Romanian Reports in Physics, Vol. 65, No. 1, P. 219–229, 2013

CELL DEATH IN V79-4 AND He-La CELL LINES, INDUCED BY CHEMICALLY ACTIVATED COLD ATMOSPHERIC PLASMA JETS

ANDREEA LUPU[1], N. GEORGESCU[2]

[1] University of Bucharest, Faculty of Biology, 91-95 Splaiul Independentei, Bucharest, Romania
E-mail: ldreea@yahoo.com

[2] National Institute for Laser, Plasma and Radiation Physics,
409 Atomistilor Str., Magurele, Jud. Ilfov, RO 077125, Romania
E-mail: n_georgescu@hotmail.com

Received June 20, 2012

Abstract. The effects of atmospheric pressure cold plasma jets on normal (V79-4) and tumoral (HeLa) cell lines are presented. Predominance of apoptotic or necrotic processes is described in relation to plasma contents and cell treatment times for two experimental setups: indirect (diffused) and direct cell exposure. Optimal treatment times for each type of cell and for each set of experimental conditions have been identified.

Key words: cold atmospheric plasma jets, inducement of cell apoptosis/necrosis.

1. INTRODUCTION

Atmospheric pressure cold (non-thermal) plasmas are nowadays extensively studied for their potential biomedical applications: treatment of certain types of skin cancers and dermatologic infections, burns, ulcers, blood coagulation inducement during surgery, sterilization of medical instrumentation, bacterial decontamination and others [1].

The conceptual possibility to adjust plasma and treatment parameters to ensure its non-invasivity, effectiveness and (ideally) selectivity regarding its action on different types of living organisms and cells has become a subject of interest and active research during the last several years [2].

Cold plasmas may possess chemical oxidative activity exposing biological material to oxidation processes mediated by the presence of reactive oxygen species (ROS) such as: singlet oxygen, hydroxyl radical, hydrogen peroxide, superoxide anion, and reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite. These radicals are generated either within the plasma itself or as a

consequence of the interaction between the plasma and the surrounding air. These reactive species are strong oxidants, acting on redox sensitive cellular components (primarily on proteins, lipids and nucleic acids).

The primary target of plasma jet is the cell membrane, where plasma treatment induces lipid peroxidation, transient pores formation, alteration of protein structure [2]. The changes in membrane structure are followed by complex cell responses including activation of intracellular signaling pathways.

Mitochondrion (pl. mitochondria) represents a cytoplasmic organelle which provide the principal source of cellular energy through oxidative phosphorylation and adenosine triphosphate (ATP) synthesis and is responsible for the conversion of food in usuable energy [3, 4].

The excess of pro-oxidant species leads to oxidative stress followed by a series of effects, including cell death. The mitochondria act like sensors for intracellular ROS and RNS. These reactive species are able to induce the loss of inner mitochondrial membrane potential. This has been described as an essential step in most apoptotic processes [5], representing the ultimate loss of defence that a cell can suffer before its death [6].

ATP is produced inside cells by redox reactions from carbohydrates or lipids. This molecule contains three phosphate groups attached to adenosine. By loosing one of the phosphate groups it turns into adenosine diphosphate (ADP), this conversion being accompanied by a high release of energy. This energy is essential for cell metabolism [7].

ADP molecules are carried in mitochondria by ADP/ATP translocase (protein from the inner mitochondrial membrane). Here (in the mitochondrial matrix), ADP is converted again to ATP, which is pumped back into the cytoplasm [7].

ATP regulates the form of cell death. Apoptosis is ATP-dependent and high amounts of ATP induce apoptotic cell death, while ATP depletion alone is sufficient to induce necrosis [8, 9, 10, 11]. In this context, the measurement of ADP/ATP ratio can offer valuable information concerning the type of cell death.

Based on our previous experience regarding the inducement of cell apoptosis under the action of chemically activated cold plasma jets (CPJ) [12, 13], we investigated the presence of apoptotic and necrotic effects within cell populations exposed to a helium-oxygen cold plasma. The experimental parameters that we have independently varied during the present study are the atomic oxygen content of the plasma and the cell exposure times to the CPJ.

In order to obtain higher quantities of atomic oxygen (which implies a stronger chemical activity), it is necessary to increase the concentration of molecular oxygen (O_2) in plasma-forming gas (He, in our case). Unfortunately, this increase brings along the decrease of the plasma current. Consequently, the quantity of atomic oxygen produced within the plasma has an initial increase along with the concentration of molecular oxygen, and then it drops as a result of the decrease of the plasma jet current. Therefore there is an optimal concentration of O_2 in He that maximizes the quantity of atomic oxygen.

2. EXPERIMENTAL SET-UP

The high voltage pulsed, cold atmospheric plasma jet generator (Fig. 1) has been presented in our previous work [14].

Three independent high voltage discharges were produced in He-O_2 gas mixture, inside a dielectric discharge chamber. Their plasma was pushed by the gas flow through the exit orifice, taking the shape of an unique plasma jet.

This structure led to higher O_2 concentrations in the gas mixture, and consequently to stronger chemical activities of the plasma jets. The optimal values of the O_2 concentrations were over 2 times higher than in the case of experiments in [15, 16] and of theoretical simulation in [17].

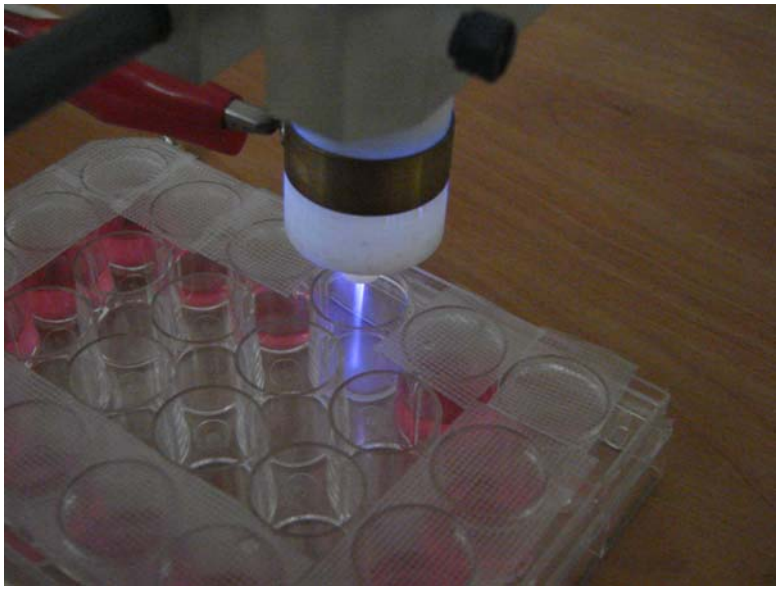


Fig. 1 – Cell treatment with the high voltage pulsed, cold atmospheric plasma jet.

Inside the discharge chamber, the plasma was produced with high voltage pulses of 25 kV amplitude, 400 ns full width at half maximum, and 100 pulses per second repetition frequency (Fig. 2).

The gas mixtures inside the discharge chamber had the compositions He(2.5L/min) + O_2(X mL/min), X taking the values 12.5, 25. These values correspond to 0.5 % and 1 %, percents of oxygen in the helium gas.

In our experiments we used the V79-4 cell line (ECACC no 93010723, normal fibroblasts isolated from Chinese Hamster Lung) and HeLa cells (ATCC no. CCL-2, human cervical cancer cell line).

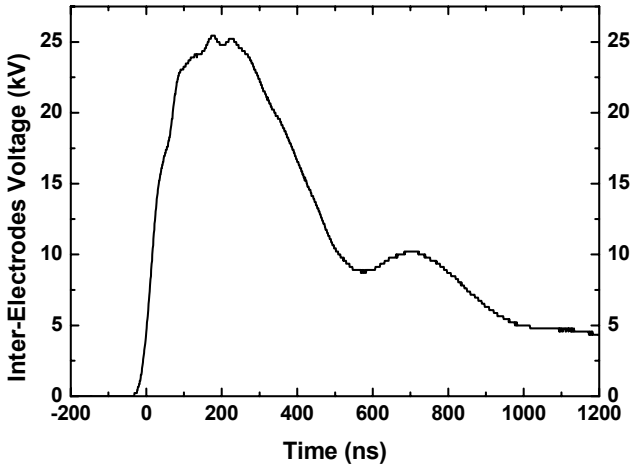


Fig. 2 – The high voltage pulse used to generate cold atmospheric plasma in He-O_2 gas mixture, inside the discharge chamber.

The V79-4 and HeLa cells were cultured (1×10^6 cells/sample in 24 well culture plates) in DMEM-F12 culture medium (Sigma) supplemented with 1mM L-glutamine (Sigma), antibiotics and 10% fetal calf serum (Biochrom). When cells formed a confluent monolayer, they were exposed to plasma jets in two experimental models: 1. Indirectly, by diffusion of reactive species in the culture medium; 2. Directly, without the presence of the culture medium. For each case, the exposure times were varied within the range 30 – 150 s with a timestep of 30 s.

Taking into account that the plasma effect is highly dependent on the amount of water surrounding the cells [18], and in order to exclude the possible effect of pH variations (data not shown), in our experiments we have removed the culture medium from above the cell monolayers immediately before the treatment and re-added it after the plasma exposure time. Another reason for removing the culture medium was the known fact that the density of active oxygen species (produced by plasma and diffusing into medium) significantly decreased with increasing distance from the liquid interface [19]. In order to avoid cell dehydration, all wells except for those being treated were covered (Fig. 1). Immediately after the culture medium was re-added, image of the cells were taken using a Sony DSC–H5 camera and an Olympus IX71 fluorescence microscope connected with a computer using Cell F dedicated software (magnification 10X). Some studies report that the cells detached by the plasma action kept their characteristics and became adherent again reentering the normal cell cycle if they were transferred to another culture plate [19]. Based on these results, in our studies we chose to use both, cells detached by the plasma jets as well as cells that remained adherent after the plasma exposure. The cells in culture medium were recovered by centrifugation and supernatant removement. The V79-4 and HeLa cells were detached afterwards using trypsin 0.25% + EDTA 0.53mM.

We have determined the cell viability by means of the MTT colorimetric method. Briefly, the cells were incubated with 300 µl 3-(4.5-Dimethylthiazol-2-yl)-2.5-dipheniltetrazolium bromide (MTT) for 4 hours at 37º C, 5 % CO_2 and 90 % relative humidity. MTT was converted to formazan by the mitochondrial enzyme glucose-6–phosphate dehydrogenase (G6PD). Then 300 µl Dimethyl sulfoxide (DMSO) were added in each well in order to solubilize the cell membranes and the formazan crystals (produced only in the presence of intact mitochondria). The absorbance of the purple formazan compound was read at 540 nm.

In order to determine the ADP/ATP ratio in samples, the cells were detached and washed twice (2 min, 2000 rpm), the samples being afterward analyzed using the ApoGlow Assay Kit (Lonza). This assay is based upon the bioluminiscent measurement of ATP, using the luciferase. ADP is indirectly determined, following its conversion to ATP, which is subsequently detected using the same dye enzyme. By means of an ADP/ATP ratio-based indicator, ApoGlow allows to distinguish between apoptosis, necrosis and cell proliferation. Apoptosis is characterized by a moderate increase of the ADP/ATP ratio vs control, while necrosis can be recognize for a significantly higher ADP/ATP ratio than the control. Each sample was analyzed in triplicate.

3. EXPERIMENTAL RESULTS

The most evident effect of plasma jet treatment was the detachment of the cells, as shown at macroscopic and microscopic level (Fig. 3, and Fig. 4, respectively).

In our studies, the variations larger than 20 % for cell viability and larger than 50 % for the ADP/ATP ratio were considered as “significant”.

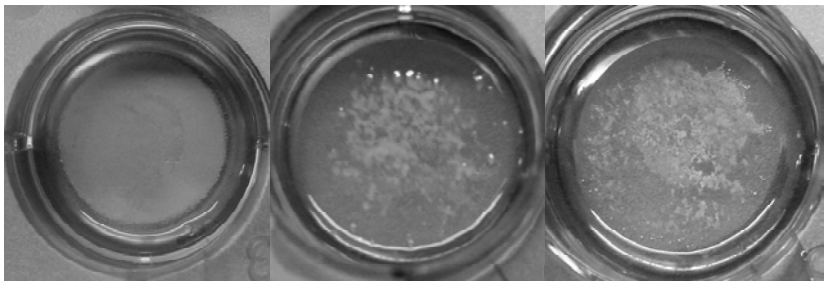


Fig. 3 – Macroscopic images. Plasma jet treatment (He: 2.5 L/min; O_2: 12.5 mL/min and 25 mL/min) was followed by V79-4 cells detachment from substrate, while the control (untreated) cells remained adherent. Plasma treatment time: 90s.

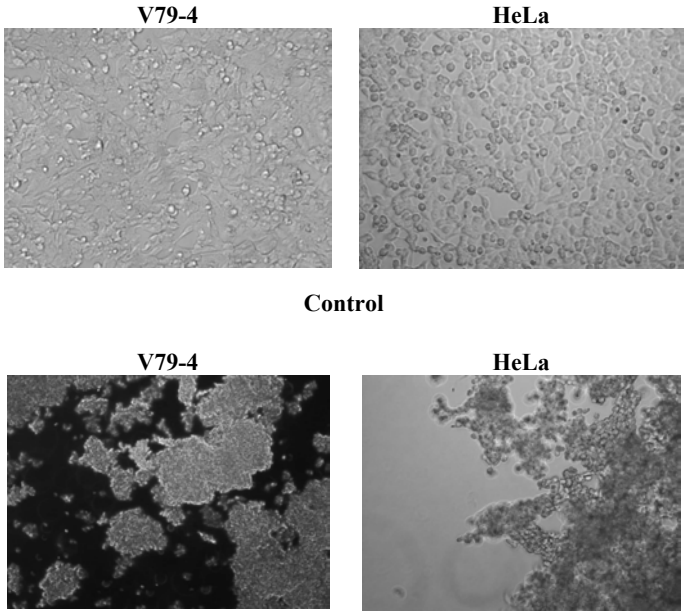


Fig. 4 – Microscopic images. Plasma jet treatment (He: 2.5 L/min; O_2: 25 mL/min) induces V79-4 and HeLa cells detachment from substrate. Plasma treatment time: 90s. Magnification: 10X.

In the first experimental model (indirectly treated cells, by diffusion of ROS and RNS in the culture medium), the V79-4 cells have been used. For 0.5 % and 1 % of oxygen in helium, the ADP/ATP ratio increased with the treatment time (Fig. 5). The first gas composition led to a significant ADP/ATP increase after 60 s of treatment, meaning the inducement of necrosis; for the second case, the necrotic process was initiated following the 30 s treatment time.

Taking into account that ROS density in cell microenvironment depends on the oxygen concentration in the gaseous mixtures and also on the treatment times, one may assume that the cell death (apoptosis/necrosis) is a consequence of the exposure to the ROS produced within the plasma jet.

For the second experimental model (directly treated cells, without the presence of the culture medium), the results obtained for the V79-4 cells are presented in Fig. 6.

Concerning the ADP/ATP ratio, in the case of 0.5 % O_2 in helium gas treatment, the graph reveals a time dependent increase for 30, 60, 90 and 120 s. After 150 s of treatment, the obtained value is less than ADP/ATP ratio corresponding to 120 s. The cell viability decreased in a time-dependent manner with respect to control.

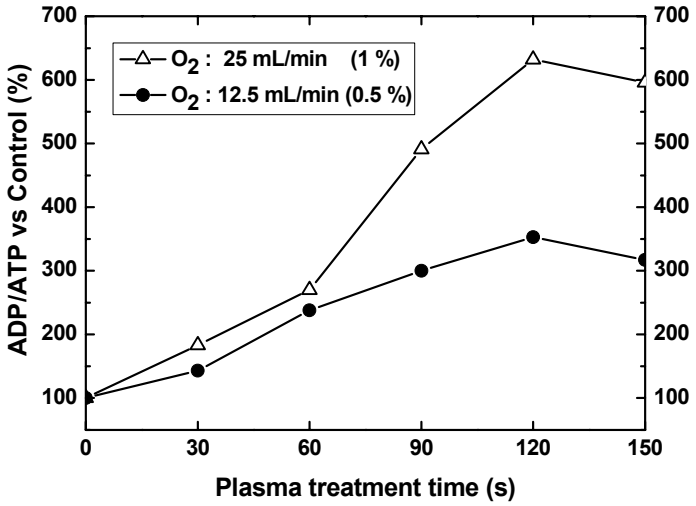
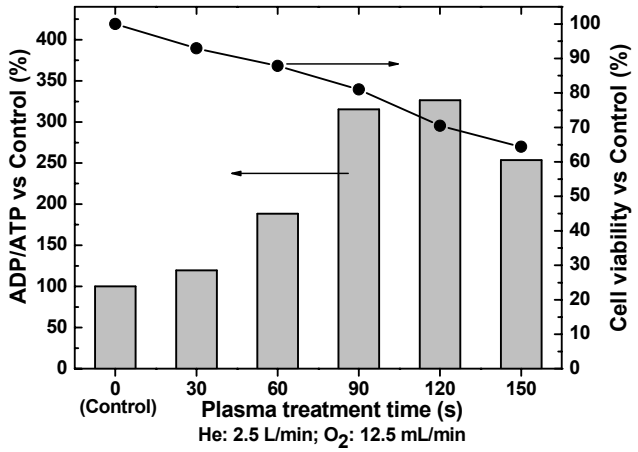


Fig. 5 – Exposure to cold atmospheric pressure plasma jets induces the death of V79-4 cells. Experimental model 1 – indirect exposure of the cells, by diffusion of reactive species in the culture medium (Coefficient of variation CV ≤ 25%).

In the case of 1 % O_2 in helium gas, the maximum value of the ADP/ATP ratio is obtained for 30 s of treatment. For 60, 90, 120 and 150 s, a time-dependent decrease has been observed. The cell viability wasn't significantly modified even for 150 s of treatment. These results may be explained based on the fast cell displacement action of the plasma jets. The images in Fig. 3 and Fig. 4 show that following jet exposure, cells are detached and pushed towards the walls of the well, plasma acting afterwards in a cell-free region. The number of detached cells appears to be proportional to the treatment time.



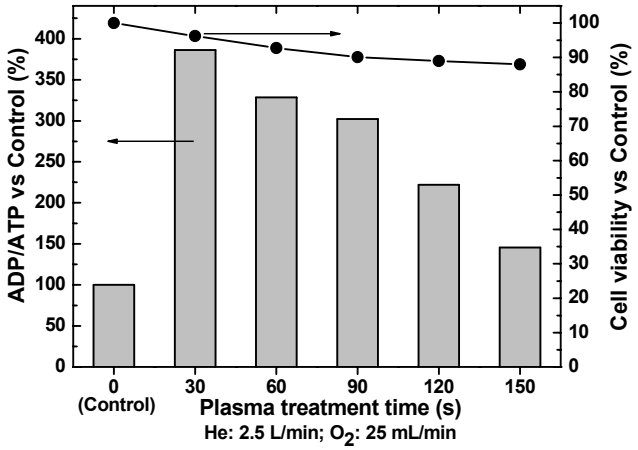


Fig. 6 – The effect of cold plasma jet treatment on V79-4 cells. Experimental model 2 – direct exposure of the cells to plasma jet. (Coefficient of variation CV ≤ 25%).

Concerning the HeLa tumoral cells, the results for the second experimental model (directly treated cells) are presented in Fig. 7. The data show a time-dependent increase of the ADP/ATP ratio (30 – 120 s for 0.5% O_2 in helium gas and 30 – 90 s for 1 % O_2 in helium gas), followed by decreased values for 120 s (1 % O_2 in helium gas) and 150 s (both gas mixtures). The cell viability shows a time-dependent decrease, with significant variations after 120 s of treatment.

In all cases – V79-4 cells (experimental model 1 and 2) and HeLa cells (experimental model 2) – the values of the ADP/ATP ratio obtained for 150 s were lower than those obtained for 120 s. A plausible explanation for these findings, would be that for the mentioned experimental parameters, the cells were destroyed/fragmented by the mechanical action of the plasma jet and could not be recovered after centrifugation (the cell pellet contains whole cells only). In consequence, samples corresponding to 150 s contain fewer cells than those associated to 120 s of plasma treatment. This implication is supported by the significantly decreased viability values revealed by the MTT tests (excepting V79-4 treated with 1% O_2 in helium gas – experimental model 2).

For data analysis and interpretation we took into account the times after the plasma treatments when the measurements for each sample were performed. Generally, the metabolic rate changes (ADP/ATP ratio variations) inside a cell exposed to a relevant stimulus, are fast processes, beginning after tens of seconds after the treatment [20]. The glucose-6 – phosphate dehydrogenase (G6PD) needs several hours to indirectly "sense" these changes and reflect them in the amount of purple formazan crystals formed [21]. Thus, the G6PD activity remains unchanged for a long time after the change of the ADP/ATP ratio.

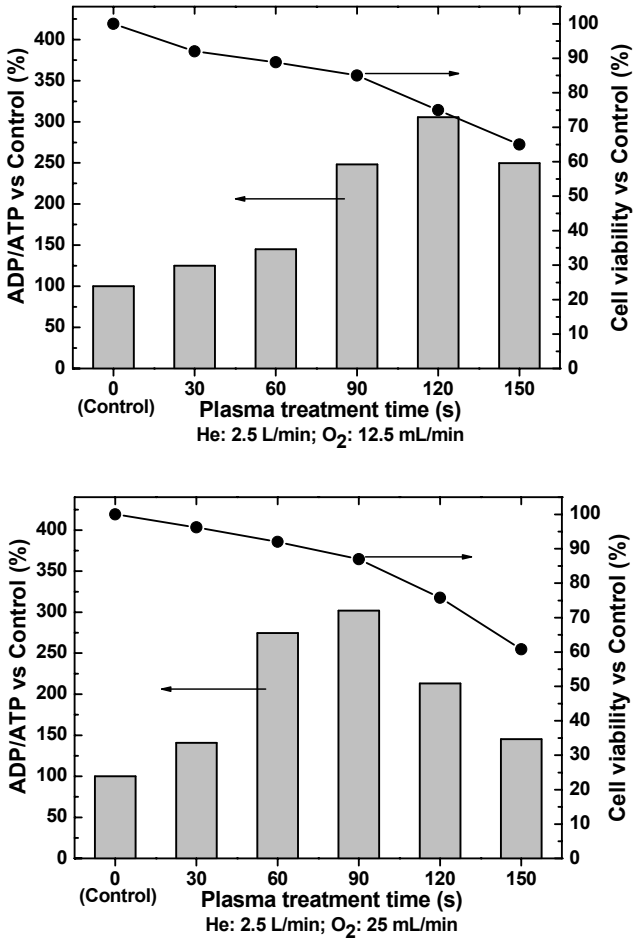


Fig. 7 – The effect of cold plasma jet treatment on HeLa cells. Experimental model 2 - direct exposure of the cells to plasma jet. (Coefficient of variation CV ≤ 25%).

Apoptosis is a "relatively" slow process (hours). An increased ADP/ATP ratio (with respect to control) not accompanied by a significant change in the cell viability would thus indicate the inducement of apoptosis in a large number of cells. Necrosis is a much faster process, affecting a smaller number of cells. Besides a high ADP/ATP ratio due to the higher ADP concentration in the cells, necrosis would also produce a significant decrease in cell viability. Based on the above considerations our results indicate that for specific treatment times, an extended but "soft" cell death in samples treated with cold plasma jet is induced.

Based on the above mentioned considerations, we conclude the following: a) a non-significant decrease of viability (cell viability *vs* control > 80 %) and a moderate increase of ADP/ATP ratio (ADP/ATP *vs* control < 150 %) mark an

apoptotic process; b) cell viability vs control > 80 % and ADP/ATP ratio significantly increased (ADP/ATP vs control > 150 %) indicate an "extended" apoptosis; c) cell viability significantly decreased (cell viability vs control < 80 %) and ADP/ATP vs control > 150 % describe a necrotic process.

By applying this algorithm to our data, we could observe that 0.5 % O_2 in helium gas plasma treatment (PT) induced apoptosis in V79-4 cells for 30 s PT, "extended" apoptosis for 60 s PT, and necrosis for 90, 120 and 150 s PT. V79-4 cells treated with 1 % O_2 in helium gas plasma suffer an extended apoptosis for all mentioned treatment times.

The results obtained for HeLa cells can be summarized as follows: a) 0.5 % O_2 in helium gas: apoptosis for 30 and 60s PT and necrosis for 90, 120 and 150 s PT; b) 1 % O_2 in helium gas: apoptosis for 30 – 90 s PT (extended apoptosis for 60 and 90 s PT), and necrosis for 120 and 150 s PT.

These data offer valuable information in order to identify the optimal treatment time for each gaseous mixture of plasma jet. For example, in the case of a concentration of 1 % O_2, for V79-4 and HeLa cells, the optimal plasma treatment times are of 30 s and 60 s, respectively.

4. CONCLUSION

The clinical use of the atmospheric pressure plasma jet may be hypothetically conceived for the treatment of tumors. For this purpose one should design treatment procedures and equipments that allow for the fine-tunning of plasma parameters, such that according to the particularities of each case, differentiated treatment could be applied to the tumor mass and tumor edges respectively (to avoid invasive effects in the healthy tissue). However, due to the lack of a clear delimitation between the tumoral and the normal tissues, this would be difficult to realize in practice. A much more realistic approach would be to use plasma jets at such parameters to obtain a maximum percent of apoptosis and a minimum amount of necrotic cells.

For some cases, the efficiency of treatment might depend on the presence of a liquid layer (hydrogel) on the plasma treated surface in order to allow for the diffusion of reactive species and for a relatively homogeneous action of plasma upon the whole region of interest. It would also be of prime importance to determine those treatment parameters, characteristic for each type of cell, that lead to a maximum apoptotic effect.

Acknowledgements. This work was supported by the European Social Fund and the University of Bucharest under Postdoctoral Program for Researcher Formation in Science (Sectorial Operational Program for the Development of Human Resources 2007 – 2013), Project POSDRU/89/1.5/S/58852.

REFERENCES

1. M. Laroussi, *The biomedical application of plasma: A brief history of the development of a new field of research*, IEEE Trans. Plasma Sci., **36**, *4*, 1612–1614 (2008).
2. M. G. KONG, G. KROESEN, G. MORFILL, T. NOSENKO, T. SHIMIZU, J. VAN DIJK, J. L. ZIMMERMANN, *Plasma medicine: an introductory review*, New J. Phys., **11**, *11*, 115012 (2009).
3. *** *The American Heritage Medical Dictionary,* Houghton Miffin Company, 2007.
4. *** *Mosby's Medical Dictionary,* 8th edition, Elsevier, 2009.
5. Z. Yang, W. Cheng, L. Hong, W. Chen, Y. Wang, S. Liu, J. Han, H. Zhou, Z. Gu, *Adenine nucleotide (ADP/ATP) translocase 3 participates in the tumor necrosis factor – induced apoptosis of MCF-7 cells*, Molecular Biology of the Cell, **18**, 4681–4689 (2007).
6. D. B. Zorov, S. Y. Bannikova, V. V. Belousov, M. Y. Vyssokikh, L. D. Zorova, N. K. Isaev, B. F. Krasnikov, E. Y. Plotnikov, *Reactive oxygen and nitrogen species: friends or foes?*, Biochemistry (Moscow), **70**, *2*, 215–221 (2004).
7. B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, in *Molecular biology of the cell*, Garland Science, 2002, 773–779.
8. Y. Tsujimoto, *Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes*, Cell Death and Differentiation, **4**, *6*, 429–434 (1997).
9. M. von Albertini, A. Palmetshofer, E. Kaczmarek, K. Koziak, D. Stroka, S. T. Grey, K. M. Stuhlmeier, S. C. Robson, *Extracellular ATP and ADP activate transcription factor NF-kB and induce endothelial cell apoptosis*, Biochem. and Biophys. Res. Communic., **248**, *3*, 822–829 (1998).
10. A. Bürkle, in *Poly (ADP-Rybosyl)ation*, Landes Bioscience and Springer Science + Business Media Ink., **143** (2006).
11. A. Garedew, S. O. Henderson, S. Moncado, *Activated macrophages utilize glycolytic ATP to maintain mitochondrial membrane potential and prevent apoptotic cell death*, Cell Death Differ., **17**, *10*, 1540–1550 (2010).
12. N. Georgescu, C. P. Lungu, A. R. Lupu, *Chemical activation of the high voltage pulsed cold atmospheric plasma jets*, Romanian Rep. Phys., **62**, *1*, 142–151 (2010).
13. N. Georgescu, C. P. Lungu, A. R. Lupu, M. Osiac, *Atomic oxygen maximization in high-voltage pulsed cold atmospheric plasma jets*, IEEE Trans. Plasma Sci., **38**, **11**, 3156–3162 (2010).
14. N. Georgescu, A. R. Lupu, *Tumoral and normal cells treatment with high-voltage pulsed cold atmospheric plasma jets*, IEEE Trans. Plasma Sci., **38**, *8*, 1949–1955 (2010).
15. N. Knake, S. Reuter, K. Niemi, V. S. von der Gathen, J. Winter, *Absolute atomic oxygen density distributions in the effluent of a microscale atmospheric pressure plasma jet*, J. Phys. D: Appl. Phys., **41**, *19*, 194006 (2008).
16. J. L. Walsh, D. X. Liu, F. Iza, M. Z. Rong, M. G. Kong, *Contrasting characteristics of sub-microsecond pulsed atmospheric air and atmospheric pressure helium–oxygen glow discharges*, J. Phys. D: Appl. Phys., **43**, *3*, 032001 (2010).
17. G. Y. Park, Y. J. Hong, H. W. Lee, J. Y. Sim, J. K. Lee, *A global model for the identification of the dominant reactions for atomic oxygen in He/O_2 atmospheric-pressure plasmas*, Plasma Process. Polym., **7**, *3–4*, 281–287 (2010).
18. D. Dobrynin, G. Fridman, G. Friedman, A. Fridman, *Physical and biological mechanisms of direct plasma interaction with living tissue*, New J. Phys., **11**, *11*, 115020 (2009).
19. E. Stoffels, I. E. Kieft, R. E. J. Sladek, L. J. M. van den Bedem, E. P. van der Laan, M. Steinbuch, *Plasma needle for in vivo medical treatment, recent developments and perspectives*, Plasma Sources Sci. Technol., **15**, *4*, S169–S180 (2006).
20. T. Nilsson, V. Schultz, P. O. Berggren, B. E. Corkey, K. Tornheim, *Temporal patterns of changes in ATP/ADP ratio, glucose 6-phosphate and cytoplasmic free Ca^{2+} in glucose stimulated pancreatic ß-cells*, Biochem. J., **314**, 91–94 (1996).
21. E. Biagiotti, L. Guidi, S. Capellacci, P. Ambrogini, S. Papa, P. del Grande, P. Ninfali, *Glucose-6-phosphate dehydrogenase supports the functioning of the synapses in rat cerebellar cortex.*, Brain Res., **911**, *2*, 152–157 (2001).