Plasma Medicine, 2(1-3): 141–149 (2012)

Comparative Study of Three Nonthermal Plasma Sources against Causative Agents of Nosocomial Infections

Svetlana A. Ermolaeva,[1,*] Oleg F. Petrov,[2] Yurii S. Akishev,[3] Viktor N. Vasilets,[4] Elena V. Sysolyatina,[1] Mikhail M. Vasiliev,[2] Boris S. Naroditsky,[1] Gregor E. Morfill,[5] Anatoly I. Grigoriev,[6] Vladimir E. Fortov,[2] & Alexander L. Gintsburg[1]

[1]Gamaleya Research Institute of Epidemiology and Microbiology, Ministry of Health and Social Development of Russian Federation, Moscow, Russia; [2]Joint Institute of High Temperatures RAS, Russian Academy of Science, Moscow, Russia; [3]Scientific Research Center of Russian Federation TRINITI, Troitsk, Moscow Region, Russia; [4]Institute for Energy Problems of Chemical Physics (Branch) RAS, Russian Academy of Science, Chernogolovka, Moscow Region, Russia; [5]Max Planck Institute for Extraterrestrial Physics, Munich, Germany; [6]Institute for Biomedical Problems RAS, Russian Academy of Science, Moscow, Russia

*Address all correspondence to: Svetlana Ermolaeva, Gamaleya Institute of Epidemiology and Microbiology, Gamaleya St. 18, Moscow 123098, Russia; Tel./Fax: +7-499-190-4375; sveta@ermolaeva.msk.su

ABSTRACT: Nonthermal plasmas (NTPs) represent a perspective class of antibacterial agents that might be an alternative to chemical disinfectants because they possess an unspecific antimicrobial activity and can be applied to decontamination of heat- and chemical-sensitive surfaces as well as to the infected skin and wounds. The resistance to NTP depends on properties of both a microorganism and a certain plasma source. Two bacterial species, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are among the most important causative agents of nosocomial infections, were tested with three different plasma sources. The ferroelectric air plasma generator, the microwave argon plasma source, and the generator of a positive and negative corona discharge in air demonstrated increasing activity toward tested bacteria. The relative resistance of the tested strains to NTPs produced by distinct plasma sources differed. *P. aeruginosa* was more resistance to the corona discharge and less resistant to the plasma generated by the ferroelectric generator than *S. aureus* while both strains demonstrated comparable resistance to microwave argon plasma. Obtained results demonstrated a role of charged particles as a primary factor in the high effectiveness of the positive and negative coronas and an importance of the NO• radical among neutral active species.

KEY WORDS: bactericidal activity, nonthermal plasma, microwave argon plasma, ferroelectric plasma generator, positive and negative corona discharge

I. INTRODUCTION

The nonthermal plasma (NTP) is an alternative to chemical disinfectants that are applied to heat- and chemical-sensitive surfaces as well as to infected skin and wounds. Available results demonstrated effectiveness of NTP as an antibacterial agent effective against bacteria, bacterial spores, and fungi.

1947-5764/12/$35.00

The most often used approach for comparison of the susceptibility of microbial cells to NTP is treatment of bacteria plated on the nutritive agar followed by registration of survivors.[1–3] Bacterial spores were shown to be more resistant to plasma than vegetative bacterial. When non-spore-forming gram-positive and gram-negative bacteria were compared, the results were controversial. In some cases, no significant differences were detected,[1] while other researchers found that gram-positive bacteria were more resistant than gram-negative bacteria, or vice versa.[2,3]

The differences in cell wall structure can be critical for the differential sensitivity of gram-negative and gram-positive bacteria. Gram-positive bacteria possess one lipid bilayer membrane surrounded by a thick cell wall, which consists of 20–80 layers of peptidoglycan transpierced with techoic acids and proteins.[4] Gram-negative bacteria have two lipid bilayer membranes separated by a periplasmic space, which is similar in composition to the intracellular cytosol. The cell wall of gram-negatives includes one to three layers of peptidoglycan and is placed within the periplasmic space between the outer and inner membranes.[5] Therefore, the cell wall of gram-positives is more rigid and appears to be more resistance to mechanical forces, while the outer membrane of gram-negatives gives them additional defense against membrane-damaging factors. Besides this fundamental difference in the cell wall structure, other features such as operation of the stress response system and/or subtle differences in cell wall composition might contribute into the intrinsic resistance of the microorganism. A considerable variability in plasma resistance between different strains of the same species is in line with this suggestion.[3,6]

One can suggest that the microbial species (strain), which is most sensitive to a certain plasma source, may be relatively resistant to another. A diverse response of microorganisms to individual plasma components and their superimposition might change the resistance of a particular microorganism to plasmas of different origin. Still, the majority of works tests activity of an individual plasma source and comparison of results is complicated because of differences in methods of microbial resistance measurements and a divergence in intrinsic sensitivity among strains.

In this work, we used two strains of nosocomial pathogens to compare microbial resistance and further establish a role of plasma composition in microbicidal properties of nonthermal plasma.

II. MATERIALS AND METHODS

A. Bacterial Strains and Cultivation Conditions

The gram-negative bacterium *P. aeruginosa* (strain Pa 103) and the gram-positive bacterium *S. aureus* (strain Sa78) were used in this study. Bacteria were kept frozen, and thawed before an experiment started. Thawed bacteria were plated on LB (*P. aeruginosa*) or brain heart infusion (BHI, *S. aureus*) agar (all nutritive media were from Becton, Dickinson and Co, NJ, USA) and incubated at 37°C for 24 h. Fresh LB or BHI broth was inoculated with a *P. aeruginosa* or *S. aureus*, respectively, and incubated with shaking at 37°C. The over-

night culture was diluted in PBS up to the concentration of 3×10^5 CFU ml^{-1}. 30 µl of the suspension was plated on 36 mm petri dishes filled with the corresponding nutritive agar and immediately subjected to plasma treatment as described below. Treatment time ranged from 10 s to 25 min. The time required to destroy 10^5 CFU was detected.

B. Plasma Sources and Treatment Conditions

Three plasma sources were used. The microwave argon plasma source MicroPlaSter β was previously described.[7] Microwave power was applied to six electrodes placed inside an aluminum cylinder 20 mm from the opening. The feeding argon gas was flowed at 2.2 slm from the base of the electrodes through a Teflon shower plate that regulates flow around the electrodes. Microwave power at 2.45 GHz and 85 W was applied to the electrodes.

The ferroelectric plasma generator produces discharges between ferroelectric $TiBaO_3$ particles placed within a cylindrical volume with two mesh electrodes (Fig. 1). Plated bacteria were treated with plasma afterglow flowed out from the cylinder. Argon and air were used as plasma-forming gases. Argon was flowed at 5 slm. The air plasma was created without external expulsion.

The positive and negative corona in air was created within a cylindrical gas discharge chamber made of Plexiglas with an inner diameter of 70 mm and a height of 50 mm. The six openings on the lateral face of the camera serve as an input of ambient air. A high-voltage rod electrode and a ground flat electrode were placed in the cylinder. The high-voltage electrode consisted of seven metal needles. The ground electrode was a flat thin disk with a diameter of 25 mm and a thickness of 1 mm, which was placed on the ground of the 36 mm petri dish and covered with an agar lay. Both positive and negative coronas were used.

Samples were placed at the distance of 20 mm from the MicroPlaSter β opening of the torch, 20 mm from the ferroelectric generator opening, and 15 mm from the high-voltage electrode in the corona discharge chamber. A plasma flow covered the surface of the petri dish for all sources.

C. Plasma Characterization

The emissive spectrum of the discharge was recorded with the monochromator MDR-6, the spectral resolution in the UV region $200 \leq \lambda \leq 300$ nm is 0.5 nm. The ozone concentration was measured by commercial ozonometer "Alpha-Photo." The lower and upper limits of ozone concentration detected by this device are equal to 10^{-4}% and 10% (1 and 105 ppm). Concentrations of nitrogen oxide radical were measured with the gas analyzer MX2100. The measurements were done by sampling of the activated air contacting the agar surface. The time-averaged discharge current was measured by the ammeter M-2044 with accuracy of the measurement equal to 2 µA. The voltage drop across the discharge gap was measured by the kilovoltmeter C-196, accuracy of measurement is 0.2 kV. The averaged electric field strength *Eag* at the agar surface was determined from the measurements of the voltage drop as described in Ref. 8.

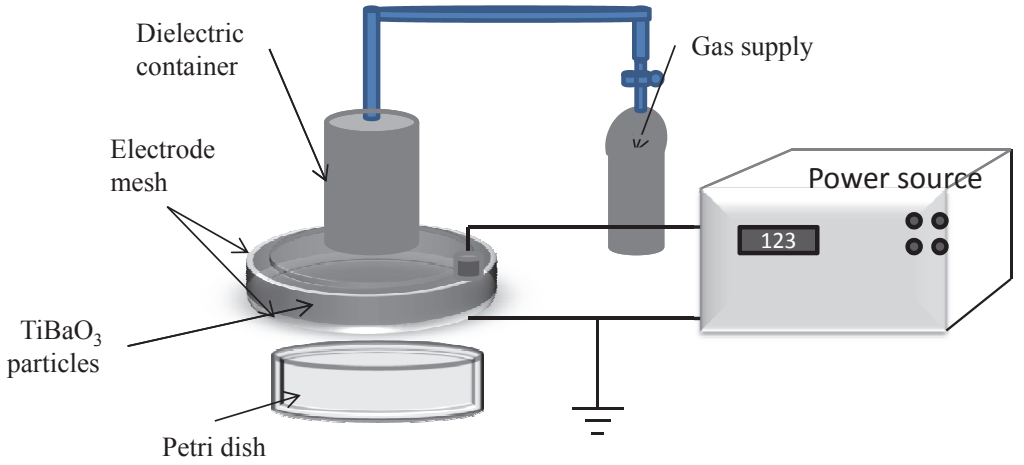

FIG. 1: Schematic representation of the ferroelectric plasma generator. The reactor comprises two metal mesh electrodes installed inside of a dielectric container. The space between electrodes is filled with spherelike particles of the alloyed barium titanate ($TiBaO_3$). The nonequilibrium plasma was generated by microdischarges that appeared at contacts of ferroelectric beads.

III. RESULTS

The time was determined that was required to kill 10^5 CFUs of *P. aeruginosa* and *S. aureus* (Table 1). The strains Pa 103 and Sa 78 demonstrated comparable sensitivity to argon plasma produced by the microwave plasma generator MicroPlaSter β. The microwave argon plasma killed 10^5 CFUs of both bacteria in ~5 min. In contrast, the argon plasma afterglow formed by the ferroelectric generator did not exert a noticeable effect on bacterial viability even after 25 min. The air plasma afterglow formed by the same ferroelectric generator was bactericidal. The treatment for 5 min gave 125- and 24-fold decrease for *P. aeruginosa* and *S. aureus*, respectively (Fig. 2). The killing of 10^5 CFUs required >25 min (the time of the experiment). The positive and negative coronas were the most effective. 45 s and 120 s were required to kill 10^5 *P. aeruginosa* CFUs with the positive corona and negative corona, respectively (Fig. 2). Only 10 s was required to kill 10^5 *S. aureus* CFUs with the both positive and negative coronas. Therefore, plasma sources not only significantly differed in their bactericidal potential against both bacterial strains tested, but the relative sensitivity of bacterial species varied for different plasma sources.

Concentrations of charged particles, UV, excited argon atoms, and neutral reactive species were established that demonstrated a great variability in plasma composition among the sources used. Both positive and negative coronas had the highest concentration of charged particles. The microwave plasma had a much lower concentration of charged particles than the coronas (10^5–10^6 versus 10^{11}–10^{12}, respectively). The after-

TABLE 1. Comparison of bactericidal activity and some characteristics of plasmas used in this study

Characteristics	Microwave argon plasma	Ferroelectiric argon plasma afterglow	Ferroelectiric air plasma afterglow	Positive corona	Negative corona
Time to kill 10^5 CFUs	300 s	—	>25 min	10–45 s[a]	10–120 s[a]
Charged particles	10^5–10^6	—	—	10^{11}–10^{12}	10^{11}–10^{12}
Ozone	> 1.2 ppm	—	≈ 1 ppm	≈ 50 ppm	≈ 10 ppm
NO radical	> 1.2 ppm	—	≈ 350 ppm	≈ 300 ppm	≈ 50 ppm
Ar*	+	?	—	—	—
UV	+	—	—	+	+
Electric field	Microwaves ≈ 0.05 W/cm^2	—		Pulsed >10^6 V/cm	Constant ≈ 10^4 V/cm

[a] Strain-dependent

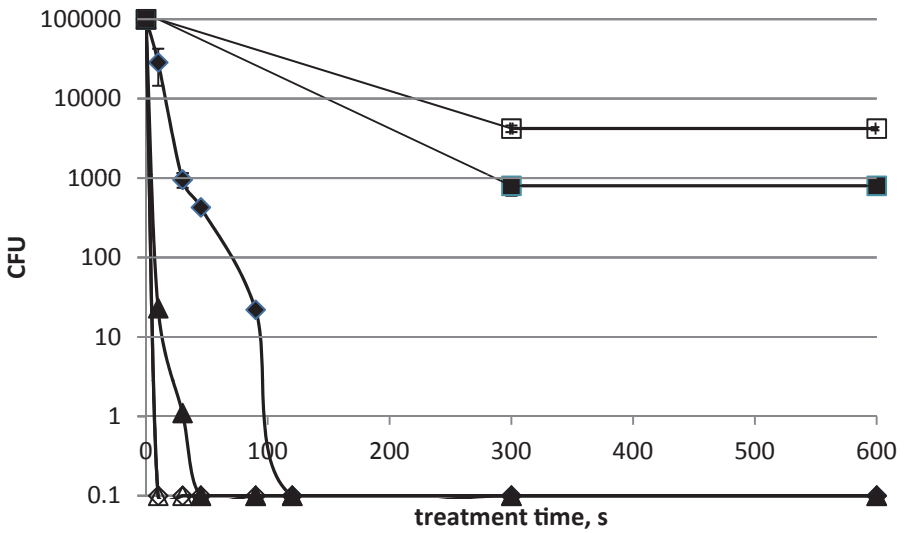

FIG. 2: Bacterial survival curves. 10^5 *P. aeruginosa* Pa 10^3 (filled figures) and *S. aureus* (open figures) were treated with positive corona (triangles), negative corona (diamonds), or with air plasma generated by the ferroelectric generator (squares). The average amounts of survivors from at least three independent experiments are shown.

glow formed by the ferroelectric plasma generator did not include charged particles in any detectable amounts.

The concentration of ozone was measurable for all sources. The exception was the ferroelectric plasma generator when argon was used as the feeding gas. In fact, the argon plasma afterglow formed by this last source did not include any detectable active particles. The possible presence of active forms of argon was not detected in this work and is questionable. Concentrations of nitrogen monoxide radical NO• did not reach bactericidal values in microwave argon plasma and negative corona. The afterglow of the air plasma formed by the ferroelectric plasma generator and positive corona included about 300–350 ppm of the NO• that exceeded a lower limit of bactericidal NO• concentrations.[9] NO• concentrations observed in ferroelectric plasma generator afterglow and positive corona are close to the working concentration of therapeutic NO sources for wound therapy that possess bactericidal and immune stimulating activities.[10]

The spectra of UV irradiation were different for the used sources in the range 200–350 nm (Fig. 3). Emission of the OH• radical gave a major peak at 308 nm in the emission spectrum of the microwave plasma produced by Microplaster β. Emission of the nitrogen (N_2) prevailed in the spectrum of both positive and negative coronas (Fig. 4). Total UV radiation of the negative corona was lower than UV radiation of the positive corona. The nitrogen monoxide radical (NO•) gave multiple bands with different shapes in the spectra of all sources with exception of the ferroelectric generator. The last source did not produce UV radiation in detectable amounts (Fig. 4).

The electrical component was one more factor that affected bacterial viability. The electrical breakdown (electroporation) of the cell membrane happens when the external electrical field E reaches a value $E^* \approx 10^6$ V/cm (the standard value used in commercial electroporators). Streamers of the positive corona when they are approaching to the

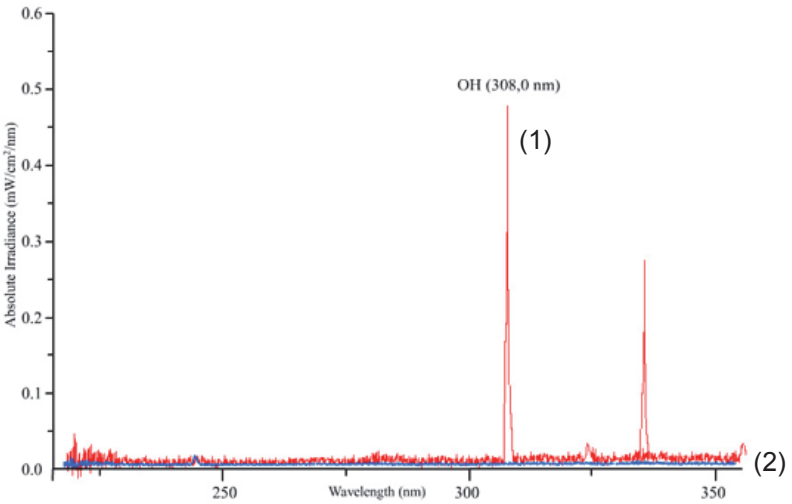

FIG. 3: Emissive spectra of the argon microwave plasma torch (1) and ferroelectric air plasma afterglow (2). Spectral resolution of the monochromator is 0.5 nm. The emission was measured at 20 mm from the plasma torch opening.

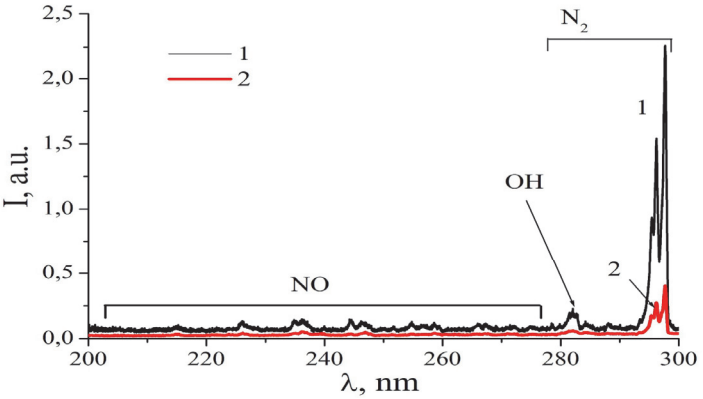

FIG. 4: Emissive spectra of positive (2) and negative (1) corona in ambient air. The discharge current and voltage are 220 μA and 13.3 kV. Spectral resolution of the monochromator is 0.5 nm. The radiation was collected from a small area near the tips of the high-voltage electrode.

agar surface at a distance closer than 1 mm create a field density between the streamer head and the surface of agar greater than the threshold value E^*. Therefore, membrane electroporation might be an active microbicidal factor in the positive corona. Electric field density produced by other plasma sources did not get the threshold value of 10^6 V/cm: the ferroelectric plasma source did not generate the electrical field outside of the volume; the electric field density near/on the agar surface in the negative corona was constant with the value of $E_a \approx 10^4$ V/cm that was not sufficient for the breakdown of the membrane; the microwave alternate field produced by the MicroPlaSter β device had the electric field density of about tens of volts per cm, which was even lower than the constant electric field of the negative corona. Therefore, electroporation could add to a whole plasma bactericidal effect only in the positive corona. This suggestion is in line with the obtained results that demonstrated the highest bactericidal activity of the positive corona. While electroporation itself is not bactericidal, it can affect bacterial survival when it repeats many times or acts in synergy with other plasma active components.

Still, an electric field could affect bacterial survival via other mechanisms besides electroporation. Cell polarization caused by an external electric field can lead to a redistribution of the positive and negative charges inside and outside the cell that in turn might interact with functioning of ion pumps and disturb influx/efflux of charged particles across the membrane. Such a mechanism can be realized in the negative corona, which creates a stationary electric field.

The microwave plasma source Microplaster β was shown to produce microwave irradiation at doses that are incapable to cause thermal effects.[11] However, the view that microwave fields are incapable of inducing bioeffects other than by heating was revised when nonthermal effects of microwaves on biological objects were document-

ed.[12,13] A transient heating of proteins and their close environment was suggested to cause a triggering of the stress response by altering the conformation of the proteins.[12] Microwaves could multiply effects of other plasma active agents via effects on the stress response system. This is particularly important for microorganisms, which are extremely vulnerable to injuries of the stress response system. Therefore, microwave irradiation might be one more factor that added to the bactericidal activity of Microplaster β but not other sources.

IV. CONCLUSIONS

Taken together, the results demonstrated a role of charged particles as an important factor in the high effectiveness of the positive and negative coronas. An electric field represents one more active factor whose impact depends on its strength and mode. Even a field with a density below the threshold required for electroporation might amplify effects of other biologically active factors of the NTP. The NO• radical demonstrated a higher potency among neutral active species. Still, neutral active species when acting alone had the lowest biological activity, which indicates an importance of synergy of plasma components in the total NTP effect. Two bacterial strains demonstrated differential relative resistance to distinct plasma sources, which supports the suggestion about a diverse response of microorganisms to individual plasma components and their superimposition. Relatively low concentrations of active agents, the absence of long-living toxic compounds, and an opportunity to optimize plasma torch parameters to destroy even more resistant microorganisms significantly broaden the sphere of its application in the hospital environment. The nonthermal plasma is applicable to treatment of hospital facilities, medical instruments, and water as well as skin and wound surfaces, which makes it a universal agent for treatment of nosocomial infections. Better understanding of the mode of action of plasma active factor superimposition will provide optimization of plasma parameters for its most effective application.

ACKNOWLEDGMENTS

The authors thank the members of their labs for their contribution to this plasma medicine research. The work was supported by the Ministry of Education and Science of the Russian Federation (Government Contracts No. 02.512.12.2023 and No. 16.512.12.2013).

REFERENCES

1. Venezia RA, Orrico M, Houston E, Yin S, Naumova YY. Lethal activity of nonthermal plasma sterilization against microorganisms. Infect Control Hosp Epidemiol. 2008;29:430–6.
2. Lee K, Paek K, Ju W, Lee Y. Sterilization of bacteria, yeast, and bacterial endospores by atmospheric-pressure cold plasma using helium and oxygen. J Microbiol. 2006;44:269–75.
3. Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, Moisenovich MM, Romanova JM, Murashev AN, Selezneva II, Shimizu T, Sysolyatina EV,

Shaginyan IA, Petrov OF, Mayevsky EI, Fortov VE, Morfill GE, Naroditsky BS, Gintsburg AL. Bactericidal effects of non-thermal argon plasma *in vitro*, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011;60:75–83.

4. Vollmer W, Blanot D, de Pedro MA. Peptidoglycan structure and architecture. FEMS Microbiol Rev. 2008;32:149–67.
5. Höltje JV. Growth of the stress-bearing and shape-maintaining murein sacculus of escherichia coli. Microbiol. Mol Biol Rev. 1998;62:181–203
6. von Keudell A, Awakowicz P, Benedikt J, Raballand V, Yanguas-Gil A, Opretzka J, Flötgen C, Reuter R, Byelykh L, Halfmann H, Stapelmann K, Denis B, Wunderlich J, Muranyi P, Rossi F, Kylián O, Hasiwa N, Ruiz A, Rauscher H, Sirghi L, Comoy E, Dehes C, Challier L, Deslys J. Inactivation of bacteria and biomolecules by low-pressure plasma discharges. Plasma Proc Polym. 2010;7:327–52
7. Tetsuji S, Steffes B, Pompl R, Jamitzky F, Bunk W, Ramrath K, Georgi M, Stolz W, Schmidt H-U, Urayama T, Morfill GE. Characterization of microwave plasma torch for decontamination. . Plasma Proc Polym. 2008;5:577–82
8. Sysolyatina S, Mukhachev A, Yurova M, Grushin M, Karalnik V, Petryakov A, Trushkin N, Ermolaeva S, Akishev Y. Role of the charged particles in bacteria inactivation by plasma of a positive and negative corona in ambient air. Plasma Proc Polym. 2014;11:315-34
9. Sulemankhil I, Ganopolsky JG, Dieni CA, Dan AF, Jones ML, Prakash S. Prevention and treatment of virulent bacterial biofilms with an enzymatic nitric oxide-releasing dressing. Antimicrob Agents Chemother. 2012 Dec;56:6095–103
10. Shekhter AB, Serezhenkov VA, Rudenko TG, Pekshev AV, Vanin AF. Beneficial effect of gaseous nitric oxide on the healing of skin wounds. Nitric Oxide. 2005;12:210–9
11. Vasiliev MM. Diagnostics of low temperature plasma at atmospheric pressure for treatment of heat-sensitive objects. Book of Abstracts XXVII International Conference on Equations of State for Matter. Moscow: JITH RAS; 2012. p. 141–2.
12. Laurence JA, French PW, Lindner RA, Mckenzie DR. Biological effects of electromagnetic fields—mechanisms for the effects of pulsed microwave radiation on protein conformation. J Theor Biol. 2000; 206:291–8.
13. Banik S, Bandyopadhyay S, Ganguly S. Bioeffects of microwave—a brief review. Bioresour Technol. 2003;87:155–9.