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Mitochondria-Mediated Anticancer Effects of Non-Thermal Atmospheric Plasma

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Abbreviations: OxPhos, oxidative phosphorylation; FCCP, carbonyl cyanide 4-(trifluoromethoxy)

Abstract

Non-thermal atmospheric pressure plasma has attracted great interest due to its multiple potential biomedical applications with cancer treatment being among the most urgent. To realize the clinical potential of non-thermal plasma, the exact cellular and molecular mechanisms of plasma effects must be understood. This work aimed at studying the prostate cancer specific mechanisms of non-thermal plasma effects on energy metabolism as a central regulator of cell homeostasis and proliferation. It was found that cancer cells with higher metabolic rate initially are more resistant to plasma treated phosphate-buffered saline (PBS) since the respiratory and calcium sensitive signaling systems were not responsive to plasma exposure. However, dramatic decline of cancer oxidative phosphorylation developed over time resulted in significant progression of cell lethality. The normal prostate cells with low metabolic activity immediately responded to plasma treated PBS by suppression of respiratory functions and sustained elevation of cytosolic calcium. However, over time the normal cells start recovering their mitochondria functions, proliferate and restore the cell population. We found that the non-thermal plasma induced increase in intracellular ROS is of primarily non-mitochondrial origin. The discriminate non-thermal plasma effects hold a promise for clinical cancer intervention.

Introduction

Prostate cancer is the second leading cause of death from cancer in North American and European men [1]. It is a slow growing cancer, but as many other types of cancer, it is generally incurable once it reaches the metastatic stage [2]. Existing chemotherapies have severe side effects and do not provide a cure for advanced stages of the disease. There is an urgent need for novel medical approaches for treating tumor types which tend to easily develop resistance to chemo- and radiation therapies [3]. Non-thermal atmospheric pressure plasma has been

phenylhydrazone; ROS, reactive oxygen species; DBD, dielectric barrier discharge.

recently identified as a potent technology for modulating the function of both prokaryotic and eukaryotic cells. Non-thermal is distinguished from thermal plasma based on the relative energetic levels of electrons and heavy species of the plasma [4]. Biomedical applications of non-thermal plasma include surface sterilization [5], wound healing and blood coagulation [6, 7], anti-bacterial treatment [8] and induction of cancer cells apoptosis [9–11], stimulation of proliferative activities of endothelial cells [12], anti-bacterial treatment [13, 14].

In biomedical applications, non-thermal plasmas are characterized by the type of discharge and method of applying the plasma products to cells and tissues. The types of discharges commonly used include dielectric barrier discharge (DBD), corona discharge, and gliding arc discharge [15]. Dielectric barrier discharge plasma is generated in the gap between two electrodes driven by *a.c.* voltage, typically in the kHz frequency range. One of the electrodes is insulated with a dielectric barrier with high breakdown strength which prevents spark formation in the discharge region, thus eliminating localized overheating. The two broad categories of plasma application modality are *direct* and *indirect* treatment. Direct plasma application is one in which the tissues or cells are in direct contact exposing the sample to both the chemical plasma products and the electric field used to generate the plasma, with cell lysis being the most drastic physical effect observed [16]. The indirect involves administration of plasma-treated liquids to cells and relies on the transfer of plasma-generated reactive species to the cells while eliminating the exposure of cells to electric field of plasma. The method of liquid-mediated indirect treatment appears to be more suitable for future clinical applications when a tumor may be not accessible for direct treatment in a patient.

To realize the full potential of non-thermal plasma treatment for cancer therapeutics, the exact mechanisms through which plasma causes cell death must be understood. It is also critical to study the side effects of non-thermal plasma on healthy cells. The primary goal of this work is to explore the effects of indirect non-thermal plasma generated by microsecond (pulse width) dielectric barrier discharge on mitochondria-mediated processes. The mitochondria orchestrate cell metabolism and signaling, and therefore, they are a promising target for cancer therapy [17]. Yet, it has been demonstrated that high doses of plasma induce apoptosis in other cancers due to massive generation of intracellular reactive oxygen species (ROS) [9, 18] and the mitochondria are one of the major intracellular sources of ROS [19]. These facts indicate that elucidating the mechanisms of non-thermal plasma effects on mitochondria is critical for learning how we can advance proof-of-concept demonstrations into a clinically-relevant method for cancer treatment.

A new antitumor drug or therapeutic treatment targeted only to cancer cells without affecting normal ones is the Holy Grail in cancer research. Achieving this kind of selectivity is very challenging which is why the side effects of chemo and radiotherapies remain a major problem. In this work, we compare the outcomes of non-thermal plasma treatment for metabolically different normal and cancerous prostate cells. It is reasonable to hypothesize that both normal and cancerous cells can be affected through the mitochondria-mediated mechanism to hopefully different extents.

Materials and Methods

Cell lines and growth conditions

Human prostate metastatic DU145 cells were purchased from ATCC at the available passage 60 and used up to passage 70 (Manassas, VA USA). Cells were maintained in RPMI 1640 growth medium supplemented with 10% FBS. Human primary prostate cells PrEC obtained at passage 2 from Lonza Inc. (Allendale, NJ USA) were maintained in manufacturer recommended PrEGM medium with all required supplements according to the manufacturer

protocol except gentamicin and used by passage 5. Both cell lines were grown at 37°C and 5% CO_2 atmosphere.

Generation of atmospheric non-thermal plasma and cell treatment procedure

To generate non-thermal plasma we used the setup described elsewhere [9, 20]. Plasma was generated by applying alternating polarity pulsed (500 Hz –1.5 kHz) voltage of 20 kV magnitude (peak to peak), 1.65 μs pulse width and a rise time of 5 V/ns between the high voltage electrodes using a variable voltage and frequency power supply (Quinta, Russia). One mm thick quartz glass was used as an insulating dielectric barrier covering the 1-inch diameter copper electrode. A grounded mesh was placed between the high voltage copper electrode and the surface of the medium to block electrons and ions and allow only uncharged gas species to reach the medium.

The plasma dose for a DBD device is often expressed as energy per unit area (Joules/cm^2), where energy is the output energy provided by the power supply (input to the electrode) and the area is the surface area of the electrode. Although this definition is useful in many applications it is not an actual measure of the plasma energy delivered to the medium. For our purposes it was more useful to characterize the plasma doses (D1-D7) through the shifts of pH values of 1 ml double deionized water in an eppendorf tube using 0.5 cm pH electrode tip upon application of plasma at certain frequency/power ratio and duration (**Table 1**). The narrowing 1 cm diameter opening of the eppendorf tube enables to minimize the contact of water surface with atmospheric CO_2, which could buffer water pH.

The use of indirect plasma mediated through plasma-treated fluids including deionized water, phosphate-buffered saline (PBS), and N-acetyl-cysteine (NAC) has been evaluated for various purposes [21]. In this work the PBS (Ca^{2+}/Mg^{2+} free) was chosen to produce plasma-treated solution as by composition it is similar to physiological solution used in clinical practices. The PBS treated with plasma dose D7 retained pH 6.8.

Cells monolayers were washed from growth medium and covered with plasma treated PBS for 1 or 10 minutes and the solution was diluted with fresh complete medium in 1:15 (plasma-PBS:RPMI medium) volume ratio.

Cytotoxicity test

Cytotoxicity was assessed by the Alamar Blue assay [22]. Cells were seeded in the 96-well plate (10,000 cells/well). Control and experimental cells were incubated with 50 μl of 3% Alamar Blue solution prepared in a complete growth medium at 37°C for 2 hours. The fluorescence signal (peak emission 585 nm) was read on BioTek Synergy 4 microplate reader (Winooski, VT, USA).

Table 1. Determination of non-thermal plasma doses as a function of pH change.

Setup parameters	Non-thermal plasma doses						
	D1	D2	D3	D4	D5	D6	D7
Hz:W:Sec	2:3:5	2:3:10	2:3:15	2:3:20	2:3:30	3:3:20	3:3:30
pH	6.4±0.13	4.9±0.11	4.5±0.13	4.1±0.11	3.9±0.14	3.7±0.14	3.2±0.01

The plasma was applied to double deionized water at different frequency/power ratio and duration. The more acidic the water, the stronger the plasma dose.

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