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Original Research Article

Study of a Cold Atmospheric Pressure Plasma jet device for indirect treatment of Squamous Cell Carcinoma

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ABSTRACT

Purpose: The possibility of obtaining plasmas at low temperatures and atmospheric conditions, the Cold Atmospheric Plasmas (CAPs), has triggered several studies on its possible medical applications. The use of CAPs devices applied to oncological treatments is still in a pre-clinical stage, but early investigations have shown promising results, both *in vitro* and *in vivo*. In this work, is reported the study of a custom made Cold Atmospheric Pressure Plasma jet device for indirect treatment of Squamous Cell Carcinoma.

Methods: The plasma plume produced by the jet device was characterized by optical emission spectroscopy and the vulnerability of two different cell lines, SCC-15 (Squamous Cell Carcinoma) and HGF-1 (non-cancer cell line) to different working parameters factors such as the volume of medium used on indirect plasma treatments, the number of cells used in the biological assays and the volume of treated medium in contact with the cultured cells were systematically tested and studied.

Results: The effectiveness of CAPs' treatments is time, volume and cell dependent. Cancer cells, specifically SCC-15 cells have shown to be more sensitive to indirect plasma treatments than the non-cancer cell line used in the present work.

Conclusions: According to the obtained results, this study showed that thein *vitro* anti-cancer capacity of the developed jet plasma device will be higher when small volumes of medium are used together with moderate plasma treatment times, keeping the non-cancerous cells almost unaffected.

1. Introduction

Plasma is a partially ionized gas composed by ions, electrons, photons and neutrals, which are active species capable of inducing different physical phenomena and chemical reactions. Considered as the fourth state of matter, plasmas can be found in nature (plasmas created in stars, the polar aurora, lightning, etc.), but can also be generated in laboratory conditions by applying an external source of energy to a neutral gas causing a breakdown, i.e., the gas becomes electrically conductive. During the plasma formation, energy is transferred to the feed gas creating large amounts of ions and electrons. These electrons will induce several reactions leading to either molecular ionizations or dissociations. Consequently, the produced plasma will be composed by a mixture of reactive species generated as a result of these interactions [1–5]. Therefore, the overall ratio between positive and negative particles is such that the produced plasma is considered neutral.

Plasmas can be divided into two different types taking into account if the electrons are, or not, in thermal equilibrium with the ions, namely, thermal and non-thermal plasmas, respectively. Thermal plasmas are those in which the electrons are at the same temperature than ions and neutrals. These plasmas require quite long discharge times in order to achieve the thermal equilibrium. On the other hand, non-thermal plasmas, also known as cold plasmas, are characterized by having electrons at a higher temperature than ions and neutrals, which are close to room temperature [2,4–8].

Within the non-thermal plasmas, there is the specific case of Cold Atmospheric Pressure Plasmas (CAPs), which offer the possibility of operate without the necessity of vacuum systems [7]. These plasmas are mainly composed by reactive nitrogen and oxygen species, UV radiation and charged particles, being their combination dependent on the type of feed gas used, the applied voltage, the type of device, the physical setup or the treatment time [9,10]. Over the past decade, CAPs have emerged worldwide as an independent field of high relevance in biomedical sciences and also in medicine since they can be directly applied to living cells and tissues offering the possibility of a minimally-invasive surgery. CAPs are the only plasma sources that may be applied to living beings since biological applications require the discharge to occur at atmospheric pressure [9,11,12]. Many reports have inclusive

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successfully described the potential use of these plasmas as a good alternative to common anti-cancer therapies. According to the literature experiments performed both *in vitro* and *in vivo*, CAPs show their ability to selectively kill cancer cells without harming normal surrounding cells [2–4,6–11,13]. Additionally, Keidar et al. [14] have demonstrated that the heat that is dissipated by the plasma can lead to an increase in skin temperature up to 2 °C, which is not enough to cause thermal damage. However, the full mechanism of action between CAPs and living cells or tissues remains not completely understood, the reason why further studies must be carried out in order to establish CAPs as a solid treatment on medicine fields.

Although conventionally CAPs have been directly applied to cancer cells and tissues over the last years, many investigation studies have used CAPs irradiated medium (indirect CAPs treatment) as a possible method of anti-cancer therapy, instead of direct irradiation by CAPs [15–16]. These indirect treatments have demonstrated similar effectiveness to direct treatments with the advantage that treated medium can be injected into the body and therefore can reach tissues inaccessible through direct irradiation [17–21].

Currently, mainly due to the change of behavior of the population favoring a greater and inadequate exposure to solar radiation, an increase in the incidence of skin cancer has been registered. According to statistics from the World Health Organization, one in each three diagnosed cancers corresponds to a skin cancer. The most common types of skin cancer can be separated into basal cell carcinoma and Squamous Cell Carcinoma (Non-melanoma skin cancers—NMSC) depending on the type of skin cells from which the cancer cells are originated. The Squamous Cell Carcinoma (SCC) represents 20%–30% of the reported cases of NMSC and compared to malignant melanoma they have an incidence about 20 times higher. The risk of developing SCC throughout life is about 15% for light-skinned individuals, which are more conducive to the onset of a sunburn and it is twice more common in men than in women. It is also 65 times more likely in individuals who have received transplants (immunosuppressed), particularly kidneys, compared to control individuals of the same age [22,23].

In this work, the main goal is to optimize the anti-cancer capacity of indirect treatments of DMEM (*Dulbecco's Modified Eagle's Medium*) w/o sodium pyruvate, using a custom-made CAPs jet device, for the treatment of Squamous Cell Carcinoma, which represents 24% of all skin cancers [24].

For that, the vulnerability of two different cell lines, SCC-15 (Squamous Cell Carcinoma) and HGF-1 (non-cancer cell line) to different factors such as the volume of medium used on indirect plasma treatments, the number of cells used in the biological assays and the volume of treated medium in contact with the cultured cells were investigated and are presented in this study.

2. Materials and methods

All chemicals used in this work were of analytical or chemical grade (Aldrich and Gibco) and were used as received.

2.1. Plasma jet device

The cold atmospheric plasma (CAP) jet device used in this research was designed and constructed in our laboratory (Plasmas and Applications laboratory, CEFITEC, Portugal) and for the presented experiments Argon was used as the carrying gas. 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 4.94 mm, with a stainless steel electrode in its center (2 mm diameter) and a copper ring around it, in accordance with the schematic representation depicted in Fig. 1. The copper ring electrode is connected to a custom made DC high voltage power supply (2.5 mA, 20 kV). The jet operates with an Argon (99% purity, Air Liquide) flow rate of 3 slm (standard liters per minute) controlled by a flowmeter (Dynamal Argon 0–15 L/min, Air Liquide), which ensures a vigorous mixing of the medium during the plasma treatments and drives the plasma stream from the top of the inner electrode to the surrounding air outside the borosilicate tube (see Fig. 1).

2.2. Optical emission spectroscopy (OES)

Cold atmospheric plasma applications are mainly based on the ability to generate and produce a sufficient amount of reactive species. In order to detect the existence of these species, Optical Emission Spectroscopy (OES) was performed. The equipment used was composed by an optic fiber (FC-UV600-2, Avantes) coupled to a spectrometer (SPEC STD, Sarspec's). The instrument's spectral range was 180–1100 nm with a resolution of 1.7 nm and the optical fiber was kept at a distance of approximately 5 mm perpendicular to the plasma plume.

2.3. Cell lines and cell culture

In the present work two cell lines were studied: an oral Squamous Cell Carcinoma SCC-15 (ATCC CRL-1623) and a normal human gingival fibroblast, HGF-1 (ATCC CRL-2014). Both cell lines were purchased from American Type Culture Collection (ATCC, LGC standards, Barcelona) and were preserved under standard conditions (37 °C in a humidified atmosphere of 5% CO_2).

Human squamous carcinoma cells (SCC-15) were maintained in DMEM-F12 (*Dulbecco's Modified Eagle's Medium: nutrient mixture F-12*; ATCC 30 2006) supplemented with 10% fetal bovine serum (FBS, Sigma, 102710), 1% antibiotic Penicillin/Streptomycin (Pen/Strep, Sigma, A5955) and 400 ng/mL of hydrocortisone (Sigma, H0888).

Human fibroblasts (HGF-1) were kept in DMEM (*Dulbecco's Modified Eagle's Medium*, Sigma), supplemented with 10% FBS, 1% antibiotic Pen/Strep and 1% L-Glutamine.

Prior to plasma treatments, the cells were washed with phosphate buffered saline (PBS) without calcium and magnesium, detached with Tryple (Gibco, 12604-021) and seeded in the 60 inner wells of a 96-well microplate with the desired concentration.

2.4. Plasma treatments

The protocol used for the two cell lines in study was identical. First, SCC-15 and HGF-1 cells were seeded in a 96-well plate with the desired cell confluence and cultured in an incubator overnight under standard conditions. The indirect non-thermal plasma treatments were performed using the CAP jet device to vertically irradiate the medium in different wells of a 12-well plate and only then the treated medium was transferred to the previously cultured cells (see Fig. 2). The treatments were performed using two different volumes of DMEM without sodium pyruvate (1 and 2 mL) for different times of exposure. For the present work, it was decided to use only DMEM without sodium pyruvate, since some authors claim that sodium pyruvate could act as a scavenger for hydrogen peroxide, which is considered one of the main agents responsible for the effects of CAP on cells [25]. Indeed, such scavenging effect was also experimentally observed in some preliminary investigations performed with this jet device (data not shown here). A distance of 2 mm from the borosilicate tube to the upper edge of the wells was kept constant during all the realized experiments. This gap was chosen based on the results of previous studies that were carried out using the same plasma jet device, in order to understand which distance ensures a better effectiveness of CAPs treatment.

After treatments, the CAP treated medium was immediately (< 2 minutes) transferred (100 or 150 μL) to the previously prepared 96-well cell culture plates in sextuplicate. Before this step, the medium which has been used to culture cells overnight was discarded and the cells were washed with PBS (*Phosphate Buffer Saline*). After that, the cells were incubated for 48 hours under standard conditions and only then

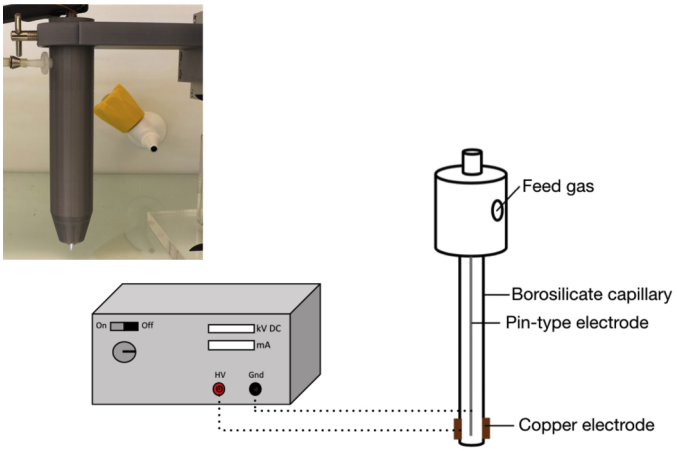

Fig. 1. Schematic representation of the custom made Argon plasma jet device. As inset, is a representative photograph of the custom made jet device.

the cellular viability was assessed by the resazurin assay. A positive control of cells in untreated DMEM without sodium pyruvate was used in all the realized experiments.

2.5. Cell viability assay

Cell viability was assessed using the resazurin assay, which is based on the ability of the dehydrogenase enzyme, present in metabolically active cells, 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 quantity of resorufin produced will be proportional to the number of viable cells, which can be easily quantified using a microplate reader. Resazurin assay compared with other viability assays, specifically with tetrazolium reduction assays, as the advantage of being slightly more sensitive and less toxic to cells [26,27]. To assess cell viability 48 hours after plasma treatments, the medium in the wells was discarded and 150 µL of resazurin was added to all the wells in study. After four hours of incubation in the dark, under the standard conditions, the absorbance was measured at an emission wavelength of 570 nm and a reference of 600 nm using a microplate reader (BioTeK Instruments, ELX800).

2.6. Statistical analysis

All data are expressed as mean ± standard deviation of at least three independent experiments. The statistical significance of the differences was evaluated using the Student's *t* test and statistical significance was recognized as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

3. Results and discussion

3.1. Optical emission spectroscopy

The emission of UV radiation by Cold Atmospheric Pressure Plasma sources is a well-known phenomenon. There are three different types of UV radiation: UV-A, UV-B and UV-C. They decrease in wavelength number from A to C but increase in intensity, being UV-C the most cytotoxic type of UV radiation due to the ability to induce mutagenesis and photochemical oxidation processes in cells [28].

Spectral characteristics of UV radiation generated by plasma can be affected by several factors as for example the feed gas used and its purity and also the addition of different gases [29]. To investigate the species generated by the plasma expanding into the air and interacting with the phase below it, the gas phase of the jet was characterized. Fig. 3 shows a typical optical emission spectrum of the Ar plasma discharge in the wavelength range from 200 to 900 nm obtained with a gas flow rate of 3 slm. Based on the analysis of the obtained emission spectrum it is possible to identify an emission peak belonging to $OH^{\cdot}$ radical in the UV-B region at a wavelength of 308 nm and some peaks between 330 and 400 nm representing the nitrogen (N_2) emission in the UV-A range. However, one can further observe that the peaks assigned to the emission of UV-A and also UV-B radiations are generated at low intensities. Therefore, possible biological damages due to these UV radiations can be excluded [29].

Since neither oxygen nor nitrogen is present in the working gas, the appearance of these emission peaks can be attributed to the interactions with the surrounding ambient air. Emission peaks representing the excited atoms of Ar lying in the range from 700 to 900 nm, VIS-NIR region were also found. Moreover, no detectable emission in the UV-C range (100–280 nm) can be found, as depicted in Fig. 3. Thus, it can be concluded that the most dangerous radiation emitted by the developed plasma jet device occurs in the UV-B region.

3.2. Cell number influence on the effectiveness of cold plasma treatments

In order to investigate the effectiveness of plasma treatments on SCC-15 cells and on HGF-1 cells, distinct cell confluences were tested, namely: 2×10^4, 3.5×10^4, 2×10^5 cells/mL for SCC-15 cells and 2×10^4 and 2×10^5 cells/mL for HGF-1 cells. In both cases three different CAPs treatment times were used (3, 6 and 9 minutes). From the analysis of the obtained results (see Fig. 4), it is clear that cell viability decreases as the treatment time increases and for the same CAP treatment time increases as the cell seeding confluence decreases. This is in

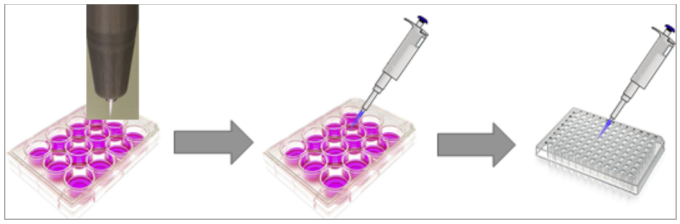

Fig. 2. Representation of the indirect plasma treatment stages. First, CAP vertically irradiate 1 or 2 mL of culture medium in different wells of a 12-well microplate. Then, 100 or 150 µL of the treated medium was transferred for the different wells of a 96-well microplate, in which the cells were previously cultured.

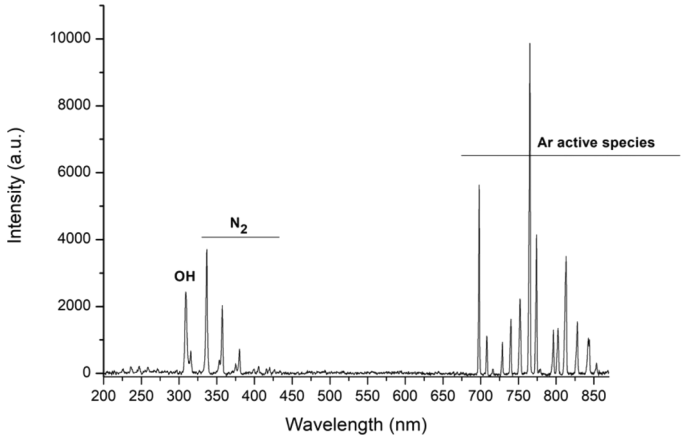

Fig. 3. The optical emission spectrum of the plasma plume of the developed CAP device, obtained using an Argon flow of 3 slm.

accordance with the studies of Dayun et al., who observed that the anti-cancer capacity of the CAPs treatments was determined by the dose of CAPs acting on a unit cell, instead of the whole treatment dose [17,19–21]. Moreover, analyzing Fig. 4 it can be detected that SCC-15 cells are more sensitive to CAPs treatment than HGF-1 cells. For example, after 3 minutes of CAPs treatment using a cell confluence of 2×10^4 cells/mL the relative cell viability of HGF-1 cells remains practically the same (0.98) as the one of the control group (1.0). However, the same does not happen with SCC-15 cells, whose viability after a CAP treatment performed under the same conditions and with the same duration decreases for less than half (≈ 0.28). These differences in relative viability (viability compared to control group) between SCC-15 and HGF-1 cells remain significant for longer treatment times and different cell confluences, as can be observed through the analysis of graphs presented in Fig. 4. This seems to prove the ability of CAPs treatment in selectively kill cancer cells leaving the non-cancerous ones almost unaffected.

3.3. CAPs effects for different medium volumes

In order to understand if the killing capacity of plasma treated liquids is volume dependent, two different approaches were tested in this study. First, the influence of using different volumes of DMEM without sodium pyruvate (1 and 2 mL) for the CAP treatments was investigated and then the influence of transfer 100 or 150 µL of the previously treated medium to the cells was compared. For that, SCC-15 cells with a confluence of 3.5×10^4 cells/mL were seeded in different wells of a 96-well plate and cultured in an incubator overnight under standard conditions, as previously referred. Next, 1 and 2 mL of DMEM w/o sodium pyruvate were vertically irradiated in a well of a 12-well plate for 3 minutes. Subsequently, 100 or 150 µL of each one of the two treated volumes was transferred to the cultured cells in sextuplicate. Finally, 100 and 150 µL of untreated DMEM w/o sodium pyruvate were used as positive control, in wells of the same plate, also in sextuplicate.

By the analysis of Fig. 5, the killing capacity of CAPs treated medium seems to decrease when larger volumes of medium were treated. According to the literature, such results can be explained by the dilution of the reactive oxygen and nitrogen species [21,25,30] that are generated during the CAP treatments in either gaseous or aqueous forms when primary plasma species (ions, electrons and other dissociated molecules) interact with the medium being treated [31]. Besides this, also the influence of transfer 100 or 150 µL of the previously treated medium to the cultured cells has been investigated. Concerning this study, the killing capacity of CAP treated mediums was found to be higher when 100 µL of treated medium per well was used instead of 150 µL (Fig. 5). Thus, it can be concluded that the effectiveness of CAPs indirect treatment is higher when cells are surrounded by a few microliters of the medium.

3.4. CAPs effects for different treatment times

Throughout this work, it was also studied the influence of using different treatment times. Treatments of 2, 2.5, 3 and 6 minutes were carried out using cell confluences of 2×10^4 and 3.5×10^4 cells/mL. In this part of the study, it was decided to work only with these two confluences, since CAPs treatment *in vitro* was more effective when smaller cell confluences were used, as shown in earlier results.

For a confluence of 2×10^4 cells/mL, it can be observed that the viability of SCC-15 cells after 2 minutes of treatment was already reduced to less than half of the viability of the control group (0.44). The same did not happen when the treatments were carried out in the same conditions, but with a confluence of 3.5×10^4 cells/mL. In this case, cell viability showed a reduction of 50% for treatments with a duration longer than 3 minutes (see Fig. 6).

On the other hand, for HGF-1 cells, when it is used the higher cellular confluence in study no significant differences were found relative to the control group in any of the tested times. When analyzing the results obtained for the 2×10^4 and 3.5×10^4 cells/mL (Fig. 6) differently to what happens with SCC-15 in neither of the tested treatment times the viability of these cells decreases to less than half of the

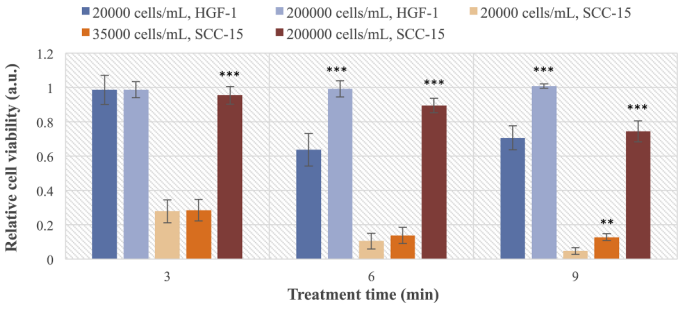

Fig. 4. The relative cell viability of SCC-15 cells and HGF-1 cells, cultured in 100 µL of CAP treated medium for different treatment times. Results are presented as mean ± s.d. of three independent experiments performed in sextuplicate. Student's *t* test was performed, and the significance compared to the first bar is recognized as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

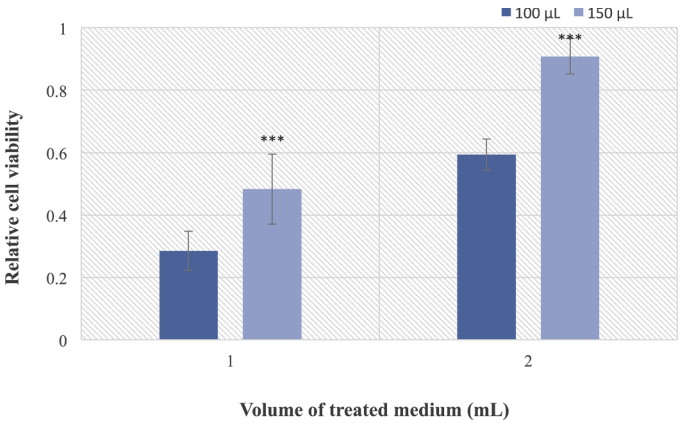

Fig. 5. The relative cell viability of SCC-15 cells after 3 minutes of CAP treatment of 1 and 2 mL of DMEM w/o sodium pyruvate. SCC-15 cells were cultured for 48 h in 100 and 150 µL of DMEM w/o sodium pyruvate previously treated. Results are presented as mean ± s.d. of three independent experiments performed in sextuplicate. Student's *t* test was performed, and the significance compared to the first bar is recognized as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

viability of the control group. Inclusively, for treatments of 2 minutes, instead of a reduction, a small increase in viability of HGF-1 cells can be observed.

Hence, it can be concluded that CAPs effectiveness is very dependent on the treatment time, being much more effective in killing SCC-15 cells than HGF-1 cells (only a small percentage of these cells is affected).

Accordingly, to the literature [20,30], this occurs due to the increase of the concentration of H_2O_2, nitrite and nitrate present on the treated medium as the treatment time increases. This will contribute to the acidification of the treated medium, which for longer treatment times could be toxic to the cells and therefore influencing the results of CAPs treatments.

The obtained viability results for all the tested parameters (cell number, medium volume, treatment time) evidence that indirect plasma treatments can be a good alternative to common anti-cancer treatments for skin cancer, such as conventional laser surgery, which is mainly based on thermal interactions and therefore often leads to accidental cell death. Also, indirect treatments have the advantage that CAPs treated medium can be stored for a few days in a refrigerator at around 4 °C without lose its anti-cancer capacity. Moreover, given its ability to specifically affect cancer-cells leaving the non-cancerous ones almost unaffected, CAPs treated liquids can be directly injected into the tissues, integrated into drug delivery systems in order to prevent tumor growth and reach tissues which would be inaccessible in another way.

4. Conclusions

The main goal of this study was to optimize the anti-cancer capacity of a plasma jet device for indirect treatment of Squamous Cell Carcinoma. The results presented point out that the effectiveness of CAPs' treatments is time, volume and cell dependent. Cancer cells, specifically SCC-15 cells have shown to be more sensitive to indirect plasma treatments than the non-cancer cell line used in this study (HGF-1). Plasma treatments were observed to be more effective in reduce the percentage of viable SCC-15 cells when smaller volumes of treated medium and lower cellular confluences were used. Since the UV radiation emitted by the developed plasma jet device seems not be strong enough to cause biological damage, it can be concluded that the anti-cancer capacity of indirect plasma treatments performed with this device could be optimized using small volumes of medium, together with small gaps between the plasma source and medium. Besides that, also using an adequate number of cells per well, will contribute to improve the anti-cancer capacity of the plasma jet device *in vitro* experiments, since it will require shorter treatment times and, consequently, no significant acidification of the treated medium will occur.

These findings are therefore meaningful and encouraging for future studies regarding the application of a Cold Atmospheric Pressure Plasma jet device for indirect treatment of Squamous Cell Carcinoma.

Conflict of interest

The authors declare no potential conflicts of interest.

Financial disclosure

The authors declare that they do not have financial and personal relationships with other persons or organizations that may have inappropriately influenced their work.

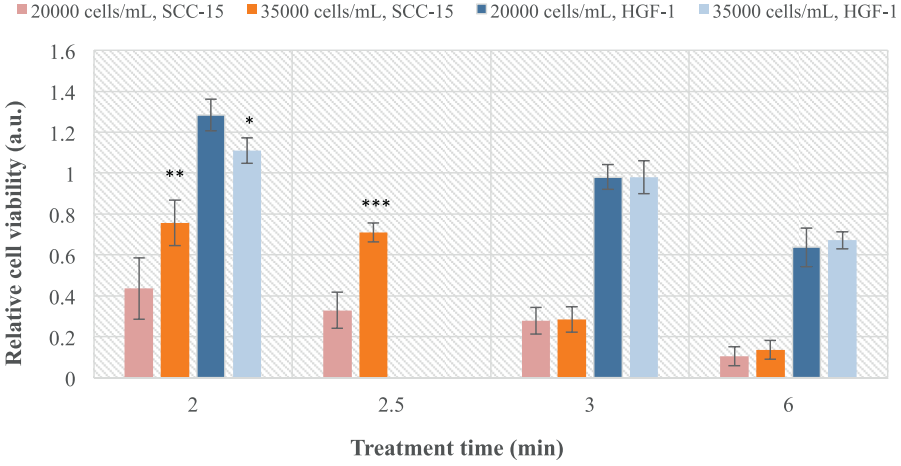

Fig. 6. The relative cell viability of SCC-15 cells and HGF-1 cells, cultured in 100 µL of CAPs treated medium for different treatment times and with two different cell confluences. Results are presented as mean ± s.d. of three independent experiments performed in sextuplicate. Student's *t* test was performed, and the significance compared to the first bar is recognized as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

Ethical statement for clinical plasma medicine

I testify on behalf of all co-authors that our article submitted to the special issue of Clinical Plasma Medicine, entitled *Study of a Cold Atmospheric Pressure Plasma jet device for indirect treatment of Squamous Cell Carcinoma,* by S. Pereira, E. Pinto, P. A. Ribeiro and S. Sério, that:

1 this material has not been published in whole or in part elsewhere;
2 the manuscript is not currently being considered for publication in another journal;
3 all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

Acknowledgments

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