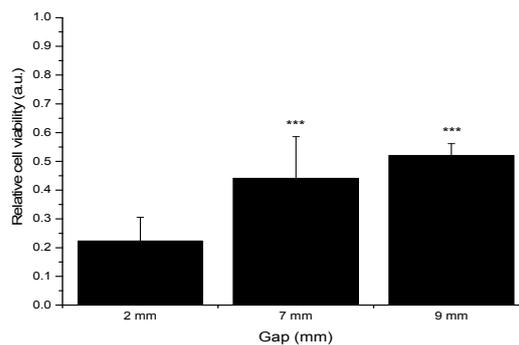


Figure 3: Plasma effects are dependent on the used gap.

present in the working gas used, the appearance of these emission bands can be attributed to interactions between the generated plasma and the surrounding ambient air (Hoentsch et al. 2014). According to Lukes and Locke 2005, the OH radical in the gas phase discharge appears as a consequence of the electron impact of H_2O molecules in the water vapor above and near the liquid surface. The near infrared (700-900 nm) emission peaks mainly represent excited molecules of Argon.

3.2 Effect of the Plasma Treatment Conditions on the Cell Viability

In order to understand the relationship between plasma treatment and cell viability, the plasma treatment conditions, namely, the distance between the jet and the liquid to be treated (gap), the treated volume, and the time of exposition to plasma were changed. First, to understand how the chosen gap can affect cell viability, using a cell concentration of 3.5×10^4 cells/mL and treatment time of 2 minutes, the used gap was varied from 2 mm to 7 mm, and to 9 mm. The results, shown in Figure 3, indicate that the viability of Met-1 cells increases as the distance from the jet increases. This means that proximity of the jet enhances its anti-tumor capacity, and for this

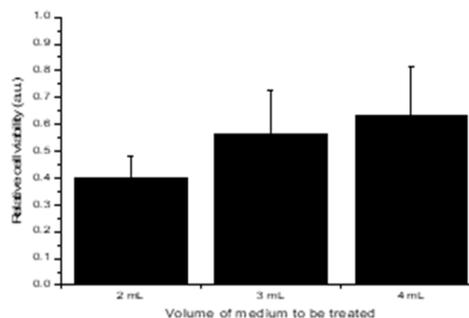


Figure 4: Influence of use different volumes during plasma treatment.

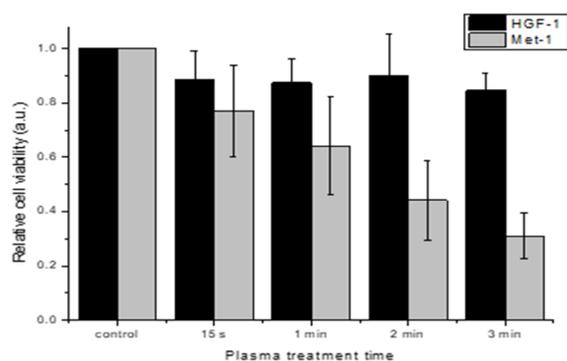


Figure 5: Plasma treatments are time dependent.

reason the gap used to carry out the treatments must be carefully chosen.

Likewise, to see if the effects of plasma treatment are volume dependent, different volumes of DMEM w/o sodium pyruvate were treated under the same conditions. After the treatment 150 μ L of the treated medium were transferred to the previously cultured Met-1 cells, and cell viability was measured 48 hours after plasma treatment. Three different volumes of medium were plasma treated: 2, 3 and 4 mL. By the analysis, of the Figure 4, it is observed that the killing capacity of CAPs treated medium seems to decrease as the total volume of treated medium increase. According to literature, these results can be explained by the dilution of the reactive species formed during the plasma exposure in the treated medium (Wende et al. 2015, 2016; Yan et al. 2015).

Moreover, using the same volume and gap, the plasma treatment was performed for different times of exposure. In this case, the assays were performed for Met-1 and also HGF-1, in order to investigate if cancerous, and non-cancerous cells have a similar response to CAPs treatment. According to the obtained results, Figure 5, Met-1 cells seems to be much more sensitive to plasma treatment than HGF-1 cells, whose viability remains almost unaffected.

For Met-1 cells, and a treatment time of 2 minutes, the viability was already reduced to less than 50% relative to the control, while for HGF-1 no significant differences were found between the different tested times. Here, it can be concluded that cancerous cells are much more sensitive to CAPs treatment than non-cancerous cells, and CAPs treatment is time dependent. Accordingly, to the literature (Chauvin et al. 2017; Wende et al. 2016), this occurs due to the increased concentration of short and long reactive species in the medium as the time of plasma exposure increase (section 3.2.2).

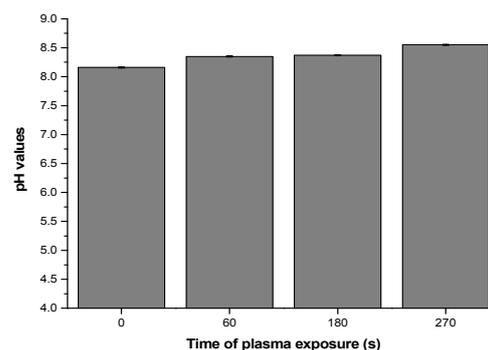


Figure 6: pH measurements of plasma treated culture medium. Results of 3 independent experiments, expressed in terms of mean \pm SD.

3.3 Study of the Liquid Phase

3.3.1 pH and Temperature Measurements

It is known that plasma treatment may alter the pH of the treated culture medium and consequently affect the cell viability. For that reason, it was measured the pH of the plasma-treated culture medium before and after the treatments. As shown in Figure 6, a slight increase in the pH could be observed as the time of plasma exposure increase. These increase in pH can be explained by the degassing effect of the carbonate buffer sodium bicarbonate, present in the treated medium (DMEM w/o sodium pyruvate) (Bundscherer et al. 2013, Rumbach et al 2015). Despite, these small differences are not significant in a statically point, however biologically they can contribute to increasing the alkaline stress, contributing to apoptosis induction.

In terms of temperature, the longest plasma exposure of 270 seconds yielded a temperature of 31 $^{\circ}$ C. Since the cellular tolerance threshold without thermal damage is around 40 $^{\circ}$ C, it can be concluded that this increase is not enough to cause cellular damage on its own. This indicates that the results obtained after the cellular assays are not a consequence of the thermal effects caused by the plasma treatments, but of the oxidative stress caused due to the presence of some reactive species, such as hydroxyl radicals, that are formed by plasma treatment of liquids.

3.3.2 Effectiveness of H₂O₂ Produced in Caps and H₂O₂ Rich Medium

Some authors claim that H₂O₂ is the main reactive species responsible for the selective effects of CAPs, due to its stability and role in a variety of multiple cellular pathways (Liedtke et al, 2017). In order to

investigate this fact, it was measured the concentration of H_2O_2 produced during the CAPs treatment and studied the response of MET-1 and HGF-1 cells to different concentrations of H_2O_2 rich medium. As shown in Figure 7, Met-1 cells are much more sensitive to H_2O_2 rich medium than HGF-1 cells ($IC_{50}=11.2\mu M$ and $IC_{50}=184\mu M$, respectively with IC_{50} corresponding to the half maximal inhibitory concentration), whose viability seems to be almost unaffected by the presence of the H_2O_2 in the tested concentrations. However, it is important to note that the real concentration of H_2O_2 rich medium is different than the one calculated for the preparation of the solutions (being lower) since H_2O_2 reacts with the proteins present in the medium, for example via Fenton reaction (Yan et al. 2015).

These results are very different from the ones observed in treatments with CAPs medium on these cell lines. In this last case, it was calculated an IC_{50} of only $2.69\mu M$ of H_2O_2 to Met-1 cells, corresponding to a treatment between 1 and 2 minutes. These differences demonstrate that H_2O_2 is not the only reactive species responsible for the anti-cancer effects of CAPs treated medium.

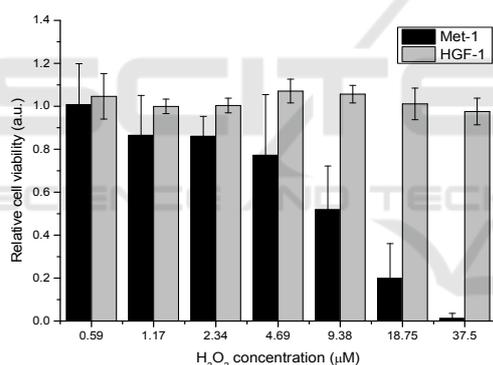


Figure 7: Vulnerability of H_2O_2 rich medium on HGF-1 and Met-1 cells. Results are present as mean \pm SD, and the significance compared to the last bar (concentration of $0.29\mu M$) is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.005$.

4 CONCLUSIONS

The aim of this work was to understand the principles behind the use of CAPs treated liquids in cancer treatment, specifically on Squamous Cell Carcinoma. For that, several parameters were studied in order to try to optimize the anti-cancer capacity of a custom-made Argon plasma jet device. The results presented in this paper, show that the effectiveness of CAPs treatment is time, volume and distance dependent. Specifically, a shorter volume of medium and a closer

distance from the jet device to the liquid to be treated, increase the effectiveness of the treatment. Regarding time, this must be carefully chosen, since longer treatments will produce high concentrations of reactive species and consequently both cancer and non-cancer cells may suffer irreversible damage. To prove this anti-cancer capacity, the vulnerability of a cancerous cell line, Met-1, was compared to the one of a non-cancerous cell line, HGF-1, for treatments performed in the same conditions. Despite a significant reduction in Met-1 viability was registered, no significant reduction in non-cancerous HGF-1 cells was observed. To exclude possible thermal damage and acidification of the medium, changes on temperature and pH were monitored. In addition, it was observed that cells respond in a different way to the H_2O_2 produced in the medium during the treatment and to H_2O_2 rich medium. This indicates that H_2O_2 is an active specie contributing to the anti-cancer ability of CAPs treated medium but is not the only one.

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