Cold plasma treatment of cholangiocarcinoma: investigating skin tissue as a barrier to electric field propagation and reactive species diffusion

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ABSTRACT – Cold atmospheric plasma (CAP) is a promising therapeutic modality for the treatment of cholangiocarcinoma (CCA), offering selective cytotoxic effects through the generation of reactive oxygen and nitrogen species (RONS) and electric field. However, the existence of biological barriers (*e.g.* epithelial tissues) between CAP and CCA may reduce the penetration of plasma-generated species and therefore the therapeutic benefits of CAP therapy. In this study, RONS are generated using an atmospheric pressure plasma jet (APPJ) to treat the upper surface of various tissue models, while the presence of long-lived reactive species is assessed on the opposite (basal) side. Particular emphasis is placed on *ex vivo* murine skin samples, which are used to mimic *in vivo* barriers and to explore the trans-barrier transport of RONS. The ultimate objective is to identify key parameters that influence species penetration and to refine CAP treatment protocols for enhanced therapeutic outcomes in CCA.

Keywords: cold plasma, cancer, cholangiocarcinoma, electric field, reactive species, tissue barriers

1. INTRODUCTION

Cold atmospheric plasma (CAP) has gained significant attention in oncology due to its ability to selectively induce tumor cell apoptosis while preserving healthy cells [1], ultimately slowing tumor progression, even for rigid and aggressive tumors such as cholangiocarcinoma, a cancer of the biliary tract [2]. One of the major assumptions supporting these findings is that the antitumor effects could result from an oxidative stress triggered by the reactive oxygen and nitrogen species (RONS) produced by plasma (*e.g.* OH, O_3, O, NO_X, O_2^- and $^1O_2$). In plasma-activated liquids, reactive species of longer lifespan can be generated, including H_2O_2, NO_2^-, NO_3^- and $ONOO^-$. While certain long-lived species, notably H_2O_2 and NO_2^- are known to synergistically promote cancer cell death [3], their ability to directly infiltrate cancer cells remains questionable as well as their involvement in the signaling pathways resulting in apoptosis.

In addition to RONS-mediated mechanisms, a complementary hypothesis posits that the CAP electric field (especially when delivered through high-voltage pulsed power at specific repetition frequencies) may itself contribute to the observed therapeutic effects. This concept aligns with the well-established domain of pulsed electric field (PEF) therapy, which has been explored for decades as a treatment modality for various cancers, including skin tumors and non-small cell lung carcinoma [4,5]. Thus, both the chemical (RONS) and physical (electric field) components of CAP may synergistically induce antitumor activity.

To better understand the interplay between these CAP components and antitumor effects, we have to consider that in most experimental settings, cancer cells are not in direct contact with CAP due to the presence of an intermediate medium which is likely to behave as a physical and/or chemical barrier. Upon *in vitro* assays, the CCA cells form a layer at the well's bottom, so that they are separated from CAP by a given thickness of culture medium. Upon *in vivo* experiments, tumors are separated from direct plasma exposure by biological layers such as skin for ectopic CCA grafted on murine models or epithelial and connective tissues for orthotopic models (*i.e.* biliary tracts).

These preclinical models raise several key questions: which extracellular RONS can penetrate biological barriers, such as mouse skin? Which physical parameters (*e.g.* time, gas flow rate, electric field magnitude) can modulate this penetration? If penetration is limited, how does CAP induce intracellular oxidative stress responsible for therapeutic effects?

Addressing these questions is crucial for optimizing CAP-based treatments to maximize their antitumor efficacy while ensuring minimal damage to surrounding healthy tissues. Therefore, the present study specifically investigates whether long-lived RONS like NO_2^- and H_2O_2 can be generated in a biological medium exposed to CAP, even when a biological barrier (*e.g. ex vivo* murine skin) is interposed between the plasma source and the medium.

2. MATERIAL AND METHODS

2.1. Experimental setup

Experiments are conducted using an atmospheric pressure plasma jet (APPJ) equipped with a double-ring electrode configuration. The lower electrode is grounded, while the upper electrode is supplied with high-voltage pulses (5-9 kV, 10 kHz frequency, 10% duty cycle). The device operates at 1 slm helium flow rate. As shown in Fig. 1, the experimental setup consists in four configurations:

(i) Negative control: culture medium without neither skin, nor plasma;
(ii) Positive control: skin covering the medium sample without plasma exposure;
(iii) PAM test: culture medium directly treated by CAP without a skin barrier;
(iv) Tissue test: CAP applied to the tissue-covered medium, exposing it to both RONS and electric field.

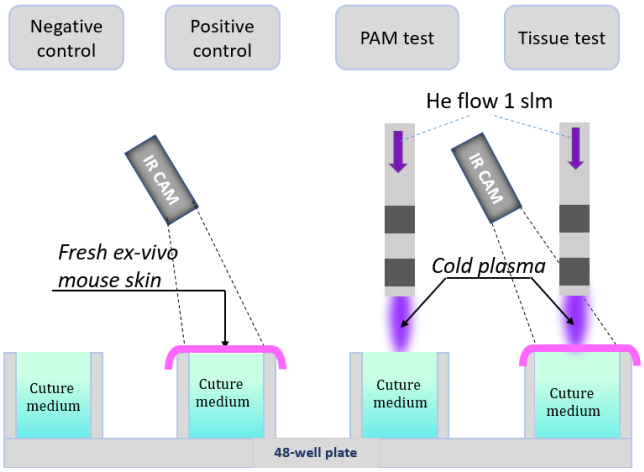


Fig. 1. Sketch diagram of the experimental setup

This experimental setup is designed to address key challenges encountered in both *in vitro* and *ex vivo* CAP-based studies. The main objective is to decipher if nitrites and hydrogen peroxide species, RONS currently produced by CAP in liquid environment, can pass throughout the mouse skin as hypothesized for *in vivo* subcutaneous tumor on murine models. Gibco™ DMEM culture medium is selected to correlate with the culture media of CCA cells used in *in vitro* experiments, without phenol red to avoid colorimetric interferences during spectrophotometry measurements [6].

To mimic the *in vivo* interface between murine skin and a subcutaneous tumor, *ex vivo* skin samples are placed in direct contact with 1700 µL of DMEM medium inside a well of a standard 48-well culture plate. Fresh dorsal skin samples from 22-week-old albino C57BL/6j-Tyr c mice are used following a dedicated protocol. Mice are shaved, euthanized and their dorsal skin are carefully isolated 10 min before plasma exposure. Each skin sample is 350 ± 50 µm thick and measures 2 x 2 cm² in order to cover a well of 48-well culture plate (12 mm in diameter).

Plasma treatments are achieved with a gap distance of 4 mm between the outlet of the APPJ device and the sample surface (skin or culture medium) during 2 min for 5 kV or 9 kV-applied voltage, 10 kHz-frequency and 10%-duty cycle.

All experiments are repeated thrice (N = 3).

2.2. Electrical characterization

The plasma's electrical properties are assessed in interaction with a liquid target, using a high voltage probe (Model P6015A 1000:1, from Tektronix, Beaverton, OR, USA) and a current monitor (Model 2877, from Pearson Electronics, Beccles, UK) connected to a digital oscilloscope (Model Wavesurfer 3054 from Teledyne Lecroy company, Chestnut Ridge, NY, USA) for precise waveform analysis. Measurements are performed at 5 kV and 9 kV applied voltages, with the plasma jet directed toward 1700 µL of culture medium contained in 48-well plates. A 100 pF capacitor, grounded and placed in series with the target, is used for monitoring voltage drops across the load. As a first-order approximation, the plasma voltage is estimated by calculating the difference between the voltage supplied by the power source and the voltage measured across the capacitor.

2.3. Thermal characterization of the samples

Sample temperatures are recorded using a VarioCAM® HD head infrared camera (InfraTec), which captures images via a microbolometer array with a resolution of 1024 × 768 and/or 640 × 480 pixels. Temperature measurements are taken at the sample surface (culture medium or mouse skin) two minutes before the beginning of plasma treatment, continuously throughout the entire treatment process, and two minutes after treatment.

2.4. Liquid phase characterization

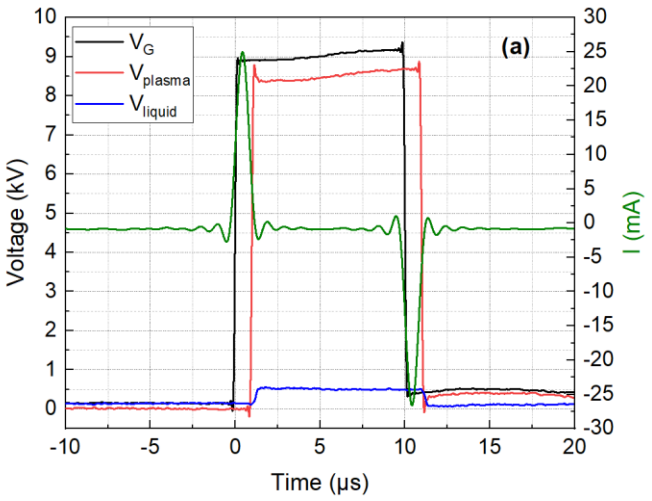
The concentrations of NO_2^- and H_2O_2 in culture medium are quantified by spectrophotometry. Preliminary experiments are conducted both in culture medium and demineralized water to assess the influence of treated medium in NO_2^- and H_2O_2 dependence on applied voltage.

Nitrite and hydrogen peroxide concentrations are measured by respectively adding 50 µL of sulfanilic acid (Griess reagent) or titanium oxysulfate ($TiOSO_4$) to a 200 µL solution of culture medium in 96-well plate, plasma-treated or not. Griess reagent reacts with NO_2^- to form a colored compound, usually pink or red, whose intensity varies with the nitrite concentration in the medium. Hydrogen peroxide (H_2O_2), when reacting with $TiOSO_4$ is reduced to Titanium (III), producing a yellow or blue color characteristic of the H_2O_2 concentration. Absorbances are measured at 515 nm after 15 min of reaction time for nitrites and at 410 nm immediately for hydrogen peroxide.

3. Results and Discussion

3.1. *Electrical characterization*

The time profiles of the high-voltage generator (V_G), the plasma voltage (V_{plasma}) and the voltage at the liquid surface (V_{liquid}) are represented in Fig. 2, as well as the time profile of the current in the whole circuit (I). It turns out that the plasma discharge is primarily delivered during the rising and falling edges of the pulses, with current peaks as high as 25 mA at 9 kV (Fig. 2a) and 6 mA at 5 kV (Fig. 2b).



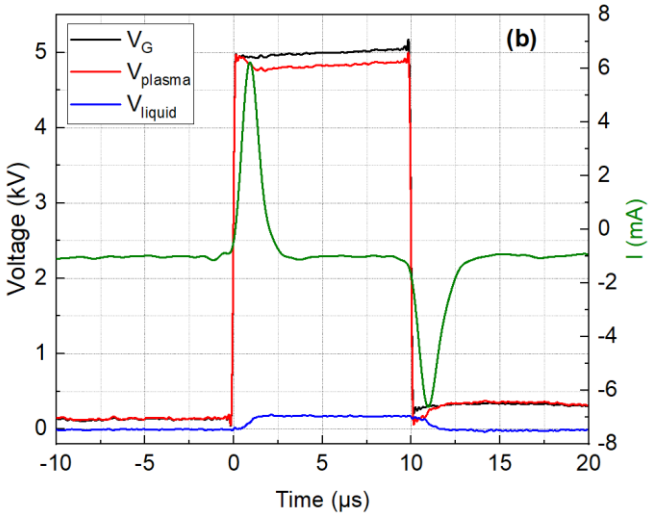


Fig. 2. Generator voltage, plasma voltage, liquid voltage and current measured for one period of the pulse in the liquid (culture medium) at (a) 9 kV and (b) 5 kV.

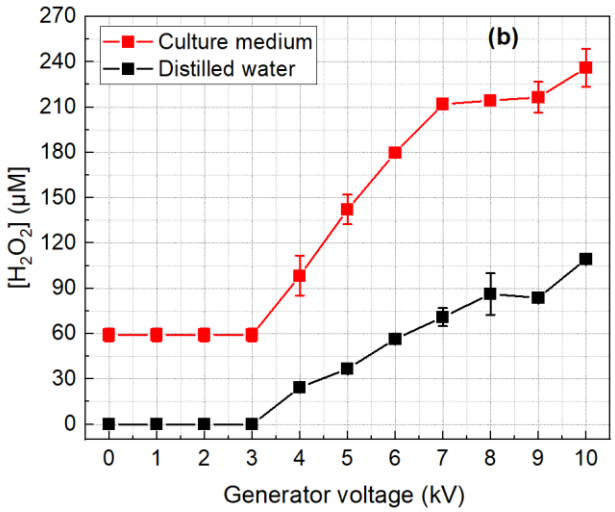
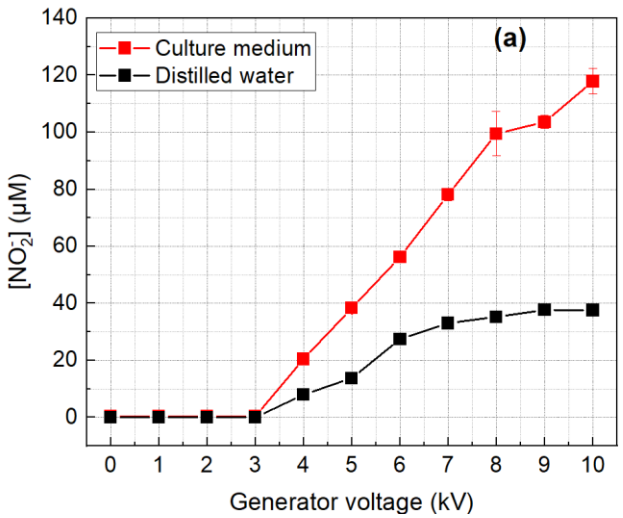


Fig. 3. (a) Nitrites and (b) hydrogen peroxide concentrations measured in a volume of 1700 µL demineralized water and culture medium sample *vs* applied voltage. Frequency = 10 kHz, duty cycle = 10 %, He flow rate = 1 slm, plasma exposure time = 2 min, gap = 4 mm

3.2. *Reactive species in liquid phase: influence of liquid-type on concentration profiles*

Temporal profiles of NO_2^- and H_2O_2 are monitored as key indicators of overall RONS production, first in demineralized water and second in culture media, under different plasma operating conditions. As shown in Fig. 3, NO_2^- and H_2O_2 concentrations increase with the applied voltage whatever the medium. In demineralized water, CAP produces more H_2O_2 than NO_2^-, reaching respectively 110 ± 3 µM and 37 ± 0.5 µM at 10 kV. Interestingly, in culture medium, nitrites are well much produced than in demineralized water, with 118 ± 5 µM at 10 kV. This is due to the medium composition, while demineralized water is only constituted by O and H atoms, culture medium is made up of amino acids, glucose, vitamins [6]. It is also noteworthy that the culture medium exhibits a non-negligible basal concentration of H_2O_2 (125 ± 25 µM), even in the absence of plasma treatment due to its composition.

Therefore, to correlate our results with the already observed *in vitro* antitumor effects of CAP, it is required to measure their proportion in culture medium rather than demineralized water.



3.3. *From gas-to-liquid media: a study on reactive species concentration profiles*

In Fig. 4, negative and positive controls correspond to culture media without and with skin layer respectively. As expected, the concentrations of H_2O_2 measured in the medium directly exposed to plasma (PAM test) at 5 kV (175 ± 12 µM) and 9 kV (174 ± 27 µM) are significantly higher than those observed in the untreated medium (125 ± 25 µM), highlighting the ability of plasma to enhance hydrogen peroxide (H_2O_2) production. When the medium is covered with mouse skin, a reduction in H_2O_2 concentration is observed in the treated wells (161 ± 10 µM at 5 kV and 165± 24 µM at 9 kV), compared to both the positive control (199 ± 26 µM) and PAM test. This decrease can be attributed to the barrier effect of the skin, whose nature and thickness (~350 µm) limit the diffusion of reactive species. It is also noteworthy that the culture medium exhibits a non-negligible basal concentration of H_2O_2 (125 ± 25 µM), even in the absence of plasma treatment.

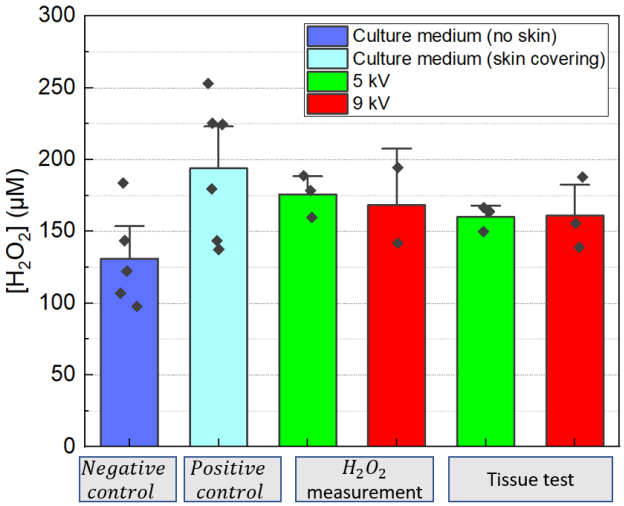


Fig. 4. H_2O_2 concentration in culture medium for the four conditions (N=3).

Fig. 5 shows the concentrations of NO_2^- in same experimental design. Unsurprisingly, the largest NO_2^- concentrations are measured in PAM condition with values close to 50 µM at 5 kV and higher than 100 µM at 9 kV. In contrast, when culture medium is covered with the skin sample, concentration of NO_2^- is no more detectable in the medium. These results suggest that for plasma treatment as short as 2 min,

the skin plays the role of an effective barrier to the penetration of NO_2^- species.

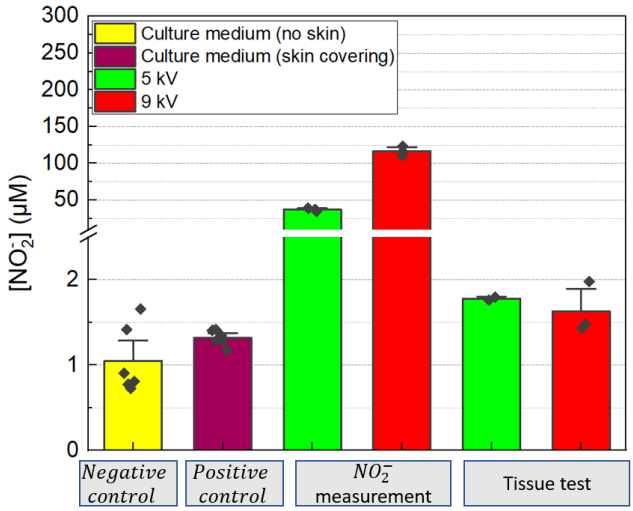


Fig. 5. NO_2^- concentration in culture medium for the four conditions (N=3).

3.3. Macroscopic observation of skin samples

The skin samples are examined to ensure the absence of deleterious effects (*e.g.* burnings). Fig. 6 shows the tissues 5 min before and 5 min after treatment. No noticeable thermal damage is observed whatever the condition.

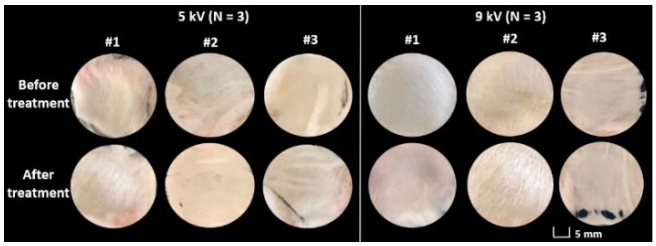


Fig. 6. Pictures of the mouse skin before and after plasma treatment, with gas flow (He) at 1 L/min, V_G = 5 kV and 9 kV, f = 10 kHz and a treatment time 2 min.

3.4. Temperature kinetics of the samples

Temperature mappings of culture medium and skin samples have been achieved at 5 kV and 9 kV. From these thermal maps, the minimum, average and maximum temperatures have been plotted as a function of time before, during and after plasma exposure, at 5 kV (Fig. 7) and 9 kV (Fig. 8).

At 5 kV, the maximum temperature during the plasma exposure is 23 °C on the liquid surface and 35 °C on the skin surface. Such difference is attributed to the higher specific heat capacity of the culture medium compared to that of biological tissue, such as mouse skin. These values are lower than 40 °C which is the temperature threshold to not overpass during experiments. However, at 9 kV, the average temperature reaches a value as high as 50 °C after 2 min of plasma exposure which makes such experimental condition inadequate to alive preclinical models.

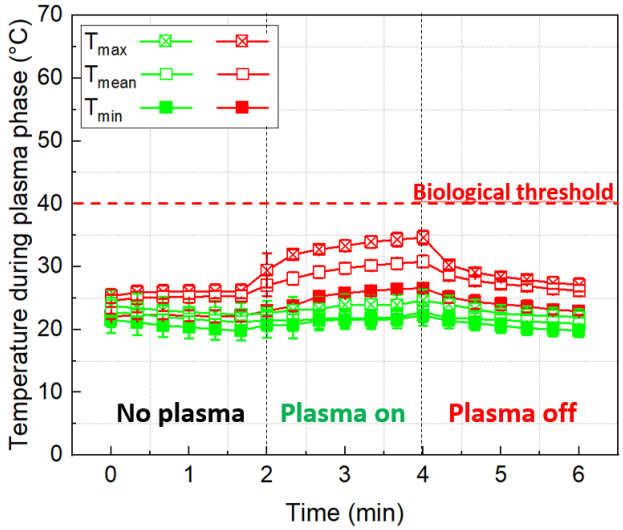


Fig. 7. Temperature values measured at the surface of the liquid and at the surface of mouse skin under a voltage of 5 kV.

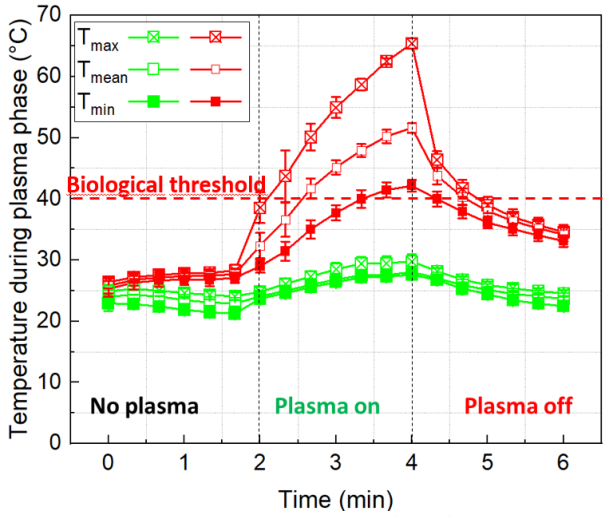


Fig. 8. Temperature values measured at the surface of the liquid and at the surface of mouse skin under a voltage of 9 kV.

4. Conclusion

This study has addressed the critical question of whether skin tissue acts as a barrier to the therapeutic effects of CAP. By focusing on the transcutaneous transport of plasma-generated species, it provides a first experimental framework to evaluate the modulatory role of biological barriers in CAP-based cancer therapy. Preliminary results indicate that, under the tested conditions (2 min of CAP), long-lived reactive species such as H_2O_2 and NO_2^- do not effectively cross murine skin, suggesting a significant limitation to passive diffusion. These results highlight the importance of optimizing key plasma parameters (including applied voltage and exposure duration) to maximize therapeutic efficacy while minimizing unintended tissue damage. Future work will aim to refine plasma delivery strategies, explore alternative mechanisms of action such as electric field effects, and assess the long-term biological impacts of CAP treatment.

5. Acknowledgments

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