Tumoral and Normal Cells Treatment With High-Voltage Pulsed Cold Atmospheric Plasma Jets

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***Abstract*—High-voltage pulsed cold atmospheric plasma jets were used to treat tumoral (B16 and COLO320) and normal (macrophage) cells. Cold atmospheric plasma jet generator and plasma jet physical characteristics are presented. The treatment of the tumoral cells intended to obtain their apoptosis. For tumoral cells, percents of up to 70% apoptosis have been obtained, using helium plasma, activated with up to 2% oxygen. In the same experimental conditions, the plasma treatment did not induce apoptosis in normal (macrophage) cells. In addition, the macrophage cells were not activated by the plasma jets.**

***Index Terms*—Cells apoptosis, cold atmospheric plasma jets, pulsed high voltage.**

I. Introduction

COLD ATMOSPHERIC plasma jets are studied as a new treatment method in the biomedical field [1]–[8]. This treatment has a strong chemical action, without the gaseous residues or dangerous radiations disadvantage and this appliance should have the advantage of a "targeted treatment," avoiding nonignorable side effects that inevitably also act on healthy cells too, by affecting their functionality. Thanks to relatively small costs and easy usability, the plasma device could be included in every hospital endowment for oncology, dermatology departments, etc.

The cold plasma jets act at the cellular level to remove diseased tissue without inflammation and damage. In cancer therapy, based on destruction of tumor cells by use of different methods, one should remember that there are two types of cellular death: apoptosis and necrosis. Apoptosis, the process of programmed cell death, involves a series of morphological processes leading to controlled cellular self-destruction. The second death cell type, necrosis, represents a form of traumatic cell death that results from acute cellular injury. Apoptosis, in contrast to necrosis, is not harmful to the host and does not induce any inflammatory reaction. This fact is very important because it is known that chronic inflammation promote tumorigenesis [9].

In order to assess the usage of the atmospheric pressure plasma jet as an effective device in tumor treatment, we studied the onset of the apoptosis. Our aim was to maximize this controlled cell death.

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At atmospheric pressure, the plasma jets are produced by electric discharges in helium or argon. However, pure helium or argon plasma jets have a low chemical activity, thus being inappropriate for biomedical applications. Their chemical activation is necessary, this implying that some chemically active species such as: oxygen atoms, OH radicals, nitrogen atoms, NO radicals, nitrogen ions, helium/argon excited atoms, etc. exist in the plasma jet. The most important chemically active species are oxygen atoms and OH radicals. That is why the introduction of the oxygen in the discharge area is of greatest importance to chemical activation. When obtaining chemically active species, the electrons produced in electric discharges have the essential contribution. The collisions between fast electrons and atoms and molecules result in enhanced levels of excitation, dissociation, and ionization, i.e., enhanced plasma chemistry. Introducing the oxygen in the discharge area implies finding a solution to the following problem: The concentration of the oxygen must be sufficiently low in order not to excessively disturb the electric discharge, but sufficiently high for a strong chemical activation of the plasma jet. That is why a very important result of our experiments was to determine optimal oxygen concentrations in view of applications in the biomedical field.

A major cause of antitumor chemotherapy failure is the development of multidrug resistance (MDR) of tumors due to MDR efflux pumps which extrude anticancer drugs from the tumor cells [10]. This paper reveals the effect of high-voltage pulsed repetitive cold atmospheric plasma jets which are chemically activated with oxygen, on B16 tumoral cells (murine melanoma cell line) and COLO320 multidrug resistant cells (human colon cancer cell line). In order to emphasize the plasma effect, we have used Verapamil as well-known MDR efflux pumps inhibitor. A comparative study between the treatment of the tumoral and a type of normal cells is presented.

Our experiments and results allow the release of an animal (murine) model for *in vivo* study of melanonma.

II. Experimental Setup

A. Cold Atmospheric Plasma Jet Generator

The cold atmospheric plasma jet generator, shown in Fig. 1, replaced our old devices, made using medical syringes [11]. This new generator is similar with the device shown in [4], which works with ac voltages. In our case, pulsed high voltages are used. The advantage of the new structure is that it allows for flexibility during the modification of various geometrical parameters.

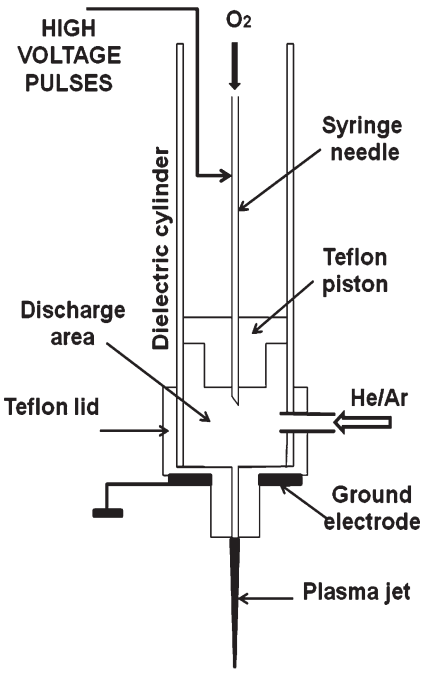

Fig. 1. Drawing of the device for generating high-voltage pulsed cold atmospheric plasma jets.

The device shown in Fig. 1 is made out of an insulating material cylinder, with an inner/outer diameter of 16/18 mm. The piston made of Teflon is placed in this cylinder. A medical syringe needle with the inner/outer diameter of 0.5/0.8 mm passes through the center of this piston. The needle is the high-voltage electrode. The chemical activation gas (oxygen, in this case) is introduced through this needle in the discharge area. The inert gas (helium, argon), facilitating the occurrence of electric discharges at atmospheric pressure is introduced through an orifice in the lateral wall of the dielectric cylinder. The lower end of the dielectric cylinder is covered with a Teflon lid. The exit channel of the plasma jet is positioned at the center of this lid. The inner diameter of the exit channel is of 1.5 mm. A metal ring working as the ground electrode of the device is stuck onto the outer part of the lid. The discharges within the cylinder are similar to the dielectric barrier discharges. The inert gas (helium, argon) flow pushes the discharge plasma out, forming the plasma jet.

High-Voltage Pulse Parameters: To obtain the discharge plasma, high-voltage pulses were applied between the high voltage and the ground electrodes. These pulses had amplitudes of 15–20 kV, durations of hundreds of ns and repetition frequencies of hundreds of pulses per second (pps). Our pulsed high-voltage generator is home made and can produce voltage pulses of up to 30-kV amplitude (Fig. 2). Similar techniques are used by some other authors [12], [13].

Gases Delivery System: The working gases were supplied by high-pressure cylinders. Gas pressure regulators were used to reduce the pressure of gases to a workable level. Then, gas flow controllers delivered the gases with the desired flow rate. For the inert gases (He, Ar), flow rates of up to 5 L/min could be controlled and measured. The oxygen flow rate was adjusted using a flowmeter (Aalborg, USA) into a range of 0–500 mL/min. Considering the flow rate of the inert gas is of a few liters per minute, these flowmeters allow for a fine adjustment of the oxygen concentrations within a range of 0%–3%. This range covers the most probable optimal concentrations.

Spectral Analysis: For spectral analysis, a low-resolution (0.75-nm FWHM) optical fiber spectrometer connected to a computer running SpectraSuite is used: HR 4000 (Ocean Optics, Inc., USA).

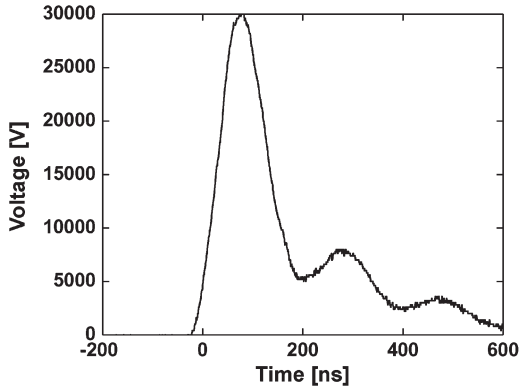

Fig. 2. High-voltage pulses obtained with the pulsed high-voltage generator.

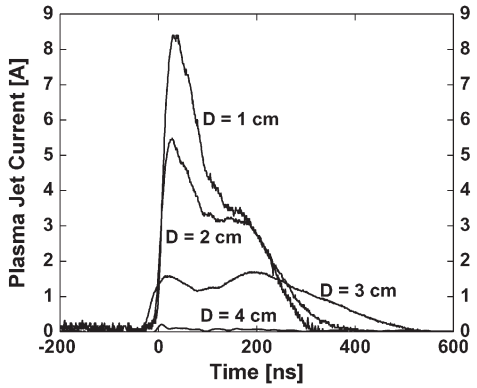

Fig. 3. Plasma jet currents for different distances (D) from the plasma exit hole. The pulsed voltage amplitude is of 20 kV. The gas is pure helium, with a flow rate of 4 L/min.

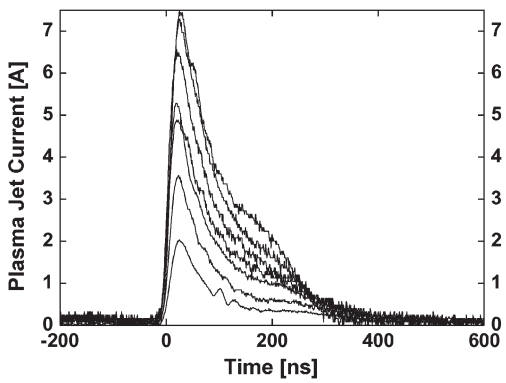

Fig. 4. Current of the plasma jet at a distance of 1 cm from the exit outlet, for various concentrations of oxygen in helium. From the most intense to the weakest current, the oxygen concentrations are the following: 0.2%, 0.4%, 0.6%, 0.8%, 1%, 1.5%, and 2%, respectively. Helium flow rate = 4 L/min. The pulsed voltage amplitude = 20 kV.

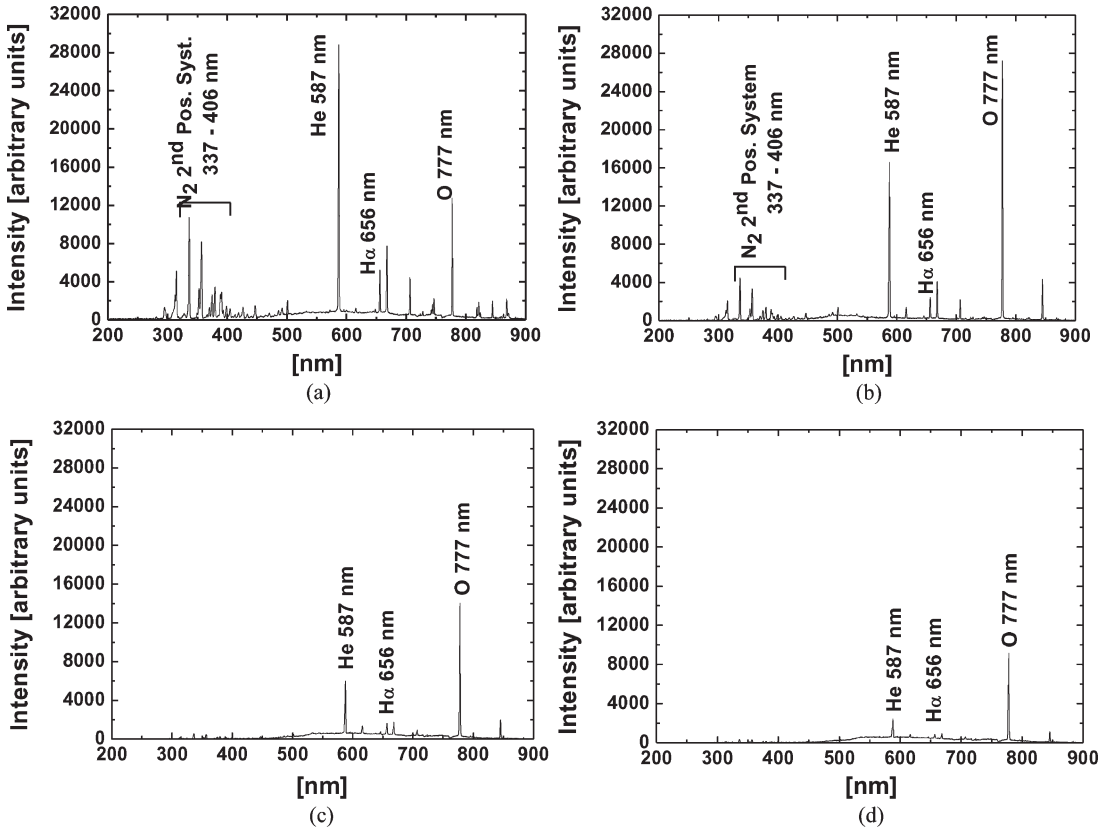

Fig. 5. Emission spectra of the helium plasma jets, for various oxygen concentrations in the discharge area: (a) 0%, (b) 0.5%, (c) 1%, and (d) 1.5%. Helium flow rate = 5 L/min. Interelectrodes high voltage amplitude = 20 kV. Pulse repetition frequency = 100 pps.

B. Cell Treatment and Analysis

In our experiments, we have used the following type of cells:

1) tumoral cells B16-F10 (skin cancer);
2) tumoral cells COLO320DM (human colon carcinoma cells, whose membranes contain high amount of MDR efflux pumps);
3) normal cells RAW264.7 (macrophages).

Jet Plasma Treatment and Apoptosis Analysis: The cells (B16-F10, COLO320DM and RAW264.7) were cultured $(1 \times 10^6$ cells/system) in complete medium (culture medium supplemented with 1 mmol L-glutamine, 10% fetal calf serum and antibiotics). When cells formed a monolayer (observed by means of optical microscopy), they were exposed to plasma jet for different time periods and using different combinations of helium and oxygen inputs. Cell culture environment (media) has been refreshed before the experiment, the cells remaining attached to the substrate. This preparation of the cells did not affect them, so that the observed results are due to plasma exposure. The plasma jets were produced with pulses of 20-kV amplitudes and 100-pps repetition frequency. Subsequently, the adherent cells were detached with trypsin, washed twice (2 min, 2000 r/min) and the samples were analyzed by flow cytometry for measuring the percentage of apoptotic cells after propidium iodide staining in hypotonic buffer, using the Nicoletti method [14]. Cells were first gated according to their scatter characteristics and then analyzed for apoptosis by WINDMI.2.7 software.

The obtained histograms (Figs. 6, 7, 9 and 11) have an apoptotic part (M1 interval) and three parts corresponding to cell evolution (M2, M3, and M4 intervals). The apoptotic percent is given by the M1 graphic area divided by the total histogram area.

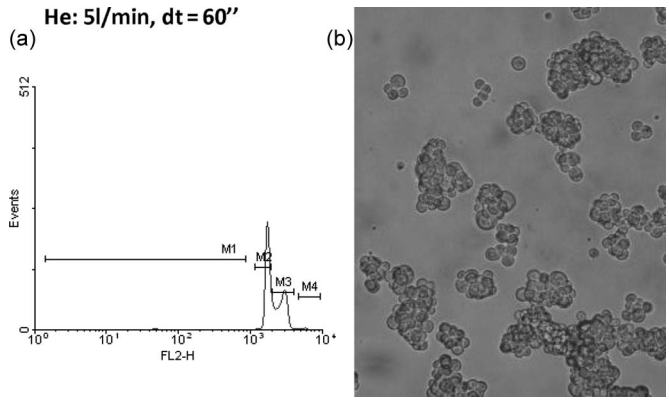

Fig. 6. B16-F10 tumoral cells treatment with pure helium (without oxygen) plasma jets. The distance between plasma jet and monolayer cells = 10 mm. Helium flow rate = 5 L/min. Treatment time = 60 s. (a) There is no apoptosis. (b) Cells detachment from substrate.

The COLO320DM tumoral cells were treated with Cyclophosphamide 1 μg as chemotherapeutic agent or Cyclophosphamide 1 μg + Verapamil 5 μg for 60 min before plasma treatment.

Morphological Modifiers Assay: The cell samples ("smears") were fixed in methanol for 5 min and then stained with Giemsa solution (10%) for 30 min. The pictures of Giemsa stain slides were collected from optical microscope

using a digital camera. The results were compared with flow-cytometry data.

Cell Activation Measurements: Activated macrophages have the ability to destroy microbes or other cells (including tumoral ones). The cells get bigger and less regular in shape and begin to produce, among other molecules, large amounts of nitric oxide. Therefore, NO quantity is a measure of cell activation intensity, but only in the cases where there is not another NO source. NO production is indirectly assessed by quantifying nitrite in the sample, knowing that NO spontaneously converts either in nitrite, or in nitrate which also converts in nitrite. The Griess colorimetric method [15] is characterized by a high sensitivity, detecting the nitrite concentration at about 0.5-μmol level. Absorbance at 540 nm presents a linear dependence toward nitrite concentration in the sample. We have tested RAW 264.7 culture cells supernates after plasma jet treatment and culture medium (used for RAW264.7 cells) exposed in the same conditions. Obtained values were related to a standard nitrite curve (0.049–200 pg/ml).

III. Experimental Results

A. Cold Atmospheric Plasma Jet Characteristics

Electrical Characteristics: The electrical current varies along the plasma jet length. Up to now, this fact has not been taken into consideration. In order to measure this parameter, the plasma jet was "captured" by a little copper plate, at different distances from the plasma exit hole. A metallic wire connected the copper plate with the experimenter's fingers. In this way, the plasma jet was electrically coupled to ground through the human body impedance. From electrical point of view, the human body is a parallel RC circuit, with $R \sim 1$ MΩ, and $C \sim 60 - 120$ pF[8]. The current through the metallic wire, equal with the plasma jet current, was measured with a current probe (Tektronix P6021).

In Fig. 3, the plasma jet currents are shown, for different distances from the plasma exit hole. The pulsed voltage amplitude was of 20 kV. The gas was pure helium, with a flow rate of 4 L/min. For the first 30 mm, the currents had significant values (greater than 1 A). Therefore, the treated objects must be situated at 10–20-mm distance from the plasma exit hole.

Once the concentration of the oxygen introduced in the discharge area is rising, the plasma jet current is getting lower and lower. Fig. 4 shows the currents of the plasma jet at a distance of 1 cm from the exit outlet, for various oxygen concentrations in helium: from 0.2% up to 2%. The helium flow rate was 4 L/min. The explanation of this phenomenon lies in the fact that the introduction of an electronegative gas (such as oxygen) in the inert gas produces a reduction of the electron density and, as a result, a reduction of the electric conductivity.

Spectral Characteristics: The optical emission spectra were observed at 1-mm distance from the nozzle exit.

A first observation with reference to the helium plasma spectra was that emission lines in 200–300-nm range were very weak. Our spectrometer managed to detect γ-NO bands at 255, 263, 276, 281, and 295 nm, but their intensities were lower than 700 units. This means that ultraviolet photons do not constitute an active species in such plasmas. This is the conclusion reached by other authors as well [16], [17]. However, the detected NO is very important for cells treatment (see Section III-B).

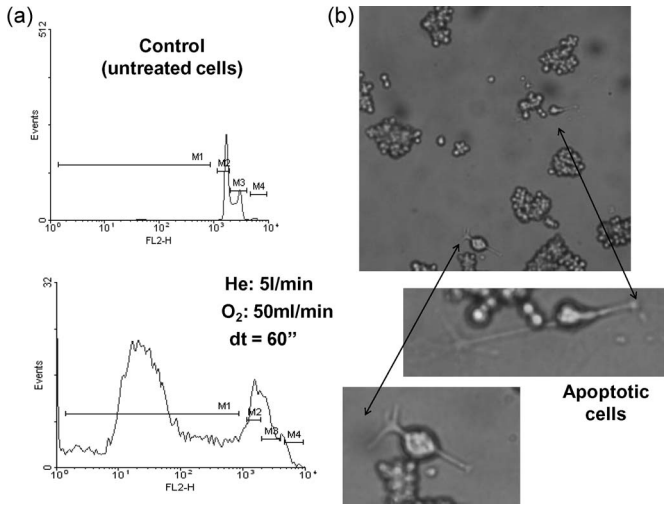

Fig. 7. (a) Helium-oxygen plasma jets (He: 5 L/min and O_2: 50 mL/min) induce apoptosis in tumoral B16—F10 cells. Apoptosis percent = 74.25%. (b) Morphological modifications. The distance between plasma jet and monolayer cells = 10 mm. Treatment time = 60 s.

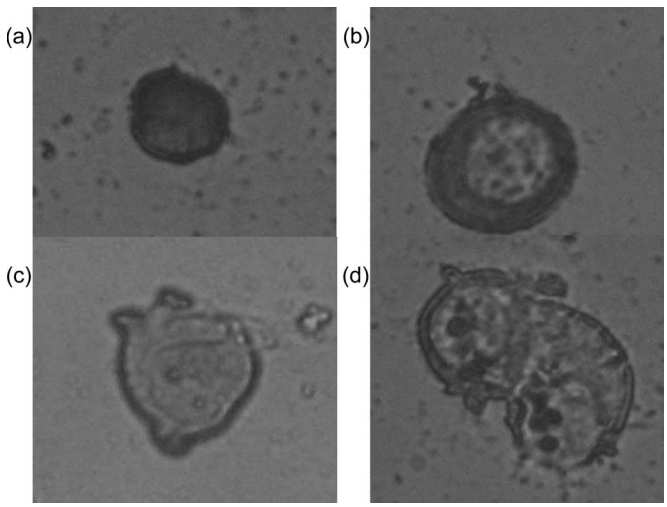

Fig. 8. Morphological aspects of B16-F10 cells treated with cold plasma jet (Flow rates: He—5 L/min; O_2—50 mL/min)—Giemsa stain: (a) Normal cell. (b), (c), (d) Apoptotic cells. The distance between plasma jet and monolayer cells = 10 mm. Treatment time = 60 s.

Fig. 5 shows the emission spectra of the high-voltage pulsed cold atmospheric plasma jets, with helium working gas, for various concentrations of oxygen introduced for the chemical activation of the plasma. The helium flow rate was 5 L/min, the amplitude of the voltage between the electrodes was of 20 kV, and the pulse repetition frequency—100 pps.

The helium 587-nm line and the atomic oxygen 777-nm line are present in all the situations shown in Fig. 5. The nitrogen lines from the air crossed by the plasma jet are obvious in the 337–406-nm spectral zone, at oxygen concentrations of up to 0.5%.

In general, the increase of the oxygen concentration in the discharge area leads to lower intensity spectral lines. The

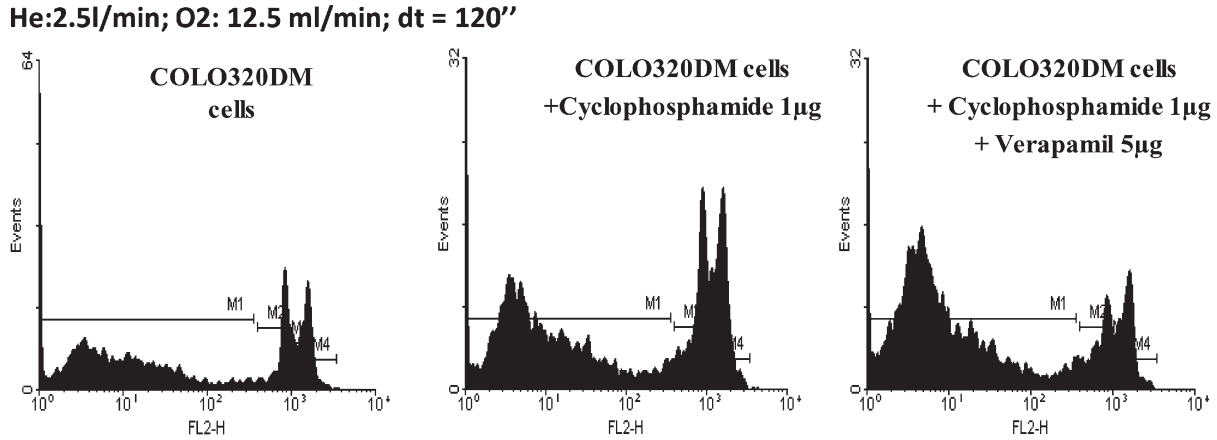

Fig. 9. Effect of cold plasma jet on COLO320DM cells. He: 2.5 L/min and O_2: 12.5 mL/min. The distance between plasma jet and monolayer cells = 10 mm. Treatment time = 120 s. Apoptosis percents: COLO320DM cells = 46%; COLO320DM cells + Cyclophosphamide 1 μg = 52.78%; COLO320DM cells + Cyclophosphamide 1 μg + Verapamil 5 μg = 55.76%.

explanation lies in the decrease of the plasma jet current when the oxygen concentration increases.

From the plasma chemical reactivity point of view, the intensity of the 777-nm line of the atomic oxygen is relevant [18]. In Fig. 5(a), we can notice that although no oxygen was introduced in the discharge area, the intensity of the atomic oxygen line was nevertheless of 13 000 units. In this case, the atomic oxygen was due to the interaction of the electrons produced by the electric discharge with the air crossed by the plasma jet. The intensity of the atomic oxygen line increased to 27 500 units when oxygen of 0.5% concentration was introduced in the discharge area [Fig. 5(b)]. However, the 777-nm line intensities dropped to 14 000, 9 200, and 5 600 units at oxygen concentrations of 1%, 1.5%, and 2%, respectively.

One more thing to observe, particularly at the spectra shown in Fig. 5(a) and (b), is the presence of the H_α line (656 nm), a result of the collision between the water molecules and electrons

$$H_2O + e \rightarrow OH + H + e. \quad (1)$$

This spectral line demonstrates the presence of OH radicals, an extremely active chemical species, in the plasma jet.

The conclusion of these experiments is that the maximum chemical activity of the helium plasma jet is obtained upon the introduction of oxygen with a concentration of 0.5% in the discharge area.

Thermal Characteristics: The plasma jet temperature was measured with a small size thermocouple, in thermal equilibrium with plasma gas. For any combination of experimental conditions (high-voltage amplitude: 15–20 kV; pulse repetition frequency: 50–200 pps; inert gas flow rate: 1–5 L/min; distance from the nozzle exit: 1–3 cm), the plasma jet temperature did not exceed 32 °C.

B. Cell Treatment Results

Apoptosis Analysis of Tumoral Cells: Apoptosis of B16-F10 cells induced by helium plasma jets was analyzed by flow cytometry. The results showed that pure helium (without oxygen) plasma jets did not induce apoptosis [Fig. 6(a)]. However, following the microscopic analysis, cells detach from the plate was observed [Fig. 6(b)], accordingly to other literature data [19]. They have demonstrated that, as a result of the interactions of plasma radicals with cell adhesion molecules, cell attachment was temporarily interrupted (the detached cells can be removed, reattached, or transferred), fine surgery applications being a possible using for plasma jet.

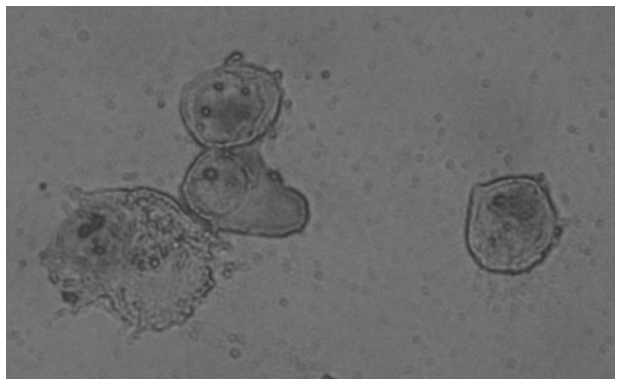

Fig. 10. Apoptotic COLO320DM cells obtained after plasma jet treatment (He: 5 L/min; O_2: 50 mL/min)—Giemsa stain. The distance between plasma jet and monolayer cells = 10 mm. Treatment time = 120 s.

By contrast, helium-oxygen treatment induces apoptosis in B16-F10 tumoral cells (in the same experimental conditions) [Fig. 7(a)], leading to an apoptosis rate of 74.25%. In Fig. 7(b), the morphological modifications are presented: loss of membrane asymmetry, chromatin condensation, apoptotic bodies, and chromosomal DNA fragmentation. These aspects have been also identified on Giemsa stain slides (Fig. 8).

Concerning the effect of cold plasma jet on COLO320DM cells, we can notice that the apoptosis percent is major (55.76%) in the case of cells treated with efflux pumps inhibitor Verapamil, relative to untreated cells (Fig. 9). Apoptotic characteristics have been identified by microscopy (Fig. 10).

Apoptosis Analysis of Normal (Macrophages) Cells: The atmospheric pressure plasma jet does not determine the onset of apoptosis in RAW 264.7 murine macrophages, not even after a 2-min treatment period (Fig. 11). During the same amount of time, an apoptosis rate greater than 30% has been obtained for the case of COLO 320DM tumoral cells.

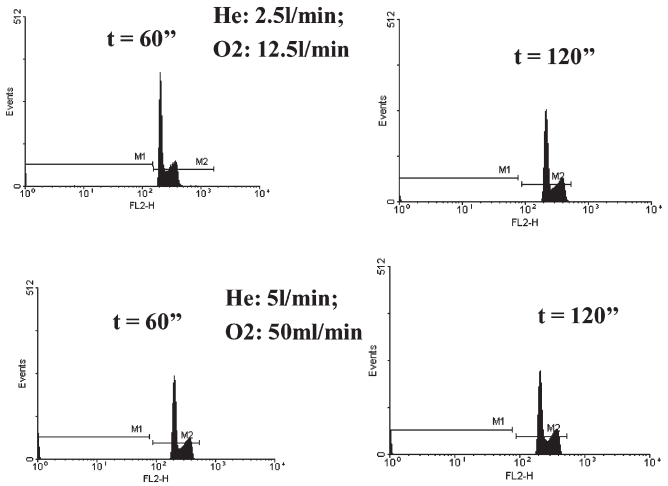

Fig. 11. Effect of cold plasma jet on RAW264.7 macrophages. There is no apoptosis.

TABLE I
NO_2 CONCENTRATIONS IN RAW264.7 CELLS SUPERNATES AND CULTURE MEDIUM AFTER PLASMA TREATMENT (GRIESS METHOD)

Experimental conditions	Treatment time [s]	NO_2 concentration (pg NO_2/mL)	
		Cellular system	Acellular system
Sample probe		2.51	<0.049
He: 2.5 L/min + O_2: 12.5 mL/min	60	103.17	>200
	120	> 200	>200
He: 5 L/min + O_2: 50 mL/min	60	35.20	32.38 +/-0.869
	120	40.48	69.60 +/- 1.268

Activation Analysis of Normal (Macrophages) Cells: Human tumors are usually subject for inflammatory cell infiltration. These cell infiltrates differ in size and chemical composition from one type of tumor to another, their presence indicating that the host organism reacts to the presence of the tumor, or more precisely that it interferes with its growth, mechanism known as immune surveillance. In this context, the existence of the inflammatory infiltrates can be considered to be an attempt to detect and eliminate the emergent tumor cells. The immune cells present inside the tumors include both, adaptive immunity cells (T lymphocytes, dendritic cells and occasionally B-type lymphocytes) and also innate immunity effectors—macrophages, PMN, leukocytes and few NK cells [20], [21]. Thus, the activation of macrophages in the early stages of the tumor is very important for the elimination of the adjacent tumor cells. Unfortunately, most of the tumors are detected in their late stages of evolution when the immune components at the tumor level have already been reprogrammed to work toward tumorigenesis and *the activation of macrophages would not be wanted in none of these situations*.

Realization of Griess technique in both cellular and acellular systems, keeping all plasma treatment parameters fixed, indicates that the increase in the medium nitrite level is a consequence of the plasma jet presence and does not follow from cell activation (Table I). The obtained data shows a good agreement with the results published in [22]. This aspect supports by means of mass spectrometry that the generation of NO is determined by the plasma jet, and *there is no macrophages activation*.

Being known that nitric oxide in large concentrations has a cytotoxic effect on all cells (including tumor cells), the obtained data may stand as a confirmation of the effectiveness of the device in tumor treatment.

IV. CONCLUSION

Tumoral and normal cells have been treated with high-voltage pulsed cold atmospheric plasma jets. The working gas was helium, with up to 5 L/min flow rate. The results obtained for the B16-F10 cells support the need to use oxygen as plasma chemical activator.

The atmospheric pressure plasma jet does not determine the onset of apoptosis in the normal cells (RAW 264.7 murine macrophages) we used. During the same amount of time, an apoptosis rate greater than 30% has been obtained for the case of COLO 320DM tumoral cells.

When the COLO320DM cells (whose membranes contain high amount of MDR efflux pumps) have been exposed to helium–oxygen plasmas in combination with Verapamil, we observed an increased apoptosis rate compared to the plasma treatment alone.

Our further experiments will aim to study cold plasma jet action upon cells treated with cytostatics and/or efflux pumps inhibitors. Optimal variables of plasma treatment (time, distance, jet parameters) will be searched, with the purpose to obtain a maximum antitumoral effect, by applying an as small as possible cytostatic dose.

The obtained data indicate that cold plasma jet may be a potential device in an animal model to study a combined treatment: atmospheric pressure cold plasma and MDR efflux pumps inhibitor, together with chemotherapy.

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