Plasma-Cell interactions mediated by liquid phase chemistry: in vitro testing methods toward therapeutic approaches

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Abstract: During the last 10 years atmospheric pressure plasmas have shown great promise for the treatment of wounds and cancer. In this paper a sealed DBD system and a controlled gas environment were used in order to produce plasma activated liquid media and address important answers to the questions: is the H_2O_2 really involved in cell apoptosis/authophagy? Has the NO and its derivatives an active role in promoting some cell responses stimulated by PALM? Is it possible to disentangle the effect of RNS on the one of ROS for in vitro testing?

Keywords: Plasma Medicine, Plasma Activated Media, cancer treatment, reactive astroglyosis

1. Introduction

Plasma medicine is an interdisciplinary field of research that studies the direct application of cold atmospheric plasma (CAP) on or inside the human body for therapeutic purposes [1,2]. Over the past decade, plasma medicine has shown promising applications in sterilization, wound healing, dentistry, tissue engineering and cancer therapy [3]. Approaches to plasma medicine include: direct plasma treatment of cells in the presence or not of liquid media and plasma treatment of liquid solutions for secondary application to cells (indirect approach). The study of chemical composition of plasma activated liquid media (PALM), beside their importance to gain new insights in mechanism of action of direct approaches, offers important advancement towards an emerging field known as Plasma Pharmacy, i.e. the systemic administration of PALM for therapeutic treatment of patient diseases. The chemical composition of PALM produced by CAP depends mainly on the composition of the gas feed, on the energy dissipated in the plasma phase or in the liquid, the volume and the chemical composition of liquid to be treated and on the distance between liquid and plasma source [3]. The observed behaviours of cells exposed to PALM were presumably due to Reactive Oxygen and Nitrogen Species (RONS) considered as main players of the process [4]. Among several factors at the base of the efficacy of PALM there are H_2O_2, which is an important signalling molecule in eukaryotic cells including cancer ones, and NO_2^- which can act therapeutically, most probably as a precursor source of nitric oxide and NO derivatives. Both H_2O_2 and NO_x play a key role in apoptotic pathway of cancer cells and on brain wound healing and affect both cell motility and colony formation [5]. In spite of the huge amount of literature produced in the last years, a full understanding of the mechanisms of action of PALM in cancer therapy and brain wound healing is yet limited. In this paper a sealed Dielectric Barrier Discharge designed as *modified Petriplas+* has been used in order to disentangle the effect of Reactive Nitrogen Species (RNS) from that of Reactive Oxygen Species (ROS) on cancer cells and primary astrocytes. The potential application of PALMs in cancer therapy with the stimulation of immunologic cell death (ICD) has been investigated by the authors as reported in a paper under revision [6]. In this abstract we reported results by broaden the study to an investigation on other cell models of pancreatic cancer and metastatic melanoma demonstrating that the potential application of PALM envisioned in the paper [6] is not dependent on the chosen cell model. Moreover, the enhancement of glial migration stimulated by PALM has been investigated as the key process sustaining the wound healing in the brain.

2. Materials and Methods

The *modified Petriplas+* source utilized in this research is composed by a DBD setup designed in the Leibniz Institute for Plasma Science and Technology (INP) in collaboration with co-authors of this paper (E.S., NANOTEC, Bari, ITA). The source consists of an aluminium housing with a ground stainless steel grid electrode 4 mm far from the high voltage electrode, which is made of a copper disk (30 mm dia) covered with quartz. The *Petriplas+* source is designed for operation with commercial Petri dishes of 57 mm diameter (TPP,

Germany). In **Figure 1** is shown a scheme of the picture of the system used.

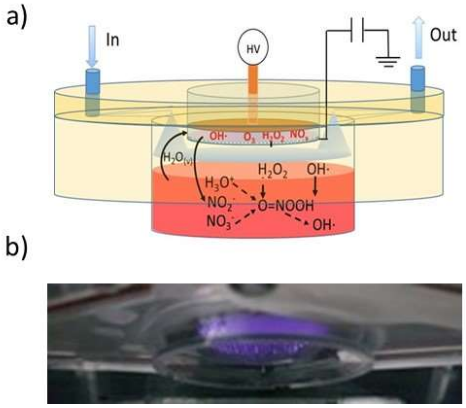

Fig. 1: a) scheme of the *Petriplas*+ system used; b) bottom view of the DBD discharge switched on in a 57mm petri dish and fed with N_2.

Experiments were carried out at 750 mTorr and room temperature, with a flow rate of 0.5 l min^{-1} of synthetic air N_2 and O_2 (Air Liquide, 99.999%). The AC (6 KHz) power supply used in this study is connected to a TG1010A programmable 10 MHz DDS TTi function generator, which allows variation of the sinusoidal voltage, amplitude and frequency of the input power for pulsing on/off the discharge. Electric parameters were monitored with a Tektronix TDS 2014 C digital oscilloscope and a Tektronix P6915A HV probe. The plasma has been pulsed with a variable duty cycle (25%-50%), over a period (t_{on} + t_{off}) of 100 ms. The dissipated energy is calculated by multiplying the mean energy per cycle by the t_{on}. Nitrites in the liquid were detected by means of the Griess assay (test kit Spectroquant) while a Spectroquant®Hydrogen Peroxide Test was used for the detection of H_2O_2 in the cell culture medium. NO detection was performed by means of ESR (VARIAN E109 ESR instrument) with iron(II) N, N-diethyldithiocarbamate used as spin trap.

MiaPaCa-2 human PDAC cells were purchased from ATCC®, EP-MM2 cells were generated starting from a metastatic melanoma patient's specimen. The antiproliferative effect of PALM against MiaPaCa-2 and EP-MM2 cells, was evaluated after 48 h of continuous incubation with the 3-[4,5-dimethylthiazol-2-yl]-2,5-phenyltetrazoliumbromide (MTT) assay. After treatment of human pancreatic and melanoma cancer cells with PALM, the protein level of CRT protein was analyzed by flow cytometry (CFM) and the release of ATP with an ELISA kit. ROS determination was carried out by H_2DCFDA staining and flow cytometry.

Primary cultured astrocytes prepared from Wistar rats were cultured in order to study glial migration. The effect of PALM on rat astrocyte adhesion and migration capability have been studied and correlated with the expression level of GFAP protein, as marker of astrogliosis. All cell lines were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) l-glutamine, 1% (v/v) penicillin/streptomycin, in a humidified atmosphere at 37 °C and with 5% CO_2.

3. Results

Hydrogen peroxide and nitrite were dosed in DMEM 10% FBS showing that the [H_2O_2] increases while the [NO_2^-] decreases with the content of O_2 in the gas feed reaching values up to 6±0.11 mg/l for H_2O_2 and up to 1±0.04 mg/l for nitrite ions. The MTT results confirmed a stronger reduction of cell viability in melanoma EP-MM2 cells rather than in the pancreatic cancer model (MiaPaCa-2) after 48 h of incubation with PALM O_2. In all samples, the reduction of cell viability was statistically significant ($p<0.001$). These results seem to be correlated with the ROS levels found in the cell lines under investigation before and after treatment with PALM. Moreover, the ATP release and the exposure of calreticulin (CRT) on cell membrane in both tumor models were found. Thus, these results confirmed our unpublished data that pancreatic and metastatic melanoma cell models exposed to PALM rich of H_2O_2 showed a reduction of proliferation and an increase of CRT exposure and of ATP release, suggesting the potential use of activated media as Immunogenic Cell Death (ICD) inducer, able to alert the innate immune system.

Finally, in case of primary astrocytes, results showed that a higher amount of ROS species including H_2O_2 negatively affect cell adhesion while a different cell migration is observed depending on [ROS] and [RNS] species in the PALM. Interestingly, GFAP protein, a typical marker of astrogliosis, resulted unaltered when plasmas from Air and N_2 were used. Thanks to the ESR investigation of PALM of PALMs, it seems that in complex system like cell culture medium the primary NO –the one coming from the discharge–would be involved in promoting certain biological responses, not directly, but only through one of its derivatives such as nitrite.

4. Conclusions

Our paper shows that indirect cancer treatment mediated by PALM can be a successful approach toward stimulation of ICD on tumor cells and that the role of ROS more than the one of RNS can be considered as pivotal. In prospective our study should strengthen the knowledge on the activity of ICD inducers, and should open the way to the identification of the reliable biomarkers for monitoring the induction of ICD by PALM that could be used in the selection of patients. In the case of investigation of primary astrocytes activity, our data, for the first time in literature, suggest that PALM can be considered a novel approach for modulating astrocyte physiological responses and the CNS scar formation, based on RONS-induced signal. These results lay the ground for new therapeutic strategies in CNS

disorders in which glial migration can be improved without generating the associated inflammatory astrogliotic response.

5. References

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