Characterization of Reactive Oxygen/Nitrogen Species Produced in PBS and DMEM by Air DBD Plasma Treatments

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ABSTRACT: Atmospheric pressure air dielectric barrier discharges were applied to phosphate-buffered saline and complete Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum to investigate their oxidative chemical modifications induced in liquids that are relevant for cell culture experiments in the field of Plasma Medicine. The study mainly identified long-living reactive species. Hydrogen peroxide, superoxide anion radicals, and nitrite and nitrate ions were detected in DMEM. The density of the reactive species was correlated with the energy dose delivered to the liquids by the discharges.

KEY WORDS: dielectric barrier discharge, plasma on liquids, culture medium, phosphate buffer serum, hydrogen peroxide, superoxide anion, nitrite, nitrate

I. INTRODUCTION

Nonequilibrium plasmas at low pressure and atmospheric pressure (AP) are acknowledged today as advanced, versatile tools for surface modification processes of materials for biomedical^{1–5} and other applications. Plasma processes also have been investigated, largely for the sterilization of materials and, most recently, for therapeutic purposes (plasma medicine).^{5,6} It was recently shown, for example, that wound healing, dentistry, and cancer treatments can potentially benefit from the direct application of air plasmas at AP onto living tissues.^{7–9} The treatments of living matter with air plasmas at AP are strongly affected by the presence of liquids (water, culture media, exudate, blood, etc.) in vivo and in vitro. A full understanding of the cascade reactions triggered by the plasma in the liquids is yet to be achieved; it is clear, though, that many short- and long-lived species are formed in water-based liquids upon exposure to plasma, depending on the plasma source and on the composition of both the feed gas and the liquid. It is also clear that plasma-generated reactive oxygen species (ROS) and reactive nitrogen species (RNS) affect cells and tissues more than other plasma species.^{10–14} For this work, phosphate-buffered saline (PBS) and the complete culture medium, Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS), were exposed to 3 dielectric barrier discharges (DBDs) in air characterized by different energy doses; superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-) species were generated in the liquids as a function of the energy delivered. The redox reaction involving cytochrome c was used to detect O_2^- anions. H_2O_2 was revealed

with a method based on 2,7-dichlorofluorescein (DCFH), kept selective by means of peroxidase enzyme. Colorimetric assays for NO_2^- and NO_3^- , already applied to PBS in our previous studies,¹⁵ were used here in DMEM + 10% FBS within 5 min. and 1 hour after the DBD treatment.

II. MATERIALS AND METHODS

A. Experimental Setup

An air DBD device with a parallel electrode geometry was designed to be mounted on top of 60-mm-diameter cell culture Petri dishes, which were filled with 3 mL of PBS or supplemented DMEM. The distance between the ground electrode and the liquid surface was 2 mm. The high-voltage (HV) copper plate electrode was covered with 1-mm-thick glass; the ground stainless steel mesh was spaced from the glass with a 1-mm-thick Teflon ring. The distance between the HV and the ground electrodes was 4 mm. DBDs were ignited with 13-kV peak-to-peak voltage at 6 kHz close to, but with no contact with, the liquids. Treatment time and plasma ignition time (t_{on} ; period, 100 ms) varied. According to our previous work, the 3 different experimental conditions investigated were set up with reference to the energy dose delivered to samples.¹⁵

ROS/RNS detection in PBS and DMEM + 10% FBS NO_2^- ions were detected using the Griess assay (Spectroquant test kit), whereas NO_3^- ions were detected via a color-forming reaction with 2,6-dimethylphenol (Spectroquant test kit). The detection of O_2^- was based on the reduction of ferricytochrome c to ferrocyclochrome c (Cyt Fe^{2+})¹⁶; the generation of Cyt Fe^{2+} increases the absorbance intensity at 520 and 550 nm. A stock solution of horse heart cytochrome c (5 mg/mL; Sigma Aldrich) was prepared and 400 μL were added to the treated liquid. To better elucidate the selectivity of the method used to detect O_2^- , superoxide dismutase (SOD) enzyme, a catalyst for the conversion of O_2^- into H_2O_2 and O_2 , was also added (40 μL of 50 $\mu\text{mol/L}$ stock SOD solution) to untreated and treated liquids.

The detection of H_2O_2 was based on DCFH, commonly used in the intracellular environment, where peroxidase catalyzes the reaction.^{17,18} When this assay was applied to PBS (Sigma Aldrich) and DMEM (Sigma Aldrich) supplemented with 10% FBS (Sigma Aldrich), peroxidase enzyme has to be added to keep the reaction selective toward H_2O_2 .¹⁸ DCFH is easily oxidized by H_2O_2 and turns to highly fluorescent species with an emission peak at 535 nm (excitation wavelength [λ_{ecc}] = 485 nm). A 10 mmol/L methanolic stock solution of DCFH diacetate (Sigma Aldrich) was prepared. To hydrolyze DCFH diacetate, 2 μL of a 100 $\mu\text{mol/L}$ carbonic anhydrase solution (Sigma Aldrich) were added. Finally, 1.4 μL of 60 $\mu\text{mol/L}$ horseradish peroxidase (Sigma Aldrich) were added before adding 750 μL of the untreated or plasma-treated sample.

All detections were performed with ultraviolet-visible spectrophotometers. The pH of the 2 buffered liquids did not change from their initial values (7.4 for PBS; 7.5 for DMEM) after the 3 different treatments.

III. RESULTS AND DISCUSSION

Figure 1A and B shows, respectively, the concentration of NO_2^- and NO_3^- detected in DMEM + 10% FBS before and after the 3 different plasma treatments. Increasing the energy dose delivered by the plasma resulted in a larger amount of both species in treated complete DMEM. Similar findings were obtained in PBS, as shown in our previous paper.¹⁵ Measurements were also repeated 1 h after exposure to the plasma, and the concentration of NO_3^- was increased, whereas that of NO_2^- had decreased. These findings give indications of the time evolution of the DBD-triggered chemistry involving nitrogen-containing species. Such behavior after discharge was revealed by Lukes et al.¹⁹ in air-plasma-treated liquid at various acidic pH values. In particular, the possible production of peroxynitrite was determined by kinetic analysis of the pseudo-second order between H_2O_2 and NO_2^- . The method was found to be valid even when decreasing the acidity of the solution.¹⁹

FIG. 1: Nitrite ion (NO_2^-) (A) and nitrate ion (NO_3^-) (B) concentrations in untreated and plasma-treated Dulbecco's modified Eagle medium +10% fetal bovine serum within 5 min. (light grey bars) and 1 hour after the plasma treatment (dark grey bars).

Fluorescence spectra of activated DCFH were registered for both untreated and plasma-treated PBS and DMEM supplemented with 10% FBS; as shown in Fig. 2, the fluorescence peak at 535 nm is very low for the untreated samples, whereas in both liquids it increases with the energy dose of plasma. A 0.2 J/cm² treatment increased the signal up to 250 arbitrary units (AU) in PBS and up to 220 AU in DMEM + 10% FBS. Both liquids, treated with 0.6 J/cm² air DBDs, shown an almost comparable fluorescence around 300 AU; after the 1.2 J/cm² treatment, the signal increases to 390 AU. This finding attests to the increased production of H_2O_2 with an increasing plasma energy dose. It is worth noting that without adding peroxidase, the same detection protocol could not detect H_2O_2 in the plasma-treated samples, leading to fluorescence signals that were almost comparable to those of the untreated samples (data not shown).

FIG. 2: Fluorescence spectra of activated 2,7-dichlorofluorescein of untreated and treated phosphate-buffered saline (A) and Dulbecco's modified Eagle medium +10% fetal bovine serum (B). Black lines refer to untreated samples; light grey lines, liquids treated at 0.2 J/cm²; grey lines, liquids treated at 0.6 J/cm²; and dark grey lines, liquids treated at 1.2 J/cm². AU, arbitrary units.

For the detection of O₂⁻, the reduction of cytochrome c was monitored in PBS and in DMEM supplemented with 10% FBS after adding ferricytochrome c; Fig. 3A and B shows the relative absorbance spectra for PBS and supplemented DMEM, respectively. Interestingly, in plasma-treated DMEM, the resulting production of Cyt Fe2+, highlighted by a peak at 550 nm, was more evident when the energy dose of the plasma was increased; however, this was not found in PBS. Such evidence allows us to hypothesize that the production of O₂⁻ in DMEM is probably cannot be ascribed only to the plasma. As emphasized by Wardman,²⁰ side reactions involving thiols or thiol-containing molecules (e.g., glutathione) generating O₂⁻ probably occur in complex media such as DMEM.^{19,21} In fact, glutathione, a strong antioxidant, is absent in PBS but is commonly present in both cell culture media and FBS. Preliminary experiments devoted to better elucidating the possible role played by glutathione and thiol-containing molecules in O₂⁻ production are in progress to confirm this hypothesis.

The insight of the presence of O₂⁻ was also strengthened by the kinetics of the cytochrome c reduction up to 1 hour after the plasma (Fig. 3C). Indeed, interesting results were obtained when the SOD enzyme was added; because of the dismutation of the O₂⁻ ions generated in the treated samples and the consequent production of H₂O₂, a decrease of the 550-nm peak intensity was observed, thus confirming the presence of such species in the plasma-treated liquids.

In our recent paper¹⁵ we showed the interesting effects of using plasma-treated DMEM for a limited duration on reducing or stimulating the viability of different cells (Saos-2, an osteoblast cell line, and primary ovine bone marrow stem cells) as a function of the energy dose delivered by the plasma to the medium and, ultimately, as a function of the ROS/RNS developed in it. We are aware that most of the promising successes of plasmas in wound healing (e.g., enhancing the viability of certain cells) and cancer treatments (e.g., triggering the death of cancer cells) are the result of the action of ROS/

FIG. 3: Absorbance spectra of cytochrome c when added to untreated and treated phosphate-buffered saline (A) and Dulbecco's modified Eagle medium + 10% fetal bovine serum (DMEM + 10% FBS) (B). Black lines refer to untreated samples; light grey lines, the 0.2 J/cm²-treated samples; grey lines, 0.6 J/cm²-treated samples; dark grey lines, 1.2 J/cm²-treated samples. (C) Evolution of the cytochrome c 550-nm peak after dielectric barrier discharges in untreated DMEM + 10% FBS (▲), 1.2 J/cm²-treated (■), and 1.2 J/cm²-treated with superoxide dismutase added (□).

RNS created by the plasma in the liquids surrounding the cells both in vitro and in vivo⁷⁻¹⁰; thus we strongly believe that a full understanding and control (by means of the plasma parameters) of the generation reactions of ROS/RNS in cell culture media and physiological exudates is essential for boosting the use of plasmas as therapeutic tools in hospitals.

IV. CONCLUSIONS

In this work 2 different buffered liquids, PBS and DMEM + 10% FBS, were analyzed after being exposed to air DBD to gain insights into both the potential of the plasma to produce ROS/RNS and the key role played by the liquid composition in this process. Long-lived species such as NO₂⁻, NO₃⁻, and H₂O₂ were detected in both liquids. O₂⁻ was

detected only in DMEM + 10% FBS, leading us to hypothesize some role for thiol-containing compounds in generating such species when , triggered by plasma. Deeper investigations are clearly needed to sharply quantify all active species generated in buffers and culture media as a function of the plasma exposure parameters, and to understand and unambiguously correlate the interaction of each kind of active species with various cells and tissues, with the aim to exploit fully the high potential of cold plasmas for therapeutic use.

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