COST-Jet and nspDBD Treatment Induces Similar Biological Responses While Generating Different Chemistry

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Abstract: The comparison of the biological response and the plasma-generated long-lived species of two plasma sources was investigated in this study. The observed reduction in viability and increased mitochondrial activity did not correlate to the resultant measured chemical species, which were ten times higher for the dielectric barrier discharge. These results indicate that plasma sources cannot be compared by their resultant chemistry when predicting the cellular response to treatment.

Keywords: nanosecond pulsed, glutathione, cancer treatment, COST-Jet

1. Introduction

Atmospheric pressure plasmas exhibit beneficial outcomes for biomedical applications in wound healing [1], cancer [2] and other diseases through their immunostimulatory effects [3]. Often, these outcomes are directly or indirectly attributed to the plasma-generated reactive oxygen and nitrogen species (RONS) [4]. This conclusion is common in studies regardless of the approach used such as directly through dielectric barrier discharge (DBD) [5], indirectly via atmospheric pressure plasma jet (APPJ) [6], or plasma-treated medium [7]. However, the concentration of plasma-generated RONS does not adequately serve as a comparison between these plasma devices or the biological effects due to the differences in amounts and contribution of UV exposure, electric field and short-lived and charged species. For example, plasma-generated hydrogen peroxide (H_2O_2) is theorized to play a major role in tumor cell killing [8]. However, studies comparing the nano-second pulsed floating-electrode DBD (nspDBD) and the kINPen (an APPJ) showed analogous reduction in viability for a colorectal cancer cell line, despite the fact that the kINPen produced twice as much H_2O_2 [9]. These observations indicate other plasma components of the nspDBD, in addition to H_2O_2, must contribute to comparable reductions in viability. Furthermore, this study supported the literature showing that exposure to exogenously added RONS was not the sole reason for the biological effect observed subsequent to plasma exposure [10]. This suggests that biological effects can be similar even if the end composition of RONS from each discharge is different. In order to compare plasma devices from a biological perspective, the resultant plasma components that interact with the cells must be measured. Understanding how plasma-generated RONS, UV, and electric fields affect the cells will allow devices to be tailored to each biological application and eventually guide the community to determine the effective "plasma dose" of each device. To this end, this work will show the comparison between the European COST-Jet and the nspDBD from both a chemical composition and biological response perspective. First, we will demonstrate comparable reduction in viability with Jurkat cells in response to plasma treatment. Then, we will discuss the plasma-generated and cell-produced chemistry as a result of plasma treatment.

2. Experimental Setup

2.1 Cell Culture

Jurkat cells clone E6 (ATCC® TIB152™) were maintained in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin in a 37°C incubator at 5% CO_2. For all experiments, cells were seeded at $3x10^5$ cells/ml in 12-well plates.

2.2 nspDBD Plasma Treatment

The nspDBD used in this work is similar to those described previously [5]. Non-thermal plasma was generated by applying a high voltage pulse to a copper electrode with a quartz dielectric barrier. As shown in **Table 1**, the treatment parameters for the nspDBD were fixed at 1 mm gap distance, 29 kV and an exposure duration of 10 seconds. The frequency of the pulses was varied to change the amount of energy deposited by the discharge. For both the colorimetric and cellular response assays, 100 µl of deionized water or media with cells were treated, respectively. All samples were treated in 12-well plates, except Raman samples which were treated in 24-well plates.

2.3 COST-Jet Plasma Treatment

The COST Reference Microplasma Jet (COST-Jet) is a capacitively coupled plasma jet. The feed gas used in this study is helium with a 0.6% oxygen admixture. The plasma is generated by applying an AC voltage at 13.56 MHz. The gases used in this experiment were 5.0 purity. The COST-Jet was operated at the ignition voltage to be comparable to reported literature [11]. The plasma treatment parameters are listed in **Table 1**. Similar to the nspDBD, viability and MitoSOX experiments were performed in 12-

well plates while the Raman samples were prepared in 24-well plates.

Table 1. Plasma Treatment Parameters

Parameter	DBD	COST-Jet
Treatment Distance	1 mm	4 mm
Voltage	29 kV	200 V - RF
Gas Flow Rate	--	He + 0.6% O_2 He – 1 SLM O_2 – 1 SCCM
Frequency	30, 45, 75 Hz	13.56 MHz
Treatment Time	10 s	0, 1, 2, 3, 4, 5 minutes
Treated Volume	100 µL	500 µL
Pulse Width	20 ns	--
Rise Time	2 ns	--
Energy/ Pulse	0.9 mJ /pulse	--

2.4 Viability Assay

Jurkat cells were exposed to nspDBD at 45 and 75 Hz for 10 s or the COST-Jet effluent for 2 and 4 mins. Immediately after nspDBD treatments, cells were supplemented with media then incubated for 24 hours prior to assessing cell viability. Dead cells were detected using propidium iodide (ThermoFisher Scientific, USA) and the percent viable cells was quantified using an image cytometer (Nexcelom Bioscience, Vision CBA, USA).

2.5 MitoSOX Red Assay

Intracellular mitochondrial superoxide production was detected in Jurkat cells 24 hours post plasma exposure. Cells were washed with PBS then stained with MitoSOX™ Red mitochondrial superoxide indicator for 10 minutes (ThermoFisher, Cat. M36008). Superoxide generation was correlated with fluorescent intensities measured using an image cytometer.

2.6 Colorimetric Assays

Plasma treated samples were analysed immediately for H_2O_2 and nitrite (NO_2^-) concentrations using colorimetric assays. The phenanthroline derivative-copper method (Millipore Sigma, USA) and Griess reagent (ThermoFisher Scientific, USA) were used for H_2O_2 and NO_2^- respectively. 150 µl of both nspDBD and COST-Jet treated deionized water were analysed.

2.7 Raman Spectroscopy

Glutathione (GSH) (ThermoFisher Scientific, USA) samples were prepared in deionized water at 10 mg/ml. For the nspDBD treated samples, 10 µl were treated at 45 and 75 Hz, frozen at -80 °C and shipped to North Carolina State University for analysis. Upon arrival, 10 µl of the sample was dried on a calcium fluoride window overnight using a desiccator. For the COST-Jet, 500 µl samples were treated for 2 and 4 minutes at North Carolina State University and followed the same drying process. Measurements were performed on a confocal Raman microscope (Senterra II, Bruker, USA) with a wavelength of 532 nm at 20 mW. All measurements were taken using a 50x objective lens (OLYMPUS BX53M). The spectra were measured from 0-3700 cm^{-1} using the OPUS software. For each sample, 10 spectra were averaged for 10 different positions with an integration time of 2 s, similar to previous studies [12]. All measurements were done in triplicate.

2.8 Statistical Analysis

Each experiment was performed in triplicate unless otherwise stated. The data are plotted as mean and standard deviation. Graphs were plotted in Origin 2018b (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1 COST-Jet and nspDBD Treated Samples Induce Comparable Reduction of Viability in Jurkats.

To compare how these two plasma sources affect the cells post discharge, we investigated the treatment parameters necessary to achieve a similar biological endpoint. We plated Jurkat cells the day before treatment and treated the cells at conditions similar to previous studies done with the nspDBD and the COST-Jet [5, 13]. First, we performed a cell-viability dose response curve with both sources to find comparable treatment conditions. We increased the frequency for the nspDBD and the treatment time for the COST-Jet samples respectively. Samples were analysed 24 hours post treatment.

In **Fig. 1**, only the comparable results were plotted. All samples are normalized to the mock treated controls: cells that were plated but not exposed to plasma or the feed gas (COST-Jet control). Jurkat cells exposed to the gas flow (He + 0.6% O_2) in the absence of plasma had comparable viability to the mock treated cells, indicating that the feed gas itself did not affect cellular viability. This result is similar to other studies using the COST-Jet [13].

The viability of the Jurkat cells was reduced with increasing frequency for the nspDBD and increasing treatment time for the COST-Jet. Two comparable treatment conditions were identified between the two plasma sources: 1) 2-minutes for the COST-Jet (90.3%) and 45 Hz for the nspDBD (86.5%) and 2) 4-minute (63.28%) and 75 Hz (59.66%). These results suggested that comparable levels of cellular stress were achieved after exposure to two different plasma sources at their respective parameters. During plasma application, the cells are exposed to varying magnitudes of UV radiation and electric fields, and concentrations of short-lived, long-lived, and charged species depending on the source. Therefore, these two discharge conditions were used as the basis for further comparative investigations to understand how the plasma chemistry played a role in reducing cell viability.

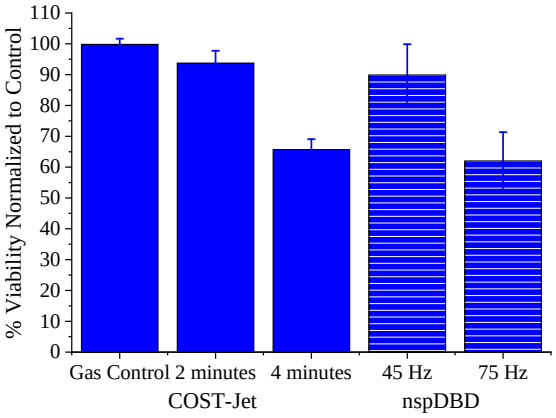

Fig. 1. Viability Comparison of nspDBD and COST-Jet Treated Jurkats.

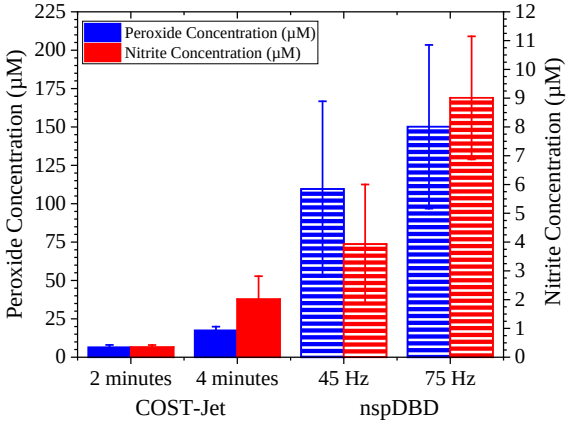

Fig. 2 Comparison of H_2O_2 and NO_2^- concentrations in plasma-treated deionized water.

3.2 Chemical Concentrations of Long-Lived Species Are Higher in nspDBD Treated Samples

The generation and delivery of RONS from the plasma to the cells is the main difference between plasma jets and DBDs. For the Jurkat cells to have similar reduction in viability between these two sources, we hypothesized that the resultant chemical species that affected the cells would have to be of comparable magnitudes. To assess this hypothesis, we measured two long-lived species, H_2O_2 and NO_2^-, in deionized water. H_2O_2 and NO_2^- were measured in deionized water treated at the conditions used in the viability experiments. For both plasma sources, the concentration of long-lived species increased with longer treatment times and higher frequencies.

The H_2O_2 and NO_2^- concentration of the deionized water exposed to nspDBD were higher than the COST-Jet samples, as shown in **Fig. 2**. The nspDBD H_2O_2 concentrations at 45 and 75 Hz were higher than those reported previously [9]. This is likely due to the higher surface area of the electrode used in this study. The concentration increased from 109.55 μM at 45 Hz to 150.11 μM at 75 Hz. The NO_2^- concentrations also increased with frequency from 3.92 μM to 9.01 μM. The COST-Jet samples showed an increase of both H_2O_2 (6.49 to 17.49 μM) and NO_2^- (1.15 to 2.01 μM) concentrations with treatment time. These concentrations are similar to those reported in the literature [11].

The higher concentrations of long-lived species resulting from nspDBD exposure indicates that the viability of the cells is unaffected by changes in concentration at these magnitudes. However, there are multiple mechanisms that result in cell death from reactive oxygen species [14]. This suggests that the cell death mechanisms observed may be different between cells exposed to the nspDBD or the COST-Jet. Furthermore, the plasma components that are unique to each source (UV, electric field for the nspDBD, and forced convection for the COST-Jet) may affect the cell-death mechanism. Taken together these observations suggest that using chemical concentrations as an indicator of biological outcomes may not be sufficient for comparing plasma sources.

3.3 Raman Spectroscopy

To understand how the solvated, plasma-generated RONS react with biomolecules, we analysed the modifications caused post plasma treatment. Similar to previous reports, DBD treatment of GSH caused a decrease in the intensity of the peak at 2560 cm^{-1}, and an increase of the 1045 cm^{-1} peak when compared to the untreated GSH as shown in **Fig. 3**. [12]. These wavenumbers correspond to the S-H (ν(S-H)) and the S=O vibrational stretching modes (ν(S=O)) respectively. These observations indicate that the –SH bonds are being oxidized. The COST-Jet treated samples had a similar reduction in the ν(S-H) band, however comparable decrease for the ν(S=O) mode was not observed when compared to the nspDBD samples. This is likely due to concentration difference of H_2O_2 produced by the nspDBD compared to the COST-Jet. The ν(S=O) band does seem to increase in the 2 and 4 minute treated samples in comparison to the gas only control. Additionally, it was shown that H_2O_2 concentration is higher when helium is the feed gas rather than a helium/oxygen admixture [11]. Therefore, future studies will investigate how admixtures and treatment time affect the resultant chemistry of the COST-Jet treated samples and the conditions that are comparable to the nspDBD.

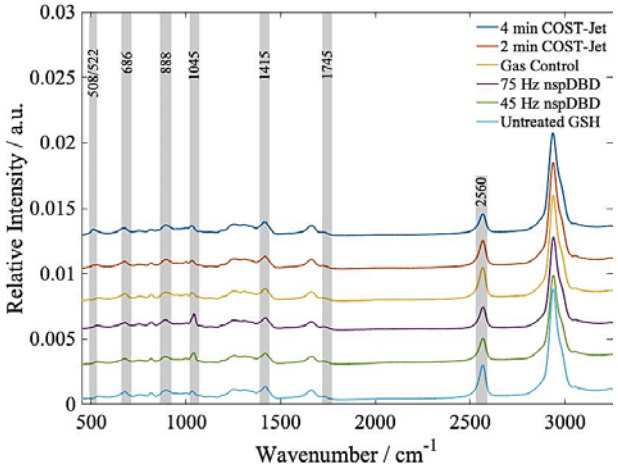

Fig. 3. Raman spectra of GSH in deionized water post plasma treatment with the nspDBD and COST-Jet.

3.4 Mitochondrial Superoxide Production Increases in Response to Plasma Treatment

Superoxide (O_2^-) production in the mitochondria is an indicator of cellular oxidative stress due to the potential formation of H_2O_2 and peroxynitrite [15, 16]. Increases in O_2^- concentration above physiological conditions were shown to negatively impact cellular functions and even result in cell death. We compared MitoSOX fluorescence in Jurkat cells treated with both plasma sources to understand if there were differences that were unique to the treatments. As shown in **Fig. 4**, the 2-minute COST-Jet and the 45 Hz nspDBD treated cells had similar magnitudes of MitoSOX fluorescence. Both treatments increased mitochondrial O_2^- production when compared to the untreated cells. These observations suggest that regardless of plasma treatment approach, mitochondrial activity increases in response to treatment. This result is unexpected due to the measured H_2O_2 and NO_2^- concentrations. However similar to the viability results, changes in RONS concentrations of this magnitude may not have a significant role on mitochondrial activity. In addition, the Raman spectra between the two sources are similar which may indicate that from a cellular perspective the treatments are similar despite the differences in concentration. Depending on the exact stimulated cellular pathways in response to COST-Jet or nspDBD treatment, the sources may be more suited for particular applications that are sensitive to exogenous RONS concentrations.

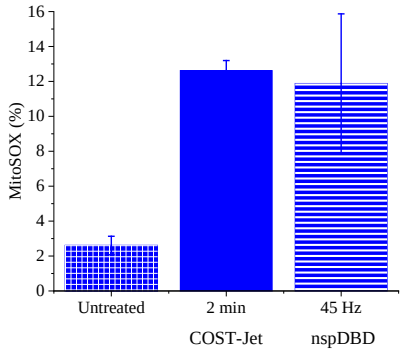

Fig. 4. Comparison of mitochondrial superoxide production in response to plasma treatment between COST-Jet and nspDBD treated Jurkats.

4. Conclusion

Comparison of the nspDBD and COST-Jet treatments has shown that plasma sources may induce similar biological responses while producing different magnitudes of chemical species. Future studies comparing these sources will focus on the individual components and their role in the resulting biological responses. Results from this study and mock treatments of GSH with exogenously added RONS will be presented.

5. References

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