Inhibition of tumor cell proliferation \textit{in vitro} using atmospheric-pressure plasma jet

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\textbf{Abstract.} The effect of atmospheric-pressure plasma jet on the proliferative activity of tumor and normal cells was studied. It was shown that under certain parameters, plasma jet inhibits the proliferative activity of tumor cells up to 75\%. However, the reaction of normal cells under the same parameters of plasma exposure is different. It was found that low-temperature plasma jet stimulates the proliferative activity of normal cells up to 25\% on the 5th day after exposure. The obtained data indicate the prospects of using this low-temperature plasma jet in biomedical research aimed to minimizing the negative impact on healthy tissues during antitumor therapy.

1. Introduction

Nowadays, many works are devoted to the use of plasma and plasma jets in medicine and biology. There are various biomedical applications of low-temperature plasma, such as wound healing, blood clotting, anti-bacterial properties of plasma for treatment, control of the physiological state of endothelial cells, and much more \cite{1, 2}. This difference in the use of plasma is explained by the relative simplicity and availability of atmospheric pressure plasma generators, as well as its efficiency.

There is quite a large amount of literature data on the potential ability of low-temperature plasma in the treatment of cancer. Recently, the use of atmospheric-pressure plasma jet as a factor that induces apoptosis in tumor cells has become more relevant. To date, the literature describes the antitumor effect of low-temperature plasma, demonstrated \textit{in vitro} on many tumor cell lines \cite{3}. There are two ways to apply plasma: direct treatment and indirect treatment using a plasma-activated medium or solution (PAM) \cite{4}. The first method is to directly apply the plasma jet product to cells \textit{in vitro}, \textit{in vivo} models or human tissue. The second strategy is to produce PAM and then use it by injecting into cell cultures or tumor tissues \cite{5}.

\textit{In vitro} experiments have shown a change in the physiological state of tumor cells, leading to apoptosis or necrosis, depending on the cell type and exposure parameters (discharge power, exposure time) \cite{6}. It was also noted that plasma can not only produce active radicals into the environment, but also induce the generation of reactive oxygen species (ROS) in cells, leading to their death \cite{7, 8}. In experiments conducted \textit{in vivo}, a decrease in the growth of subcutaneous tumors in mice was recorded, as well as stimulation of the immune response to tumor progression \cite{9}.
Considering the effect of reactive oxygen species on cells, it is necessary to mention the ozone therapy of tumors, scientific works on this topic have been published for many years. It is based on the use of ozone gas (O\textsubscript{3}), which frequently is generated by special gas-discharge devices. The main target of this technique is the microenvironment of the tumor, which is directly affected by ozone, resulting in the death of tumor cells. The microenvironment of the tumor is mainly represented by immune cells, for example, lymphocytes, tumor-associated macrophages. The main function of the microenvironment is the ability to influence the progression and growth of the tumor or its regression [10]. However, when using ozone \textit{in vivo} experiments, indirect effects occur that stimulate adaptive mechanisms responsible for modulation in the body [11]. In experiments \textit{in vitro} on cell cultures, it has been shown that ozone can cause opposite effects depending on its concentration. Antitumor effect is associated with intracellular production of ROS and free radicals. ROS can both stimulate cell proliferation and survival, and inhibit their growth. Free radicals cause damage to genetic material, resulting in cell death [12]. Animal studies have demonstrated the antimetastatic and antitumor effects of ozone depending on its concentration [13, 14]. The above data prove the relevance of using discharge plasma in oncological practice. Based on this, the purpose of this work was to study the effect of atmospheric-pressure plasma jet in airflow on the proliferation of various types of cells.

2. Materials and methods
Experiments were performed on cervical cancer tumor cells (HeLa), breast cancer cells (MCF-7) and normal rat fibroblast cells (3T3). The cells were incubated in Petri dishes in a humid environment containing 5% CO\textsubscript{2} at a temperature of 37 °C. Treated cells were exposed to a plasma jet in Petri dishes with an area of 9.2 cm\textsuperscript{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.

2.1. 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 figure 1.

![Figure 1](image)

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

The plasma jet device 1 consist of a 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 power supply 2 provides the pulsed voltage with the magnitude up to 15 kV and a pulse duration about of 1 \textmu s. The pulses repetition frequency was able to set in range from 1.5 kHz to 4.2 kHz. An airflow up to 0.05 gm/s generated by micro-compressor 3 allows to forms plasma jet and provide the transferring of plasma-activated medium via the nozzle 4 to the substrate 5.

The curved nozzle 4 is made of a 15 cm long silicone tube with an inner diameter of 10 mm. Thus, the discharge plasma region is located at a considerable distance from the nozzle outlet. Therefore, at the output of our system, we have no radiation, electrical fields or charged particles and get mainly...
ozone (estimated concentration less than 0.1 ppm) and a low concentration of nitrogen oxides. Therefore, at the output of our system, we get mainly ozone and nitrogen oxides. These compounds are chemically active and can change the physiological state of cells [7].

In the experiment, we varied the frequency of repetition of voltage pulses from 2 to 4 kHz, as well as the exposure time: 120 and 240 s per session.

2.2. Determination of cell viability
The proliferative activity of cells was assessed using the MTT test on days 1 and 5 after exposure to low-temperature plasma. This analysis is based on measuring the metabolic activity of cells [15]. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) was added to the cells at a final concentration of 5 mg/ml, then cells were incubated for 4 hours. During this time, the metabolically active cells restore tetrazolium salts (MTT) to purple-colored formazane compounds that are insoluble in water and are deposited in the cells. After incubation, the supernatant was removed and the precipitate was dissolved with dimethylsulfoxide (DMSO). Then the optical density of the obtained solutions was measured using a spectrophotometer (Thermo Scientific Multiskan FC, USA) at a wavelength of 620 nm. The proliferative activity of cells is represented as a percentage.

Statistical comparisons of the obtained data were performed using the nonparametric Mann-Whitney test in the Statistica 8.0 software. All of obtained data is statistically significant differences at p<0.05 compared to the sham group.

3. Results and discussion

3.1. Proliferative activity of HeLa tumor cells after exposure
Atmospheric-pressure plasma jet exposure leads to inhibition of the proliferative activity of HeLa cells (Figure 2). On the first day after exposure, cell growth inhibition was 45-50% compared to the sham group. The effect was weakly dependent on the frequency of the voltage pulses and the time of exposure. The maximum effect up to 70% on the 5th day was observed when frequency of electric discharges was 2 kHz and treatment time was 120 seconds. Longer exposure (240 s) did not lead to increasing inhibition effect, as well as an increase in the frequency of repetition of voltage pulses to 4 kHz also did not lead to a more pronounced inhibition of cell growth.

![Figure 2](image-url)

Figure 2. Proliferative activity of HeLa 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.

3.2. Proliferative activity of MCF-7 tumor cells after exposure
The response of breast tumor cells was different from the response of cervical cancer cells after exposure of atmospheric-pressure plasma jet. As in the case of HeLa, there was an inhibition of the
proliferative activity of MCF-7 cells but only up to 20-25% at the first day (Figure 3). The effect was also weakly dependent on the frequency of voltage pulses and the duration of exposure. When using a frequency of 2 kHz, the effect depended on the time of treatment. Exposure for 120 s resulted in a 35% inhibition of cell growth compared to the control group, while longer-term exposure (240 s) inhibited cell proliferation up to 75%. When using electric discharges with a frequency of 4 kHz, the inhibition of breast cancer cell growth was 55-60% and change slightly with increasing exposure time.

Figure 3. Proliferative activity of MCF-7 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.

3.3. Proliferative activity of 3T3 normal cells after exposure

The reaction of normal rat fibroblast cells is the opposite of previous experiments (Figure 4). As in cases with tumor cells, the first day showed inhibition of cell proliferative activity, but after 5 days their ability to divide was restored. In addition, treatment with a plasma jet (gas-discharge powering at a frequency of 4 kHz and an exposure time of 120 seconds) stimulated proliferative activity of normal cells up to 25%. The effect depended on the frequency of voltage pulses and the duration of exposure. Applying electric discharges with a frequency of 2 kHz stimulated fibroblast growth according with increasing exposure time. Thus, 120 seconds exposure did not stimulated cell division relative to the sham group. However, when the exposure time was increased to 240 seconds, the cell proliferative activity was stimulated by up to 18%.

Figure 4. Proliferative activity of 3T3 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.
The above results may be due to the presence of reactive chemical compounds that are formed during the passage of air through a discharge plasma region [3]. Such a complex of reactive compounds can affect the metabolism of biological objects, disrupting their redox balance. The obtained data indicate that tumor cells are more sensitive to the action of reactive oxygen species than normal cells. Analysis of the literature data suggests possible mechanisms of such selective decrease in the viability of different cell types [16, 17, 18]. The main features of tumor cells responsible for this selectivity are their basal intracellular level of ROS, expression of aquaporins, and the cholesterol composition of the membrane [4].

4. Conclusion

Thus, it was shown that atmospheric-pressure plasma jet treatment in the range of 2-4 kHz voltage pulse repetition frequencies and exposure time in a range 120 - 240 seconds can inhibit the proliferative activity of human tumor cells in vitro. Inhibition of the proliferative activity of HeLa cells was maximal after exposure with a frequency of 2 kHz and a duration of exposure of 120 s and was most pronounced on the 5th day after exposure (up to 70%). Exposure for 240 seconds and electric discharges with a frequency of 2 kHz causes inhibition of MCF-7 cell growth by up to 75% 5 days after exposure. Treatment of normal cells using a plasma jet with a similar operating mode and duration stimulated the proliferative activity of normal rat fibroblast cells (3T3) by up to 25%. However, the chemistry of plasma discharge modes requires detailed study, since to implement an adaptive plasma system, an effective multiparametric feedback system based on cellular reactions must be developed.

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