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Selective treatments of prostate tumor cells with a cold atmospheric plasma jet

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Abstract

A cold atmospheric plasma torch was developed to treat different tumor prostate cells. Parameters like electric voltage, gas flow, cell/torch distance, and exposure time were studied to improve the effects of plasma onto the various prostate tumor cells p69, LNCaP and PC-3. The DBD Plasma torch operated by a voltage up to 10 kV, without any risk of arcing, exhibited a long visible plasma plume up to about 70 mm with a temperature near of 27°C. It is shown in this paper how the optimized protocol lead to the tumor cells death with regards to cell viability. Cold plasma treatment exhibits differential effect regarding tumor status of cells. The results show that P69 cells are less sensitive to cold plasma exposition compared to metastatic tumor cell lines LNCaP and PC3.

Keywords: Low temperature plasma, Dielectric barrier discharge, Plasma torch, Prostate cancer cells, Selectivity

1. Introduction

1 Medicine plasma applications started in the 2000's years, exploiting es-
2 pecially their thermal effects for coagulation, cauterization sterilization or
3 ablations [1, 2]. In those cases, the plasma effects on cells were only necrosis.
4 The cold atmospheric plasma technologies (CAP) appeared and has been
5 widely developed for extended plasma medicine. The using of such technolo-
6 gies showed that cold plasma could activate the natural destruction of cells,
7

8 via apoptosis process instead of necrosis [3, 4, 5, 6, 7]. Many papers deal with
9 the description of all different devices, for instance [8]. This particularity of
10 CAP's are very promising in the fight against cancer. Indeed, several studies
11 showed that plasma treatments could show selectivity, i.e that they might
12 ignite the apoptosis of tumor cells with much less effects on healthy cells. An
13 explanation of such selectivity was proposed by Yan et. al [9]. It is known
14 that the CAP forms mainly reactive oxygen and nitrogen species (ROS and
15 NOS) and combining to the medium, H_2O_2 is also formed whatever is the
16 plasma gas used. Since it has been shown that cancer cells exhibit much more
17 aquaporins (AQP) and less catalase than the normal cells, H_2O_2 penetrate
18 much easier into the cells [10, 11, 12, 13], leading to a high rise of ROS into
19 them, igniting apoptosis. A review of the use of CAP's written by Dubuc
20 et. al in 2018 [14] claims that only 4.2 % of related publications focused
21 on prostate. Moreover the most used plasma gas in publications is Helium
22 at 36 %. In a review published in 2016, Gay-Mimbrera et. al [15], only few
23 publications was found to be focused on prostate cancer [16, 17, 18, 19]. Gib-
24 son et.al showed that the using of a radio-frequency driven helium plasma
25 induced intrinsic pathway of apoptosis for the PC-3 cells (cancer cells of
26 prostate)[18]. Authors also proved that plasma treatments activated initia-
27 tor caspase 9 and effector caspase 3, but also deteriorated the PARP enzyme,
28 leading to the inability of the cells to repair DNA damages. Nareksi et.al
29 [19] focused on DU145 cancer cell and also showed the efficiency of a pulsed
30 RF helium plasma. Weiss et. al [20, 21] also proved the efficiency of CAP's
31 with argon plasma on PC3 and LNCaP cells, demonstrating the attenuation
32 of their proliferation. All the publications about the treatment of prostate
33 cancer using CAP's show the destruction of cancer cells by the pathways of
34 apoptosis but as far as we know, no one compared the plasma treatment
35 given the tumor status of cell lines. In this paper is presented an optimiza-
36 tion of a high frequency helium plasma jet to treat LNCaP and PC3 prostate
37 cancer cells. The effects of such plasma are also compared onto immortalized
38 prostate cells P69. Like Judée et.al [22], a peculiar attention is paid to the
39 plasma electrical properties.

40 **2. Materials and methods**

41 *2.1. Experimental setup and electrical analysis*

42 Figure 1 represents a schematic view of plasma torch based on a high
43 voltage dielectric barrier discharge configuration already described in the

44 literature [22]. An inner high voltage hollow electrode (3 mm inner diameter)
45 is inserted into a pyrex capillary, 6 mm inner diameter, 1 mm thick. An
46 outer electrode, 10 mm long, connected to the ground, is placed around the
47 capillary in front of the end part of the inner electrode. The inner electrode
48 is supplied by helium (purity 4.5) and powered by a mono-polar square pulse
49 high voltage generator (Spellman, SLM 10 kV 1200 W) coupled with a HV
50 pulses generator (RLC electronic, NanoGen1 10 kV).

51 Electrical power is calculated using $Q - V$ plot of electrical charge trans-
52 ferred between pulse generator and the plasma torch, in function of genera-
53 tor's voltage measurement V_{gene} . Voltage and electrical current are obtained
54 using voltage probes (Testec, TT-HVP 2739, 1000:1), current transformer
55 (Stangenes, 0.5-1 W, 1:1) and are monitored with a digital oscilloscope (Tek-
56 tronix, MDO3024). Electrical charges are deduced from the first integral of
57 electrical current.

58 2.2. Thermal imaging

59 The temperature of the plasma is defined from the average of the kinetic
60 energies of the particles in the discharge. In cold plasma, electrons and heavy
61 particles are out of thermodynamic equilibrium, i.e . a different temperature
62 can be associated to the both populations. For a thermal analysis, the plasma
63 was generated with the following conditions: voltage 6 kV, duty cycle 20 %,
64 frequency 20 kHz and height 15 mm. The temperature change of the culture
65 medium surface during plasma treatment was measured in dynamic mode
66 by infrared thermography, which is a non-contact technique. A Cedip Jade
67 III-MWIR camera (Flir, Torcy, France) featuring a wavelength range of 3-
68 5.2 μm and 320 \times 240 detectors was used to capture the temperature fields at
69 the culture medium surface. The integration time and acquisition frequency
70 were set to 1500 μs and 100 Hz, respectively. The Noise Equivalent Tem-
71 perature Difference (NETD) of the camera, corresponding to the thermal
72 resolution, was equal to 0.02 $^{\circ}\text{C}$ at ambient temperature (here 21 $^{\circ}\text{C}$). Only
73 the temperatures measured at the contact point between plasma and culture
74 medium are reported in the present paper.

75 2.3. In vitro experimentation

76 2.3.1. Cell culture

77 Human prostate cancer cells LNCaP [23] and PC3 [24] and human prostate
78 epithelial cells P69 [25] were cultured such as monolayer adherent cells in
79 RPMI-1640 medium (Invitrogen) with penicillin/streptomycin (100 mg/ml)

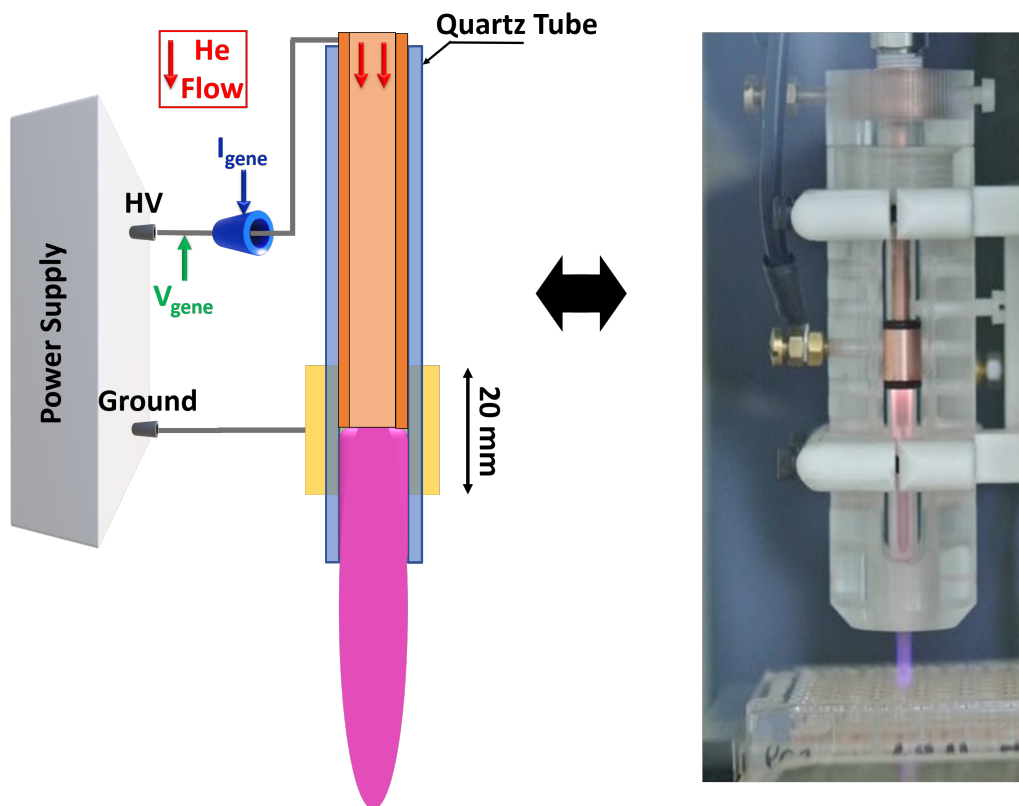


Figure 1: The working He plasma torch.

80 (Invitrogen), l-glutamine (2mM) (Invitrogen) and supplemented with 10 per-
 81 cent of fetal calf serum (Eurobio, France). All cells were grown at 37 °C in
 82 a humidified chamber with 5 % CO₂. Cold plasma exposition have been
 83 performed onto sub-confluent cell monolayer. Microscopy analysis have been
 84 performed using ZEISS Axio Vert.A1 and ZEN software.

85 2.3.2. Plasma treatment

86 Plasma treatment of cells was carried out 24 hours of cells incubation
 87 on 96 wells plates and treated with the parameters shown in table 1. A
 88 scheme view of the distances between plasma and medium is shown in figure
 89 2. On each 96 wells plates, reference treatment were done, consisting in just
 90 letting the helium flow above cells without igniting the plasma during 120 s.
 91 Plates containing cells and medium are placed on a XY table (OpenBuilds).

Parameter	Constant
Electrical frequency in kHz	20
Duty ratio in %	20
Helium Flow in L/min	5
Distance torch exit / medium in mm	10
Parameter	Variable
Voltage in kV	5-6-7-8
Exposure time in s	0-5-10-30-60-120

Table 1: Plasma parameters used in the treatment of cells

92 A Matlab interface was developed to write specific G-codes lines used by
93 the table interface. This program manages the displacement protocol of the
94 treatment, i.e the moves and treatments times.

95 *2.3.3. Cell viability*

96 The quantification of cell viability after plasma torch treatments were per-
97 formed by the MTT assays (3[4,5-dimethylthiazol-2-yl]-diphenyltetrazoliumbromide)
98 (Sigma-Aldrich, USA) [26] In briefly, for the different cell lines used, 20,000
99 cells/ml were seeded for 24 h in 96-well plates. Then, the cells were exposed
100 to helium flow (such as control) or cold plasma torch as described in the
101 previous section. The parameters that were mainly challenged to measure
102 cell viability were the voltage and exposure time. After 72 h of incubation,
103 the percentage of cell viability was quantified compared to the control treat-
104 ment using a spectrometer Multiskan GO (Thermo Scientific, USA) with an
105 intensity at 570 nm. All experiments were done in quadruplicate, using a
106 minimum of 3 independent biological replicas.

107 *2.3.4. Statistical analysis*

108 Results were analyzed using the GraphPad Prism 8.0 statistical software.
109 Data are reported as means \pm standard error of the mean (SEM) of four
110 independent experiments. For comparisons between two groups ordinary one-
111 way ANOVA test were used. Differences were considered to be significant
112 when *p-values* were less than 0.05.

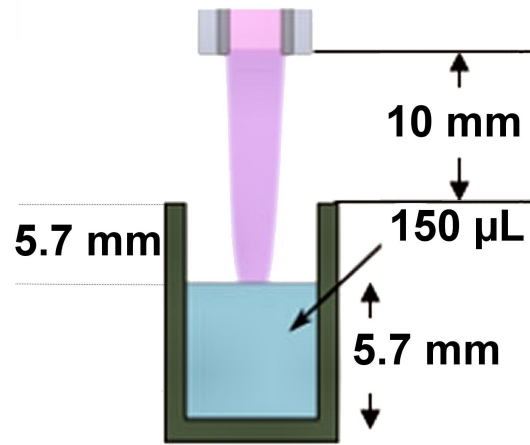


Figure 2: Schematic view of the 96 wells treatment

113 3. Results

114 3.1. Thermal effects

115 To ensure that the effect of the plasma on the cells is not thermal, the
116 temperature change ΔT with respect to ambient temperature was measured
117 during plasma excitation by using infrared thermography. Results are shown
118 in figure 3 for various helium flows. Starting from ambient temperature, the
119 culture medium surface first cold down because of the gas flow (before plasma
120 ignition): see curves in the gray-shaded zone of the graph. The plasma ex-
121 citation then resulted in temperature rises. The thermal evolution differed
122 depending of the gas flow intensity. Temperature remained lower than ambi-
123 ent temperature ($\Delta T < 0$) for the flows of 6 L.min⁻¹ and 8 L.min⁻¹, whereas
124 it exceeded ambient value ($\Delta T > 0$) for the flows of 2 L.min⁻¹ and 4 L.min⁻¹.
125 The lower the flow, the higher the specific enthalpy of the plasma, leading
126 thus to more energy provided to molecules and atoms of the culture medium.
127 Thermal equilibrium between gas flow effect and plasma effect ($\Delta T = 0$) can
128 be estimated at about 5.8 L.min⁻¹. Note that for 8 L.min⁻¹, temperature
129 slowly decreased with exposure time, whereas it increased for 2 L.min⁻¹.
130 However, even in this latter case, temperature change ΔT did not exceed
131 2° after 60 s. For further *in vitro* experiments, feed gas flow should be set at
132 about 5.5 - 6 L.min⁻¹ to avoid significant temperature change during treat-
133 ment.

134 3.2. Total electrical power and plasma length

135 The total electrical power of plasma torch is measured as a function of
136 the plasma voltage and helium flow, in free jet configuration. As shown in
137 Figure 4.a , the plasma torch electrical power increases linearly with voltage
138 between 6.6 W and 14.3 W respectively for 5 and 8 kV. Helium flow does
139 not impact electrical power consumption between 2 and 8 L/min. Inversely,
140 plasma length increases with gas flow, see Figure 4.b, whereas electrical volt-
141 age has no influence. In our conditions, plasma length can reach 70 mm
142 in surrounding air (i.e. after the end part of the capillary) at gas flow of
143 8 L/min.

144 The light emitted by the plasma torch is mainly produced by an ionization
145 wave propagating in the helium-rich region past the exit of the tube. The air
146 surrounding the helium ow is much more difficult to ionize due to difference
147 of ionisation potential, and consequently the propagation of the ionization
148 wave is conned into the volume of the helium channel. The gradual dilution of

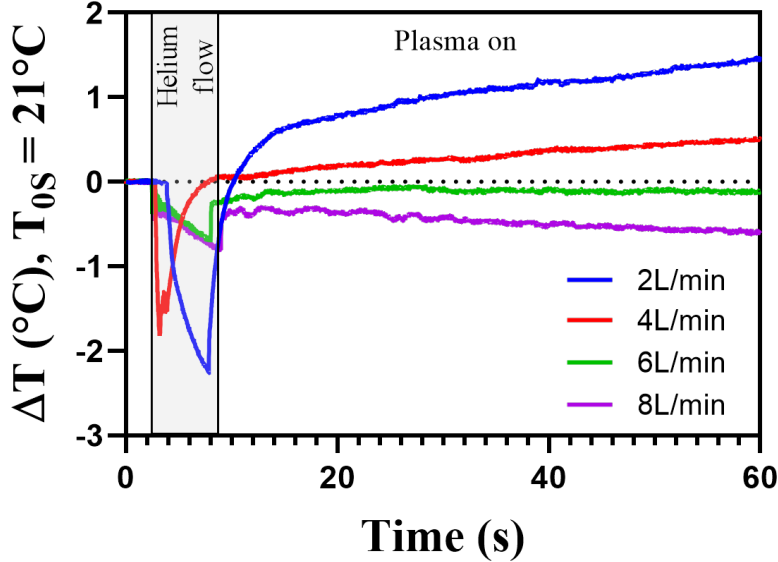


Figure 3: Temperature evolution of the medium surface during plasma treatment with different He flows at 6 kV voltage

149 helium in surrounding air is related to gas flow as predicted by hydrodynamics
 150 laws [27] and so increase helium flow increase the plasma length.

151 3.3. Plasma induces cell stress and cell death

152 Figures 5 and 6 sum up several microscopes images obtained on various
 153 cell lines 72 hours after treatments of 10, 30 and 120 s respectively at 5 and
 154 7 kV with an helium flow of 5 L.min⁻¹.

155 As expected, microscopy analysis confirm that long exposure (up to 120 s)
 156 results in cell death whatever the cell line. However, each cell line exhibits
 157 differential sensitivity to plasma treatment. Actually, focusing on 10 s treat-
 158 ment exposition, PC3 cells are more resistant to cold plasma treatment that
 159 LNCaP cells, as already depicted by Blagosklonny et. al [28]. Next, we
 160 choose to challenge non-tumor immortalized prostate cell line P69 in order
 161 to compare the effect of cold plasma treatment according to tumor status of
 162 each cell lines. Interestingly, we observed that p69 did not showed any cell
 163 death features up to 30 s exposure while LNCaP and PC3 cells are signifi-
 164 cantly impaired in similar conditions (see figure 6). These observations have

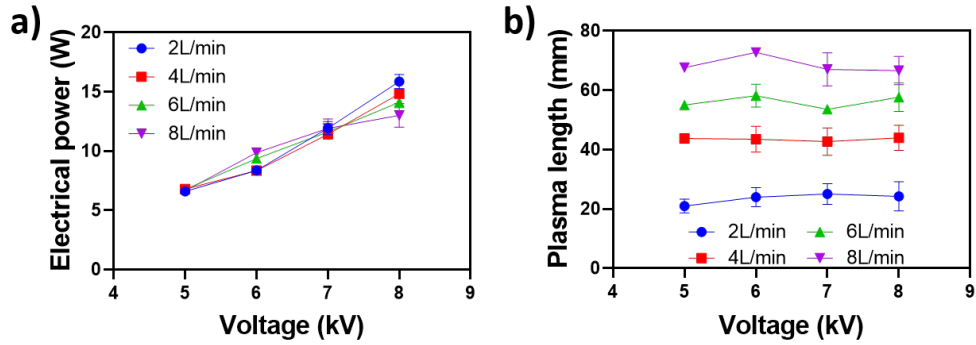


Figure 4: (a) Electrical power per pulse and (b) plasma length as a function of the plasma voltage and helium flow. Experimental conditions: Voltage: 5 to 8 kV, helium flow rate : 2 to 5 L/min, frequency : 20 kHz, duty cycle : 20 %, free jet configuration

165 been observed for 5 kV as well as 7 kV indicating that exposition duration
 166 more than electrical voltage seems to be the critical parameter. Together,
 167 these results indicate that tumor status of cell lines is correlated with sensi-
 168 tivity to cold plasma treatment.

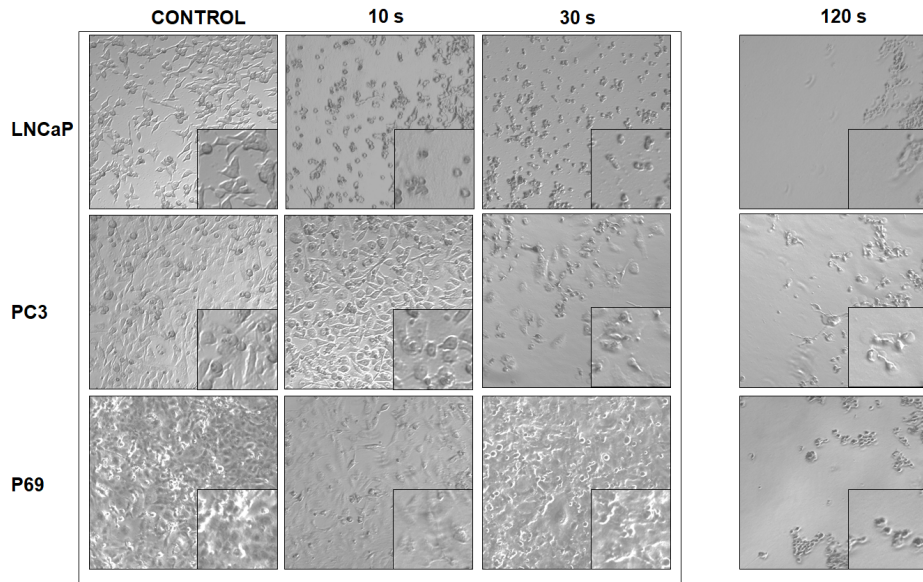


Figure 5: Microscopy pictures of control and treated cells 5 kV after 72 hours

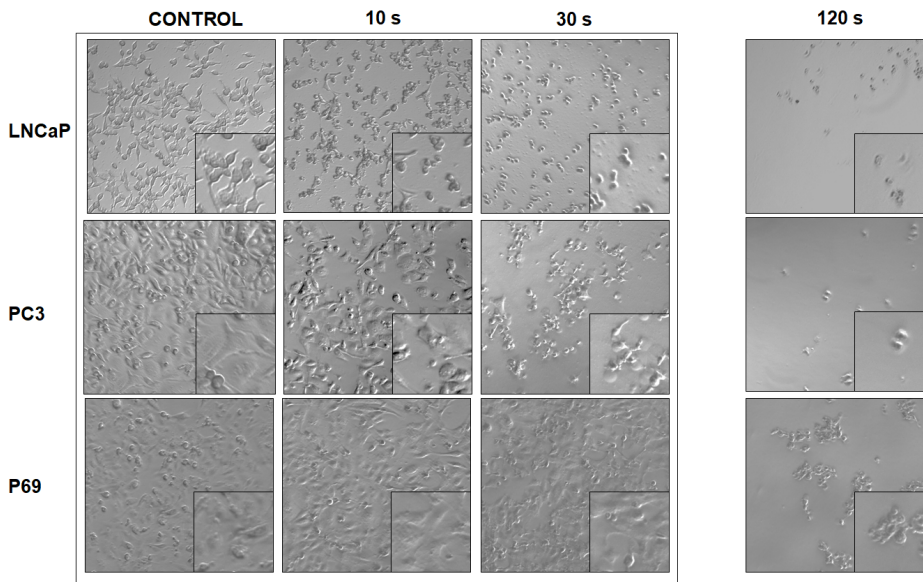


Figure 6: Microscopy pictures of control and treated cells 7 kV after 72 hours

169 *3.4. LNCaP and PC3 cells viability*

170 In order to quantify cold plasma effects on cell viability, we performed
171 MTT analysis. Figure 7 and 8 aggregate the results obtained 72 h after
172 cold plasma exposure of LNCaP and PC3 cells at various pulse duration
173 and voltage, helium flow was fixed to 5 L/min. First, we confirmed that
174 exposure duration is the critical parameter given that we observed significant
175 decrease of cell viability whatever the electric voltage used for the torch both
176 using LNCaP or PC-3 cells. However, we showed that electric voltage is
177 notwithstanding associated with cell viability. Indeed, viability percentage is
178 significantly decreased for 30 s exposure condition only when the threshold
179 of 7 kV is apply to the cell monolayer. Similar observations have been made
180 either on LNCaP cells and PC3 cells. These findings did not recapitulate
181 sensitivity differential between LNCaP and PC3 cells that have been observed
182 in figures 5 and 6 regarding 10s exposure condition. We could postulate
183 that morphology changes results in cell stress differential between both cell
184 lines without any effect on viability. Unexpectedly, we observed for both
185 cell lines that low electric voltage is associated with an increased in cell
186 viability for short pulse exposure (i.e. 5 s and 10 s). These results suggest
187 that cell exposition to cold plasma without enough injuries to trigger cell
188 death mechanism could conversely stimulate proliferation. Finally, we could
189 conclude that LNCaP and PC-3 cells exhibit similar response to cold plasma
190 treatment and that the critical parameter is the duration of pulse exposition.

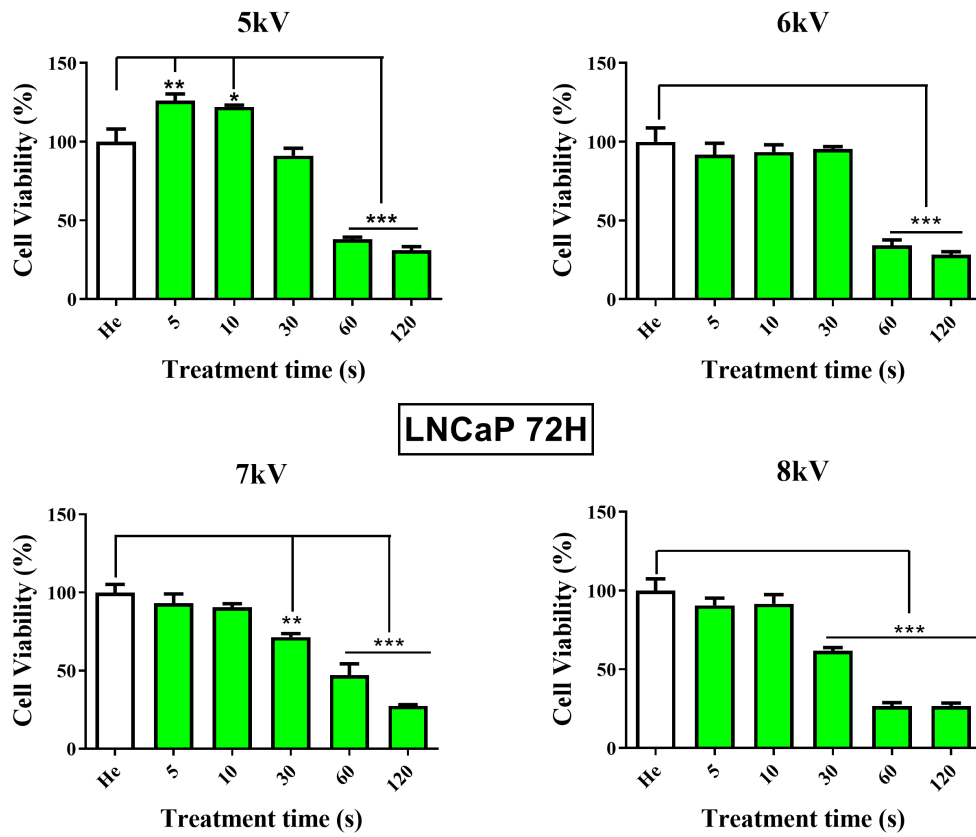


Figure 7: MTT analysis of LNCaP cells

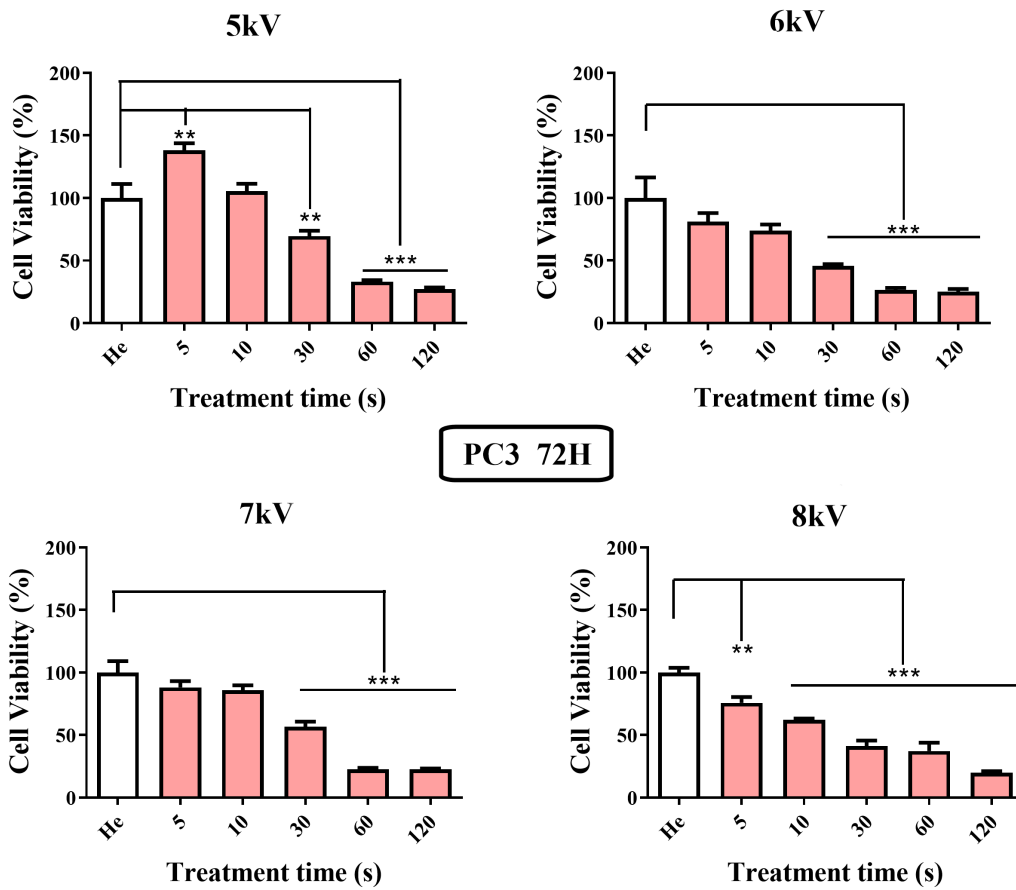


Figure 8: MTT analysis of PC3 cells

191 *3.5. P69 cells viability*

192 The results of MTT analysis on P69 healthy cells are shown in figure 9.
 193 According to previous findings using LNCaP and PC3 cells, cell viability is
 194 tightly correlated with the cold plasma pulse duration. Conversely to LNCaP
 195 and PC3 cells, P69 are significantly impaired in terms of viability at least
 196 upon 10 s of exposure. Nevertheless, as depicted in Figure 9, P69 cell line
 197 reveals to be more resistant to cold plasma treatment compared to LNCaP
 198 and PC3 cells. In particular, this is strongly marked for long pulse exposure
 199 such as 120 s. This last finding suggest that the tumor status of the cell line
 200 is associated with the sensitivity to cold plasma treatment. Together, these
 201 results support the idea that the malignant phenotype of the cell lead to en-
 202 hance the sensitivity to cold plasma opening new perspectives for therapeutic
 203 issues.

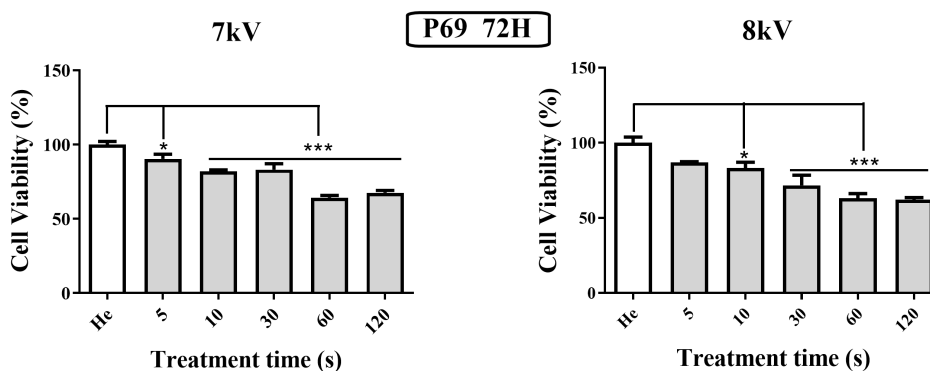


Figure 9: MTT analysis of P69 cells

204 **4. Conclusion**

205 In this paper we have shown the efficiency of the CAP treatment with he-
 206 lium gas on the prostate cancer cells like LnCaP and PC3. We demonstrated
 207 that exposure duration is the critical parameter, more than electrical voltage
 208 or helium flow, given that we observed significant decrease of cell viability
 209 whatever the cell line. Also we demonstrated that cold plasma treatment
 210 exhibits differential effect regarding tumor status of cells. The results show
 211 that healthy cells P69 are less sensitive to cold plasma exposition compared
 212 to metastatic tumor cell lines as summarized in figure 10. Forthcoming work
 213 will have to be devoted to investigate the mechanisms responsible of the cold

214 plasma selectivity. In the same way, more cell lines need to be treated with
215 plasma torch for demonstrate if selectivity always exist between tumor and
216 non-tumor cells.

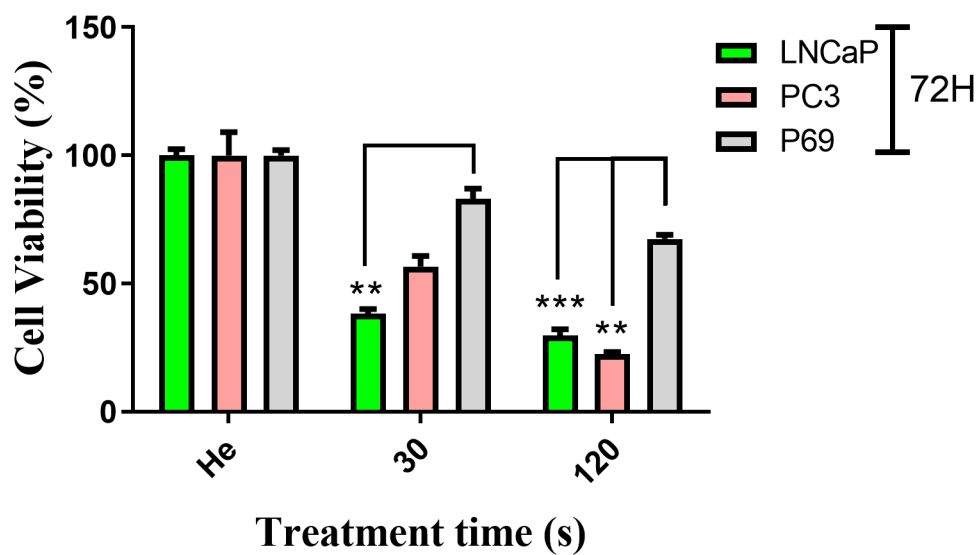


Figure 10: Comparison of cell viability 72 hours after plasma treatment

217 Appendix A. protocol used on a 96 wells plates

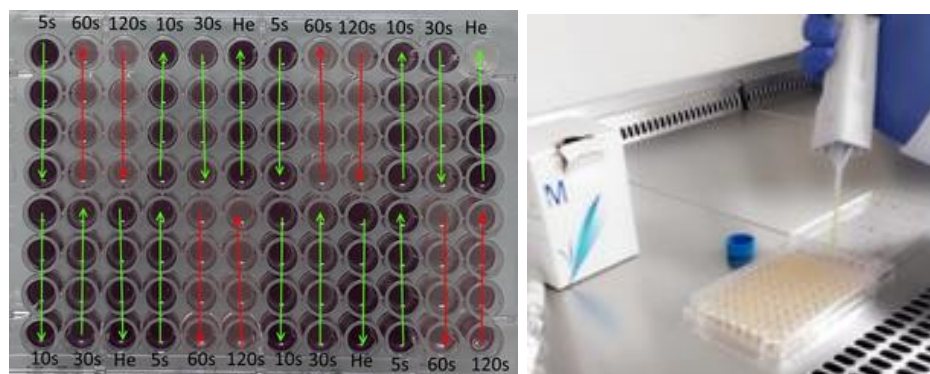


Figure A.1: Example of a protocol used on a 96 wells plates and the following MTT tests.

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