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Effect of Atmospheric-pressure Plasma Jet on Normal and Tumor Cells *in vitro*

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***Abstract*—The purpose of this work is to investigate the effect of low-temperature plasma on tumor and normal cells. As a result of *in vitro* experiments, plasma-exposed tumor and normal cells demonstrate several effects such as cell detachment, apoptosis or necrosis according to cell type and exposure parameters (power, time of exposure, frequency). In experiments, the inhibition of tumor cell growth was observed up to 70% on the 5th day after exposure. The effect of gas discharge plasma on normal cells was the opposite, and by 5 days there was a stimulation of cell proliferation. The obtained data demonstrate the prospects of using this atmospheric-pressure plasma jet in biomedical research aimed at the treatment of cancer.**

Keywords—plasma jet, atmospheric-pressure discharge, tumor cells, hela, cell proliferation, normal cells.

I. Introduction

The use of low-temperature atmospheric plasma in biology and medicine is becoming more popular. It is used in various applications, such as disinfection, wound healing, an antibacterial agent and a means of controlling the physiological state of cells [1–4].

It is worth noting that using of low-temperature plasma is considered as a new method of treating cancer [5]. Many researchers have shown the anti-cancer effect of plasma in vitro of various types of tumor cells, including skin, pancreatic, lung, head and neck, etc. [6, 7]. The effect of gas discharge plasma changed the metabolism of tumor cells and led to apoptosis and necrosis, depending on the cell type and exposure parameters (power, exposure time, frequency) [8]. It is shown that one of the mechanisms of plasma action is based on the production of active radicals into the environment, as well as the induction of generation of reactive oxygen species (ROS) in cells, causing their death [9].

In this regard, it should be mentioned that the selective action of plasma, which actively inhibits the growth of tumor cells, leaving normal cells almost not susceptible. This is most pronounced in *in vivo* experiments, in which scientists demonstrate a reduction in tumor size without significant damage to surrounding normal tissues [10]. Stimulation of the immune response to tumor progression was also recorded [11].

In addition to plasma therapy of tumors using reactive oxygen species, there is also ozone therapy of tumors. It is based on the use of ozone gas (O_3) generated by special gas-discharge devices. *In vitro* studies have shown that ozone can cause opposite effects depending on its concentration. The antitumor effect depends on the concentration of intracellular ROS and free radicals, since ROS can both stimulate cell viability and suppress it by damaging genetic material [12]. In vivo studies have also shown antimetastatic and antitumor effects of ozone depending on its concentration [13, 14].

Thus, plasma jet can be a promising tool in the treatment of tumors, but not all the molecular and cellular mechanisms of its action are clear. Based on this, the purpose of this study was to study the effect of an atmospheric-pressure plasma jet on the proliferation of tumor and normal cells.

II. Experimental Setup

This study were performed on HeLa cervical cancer cells and 3T3 normal rat fibroblast cells. The cells were incubated in a nutrient medium at 37° C in an atmosphere of 5% CO_2. The study subjects were exposed to a plasma jet in Petri dishes with an area of 9.2 cm^2. In all experiments sham cell cultures were used as controls. The sham cells were subjected to the same manipulations as the treated ones, but without activating the plasma source.

A. Experimental Setup

In this work, a discharge in the airflow was used to produce the non-thermal plasma jet. The circuit of the experimental setup is shown on the fig. 1.

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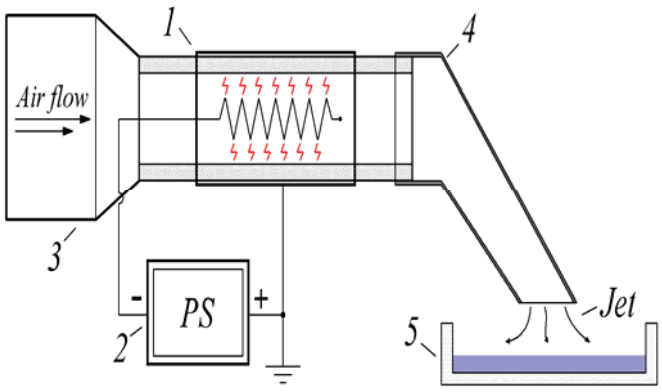

Fig. 1. The simplified circuit of experimental setup for cell treatment by the atmospheric-pressure plasma jet.

The plasma jet source consist of a coaxial plasmatron *1*, a high voltage power supply *2* and a gas-feeding device *3*. The plasmatron *1* consist of quartz tube (inner diameter 10 mm, wall thickness about 1 mm) that has the spiral-like inner electrode made from stainless steel (wire diameter 0.5 mm, coil diameter 5 mm, coil length 25 mm) and outer electrode made from aluminum foil. The length of the gas-discharge gap is about 2 mm, and the electric capacity between the electrodes in the absence of plasma is about 5 pF.

The power supply *2* provides the pulsed voltage with the magnitude up to 15 kV and a total pulse duration about of 1 μs. In this work the negative polarity of the excitation voltage is used for initiate the discharge and sustain the plasma. The pulses repetition frequency was able to set in range from 1.5 kHz to 4.2 kHz. Thus, under these conditions, the maximum electrical power dissipated in the discharge plasma is limited by a dielectric barrier at about a few watts.

The gas supply device 3 generates an airflow with a mass flow rate of up to 0.07 gm/s. It allows the transfer of plasma-activated gas from the plasma region through the nozzle 4 to the substrate 5. The experiments use atmospheric air with a relative humidity of 30–40%.

Nozzle *4* is made of silicone tube 15 cm long and inner diameter of 10 mm. Thus, the plasma discharge region is located at a considerable distance from the nozzle outlet. Therefore, at the output of our system, we do not have any radiation or electric fields and get mostly ozone (the calculated concentration is less than 0.1 ppm) and a negligible concentration of ions and nitrogen oxides. These compounds are chemically active and can change cell metabolism [9]. In experiments, the temperature at the outlet of the nozzle outlet does not exceed 40°C. This eliminates the thermal effect on cell proliferation.

B. Determination of Cell Viability

The proliferative activity of cells was measured using the MTT-test on days 1 and 5 after exposure to low-temperature plasma. The MTT-test is a colorimetric method for evaluating cell viability. It is based on the ability of mitochondrial dehydrogenases to convert water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into non-soluble formazane, which has a purple color and crystallizes in the cytoplasm of cells [15]. Using an organic solvent dimethyl sulfoxide (DMSO), the formazane is converted to a dissolved state. By changing the optical density of the solution, it is possible to estimate the number of living cells. In this paper, the optical density of solutions was determined using a spectrophotometer (Thermo Scientific Multiskan FC, USA) at a wavelength of 620 nm. Then the received data were presented as a percentage.

Experiments on colony formation were conducted to assess the viability of cells. The cells were placed on Petri dishes with an area of 60.1 cm^2. After a day, the cells were exposed to gas discharge plasma in various modes, followed by culture in the same dishes for 120 hours. When visible colonies appeared, the cells were washed twice with PBS, fixed in 2% formaldehyde for 15 minutes, and stained with 5% Giemsa solution for 20 minutes. After that, the Petri dishes were washed with water and dried in the air. The number of visible colonies was calculated using a microscope (Carl ZEISS Primo Star, German). The rate of colony formation was calculated using the formula: (number of colonies/number of cells planted)·100% [16].

Statistical comparisons of the obtained data were performed using the nonparametric Mann-Whitney test in the Statistica 9.0 software. All of obtained data is statistically significant differences at $p < 0.05$ compared to the sham group.

III. RESULTS

A. The Viability of HeLa Tumor Cells after Exposure, Measured Using the MTT Test

Experiments have shown that exposure to the atmospheric-pressure plasma jet leads to inhibition of the proliferative activity of HeLa cells (Fig. 2). On the first day after exposure, the decrease in cell growth was 45–50% compared to the sham group. This effect was weakly dependent on the frequency of voltage pulses and the time of exposure. The maximum effect of the plasma jet was observed on the 5th day at a frequency of electric discharges of 2 kHz and an exposure time of 120 seconds and reached 70%. A longer exposure (240 s) did not lead to an increase in the inhibitory effect, as well as an increase in the frequency of voltage pulses repetition to 4 kHz.

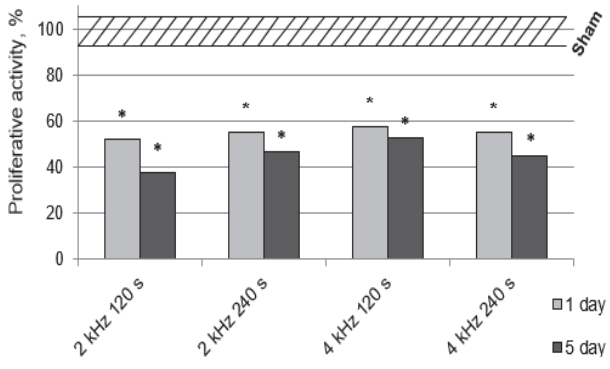

Fig. 2. Proliferative activity of HeLa tumor cells at 1st and 5th days after exposure to atmospheric-pressure plasma jet with discharge frequencies of 2 and 4 kHz. Note: * – statistically significant differences at $p < 0.05$ compared to the sham group.

Since our study does not use direct contact with plasma during processing, it was decided to conduct an experiment with changing the height of the nozzle from which the plasma jet products (free radicals) come out. The study used three height options: 8, 12 and 16 mm from the nozzle outlet to the substrate surface (Fig. 3). The exposure mode of 120 s at a frequency of 2 kHz was chosen based on the previous experiment, where the nozzle height was about 16 mm.

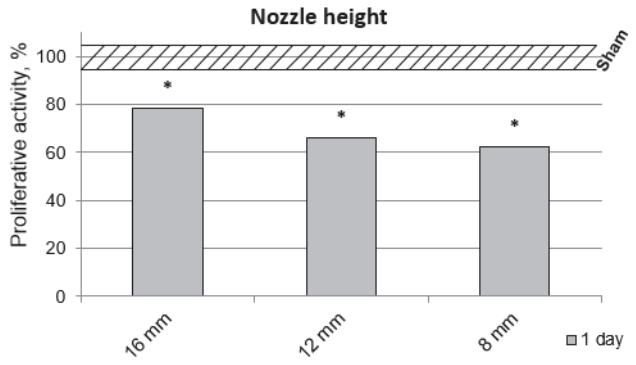

Fig. 3. Proliferative activity of HeLa tumor cells at 1st day after 2 kHz 120 s exposure to atmospheric-pressure plasma jet at nozzle height variation. Note: * – statistically significant differences at $p < 0.05$ compared to the sham group.

Experiments have shown that the difference in the nozzle height affects the inhibitory effect of atmospheric-pressure plasma jet. This effect was observed to increase by 20% at a height of 8 mm relative to the group at a height of 16 mm. The obtained data indicate that the cell proliferation inhibiting effect decreases with increasing distance from the nozzle outlet to the substrate surface. This may be due to the fact that the jet mixes with the surrounding air and the concentration of active particles and radicals in the area of the substrate surface decreases.

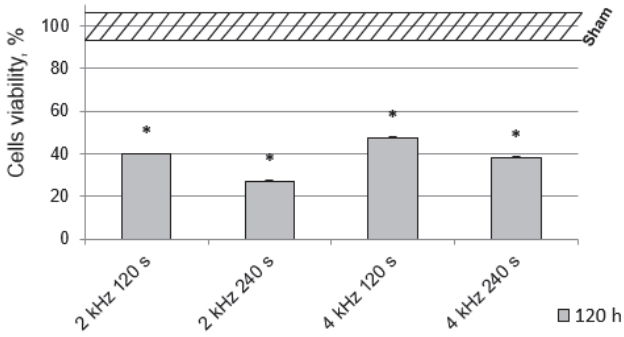

Fig. 4. The viability of HeLa tumor cell colonies after exposure to atmospheric-pressure plasma jet. Note: * – statistically significant differences at $p < 0.05$ compared to the sham group.

B. The Viability of HeLa Tumor Cells after Exposure, Measured Using the Colony Test

To assess the propagation of ROS and plasma free radicals, the formation of colonies on large Petri dishes was studied. The cells were exposed to plasma jet in various operating modes of plasma source. The obtained results showed that the inhibitory effect of plasma action persists even with a significant increase in the area of the irradiated object. The maximum effect was observed when the culture was exposed to a plasma jet with an electrical discharge frequency of 2 kHz and an exposure time of 240 seconds and reached 73%. It was most pronounced after 120 hours (Fig. 4).

In all groups, cell growth was observed after exposure, and after 24 hours, a decrease in the rate of cell division relative to the sham group was shown (Fig. 5).

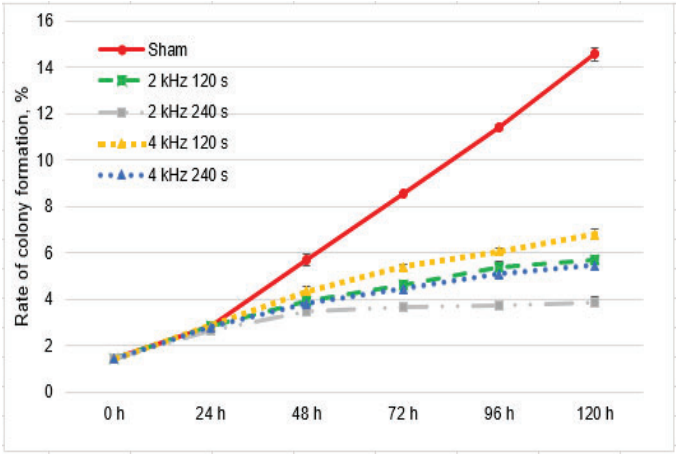

Fig. 5. The growth rate of HeLa tumor cell colonies after exposure to atmospheric-pressure plasma jet depending on time. All values are statistically significant.

C. The Viability of 3T3 Normal Cells after Exposure

Experiments on normal fibroblastoid cells were performed to confirm the unique properties of plasma that ensure high viability of normal cells as opposed to tumor cells. The reaction of normal cells was the different of previous experiments (Fig. 6). Despite the fact that the first day there was an inhibition of proliferative activity of cells, however, on the 5th day their ability to divide was restored. In addition, treatment with a plasma jet with an electrical discharges frequency of 4 kHz and an exposure time of 120 seconds stimulated the proliferative activity of normal cells by up 25%. This effect depended on the frequency of voltage pulses and the duration of exposure. Using a lower frequency of voltage pulses (2 kHz) caused less stimulation of cell proliferation. However, with increasing exposure time, cell growth exceeded the corresponding values of the sham group by 18%.

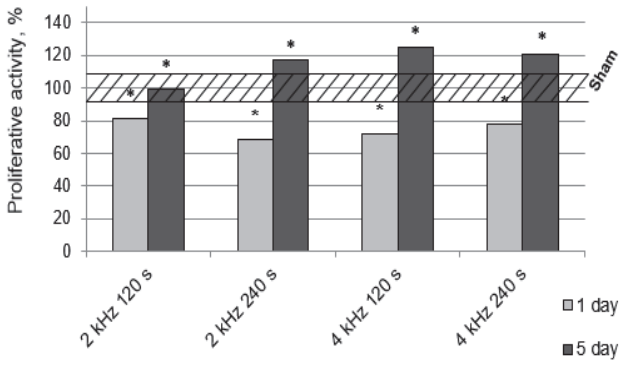

Fig. 6. Proliferative activity of 3T3 normal cells at 1st and 5th days after exposure to atmospheric-pressure plasma jet with discharge frequencies of 2 and 4 kHz. Note: * – statistically significant differences at $p < 0.05$ compared to the sham group.

IV. Discussion

The inhibition of proliferative activity of tumor cells shown by us can be caused by the presence of reactive chemical compounds. They are formed when air passes through the plasma region of the discharge [6]. This complex of reactive compounds changes the physiological state of biological objects, disrupting their redox balance. The obtained data indicate that tumor cells are more sensitive to the action of atmospheric-pressure plasma jet products than normal cells. The analysis of the literature data suggests possible mechanisms of such selective decrease in the viability of various cell types [17–19]. The main features of tumor cells responsible for this selectivity are their basal intracellular ROS level, aquaporin expression, and membrane lipid composition [5]. Reactive oxygen species can damage cell components, disrupt the function of mitochondria with a decrease in ATP synthesis, and cause a state of oxidative stress, which together leads to the death of tumor cells [20].

V. Conclusion

Thus, sensitivity of tumor and normal cells to the effects of atmospheric-pressure plasma jet was investigated. Exposure to low-temperature plasma in the voltage pulses repetition frequency range of 2–4 kHz and exposure time in the range of 120–240 seconds can inhibit the proliferative activity of tumor cells *in vitro*. Inhibition of the proliferative activity of HeLa cells was maximal after exposure with a frequency of 2 kHz voltage pulses and an exposure time of 120 seconds and reached 70% on day 5. The resulting effect depended on the frequency of the voltage pulses, nozzle height and the time of exposure.

The effect of atmospheric-pressure plasma jet with a similar mode of operation and duration of exposure to normal cells is opposite and after 5 days causes stimulation of proliferation. The effect also depended on the frequency of the voltage pulses and the time of exposure. The greatest stimulation of cell growth was observed when exposed to a plasma jet powered by frequency of 4 kHz and an exposure time of 120 seconds, on the 5th day it reached 25%.

The complexity of the resulting cellular response to atmospheric-pressure plasma jet requires further study with various types of tumor cells. The above results demonstrate the prospects of using this low-temperature plasma in biomedical research aimed at treating cancer both *in vivo* and *in vitro*.

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