THE EFFECTS OF COLD ATMOSPHERIC PLASMA JETS ON B16 AND COLO320 TUMORAL CELLS

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ABSTRACT

Cold atmospheric plasma treatment acts at the cellular level to remove diseased tissue without inflammation and damage, to suppress infections and to modulate the viability (apoptosis/necrosis) of tumoral cells. It is also known that, a major cause of anti-tumor chemotherapy failure is the development of multidrug resistance (MDR) of tumors. This study 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 COLO320DM multidrug resistant cells (human colon cancer cell line).

The tests have been performed on human colon cancer cell line COLO320DM and murine melanoma cell line B16-F10. These cell lines have been treated with cold helium or helium-oxygen generated plasma jets and the consequent apoptosis has been analyzed by means of flow cytometric method. A treatment time-dependent apoptosis has been observed only in the case of B16-F10 cells interacting with helium-oxygen plasma and no apoptosis has been identified when the cells were treated only with helium plasma jets. These results indicate the need of oxygen for the chemical activation of plasma.

The COLO320DM cells (that over-express the MDR efflux pumps) have been exposed to helium-oxygen plasmas only, or in a combination with vegetal extract MCS D161 as MDR efflux pumps inhibitor. For the secondly mentioned case the results have showed an increased apoptosis rate compared to the plasma treatment alone.

The obtained data represent a starting point for the study of a possible combined treatment (atmospheric pressure cold plasmas and a MDR efflux pumps inhibitor applied with chemotherapy).

Key words: *cold atmospheric plasma jets, tumoral cells, efflux pumps, flow-cytometry*

INTRODUCTION

Cold atmospheric plasma jets have multiple practicabilities both in civil and military area, by using them as light sources, decontaminating devices, cleaning and etching media, surgery, surface treatments (without any thermal damage) and so on [1]. Its small size guarantees a low gas temperature, and in addition it allows for a high precision local surface treatment.

Decontamination capacity regards either bacteria killing by sterilization at low temperatures [2-8,10], or eliminating some pollutants e.g. benzene, toluene, acetone, freon, xylene, trichloroethylene, trichloroethane and perchloroethylene, each of them being able to generate acid rains [9]. Intermediate pressure (1-10mbar) and sub-atmospheric discharges have proven to be efficient in bacterial decontamination of skin and dental cavities [11]. Reaching maximal efficiency in a small time period (minutes) is very important, taking into account that contamination problems related to chemical and biological warfare agents assess imediate decontamination of the source and neutralization of the environment contaminated with toxic materials [12].

Gas discharges in argon have large aplicability in electrosurgery: argon plasma coagulation (APC) is the most used endoscopic coagulation technique [13]. Plasma surgical treatment is preferable because is a non-contact method and assumes a low risk for mechanical lesions and secondary infections [14]. By association with some growth factors, plasma jet is successfully used for venous ulcers and diabetic ulcers treatment [15].

Bio-medical applications for surfaces treatment comprise surface properties modifications besides sterilization, in order to modify bio-interactions (cell adhesion, inflammatory response etc), formation of a barrier film to decrease diffusion of small molecules into or out of substrates and creation of reactive sites for subsequent immobilization of bio-molecules [16].

Low pressure plasmas are being succesfully used in biomedical engineering, e.g. for surface patterning to control cell adhesion [17] or spraying of bio-compatible materials to improve the performance of artificial implants [18]. Yonson & al. [19] showed that the miniature plasma torch could be used in biological micropatterning by reducing contact angle and promoting cell adhesion, as it does not use chemicals like the present photolithographic techniques. Due to its small size and manouvrability, the torch also has the ability to pattern complex 3D surfaces. At the laboratory level, researchers have investigated various plasma treatment methods to micropattern surfaces for the study of neuronal networks [20], the manufacture of biosensors and the imitation of *in vivo* cell patterning on implants to improve biocompatibility.

The potential oxidative effects on the cell due to plasma treatment include lipid peroxidation, protein oxidation and cell death due to an imbalance of reactive oxygen and nitrogen species (ROS and RNS). The membranes are etched by active radicals species from the plasma. These reactive species include oxygen atoms (O), oxygen negative ions (O_2^-), ozone (O_3), hydroxyl radicals (OH), nitric oxide radicals (NO) and hydrogen peroxide (H_2O_2). Bio-compatibility of plasma jet is mainly reached by temperature. Generally temperature increase above 43°C produces cell destruction. Besides, it has to be taken into account the fact that plasma jet treatment at 43°C releases painful reactions, probably by TRPV1 nociceptors activation, knowing that they are activated at temperatures above 43°C [21]. This process is not available for cold plasmas.

The plasma jets devices for biomedical applications are obtained with excitation voltages either in the several tens of kHz AC range or in the radiofrequency (RF) range [22] or in the pulsed mode [23, 24]. Energetic electrons inside the plasma disintegrate the O_2 and H_2O molecules in the air, obtaining O atoms and OH radicals which are chemically very active. The most efficient chemical activation method is the introduction of a substance in the discharge area which, under the action of the electrons inside the plasma generates chemical active agents.

ABC transporters (ATP-binding cassette) are efflux pumps transporting a variety of compounds through cellular membranes against the concentration gradient using energy resulting from ATP hydrolysis. Toxic compounds delivery through the liver and kidney, haemato-encephalic barrier functioning, isolation of germ cells from the systemic circulation and stem cells protection are processes mediated by ABC transporters. By xenobiotics elimination, ABC transporters are responsible for drug resistance appearance either at prokaryotes level (bacteria antibiotics resistance), or at eukariotes level (plants herbicides resistance, animals tumoral cells resistance to cytostatics) [25].

A major cause of failure in anti-tumor chemotherapy is the development of multidrug resistance (MDR) of tumors, due to (MDR) efflux pumps which operate against the concentration gradient using energy resulting from ATP hydrolysis and extrude anticancer drugs from the tumor cells [26].

PI3K/PTEN/Akt signaling cascade plays a critical role in signal transduction from growth factor receptors to nucleus in order to regulate gene expression and prevent apoptosis. Components of this pathway are mutated or aberrantly expressed in human cancer. Activated Akt confers a drug-resistant phenotype to tumoral cells. PI3K/PTEN/Akt pathway appears to be involved in prostate cancer drug resistance, and is associated with resistance to doxorubicin and 4-hydroxyltamoxifen, a chemotherapeutic drug and estrogen receptor antagonist used in breast cancer therapy [27].

MCS Ab162 and MCS D161 are natural products isolated and purified from *Helleborus purpurascens*. MCS Ab162 is a possible TLR2 (Toll-like receptor 2) agonist and possesses analgesic properties both *in vitro* and *in vivo* (data not shown). MCS-D161 inhibits the mRNA-COX-2 expression and COX-2 activity [28]. MCS D161 could also contain a PI3K inhibitor [29] and it is able to act on the immune components of the neuro-immuno-endocrine system by decreasing some inflammatory processes.

Cold plasma jet treatment should represent a non-invasive and non-painful method for cutaneous cancer treatment, as well as for other tumors types present on the skin surface; this plasma treatment could complete or even replace radiotherapy. Cold plasma jet 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 non-ignorable side-effects that inevitably also act on healthy cells too, by affecting their functionality. The hospitalization time should be reduced. The plasma device obtaining costs being relatively small and being easy usable, it could be included in every hospital endowment for oncology, dermatology departments etc.

MATERIAL AND METHODS

Plasma jet generator

In our study we have used atmospheric pressure plasma because it is known that this kind of plasmas are more flexible and less expensive in operation, because they do not require costly vacuum systems [1]. In our experiments the cold atmospheric plasma jets are produced by using high voltage pulses (tens of kV), which have limited duration (tens, hundreds of nanoseconds) and are repeated (tens, hundreds of pulses per second). The pulses are applied between two metallic electrodes of various shapes, separated by a dielectric barrier. An electrical discharge takes place in a cavity through which the inert gas flows at normal atmosphere. The cavity has an aperture through which discharge plasma is pushed out. Under optimal conditions, plasma is emitted as centimeter-long jets, just millimeters in diameter or even smaller.

The device used for the experiments presented here (Fig.1) is made out of an insulating material cylinder. The piston made of Teflon is placed in this cylinder. A medical syringe needle passes through the center of this piston and functions as a high voltage electrode. At the same time the needle works as the device, which introduces the chemical activation gas (oxygen, in this case) in the discharge area. The mostly inert gas (He) is introduced through an orifice in the lateral wall of the dielectric cylinder. The exit channel of the plasma jet is positioned at the center of a Teflon lid which covers the lower end of the dielectric cylinder. A metal ring working as the mass electrode of the device is stuck onto the outer part of the lid. The high voltage pulses had 18 - 20 kV amplitude, and a frequency of 100 pulses per second.

The strongest reactive component obtained in the plasma jet is the atomic oxygen, produced by electrons from electrical discharge.

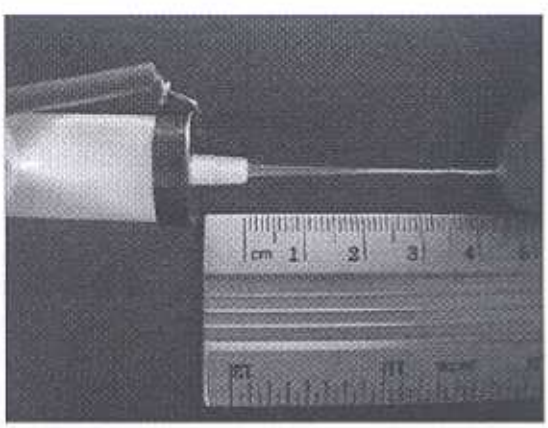
Scavenging capacity for oxygen reactive species

In order to measure the antioxidant capacity against peroxyl radicals, we used a modified and validated ORAC protocol (Oxygen Radicals Absorbance Capacity), based on Prior & al. method [30]. This technique figures on fluorescein (marker molecule) denaturation by peroxyl radicals, process showed by time-dependent fluorescence intensity decrease. An antioxidant sample inhibits this process and maintains the fluorescent signal. A Trolox calibration curve has been used and the data were expressed as Trolox equivalents (TE) per gram of tested product, (1 TE = 1 mM Trolox).

Total Antioxidant Capacity was measured according to the spectrophotometric method of Preito [31]. Briefly, the extract dissolved in water was combined with a buffer containing: 0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The samples were incubated at 95°C for 90 min, centrifugated at 13000 rpm for 5min, and the supernatants absorbance was measured at 695 nm against a blank. Ascorbic acid was used as standard and the total antioxidant capacity was expressed as equivalents of ascorbic acid (1 ascorbic acid equivalent = 1 mM ascorbic acid) per gram of tested product.

Griess colorimetric method - non-cellular system

Nitrites in MCS Ab162 and MCS D161 solutions (1mg/ml) react in an acid medium with a diazotization agent - Sulfanylamide (SA), an instable diazonium salt resulting as a reaction product. This interme-



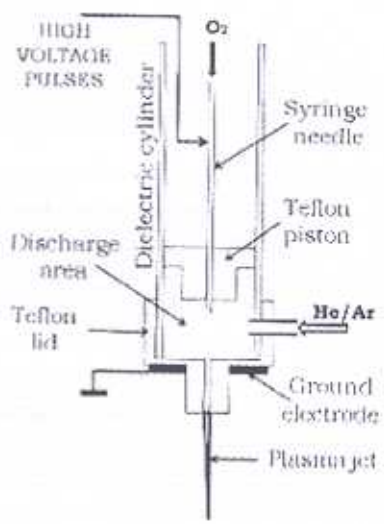


Fig.1. The geometrical configuration which has been used to treat biological objects with high voltage pulsed, repetitive, cold atmospheric plasma jets.

Table 1. B16-F10 cells experimental model

Treatment time (secondes)	He:2.5l/min	He:5l/min	He:5l/min+O_2:100ml/min
20	-	-	+
40	-	-	+
60	+	+	+

Table 2. COLO320DM cells experimental model

	O_2:12.5ml/min	O_2:25ml/min	O_2:37.5ml/min	O_2:50ml/min	O_2:75ml/min
He:2.5l/min	+ (O_2/He = 0.5%)	+ (O_2/He = 1%)	+ (O_2/He = 1.5%)	-	-
He:5l/min	-	+ (O_2/He = 0.5%)	-	+ (O_2/He = 1%)	+ (O_2/He = 1.5%)

Treatment time was 60 seconds in all cases

diate reacts with a coupling agent - N-naphthyl-ethyl-enediamine (NED) with the purpose to form a stable compound. 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 method [34] is characterized by a high sensitivity. Absorbance at 540 nm presents a lineal dependence towards nitrite concentration in the sample. Obtained values were related to a standard nitrite curve.

Cell lines

In our experiments we have used murine melanoma cell line B16-F10 (ATCC: CRL6475) and COLO320DM (JCRB0225) cell line. Human colon carcinoma COLO320DM cells overexpress MDR efflux pumps.

MTT viability test

The COLO320DM cells were cultured (1 x 10^6 cells/1 ml RPMI1640 culture medium supplemented with 1mM L-glutamine, 10% fetal calf serum and antibiotics) to adhere for 4 hours and then treated with 25ig MCS Ab162 or MCS D161. After 72 hours, the cell viability was tested using the MTT colorimetric method [32, 33] which measures a purple formazan compound produced only in the presence of living cells. Cell viability was expressed as percent towards control (untreated cells). For each sample, four wells have been used. Variation coefficient for each sample was <20%.

Efflux pumps inhibition (flow cytometric analysis)

1 x 10^6 COLO320DM cells were incubated for 30 min at room temperature with MCS Ab162 or MCS D161 (5 and 10μg) or the specific MDR pumps inhibitor Verapamil, 5μg/system. Samples were incubated with Rhodamine 123, 10 µg, for 20 min. in a water bath (37°C), washed twice (2 min, 2000 rpm), re-suspended in 0.5 ml phosphate buffer solution. Cells populations were gated and data were acquired with a FACSCalibur (Becton-Dickinson, San Jose-USA) and analysed using CellQuest software.

Plasma Jet treatment and apoptosis analysis

The B16-F10 and COLO320DM cells were cultured (1 x 10^6) in culture medium (DMEM-F12 and RPMI1640 respectively) supplemented with 1 mM L-glutamine, antibiotics and 10% fetal calf serum). When cells formed a monolayer, they were exposed to plasma jet for different time periods, using different combinations of helium and oxygen inputs. Subsequently, the adherent cells were detached with trypsin, washed twice (2 min, 2000 rpm) and samples were analyzed by flow-cytometry for measuring the percentage of apoptotic cells after propidium iodide staining in hypotonic buffer, using the Nicoletti method [35]. Cells were first gated according to their scatter characteristics and then analyzed for apoptosis by WINDMI.2.7 software. B16-F10 cells treatment is presented in Table 1.

The COLO320DM cells have been exposed to helium-oxygen plasmas only (Table 2), or in combination with vegetal extract MCS D161 (25 µg/system, 60min) as MDR efflux pumps inhibitor.

Statistical analysis

Data presented here have been calculated as the average of three identically repeated experiments. Only those values for which the variation coefficient has been found to be <10% for non-cellular systems and <20% for the cellular systems ones have been considered.

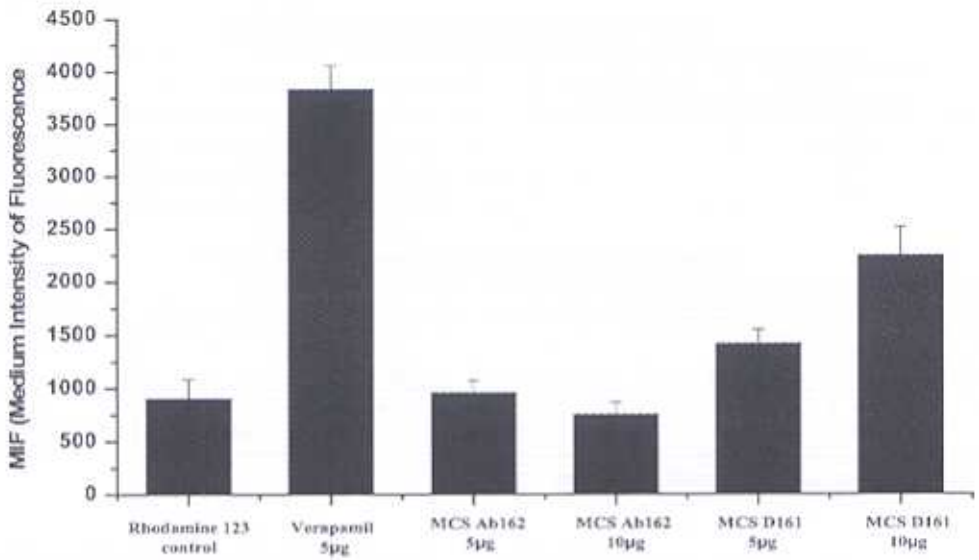


Fig. 2. MCS D161 inhibits MDR pumps efflux activity (flow-cytometry analysis)

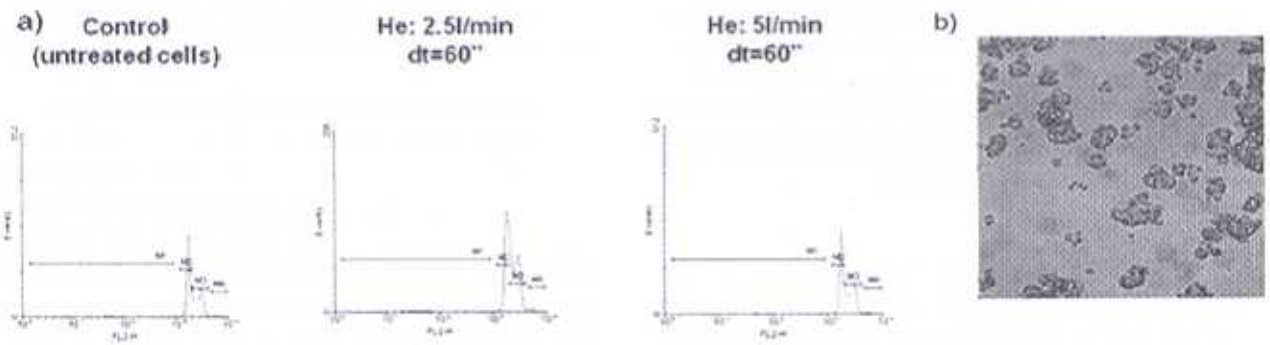


Fig. 3. Helium treatment (without oxygen) of B16-F10 cells does not induce apoptosis (a); cells detachment from substrate (b). The distance between plasma jet and monolayer cells = 10mm; treatment time = 60 seconds

Calibration curves have been determined for each experiment (if necessary) and only those cases for which a value of $R^2 > 0.95$ have been declared as valid.

RESULTS AND DISCUSSION

Scavenging capacity for oxygen and nitrogen reactive species

Using ORAC method, we have obtained a non-significant scavenging capacity for peroxyl radicals for both MCS Ab162 and MCS D161 - 689 TE/g and 763 TE/g respectively. Concerning the total antioxidant capacity of these compounds, our results showed low capacity to scavenge reactive oxygen species (2.8 ascorbic acid equivalents/g for MCS Ab162 and 3.4 ascorbic acid equivalents/g for MCS D161). The correlation factor (R^2) for the standard curves (both ORAC and Total Antioxidant Capacity methods), was > than 0.95. The variation coefficient for each sample was less than 10%.

Values obtained by Griess method were lower than the inferior limit of the calibration curve (< 0.049 pg/ml), for both MCS Ab162 and MCS D161. The correlation factor (R^2) for the standard curves, was > than 0.97. The variation coefficient for each sample was less than 10%.

We can conclude that the two tested compounds do not influence the culture medium composition concerning oxygen and nitrogen radicals content.

MTT viability test

In order to find out if a possible cell viability alteration could be due to plasma jet effect or to the tested compounds, it is necessary to test if MCS Ab162 and MCS D161 are cytotoxic.

Using COLO320DM tumoral cells, we observed that both MCS Ab162 and MCS D161 (25 μg/system)

Table 3. The effect of MCS Ab162 and MCS D161 on MDR (COLO 320) tumoral cells viability

	Control (untreated cells)	MCS Ab162 25 µg	MCS D161 25 µg
Cells viability vs control	100.000	95.740 +/- 1.884 (CV = 1.968)	96.823 +/- 0.625 (CV = 0.646)

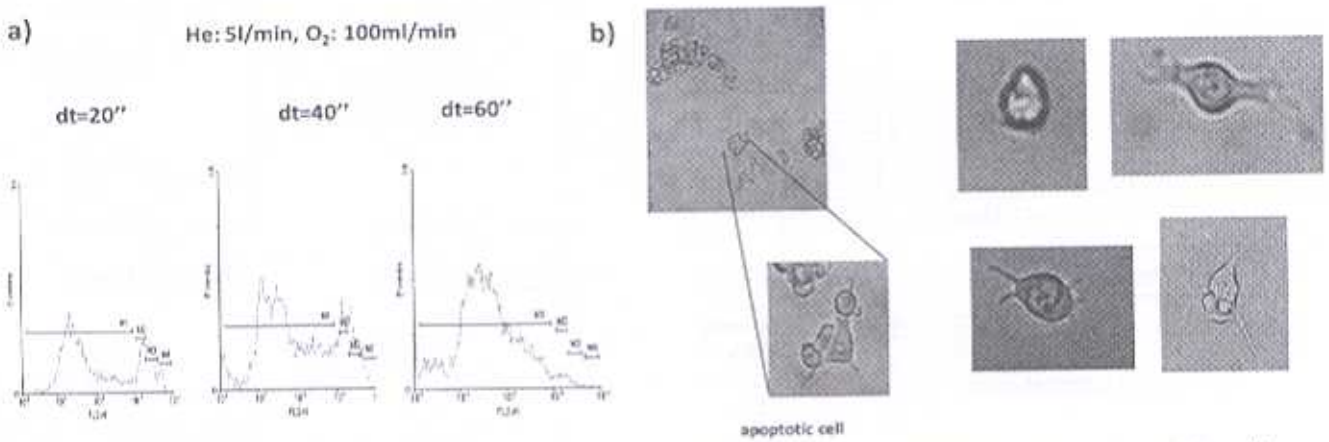


Fig. 4. Helium treatment with oxygen (He: 5l/min and O_2:100ml/mi) induces apoptosis in tumoral B16-F10 cells (a); morphological modifiers (b). The distance between plasma jet and monolayer cells = 10 mm, treatment time = 20, 40, 60 seconds respectively.

did not produce significant alterations of cell viability (Table 3).

MCS Ab162 and MCS D161 capacity to inhibit MDR efflux pumps activity

Accordingly to data shown in Fig.1, it is obvious that MCS D161 induced a dose-dependent efflux pumps inhibition by comparing with Rhodamine 123 control and with the well known efflux pumps inhibitor - Verapamil. In the same time, one can be noticed that MCS Ab162 is not an efflux pumps inhibitor, concluding that the sole interest compound for our experiments remains MCS D161.

Apoptosis analysis

Apoptosis of B16-F10 cells induced by helium was analyzed by flow-cytometry. The results showed that He: 2.5l/min and He: 5l/min (without oxygen) treatment did not induce apoptosis (Fig.2a). But, following the microscopic analysis, cells detach from the plate was observed (Fig. 2b), accordingly to other literature data [14, 36]. 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 beeing a possible using for plasma jet.

Helium cells treatment with oxygen induces apoptosis in B16-F10 tumoral cells, depending on the treatment time (Fig.3.a). In Fig. 3b were presented the morphological modifiers: loss of membrane asymmetry, chromatin condensation, apoptotic bodies and chromosomal DNA fragmentation.

Concerning the effect of cold plasma jet on COLO320DM cells, we can notice that (with few exceptions) the apoptosis percent is major in the case of cells treated with efflux pumps inhibitor MCS D161, relative to untreated cells. Besides, in the case of He flow of 5l/min, a more accentuated disorganization of the cell cycle can be observed (Fig. 4).

In our previous studies [37] regarding the effect of plasma needle on tumoral cells (BrSk and B16-F10 cell lines) we demonstrated that apoptotic cells percent over 50% was obtained. Plasma needle action did not produce necrosis higher than 7%. These data are important because, it is known that chronic inflammation is associated with tumor development and progression [38,39]

CONCLUSIONS

The results obtained for the B16-F10 cells support the need to use oxygen as plasma chemical activator.

When the COLO320DM cells (that over-express the MDR efflux pumps) have been exposed to helium-oxygen plasmas in combination with vegetal extract MCS D161 as MDR efflux pumps inhibitor,

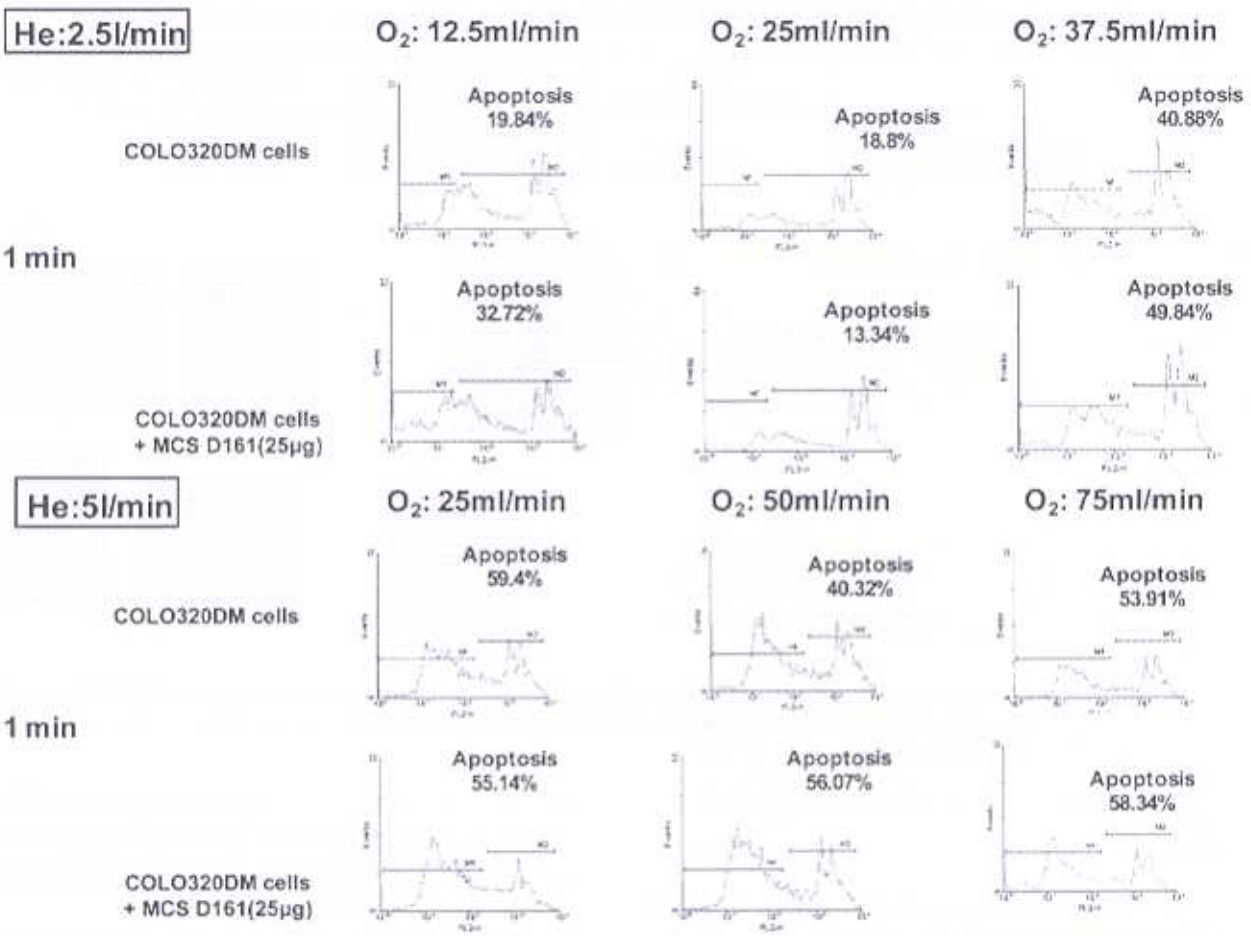


Fig. 5. Effect of cold plasma jet on COLO320DM cells with/without MCS D161 efflux pumps inhibitor.

we observed an increased apoptosis rate compared to the plasma treatment alone. 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 efflux pumps inhibitor, together with chemotherapy.

This experimental model should presume cutaneous tumors inducing by injecting tumoral B16-F10 cells in *C57/Bl* mice; afterwards, an exterior intervention upon the tumor should be made using plasma jet for induction of apoptosis in tumoral cells and for tumor reducing without inflammatory processes. Besides, an interior intervention should target the tumor by cytostatics systemic administration, combined with local administration of efflux pumps inhibitors which are able to block the chemotherapeutic agent inside the tumoral area.

Our further experiments will also 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 composition) will be searched, with the purpose to obtain a maximum anti-tumoral effect, by applying a cytostatic dose, as small as possible.

ACKNOWLEDGEMENTS

This study was supported by the National Research Program IDEI (Grant 19/2007).

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