ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Morphological Changes In Infected Wounds Under The Influence Of Non-Thermal Atmospheric Pressure Plasma .

Rybalchenko OV[1], Orlova OG [1], Astaf'ev AM[1,2], Pinchuk M E[1,2], Stepanova OM [1], Pariyskaya EN[1], and Zakharova LB[1].

[1]Saint-Petersburg State University, St. Petersburg, 199034, Russia.
[2]Institute of Electrophysics and Electric Power RAS, St. Petersburg, 191186.

ABSTRACT

Helium non-thermal atmospheric pressure plasma is now increasingly widely used in medicine for the disinfection of wounds, local treatment of cancer and skin diseases of bacterial nature. The inhibitory effect of non-thermal plasma on the growth of microorganisms with simultaneous stimulation of proliferation of wound skin cells was shown. The aim of this study was to determine the effect of helium plasma jet atmospheric pressure on the morphophysiological properties of bacteria and bacterial biofilms in infected skin wounds of animals. For the generation of non-thermal plasma, the author's development of a barrier discharge source was used. Experiments were carried out on white mongrel mice. Antimicrobial and wound healing activity of non-thermal plasma was determined at the electron microscopic level. As a result of the impact of non-thermal plasma on the skin wounds of animals shows the microbial death on the surface and in the thickness of the scab, as well as the absence of bacterial biofilms in the upper layers of the wound surface.
Keywords: non-thermal plasma; barrier discharge; helium plasma jet; infected wounds; antibacterial properties; bacterial biofilms; electron microscopy; wound healing.

***Corresponding author**

INTRODUCTION

In recent years, the range of applications of non-thermal atmospheric pressure plasma (NTAPP) in Biomedicine has been expanding worldwide: in wound healing, blood coagulation [1], angiogenesis suppression [2], cancer treatment [3, 4] and inhibition of microbial growth [5, 6] and viruses [7]. Most studies revealed positive effects of non-thermal plasma on skin healing in acne [8] and eczema [9], chronic lower limb ulcers [10, 11], burns [12, 13], as well as in complicated skin wounds in diabetes [14, 15]. However, the deep mechanisms of interaction of non-thermal plasma with living cells or tissue are still far from complete understanding. At the present time of research it is shown that plasma induces migration and proliferation of fibroblasts [16], promotes proliferation of endothelial cells [17] and keratinocytes [18], and stimulates the growth of epithelial cells [19]. The revealed effects are similar to the observed changes in the natural process of wound healing. That is why plasma therapy can be a promising new applied treatment for chronic and infected wounds.

This study is one of the few in which the effect of non-thermal plasma on skin wounds at the electron microscopic level was analyzed. To compare the wound healing process in plasma-treated and control samples, morphometric parameters were measured, which were used to estimate the reduction of the wound zone during the specified period of time.

MATERIALS AND METHODS

To study the impact of NTAPP on the skin wounds of animals used the model of the complicated course of the wound healing process in mice on the background of immunosuppression caused by intramuscular introduction of hydrocortisone in the dose of 25 mg/kg of body weight during 7 days of experiment, for the first time the day before the injury [20]. The study used 32 mongrel white male mice aged 7-8 weeks and weighing 20-22 grams (laboratory animal nursery "Rappolovo", Russia). Mice were divided into two groups of 10 individuals each. In the experimental group on the first, second and third days of the experiment skin wounds were treated with non-thermal helium plasma. Skin wounds were applied with a dermo-punch skin biopsy stylet with a diameter of 5 mm ("Sterylab", Italy). The procedures were performed in accordance with the requirements of international standards of work with experimental animals.

To infection of skin wounds used gram-positive bacteria Staphylococcus aureus 6 and gram-negative bacteria Pseudomonas indica 3 from the collection of Institute of Epidemiology and Microbiology named after N. F. Gamalei. Bacterial cultures were grown in liquid nutrient medium - broth BHI (Brain Heart Infusion Broth, "GibcoDiagnostics", USA). All bacteria were incubated under aerobic conditions at 37° C for 18-24 hours. Those wounds were made in 0.1 ml suspension of S. aureus 6 and P. indica 3 in the amount of $1x10^8$ CFU/ml.

A NTAPP source based on the atmospheric pressure barrier discharge in the helium stream was used to treat skin wounds [21]. The treatment was carried out for 4 minutes at an average discharge power of ~200 mW on the first, second and third day of the experiment. The distance to the wound surface was about 3 cm Mode of operation of the plasma source and the processing conditions were chosen based on earlier experiments on cultures of bacteria [22].

Biopsy of wounds was performed on the third and sixth days of the experiment. For electron microscopic studies using transmission electron microscopy (TEM), the material was prepared by the author's method [23]. The finished preparations were examined in a transmission electron microscope JEM-100C (JEOL, Japan).

Throughout the experiment, the wounds were photographed daily. The resulting images were transferred to a computer, calibrated and measured the area of wound lesion using the program Scion Image (NIH, USA). After that, we calculated the change in surface area for each day (as a percentage of the original area) and the rate of these changes (mm 2 per day). The results were expressed as a percentage of the original area.

Statistical processing of the data was performed using the program Statistics 19. Compliance with the normal data distribution was analyzed using the Shapiro-Fork test ($p > 0.05$). Repeated measures analysis (to compare data in each group) and independent t-test (to compare data between two groups) were used to measure wound area. The parameters were also compared between the groups using the variance analysis (ANOVA) followed by the Tukey test. Differences with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Morphometric analysis

Morphometric analysis was used to study the rate of healing of skin wounds. It is shown that the state of immunosuppression, induced by the hydrocortisone injections, as well as the additional infection of the above - mentioned factors with gram-positive bacteria S. aureus 6, and the gram-negative bacteria P. indica 3 of the experiment, led to a significant decrease in the rate of the defect from the first to the third day of the experiment. In mice of the control group, by the third day of the experiment, the wound defect area was (67±5)% (n=8) of the initial wound area. In the group of animals after hydrocortisone administration wounds with area (69±5)% (n=8) were registered, after infection (63±5)% (n=8) and with simultaneous hydrocortisone administration and infection (77±5)% (n=8). Differences in the rate of wound healing in the study groups began to appear on the fifth day of the experiment. Measuring the area of the wound defect in the morphometric study showed that the rate of wound healing to the greatest extent was reduced by simultaneous administration of hydrocortisone and additional infection: mice of this group the area of the wound area was (82±5)% (n=8) from the original area of the wound, which was confirmed by the development of immunosuppression and complication of a wound process. While the animals of the control group had the best healing and wound area (45±5)% (n=8). Animals in the groups with hydrocortisone administration were characterized by the presence of wounds (63±5)% (n=8), with additional infection (59±5)% (n=8).

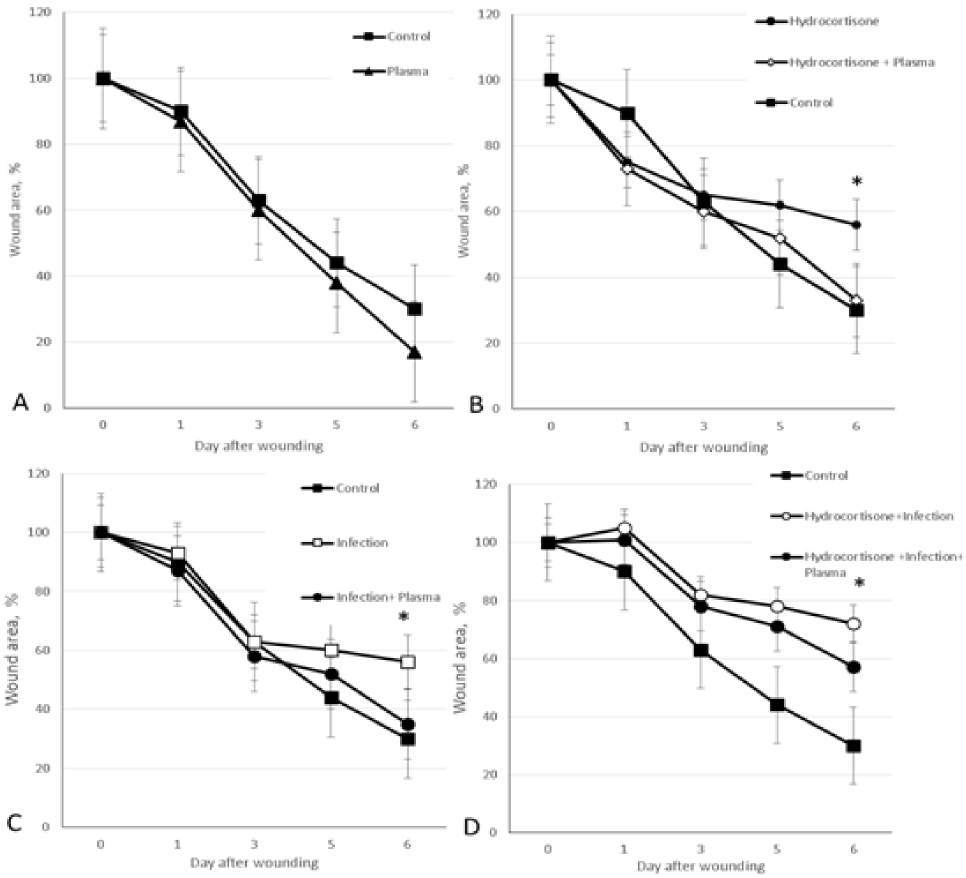

Figure 1: Dynamics of wound surface healing in mice of different experimental groups: wounds treated with non-thermal plasma (A), introduction of hydrocortisone (B), infection of wounds (C), simultaneous introduction of hydrocortisone and additional infection of wounds (G).

* - $p<0.05$ relative to other groups on the sixth day.

Morphometric analysis of the wound area in the group of animals after treatment of skin wounds with non-thermal plasma did not reveal a statistically significant decrease in the rate of reduction of the wound defect until the sixth day after the wound. However, the rate of wound healing in the groups treated with non-thermal plasma was higher than in the groups without treatment (Fig.1A).

When animals were administered hydrocortisone, the area of NTAPP treated wounds was (33±4)% (n=16), which practically corresponded to the control parameters (30±3)% (n=16)). The wound healing area in untreated animals was (56±5)% (n=16) (Fig.1 B). This trend continued in the treatment of NTAPP of infected wounds: on the sixth day of observation, the area of plasma-treated wounds was comparable to the control parameters and was (21±2)% (n=16) less than the area of untreated plasma wounds (Fig.1B).

With the combined action of hydrocortisone and bacterial infection, wound healing significantly worsened in both plasma-treated and untreated NAPAD wounds and was on the sixth day of the experiment (57±5)% (n=16) and (72±6)% (n=8), respectively. In animals of the control group on the sixth day were observed wounds area (31±7)% (n=16).

Comparative electron microscopic analysis of ultrathin structure of wounds

Morphophysiological properties of tissues of skin wounds of mice were investigated on ultrathin sections by transmission electron microscopy. On the third day of the experiment, a significant number of destroyed bacterial cells located in the form of fragments of bacterial biofilms between multilayered horny scales were detected in the wound tissues of mice with additional infection against the background of hydrocortisone application. At the same time, a significant area of unstructured epidermal tissue under the Horny scales revealed a violation of the tissue healing process in this area of wounds (Fig. 2A).

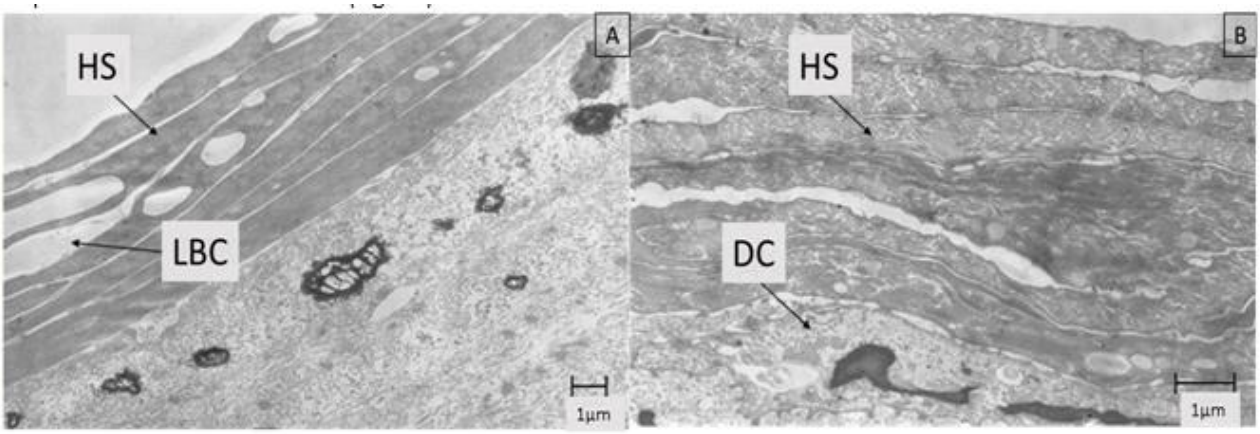

Figure 2: TEM. Ultra-thin slice of area infected (A) and uninfected (B) the wounds of mice in the region of the edges of the wound on the third day of the experiment. Designations: HS-horn scales, LBC-lysed bacterial cells, DC-degenerating cell. Marker length-1 µm

On the ultrathin section of the fragment of an uninfected wound from the control group, multilayered forming scales are visible. Between the scales are a small number of destroyed bacterial cells. Under the scales, structured cells-degrading cells of the granular layer of the epidermis (Fig. 2B).

In the study of ultrathin sections of wound preparations treated with NTAPP, on the third day of the experiment, no significant differences in the structure of cells of the growing epithelium in the tissues of animals of all the studied groups were revealed (Fig. 3). Epidermal cells had an irregular or rounded shape, the cytoplasm contained a large nucleus with heterochromatin. The cells also revealed a light zone of cytoplasm containing free ribosomes. The results of the study of the ultrathin structure of cells of the growing epithelial layer showed a high level of their biosynthetic activity.

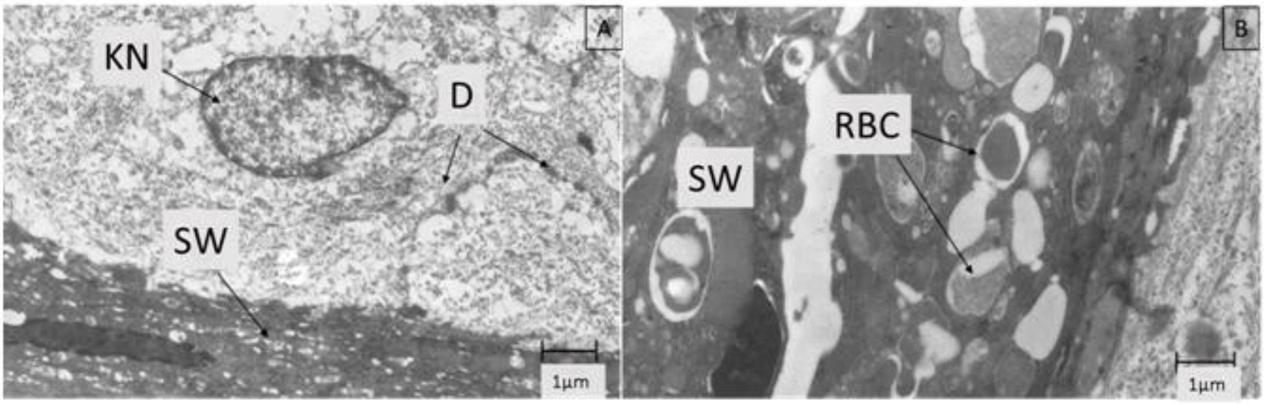

Figure 3: TEM. Ultrathin section of the mouse wound area of the experimental (A) and control (B) groups in the region of the wound edge on the third day of the experiment. The magnification of ×6000. Designations: KN – keratinocyte nucleus, SW – scab wound, D – desmosomes, RBC – remnants of bacterial cells. Marker length-1 µm

Abundant growth of physiologically active bacterial cells and their clusters in the form of colonies forming biofilms on the third and sixth days of the experiment was noted on the surface, in the thickness of the scab and under the scab in the control group (Fig. 3B). The obtained data showed the active formation of biofilms on the background of immunosuppression by hydrocortisone. On ultrathin sections of wound fragments from a group of mice after treatment with non-thermal plasma on the third day of the experiment, destroyed bacterial cells were detected in the form of bacterial cell wall residues located between the layers of Horny scales. In the thickness of the scab on the sixth day of the experiment, clusters of destructured bacterial cells were detected, while in the thickness of the Horny scales, lysed bacterial cells alternating with oval-shaped voids remaining after the destruction of bacteria and filled with cellular detritus were also detected. Development and distribution of bacterial biofilms between Horny scales after exposure to non-thermal plasma was not noted, which may indicate its bactericidal effects.

On the sixth day of experiments in the perinuclear zone of granular tissue cells in the experimental group of animals noted multiple large inclusions of irregular shape with high electron density, located in the form of a chain, in all probability, representing keratogialin granules (Fig. 4A). Also, the cells had a large number of desmosomes arranged in a row. Thus, the comparative analysis of ultrastructural organization of tissue samples of control and experimental animals testified to a sufficiently high level of metabolic activity and differentiation of cells under the influence of NTAPP. In the control group keratogialin granules were expressed to a much lesser extent (Fig. 4B).

NTAPP. In the control group keratogialin granules were expressed to a much lesser extent (Fig. 4B).

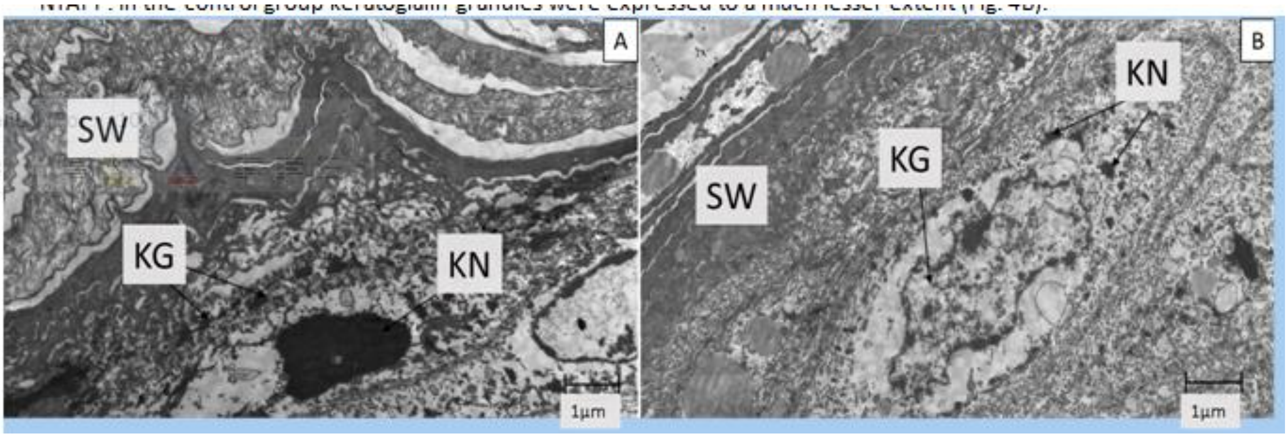

Figure 4: TEM. Ultrathin section of the mouse skin after exposure to non-thermal plasma (A) and mouse control group (B) on the sixth day of the experiment. Granular layer. Increase ×5000. Designations: KN – keratinocyte nucleus, KG – keratohyalin granules, HS – horny scales. Marker length-1 µm

CONCLUSION

The results of the study showed that the effect of non-thermal plasma on the wound surfaces can positively affect their healing. The obtained data indicate the acceleration of the healing process of complicated wounds under the influence of NTAPP in contrast to the control samples. Under the action of cold plasma of particular note are revealed electron-microscopic methods not only antibakterialnuyu activity in the biofilm, but also the attributes of an effective re-epithelialization of the tissues in the wounds.

Confirmation of the results can serve as a comparison with the data presented in the studies of Nasruddin N. et al. [24] and Ngo Thi M.-H. et al. [25] on the ability of cold plasma to accelerate re-epithelization of complete and burn wounds. Moreover, the influence of cold plasma on the acceleration of angiogenesis as an important physiological factor of wound healing is reported in the work of Haerte et al. [18]. The authors suggest the ability of plasma-produced reactive oxygen species to induce angiogenesis and epithelialization by increasing cell proliferation and migration. Therefore, the applied use of non-thermal plasma can be expressed not only as a strategic impact on bacterial biofilms in inflammation of wounds, but rather as a significant optimization factor in the care of chronic complicated wounds.

This assumption is based on observations obtained in the course of experiments, taking into account the comparative analysis of inflammation in wounds treated with cold plasma, and control samples. Analysis of the data obtained in all the studied groups leads to the conclusion that in the early stages of wound healing (the third day since the beginning of treatment of NTAPP) found approximately the same patterns of the dynamics of the wound healing process. Thus, for the first time, on the basis of electron microscopic studies found that the effect of non-thermal plasma has no significant effect on the early stage of the inflammatory process in the wounds. On the sixth day since the formation of wounds, the inflammation index in the treated cold plasma zone significantly decreased compared to the control samples. These observations confirm the assumption that non-thermal plasma causes a more active acceleration of wound healing in the late phase of inflammation. Similar results are given in the work of Nasruddin et al., where the hypothesis of the connection of this event with the proliferative effect of non-thermal plasma and, in accordance with this, the early appearance of myofibroblasts in wounds [24] is put forward.

The present study showed stimulation of the wound healing process by non-thermal plasma treatment, and it was found that the rate of wound closure significantly increased, compared with the control, by the sixth day from the beginning of the wound appearance. The authors of the article of Jacofsky M. C. put forward such assumptions, however, in this work there are no morphometric analysis data confirmed by electron microscopic studies [12].

SUMMARY

Electron microscopic studies have shown that the effect of NTAPP on the skin wounds of animals leads to the death of bacteria on the surface of the wound and in the thickness of the scab, and prevents the development of bacterial biofilms in the upper layers of the wound defect.

The lowest rate of regeneration of the wound defect in animals was observed on the third day of exposure to NPA with the combined use of immunosuppression caused by the introduction of hydrocortisone and infection of the wound.

At the ultrastructural level, the appearance of large inclusions of keratohyalin granules, a large number of desmosomes were observed in the tissues of animal wounds on the sixth day of treatment of NTAPP, while a number of morphological properties of cells testified to a high level of metabolic activity of cells and their differentiation.

Electron-microscopic and morphometric methods found that the effect of NTAPP on wounds promotes activation of epithelial cell growth and accelerates the regeneration of tissues of the skin of animals in the later stages of wound healing.

The work was supported by the grant of SPBU №0.37.218.2016

REFERENCES

[1] Fridman A. & Friedman G. Plasma Medicine. Plasma Medicine. 2012; 18. doi: 10.1002/9781118437704.

[2] Gweon B., Kim H., Kim K., Kim M., Shim E., Kim S., Choe W., Shin J.H. Suppression of angiogenesis by atmospheric pressure plasma in human aortic endothelial cells. Appl. Phys. Lett. 2014; 104: 133701. doi: 10.1063/1.4870623.

[3] Kang S.U., Cho J.-H., Chang J. W., Shin Y. S., Kim K. I., Park J. K., Yang S. S., Lee J.-S ., Moon E., Lee K., Kim C.-H. Nonthermal plasma induces head and neck cancer cell death: the potential involvement of mitogen-activated protein kinase-dependent mitochondrial reactive oxygen species. Cell Cell Death Dis. 2014; 5: e1056 doi: 10.1038/cddis.2014.33.

[4] Panngom K., Baik K.Y., Nam M.K., Han J.H., Rhim H., Choi E.H. Preferential killing of human lung cancer cell lines with mitochondrial dysfunction by nonthermal dielectric barrier discharge plasma. Cell Death Dis. 2013; 4: e642 doi: 10.1038/cddis.2013.168.

[5] Matthes R., Bender C., Schlüter R., Koban I., Bussiahn R., Reuter S., Lademann J., Weltmann K.D., Kramer A. Antimicrobial efficacy of two surface barrier discharges with air plasma against in vitro biofilms. PLoS One. 2013; 8: e70462 doi: 10.1371/journal.pone.0070462.

[6] Ehlbeck J., Schnabel U., Polak M., Winter J., Von Woedtke Th., Brandenburg R., Von T., Dem Hagen, Weltmann K.-D. Low temperature atmospheric pressure plasma sources for microbial decontamination. J. Phys. D. Appl. Phys. 2010; 44:13002 doi: 10.1088/0022-3727/44/1/013002.

[7] Ahlfeld B., Li Y., Boulaaba A., Binder A., Schotte U., Zimmermann J.L., Morfill G., Klein G. Inactivation of a foodborne norovirus outbreak strain with nonthermal atmospheric pressure plasma. MBio. 2015. 6: e02300–14. doi: 10.1128/mBio.02300-14.

[8] Chutsirimongkol C., Boonyawan D., Polnikorn N., Techawatthanawisan W., Kundilokchai T. Non-Thermal Plasma for Acne and Aesthetic Skin Improvement. Plasma Med. 2014; 4(1-4): 79-88.

[9] Tiede R., Hirschberg J., Daeschlein G., von Woedtke T., Vioel W., Emmert S. Plasma applications: a dermatological view. Contrib. to Plasma Phys. 2014; 54: 118–130 doi: 10.1002/ctpp.201310061.

[10] Isbary G., Heinlin J., Shimizu T., Zimmermann J.L., Morfill G., Schmidt H.U., Monetti R., Steffes B., Bunk W., Li Y., Klaempfl T., Karrer S., Landthaler M., Stolz W. Successful and safe use of 2 min cold atmospheric argon plasma in chronic wounds: Results of a randomized controlled trial. Br. J. Dermatol. 2012; 167:404–410. doi:10.1111/j.1365-2133.2012.10923.x.

[11] Ulrich C., Kluschke F., Patzelt A., Vandersee S., Czaika V.A., Richter H., Bob A., Hutten Jv., Painsi C., Hüge R., Kramer A., Assadian O., Lademann J., Lange-Asschenfeldt B. Clinical use of cold atmospheric pressure argon plasma in chronic leg ulcers: A pilot study. J. Wound Care. 2015; 24(5):196, 198-200, 202-203. doi: 10.12968/jowc.2015.24.5.196

[12] Ngo Thi M., Shao P., Liao J., Lin C.K., Yip H. Enhancement of Angiogenesis and Epithelialization Processes in Mice with Burn Wounds through ROS/RNS Signals Generated by Non-Thermal N2/Ar Micro-Plasma. Plasma Process. Polym. 2014; 11: 1076–1088 doi: 10.1002/ppap.201400072.

[13] Lee O.J., Ju H.W., Khang G., Sun P.P., Rivera J., Cho J.H., Park S.J., Eden J.G., Park C.H. An experimental burn wound-healing study of non-thermal atmospheric pressure microplasma jet arrays. J. Tissue Eng. Regen. Med. 2016; 10(4): 348–357. doi: 10.1002/term.2074.

[14] Jacofsky M.C., Lubahn C., McDonnell C., Seepersad Y., Fridman G., Fridman A.A., Dobrynin D. Spatially resolved optical emission spectroscopy of a helium plasma jet and its effects on wound healing rate in a diabetic murine model. Plasma Med. 2014; 4: 177–191. doi: 10.1615/PlasmaMed.2015012190.

[15] Fathollah S., Mirpour S., Mansouri P., Dehpour A.R., Ghoranneviss M., Rahimi N., et al. Investigation on the effects of the atmospheric pressure plasma on wound healing in diabetic rats. Sci. Rep. 2016; 6:19144. doi: 10.1038/srep19144.

[16] Brun P., Pathak S., Castagliuolo I., Palù G., Brun P., Zuin M., Cavazzana R., Martines E., Helium generated cold plasma finely regulates activation of human fibroblast-like primary cells. PLoS One. 2014; 9(8):e104397. doi: 10.1371/journal.pone.0104397.

[17] Kalghatgi S., Friedman G., Fridman A., Clyne A.M. Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release. Ann. Biomed. Eng. 2010; 38: 748–757. doi: 10.1007/s10439-009-9868-x.

[18] Haertel B., von Woedtke T., Weltmann K.-D., Lindequist U. Non-thermal atmospheric-pressure plasma possible application in wound healing. Biomol. Ther. (Seoul). 2014; 22: 477. doi: 10.4062/biomolther.2014.105.

[19] Hoentsch M., von Woedtke T., Weltmann K.-D., Nebe J.B. Time-dependent effects of low-temperature atmospheric-pressure argon plasma on epithelial cell attachment, viability and tight junction formation in vitro. J. Phys. D. Appl. Phys. 2011; 45:25206. doi: 10.1088/0022-3727/45/2/025206.

[20] Varyushina E.A., Antsiferova M.A., Aleksandrov G.V., Minaeva E.N., Pigareva N.In. Petrov A. V., Konusova V.G., Isaev E.N., Bokovanov V.E., Kotov A.Yu., Kazakov A. A., Dem'yanov A.V., Simbirtsev A.S. Model of the complicated course of the wound process in mice on the background of immunosuppression caused by the introduction of hydrocortisone. Cytokines and Inflammation. 2004; 3 (4):14-20. Russian.

[21] Patent of the Russian Federation RU 2638797, 19.12.2016. Russian.

[22] Stepanova O., Rybalchenko O., Pinchuk M., Astafiev A., Orlova O., Podobin V., Kudryavtsev A. Bactericidal effect of a dielectric barrier discharge plasma jet generated in laminar and preturbulent helium flows. Plasma Medicine. 2017; 7(3):187-200. doi: 10.1615/Plazmamed.2017019064.

[23] Rybalchenko O.V. The electron microscopic study of cell-to-cell interactions between antagonistic microorganisms. Microbiology. 2006; 75(4):550-555. doi:10.1134/S0026261706040187. Russian.

[24] Nasruddin N. Y., Mukai K., Rahayu H.S.E., Nur M., Ishijima T., Enomoto H., Uesugi Y., Sugama J., Nakatani T. Cold plasma on full-thickness cutaneous wound accelerates healing through promoting inflammation, re-epithelialization and wound contraction. Clin Plasma Med. 2014; 2:28–35. doi: 10.1016/j.cpme.2014.01.001.