THE STUDY OF BIOCIDAL MECHANISMS OF SPARK DISCHARGE PLASMA RADIATION

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The aim of the investigation is to study the biocidal mechanisms of spark discharge plasma radiation.

Materials and methods. The suspensions of studied bacterial strains were treated in optimal discharge conditions: pulse capacitor capacity C=3.3 nF, ballast resistance R=10 MOhm, power supply voltage U_{PS}=11 kV, pulse recurrence frequency — 10 Hz.

Biocidal effect of plasma radiation was estimated by the number of colony-forming units. The analysis of oxidative process intensity in procariotic cells after plasma radiation exposure was performed according to relative concentration of lipid peroxidation products and fluorescence level of bityrosine, tryptophan and glycated proteins, cell membrane hydrophoby change was studied by fluorescence intensity of 1.6-diphenyl-1,3,5-hexatriene. The character of metabolic changes in cells after plasma radiation exposure was studied by pyridine nucleotides fluorescence intensity, surface structures condition — by the concentration of sialic acids in extracellular medium. Extracellular pH change was assessed pH-metrically, and intracellular pH was analyzed by means of fluorometry, using fluoroscein probe.

Results. 100% gram-positive and gram-negative bacteria growth inhibition was found after plasma radiation exposure within 60 s. Surface carbohydrate structures of gram-positive bacteria were revealed to be destroyed to a greater degree. There was observed the increase of membrane and intracellular pH hydrophoby after the treatment of bacterial cells suspension. The level decrease of molecular products of lipid peroxidation was found. The proteins of gram-negative bacteria were shown to be exposed to more pronounced oxidative modification than those of gram-positive ones. Pyridine nucleotides in oxidized condition were found to prevail in cells after plasma radiation exposure.

Key words: spark discharge plasma radiation, S. aureus, E. coli, biomolecules oxidation, intracellular pH.

The application of gas-discharge cold plasma in biology and medicine is being discussed for about 20 years, since there have been accessible for scientists the devices for plasma generation under laboratory conditions. One of the attractive features of non-equilibrium plasma is the possibility to use in biological processes electron energy that is rather higher than that of ions and neutral particles formed in gas phase. The electrons with high energy run into background gas resulting in the initiation of dissociation, excitation and ionization processes [1, 2]. Since ions and neutrals stay relatively cold, plasma does not cause thermal damage [3]. It opens possibilities for using plasma for temperature-sensitive materials sterilization including biological objects such as cells and tissues [4–6]. However, plasma comes in contact with the object surface only, while non-coherent spark discharge plasma radiation can penetrate into an object. From all possible factors generated by plasma, the following can participate in biological effects: heat, charged particles, reactive neutrals, and electromagnetic radiation, but mainly — long-lived radicals interacting directly with biological substrates [7]. In spite of active study of biocidal effect of low-temperature plasma, the mechanisms of its cytotoxic effect are still unstudied.

The aim of the investigation is to study the biocidal mechanisms of spark discharge plasma radiation.

Materials and Methods. The experiment was carried on bacterial strains of antibiotic-resistant gram-positive microorganisms of Staphylococcus aureus 5913 and gram-negative microorganisms of Escherichia coli 775-3. Bacterial strains were taken from the museum of the Department of Microbiology and Immunology, Nizhny Novgorod State Medical Academy. For analyses there were preprepared

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daily culture. Then bacterial cells were resuspensed in Hanks' solution up to the concentration of (10–15)·10^6 cells per 1 ml.

Impulse spark discharge generating low-temperature plasma radiation was formed using experimental device ПИЛИМИН series ИР-10. The device was developed in Scientific Research Institute of nuclear physics named after D.V.Skobeltsyn, M.V.Lomonosov Moscow State University, in 2011. The characteristics of the discharge used: pulse capacitor capacity C=3.3 nF, ballast resistance R=10 MOhm, power supply voltage U_{PS}=11 kV, pulse recurrence frequency — 10 Hz.

In the first stage of the experiment there was assessed the bacterial cell growth inhibition after plasma radiation. For this purpose the plating on stiff agar was carried out, then Petri dishes were exposed to plasma radiation within 5, 10, 15, 30, 60, 120, 300, 600 s. After that the samples were placed in a oven for a day at 37°. Biocidal effect of plasma radiation was estimated by the number of colony-forming units (CFU).

In the second stage of the study there were estimated structural and functional changes of bacterial cells after plasma radiation effect. Cell suspension, 4 ml in volume, was exposed to plasma radiation within 15, 30 and 60 s, the layer thickness being about 3.5 mm. The distance to the discharge area was 2 cm. In cell suspension there was determined intracellular concentration of sialic acids, lipid peroxidation (LP) intensity — diene conjugates (DC), triene conjugates (TC), Schiff bases (SB), fluorescence level of bityrosine, tryptophan and glycated proteins, NADN and lipophilic probe 1.6-diphenyl-1,3,5-hexatriene (DPH), and the change of extracellular and intracellular pH.

The extraction of lipids from the analyzed material was performed according to Folch partition [8]. The level of total lipids was determined using the set TOTOL LIPIDS BIO-LACHEMA-TEST (PLIVA-Lachema Diagnostica, Czechia) spectrometrically, in wave length being 540 nm. Diene and triene conjugates were determined using spectrophotometry, by the maximum absorption of conjugated double links, in wave length being 233 and 275 nm respectively [9]. The level of Schiff bases was assessed by fluorescence intensity, in excitation wave length 345 nm, registration wave length 450 nm [10]. The levels of DC, TC and SB were referred to the number of lipids and stated in relative units.

The concentration of sialic acids in supernatant fluid was determined spectrophotometrically, in wave length 532 nm, using a reagent set Sialotest-80T (Scientific production center "Eco-Service", Saint Petersburg, Russia).

The total protein concentration was determined by biuretic method using Total Protein «FL-E» (Vital Diagnostics, Saint Petersburg, Russia). Oxidative modification of proteins was estimated by the accumulation of bityrosine, products of non-enzymatic glycol-oxidation of proteins and fluorescence of tryptophan residues. Bityrosine fluorescence was calculated by excitation wave length 325 nm and emission wave length 416 nm, tryptophaniles — in excitation wave length 297 nm and emission wave length 336 nm [11, 12]. The content of glycated proteins was determined using fluorimetrical method, in excitation wave length 370 nm and emission wave length 445 nm [13]. The level of tryptophan, bityrosine, and glycated proteins was referred to total protein count, and stated in relative units.

The intensity of NADN fluorescence was studied in excitation wave length 340 nm, fluorescence wave length 460 nm [14].

The state of lipid biolayer was estimated by fluorescence intensity of lipophilic probe of DPG (Sigma Aldrich, USA) that was added to the suspension up to the final concentration 2 mcmol. After 30-minute incubation at 37° there was registered the fluorescence intensity in wave length 430 nm, and wave length in excitation — 360 nm [15].

The change of extracellular pH was studied pH-metrically. To assess intracellular pH, as a probe there was used fluorescein in final concentration of 5 microgram/ml. The values of intracellular pH were determined by the ratio of fluorescence intensity of a probe in wave length of 516 and 570 nm, excitation wave length — 488 nm [16].

Extracellular pH was measured using pH-meter m-150 (Antekh Company, Gomel, Belarus, 2002). The other measurements were performed using spectrofluorometer «Флюорат-02 Панорама» (Lumex Company, Saint Petersburg, Russia, 2009).

The data obtained in the experiment were statistically processed using application program package EXCEL, Statistica 6.0. Statistical significance of the average differences was determined using Mann–Whitney test.

Results and Discussion. At the first stage of the experiment, spark discharge plasma radiation was stated to have bactericidal effect; and the exposure even within 60 s results in 100% growth inhibition of both gram-positive and gram-negative microorganisms. However, inactivation of gram-positive bacteria is expressed as early as in short modes of exposure (Table 1), therefore, at the second stage of the experiment, and it was of interest to assess structural and functional changes of prokaryotic cells under plasma radiation, and carry out the comparative analysis of the changes depending on the type of bacterial cell wall.

Table 1

The number of colony-forming units after the exposure of plasma radiation on bacterial strains under study

Cell type	Exposure time, s								
	Control	5	10	15	30	60	120	300	600
S. aureus	86±5	86±4	60±3*	16±3*	17±3*	0	0	0	0
E. coli	28±3	29±4	27±2	18±4*	7±2*	0	0	0	0

* — statistically significant difference of the values compared to the control group, p<0.05.

Table 2

The concentration of LP molecular products in cell suspension after the exposure of spark discharge plasma radiation, relative units/mg lipids

Exposure time, s	S. aureus			E. coli		
	DC	TC	SB	DC	TC	SB
No exposure	0.18±0.04	0.05±0.01	0.150±0.008	0.012±0.003	0.010±0.003	0.09±0.005
15	0.020±0.005*	0.010±0.002	0.10±0.004*	0.016±0.003	0.010±0.003	0.095±0.006
30	0.04±0.01*	0.010±0.005*	0.090±0.004*	0.004±0.0015	0.008±0.002	0.060±0.006*
60	0.030±0.005*	0.010±0.002*	0.080±0.002*	0.0020±0.0004*	0.005±0.002	0.030±0.0006*

* — statistically significant difference of the values compared to the control group, p<0.05.

Table 3

Fluorescence intensity of bityrosine, tryptophan, and glycated proteins in cell suspension after the exposure of spark discharge plasma radiation, relative units/mg of protein

Exposure time, s	S. aureus			E. coli		
	Bityrosine	Tryptophan	Glycated proteins	Bityrosine	Tryptophan	Glycated proteins
No exposure	0.34±0.01	2.5±0.2	0.28±0.02	0.32±0.02	2.6±0.2	0.32±0.01
15	0.36±0.02	1.80±0.05*	0.28±0.01	0.280±0.005	3.90±0.06*	0.230±0.004*
30	0.30±0.006	1.60±0.02*	0.230±0.006	0.270±0.005	4.10±0.01*	0.220±0.006*
60	0.50±0.04*	2.7±0.2	0.50±0.04*	0.31±0.02	2.1±0.2	0.32±0.03

* — statistically significant difference of the values compared to the control group, p<0.05.

Low-temperature plasma generates a great number of physically and chemically active factors, when dealing with bacterial cell they can cause its oxidative damage, and as a consequence — death [17]. In low intensity, free radical oxidation is one of the types of normal metabolic processes [18]. In particular, chain LP is of great concern for normal cell functioning. LP products perform specific functions in a cell: regulate renewal processes of biological membranes, have effect on their permeability, and regulate the composition of membrane lipids [19]. Normally, the reactions of peroxides formation and expenditure are balanced, and LP proceeds at a certain stationary level. In diverse effects such a balance can be changed and cause serious damages of biological membranes [18]. The experiment has shown after the exposure of plasma radiation there is the decrease of relative concentration of LP molecular products in suspensions of both strains of bacterial cells. However, gram-positive bacteria respond on the exposure by sharper fall of LP intensity than gram-negative bacteria (Table 2). It can be expected that the LP intensity level necessary for normal cell functioning, in gram-negative bacteria is maintained due to A lipid being a part of lipopolysaccharide of cell wall and serving as a substrate for oxidation [20].

Functional activity of protein structures is also one of the key factors taking part in cell activity regulation. Free radical oxidation of proteins leads to the formation of various derivatives of amino acids such as modified residues of tryptophans, products of non-enzymatic glycosylation of proteins, bityrosine, contents levels of which can be used to estimate the degree of oxidative protein modification (OPM) [11, 21]. Bityrosine forms in the process of one-electron tyrosine oxidation, when originating long-lived tyrosil-radical in interaction with the same radical forms bityrosine cross-links. The increase of bityrosine level is commonly supposed to be a reliable OPM marker. Amino acid residues of tryptophan are exposed to oxidizing action of oxygen radicals as well. The oxidation leads to its degradation demonstrating the decrease of fluorescence intensity. The increase of tryptophan intensity can be due to protein molecule unfolding accompanied by the change of amino acid residues from a latent form to “exposed” one [22]. The processes of protein glycosylation are closely connected with free radical processes. The products of non-enzymatic glycosylation form as a result of glucose molecule adjoining to protein amino group and further changes of the newly formed compound proceeding without enzymes [13]. So, after the exposure of plasma radiation on the suspension of gram-positive microorganisms, the fluorescence level of bityrosine and glycated proteins has increased by the 60th s, and that of tryptophan — has decreased within 15–30 s of exposure. In gram-negative bacteria there were no changes in bityrosine fluorescence level, and in tryptophan it increased under the exposure within 15–30 s, while in glycated proteins in the same time mode it decreased. Thus, the proteins of gram-positive bacteria undergo the

Table 4

The concentration of sialic acids in extracellular medium after the exposure of spark discharge plasma radiation on cell suspension, mmol/l

Exposure time, s	S. aureus	E. coli
No exposure	0.28±0.08	0.64±0.08
15	0.52±0.16	0.60±0.08
30	0.52±0.12	0.60±0.08
60	0.56±0.04*	0.680±0.028

* — statistically significant difference of the values compared to the control group, p<0.05.

most significant oxidative modification in plasma radiation exposure (Table 3).

Sialic acids located at the ends of carbohydrate side chains are responsible for the total charge of cell surface. In their abstraction, there is the change of adhesion properties of a cell and a surface charge [23]. After the exposure of plasma radiation on the suspension of gram-positive bacteria, the level of sialic acids in extracellular medium increases with the growth of time exposure, for gram-negative cells no statistically significant changes have been found (Table 4).

It can be supposed that the absence of evident oxidative modification of proteins and the degradation of surface structures of gram-negative microorganisms after the exposure of plasma radiation is due to the fact that active forms generated by plasma first of all react with of a cell wall lipopolysaccharide.

The state of lipid biolayer was estimated by fluorescence intensity of DPG lipophilic probe embedded into membranes. DPG being highly hydrophobic and located deep in phosphobilipid layer is noncovalently linked with most hydrophobic parts. The fluorescence intensity of the probe gives the evidence of the size and degree of hydrophobic nature of the areas occupied by the probe: the higher the fluorescence intensity, the more the hydrophobic nature of the corresponding part [15]. The experiment has shown the hydrophobic nature of the membranes of gram-positive cells after the exposure of plasma radiation on bacterial cell suspension to increase within 60 c, and that of gram-negative — within 15–30 s (Fig. 1). The change of membrane hydrophobic nature can result in the change of the charge of cell surface and cell destabilization.

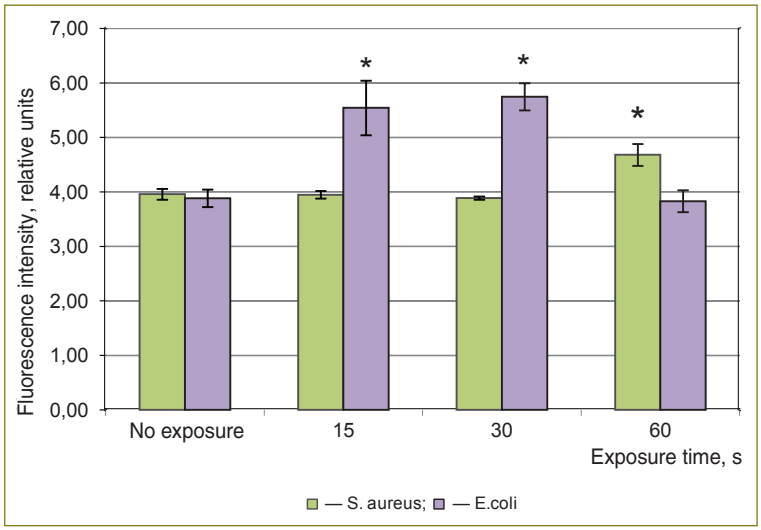

Fig. 1. Fluorescence intensity of DPG probe after the exposure of spark discharge plasma radiation on cell suspension. * — statistically significant difference of the values compared to the control group, p<0.05

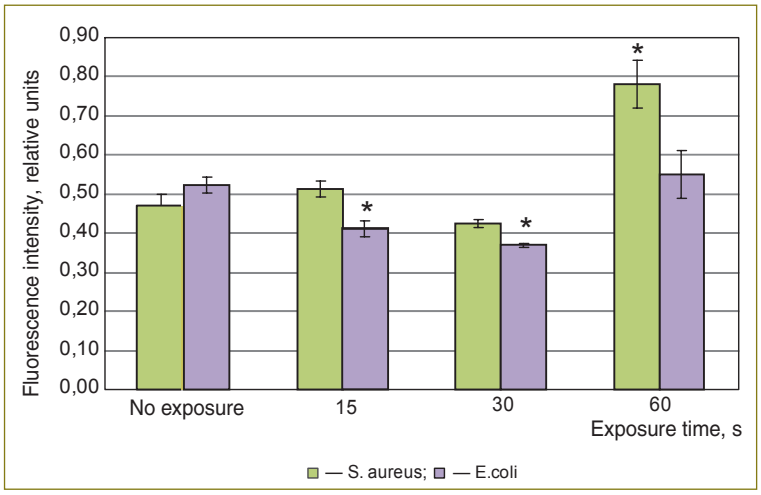

Fig. 2. NADN fluorescence intensity in cell suspension after the exposure of spark discharge plasma radiation. * — statistically significant difference of the values compared to the control group, p<0.05

Fluorescence analysis of pyridine nucleotides enables to assess the character of metabolic changes in cells. NADN is co-factor for more than 250 dehydrogenases and plays key role in electron transfer from a donor to acceptor in electron-transport chain [23]. The fluorescence analysis of pyridine nucleotides is rooted in the effect of redox state of these molecules on their fluorescence — deoxidized NAD(PH)N form has fluorescence, while oxidized NAD(PH) form does not fluoresce [14]. Thus, even minimum or short-term changes of redox-state of the molecules result in their fluorescence change. Oxidized NAD^+ form was found to predominate in cells after the exposure of plasma radiation within 60 s on bacterial strains; and by the moment of cell death there was the transfer into deoxidizing form (Fig. 2). It can be supposed that after the exposure of plasma

Table 5

The level of extracellular and intracellular pH after the exposure of spark discharge plasma radiation on cell suspension, relative units

Exposure time, s	S. aureus		E. coli	
	Intracellular pH	Intracellular pH	Intracellular pH	Intracellular pH
No exposure	6.43±0.10	6.3±0.2	5.8±0.1	5.80±0.08
15	6.17±0.08*	6.8±0.1*	5.36±0.06*	6.3±0.1*
30	5.18±0.10*	6.10±0.09	6.04±0.1	6.20±0.07
60	4.25±0.05*	5.80±0.06*	5.02±0.05*	6.20±0.09

* — statistically significant difference of the values compared to the control group, p<0.05

radiation in bacterial cells there is the decline in efficiency of dehydrogenases and electron-transport chain.

According to research data [24, 25] the effect of plasma radiation leads to pH decrease in water samples. However, in spite of the fact that bacterial cells are able to survive in rather wide pH range [26], the change of hydrogen ion concentration can have an effect on catalytic activity of intracellular enzymatic processes [27]. The obtained in our study data on the change of extracellular pH in the suspension of bacterial cells after the exposure of plasma radiation (Table 5) correspond to those in literature. Alkalization of intracellular medium is likely to be related to adaptive reactions of cells in response to pH change beyond a cell. But a significant decrease of extracellular pH within a short period of time can result in unbalance of metabolic processes and have an effect on cell viability [26].

Conclusion. Spark discharge plasma radiation within 60 s has one hundred percent bactericidal effect on both gram-positive and gram-negative bacteria. Plasma radiation treatment results in the decrease of concentration of lipid peroxidation products that can cause cell malfunction and cell death. Degradation of surface structures and oxidative modification of proteins are to a greater degree expressed in gram-positive microorganisms. Under plasma radiation hydrophobic nature of membranes increases, and in cells there predominate pyridine nucleotides in oxidized state that can lead to the blocking of dehydrogenases and electron-transport chain. As a whole, all these changes in cell metabolism have cytotoxic effect.

The research findings show the mechanisms of cytotoxic effect of spark discharge plasma radiation and enable to provide recommendations for its usage in biological and medical studies.

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