THERAPY OF PRESSURE SORES VIA ACTIVATION OF REGENERATIVE PROCESSED IN TISSUES BY LOW-TEMPERATURE GLOW-TYPE PLASMA DISCHARGES OF GLOW TYPE

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Abstract. Pressure sores remain an important clinical problem with significant socioeconomic implications. The pressure sores treatment via reparative processes activation in tissues by low-temperature plasma discharges was studied in the present work. Low-temperature plasma discharges were initiated by high-frequency 0.11, 2.64, 6.78, and 13.56 MHz current. It is shown that the optimal current frequency for the generation of the cold plasma is 6.78 MHz. This current frequency was used in clinical studies of pressure sores treatment with cold plasma discharges of the glow type. The efficiency of treatment was evaluated by analysis of histological samples, histochemical and bacteriological methods. Low-temperature plasma discharge treatment improved the dynamic of pressure sore healing, activated reparative processes in injured tissues, and decreased bacteria numbers in a wound. The most pronounced effect was observed after 14-21 days. The low-temperature plasma discharges accelerated pressure sores healing from 14 to 16% compared with non-treated by cold plasma wound. The effect depended on the pressure sores etiology. Low-temperature plasma discharges of glow type may be considered as an effective approach to pressure sores therapy.

Keywords: activation of reparative processes, biological tissues, low-temperature plasma, electrolyte matrix, histology, morphological assessments, antibacterial activity.

List of Abbreviations

AE – active electrode
DAB – 3,3-diaminobenzidine
IHC – immunohistochemical
LTP – low-temperature plasma
NE – neutral electrode
ROS – reactive oxygen species
SMA – smooth muscle actin
VEGF – vascular endothelial growth factor

Introduction

Pressure sores and decubitus ulcers remain an important clinical problem with significant socioeconomic implications. Pressure sores are individual forms of chronic wounds with different proximal pathogenic causes. To date, a large percentage of stage 3 and 4 bedsores become chronic and lead to patient death due to ulcer complications such as sepsis or osteomyelitis. Despite the medical and social problem posed by pressure ulcers, treatment is still not effective enough due to the lack of clinical characteristics of regenerative processes at the cellular level (Coleman *et al*., 2013).

Pressure ulcers or decubitus ulcers are associated with many severe post-traumatic and systemic diseases, such as stroke, heart attack, diabetes mellitus, Parkinson's disease, paraplegia, and malnutrition (Cao *et al*., 2013; Coleman *et al*., 2013). Medical statistics show that starting from the second stage the pressure ulcer becomes the main source of surgical infection with a high risk of developing dystrophic, dysfunctional and septic conditions (Ayello & Sibbald, 2019; Boyko *et al*., 2018; Dibirov, 2016; Sumarno, 2019). Effective treatment of pressure ulcers is very difficult, since it is not always possible to completely eliminate their causes (Boyko *et al*., 2018; Damert *et al*., 2015; Supilnikov *et al*., 2016). Patients with pressure sores, as a rule, are weakened by the underlying disease, and the phases of the wound process are sharply extended in time and can last for a long time in the absence of any healing dynamics (Westby *et al*., 2017).

Severe pressure sores cause of sepsis with a significant lethal outcome. In these cases radical surgical methods are often used (Baskov, 2000; Dibirov, 2016). These methods are laborious and expensive, because they require additional dressings, antiseptics, ointments, wound dressings, and physicochemical technologies (Dibirov, 2016; Westby *et al*., 2017). However, the effectiveness of procedures does not always meet expectations, and their high cost and labor intensity are not always justified. At the same time, most drugs used in conservative treatment of pressure sores can cause an allergic reaction (Demoly *et al*., 2014; Gómez *et al*., 2012; Marzano *et al*., 2016; Simons *et al*., 2015; Stone *et al*., 2014; Uetrecht &Naisbitt, 2013; Żukiewicz-Sobczak *et al*., 2015), which significantly limits the possibility of using drugs. For this reason, non-drug methods of therapy based on physical factors, as an integral part of the complex treatment of pressure ulcers, are extremely relevant.

It was previously shown by the results of clinical studies, that the activation of reparative processes in injured by pressure ulcers tissues by discharges of low-temperature plasma (LTP) is a promising method of non-drug therapy in the complex treatment of bedsores. The activation of reparative processes by LTP discharges initiated by radio frequency current was first tested in the Federal State Budgetary Institution «Federal Scientific and Clinical Center of Intensive Care and Rehabilitation» in the treatment of pressure sores in clinical trials (Belov *et al*., 2022b; Belov *et al*., 2021; Belov *et al*., 2022c). The studies were carried out on the skin and muscle tissues of the bedsore zone (with the consent of patients) in chronic critical illness (CCI) of cerebral etiology. The results of preliminary testing of the reparative processes activation by dosed exposure to radiofrequency current confirm the relevance of further research of the method as a new method of non-drug treatment of pressure ulcers. Exposure to LTP induces a complex of adaptive and compensatory reactions in the tissues, which are responsible for the activation of reparative processes. Effect of LTP on tissue regeneration depends on properties of LTP (Ashurov *et al*., 2020; Frolov *et al*., 2010; Morozov *et al*., 2020; Zelickson *et al*., 2004).

The aim of present study is evaluation of the effectiveness of minimally invasive therapy for pressure ulcers by reparative processes activation in patients with CCI of various etiologies as part of the complex treatment of pressure ulcers using a specialized high frequency generator.

Materials and Methods

Patients

Studies of the effectiveness of therapy for pressure ulcers in patients with CCI of various etiologies were carried out in Federal State Budgetary Institution "Federal Scientific and Clinical Center of Intensive Care and Rehabilitation" (Belov et al., 2022b). The research protocol corresponds to the World Medical Association Declaration of Helsinki and was approved by the Local Ethic Committee. Thirty-eight patients (16 men and 22 women) aged 31 to 76 years were selected for the study. The demographic distribution was asymmetrical: the mean age was 47.5 years. The general condition of all patients in the sample was initially regarded as severe due to traumatic brain injury in the results of massive ischemic stroke, severe traumatic brain injury, or less commonly radical surgery to remove brain tumors. The main localization of the bedsore defect was the sacral and gluteal regions (32 observations in total); less commonly greater trochanter area (3 observations), scapula (2 observations) or heel (1 observation). Several bedsores of different localization were found in 7 cases at once. In this situation, we took into account the dynamics of the treatment of the largest of the defects. The key inclusion criterion was grade III bedsores: complete loss of the thickness of the integumentary tissues in the zone of constant compression, but not deeper than the own fascia. The area of the defect varied from 5 to 20 cm^2 (average 14.6 cm^2). Comparative study of the key parameters of wounds reparative processes was carried out in 29 patients aged 29 to 73 years (average 50.4 years) with pressure sores of the sacrum, with an area of 5 to 20 cm^2 treated according to the traditional method (sanation with antiseptics, dressings with polyethylene oxide-based ointment) without LTP treatment. They formed a control group comparable in demographic and clinical criteria to the main category.

Low-temperature plasma generation

At the first stage of the study an experimental high-frequency current generator to initiate LTP discharges was developed and tested. The choice of parameters of the high-frequency current generator was based on the study of the behavior of LTP in the electrolyte matrix of biological tissue at different frequencies. The technique of such an experiment is shown in Figure 1. The main functional elements of the LTP generator are a cell with a weakly concentrated solution of a strong electrolyte – 1, an oscilloscope – 2, a high-frequency current generator – 3. Active (AE) and neutral (NE) electrodes were placed in a cell with an electrolyte. The saline solution of 1% gelatin was used as a macroscopic model of the electrolyte matrix of the biological tissue. LTP discharges were initiated with an electric current frequency of 0.11, 2.64, 6.78, and 13.56 MHz. An electronic oscilloscope was used as a recording device (LeCroy, USA).

Based on the results of selecting the parameters of the high frequency current generator, a specialized device (hereinafter referred to as the device) was developed to generate LTP and reparative processes activation in clinical study. The block diagram is shown in Figure 2. Brief description of the device can be found below.

The feature of the high frequency current generator (1) is the ability to briefly transfer high power required to initiate LTP discharges on a needle electrode in the electrolyte component of the biological tissue. The voltage pulse shaping unit (2) provides the generation of short high-voltage pulses on a low-resistance load. The exposure parameters generation unit (3) generates an high frequency current with a power of 1.0–15.0 W at a load of 50 Ohms and a duty cycle of 1.0 to 10.0 at a modulating pulse frequency of 10 kHz. The dosing block (4) allows the operator to set the exposure intensity for 8 output power levels in the range from 1 to 10 W and the exposure within 0.5–5.0 sec. Activation elements (5) ensure the inclusion of high frequency current with the exposure parameters set by the operator using a floor pedal or a button on the electrode holder. The applicator (6) is a holder of replaceable needle electrodes, which were used as needles for mesotherapy and microinjections Mesoram RI.MOS 30G (0.3 x 13).

Clinical study

Evaluation of the effectiveness of the activation of reparative processes in the tissues of the LTR treatment by LTP discharges was carried out at the Federal State Budgetary Institution «Federal Scientific and Clinical Center of Intensive Care and Rehabilitation» using a specialized device described above. Local therapy was of an urgent complex nature, was carried out taking into account the phase of the complicated wound process and, in addition to standard preventive measures, was focused on accelerated cleansing (necrolysis) and stimulation of wound defect healing. The reparative processes activation procedure is carried out by chipping the tissue covers of the borders of bedsore (Fig. 3). The chipping technique was carried out according to the previously described method (Belov *et al*., 2021). The electrodes were inserted to the full length into pre-designated zones.

An electrically conductive plate with an area of 120.0 cm^2 was used as a neutral electrode, or the role of a neutral electrode was performed by external elements of the patient circuit at a frequency of 6.78 MHz due to their electrical capacitance to ground. The needle electrode was inserted into the tissue, and a high-frequency

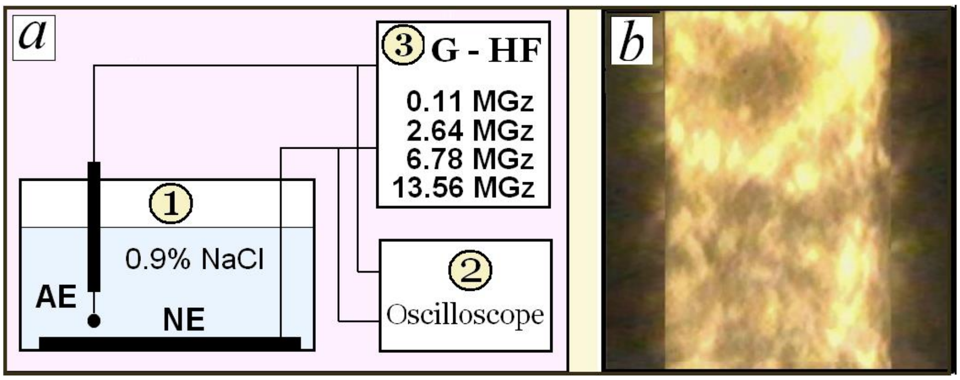


Fig. 1. Experimental sample of LTP generator for studying LTP generation. Scheme of generator (a) and photo of LTP (b). Cell with electrolyte solution – 1, an oscilloscope – 2, a high-frequency current generator – 3. AE and NE were placed in a cell with an electrolyte

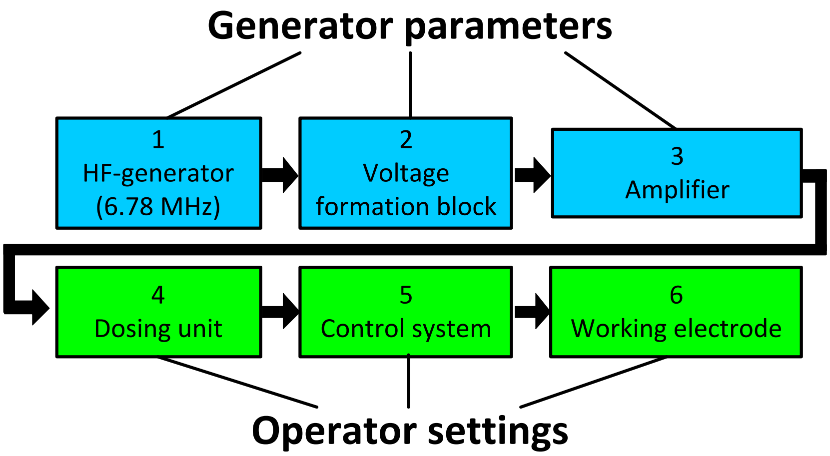


Fig. 2. Function block diagram of the device for activation of reparative processes

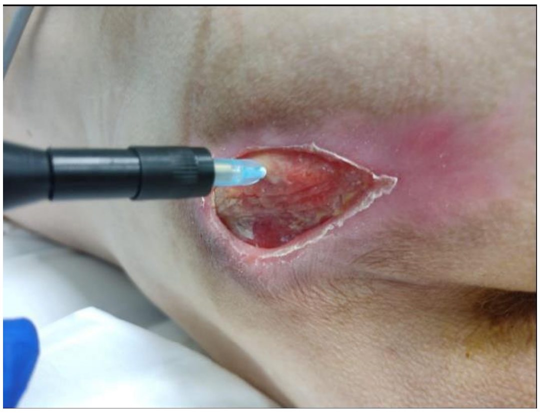


Fig. 3. The procedure for treating the bedsore area with a needle electrode

voltage was applied. High-frequency current passing through the tissue causes the evaporation of tissue fluid around the needle electrode, which leads to the appearance of a gaseous shell from water vapor. The applied voltage induces discharges of a low-temperature (average temperature does not exceed 100 °C) highly nonequilibrium plasma in the gas shell. The parameters of the resulting plasma are close to those of the plasma used in plasma electrosurgery (Belov *et al*., 2011). The exposure parameters were set by the level of the installed output power, the operating cycle and the duration of the exposure. The range of parameter values made it possible to choose the current and voltage values that ensure the optimal process of generating plasma discharges according to the criterion of the minimum destructive effect of a thermal nature. In these clinical studies, LTR treatment was carried out with an exposure of 1.0–3.0 s and a set power of 5.0 ± 1.5 W. LTR treatment performed in first day of bedsore healing (initial reparative processes activation) and every 3-4 days in addition to standard treatment (sanitation with antiseptics, dressings with polyethylene oxide ointment, etc.). Histological study of scrapings from the wound surface, bacteriological control of the microbial landscape and wound planimetry were performed the next day after reparative processes activation by LTP discharges. The usual assessment of pressure ulcer area was carried out by visual observation with photographic control. The dynamics of the course of reparative processes was additionally evaluated using the 13 criteria Bates-Jensen scale (1992) (Bates-Jensen *et al*., 1992). All patients (38 people in the experimental group and 29 people in the control group without LTP treatment) took part in this part of the study.

Histological assay and immunohistochemistry

Tissue biopsy specimens from the edges and bottom of bedsores (1 sample from each zone) were fixed in 10% formalin solution and then embedded in paraffin. Sections 3–4 μm thick were stained with hematoxylin and eosin before viewing. The sample size was 6 (15.7%) observations in the experimental group and 4 (13.7%) in the control group. Histological examination in each observation was performed three times - before the start of treatment, on the 14th and 28th days from the start of the procedure for activating reparative processes in the pressure sores. For immunohistochemical (IHC) studies, serial paraffin sections 4 μm thick were made on glasses coated with a polylysine layer (Menzel Glaser Polylisine, Germany). Unstained sections were processed using a standard IHC method with thermal antigens unmasking. Antigens unmasking was performed in a water bath with a PT Module microprocessor (Thermo Scientific, USA). Polylysine slides with tissues sections were deparaffinized according to the standard procedure. All further stages of the ICH study were carried out in a humid SlideMaster chamber (Bio Optica, Italy) to prevent drying of the sections. Slides were incubated during 10 minutes with a peroxidase inhibitor to block endogenous peroxidase, after which the sections were rinsed in phosphate buffer (pH 7.0–7.6) (Cell Marque, USA) and incubated with Ultra-V-Block buffer (LabVision, USA) within 30 minutes to block nonspecific antibodies binding. At the end of the incubation, excess reagent was removed from the slides and primary antibodies were added. Mouse monoclonal antibodies to collagen type I (clone 3G3, Santa Cruz, dilution 1:100), collagen type III (clone B-4, Santa Cruz, dilution 1:50), collagen type IV (clone COL-94, Santa Cruz, dilution 1:50), MMP-1 (clone 3B6, Santa Cruz, dilution 1:100), SMA (clone 1A4, Dako Agilent, dilution 1:100) and TGFβ (clone 3C11, Santa Cruz, dilution 1:100) were used. Sections were incubated with primary antibodies for 30 minutes according to the manufacturer's specification for the antibody. After incubation sections were rinsed by phosphate buffer (pH 7.0–7.6) to remove primary antibodies that did not bind to epitopes. Histofine® Simple Stain MAX PO (MULTI) universal polymer system (Nichirei, Japan) was used to detect primary antibodies bound to the corresponding antigens. Polymer system containing a dextran framework with multiply attached molecules of the horseradish peroxidase enzyme and secondary antibodies to anti-mouse and anti-rabbit immunoglobulins. The time of incubation of sections with polymer detection system in a humid chamber was 30 minutes. After incubation the sections were rinsed in phosphate buffer (pH 7.0–7.6). Visualization of antigen-antibody complexes was carried out by oxidation of 3,3-diaminobenzidine (DAB) by hy-drogen peroxide in the presence of horseradish peroxidase (N-Histofine® DAB-2V, Nichirei, Japan) with the formation product, which was visible as brown staining of specific cell structures. Sections were incubated with DAB for 5–10 minutes depending on the required staining intensity. The slides were washed in distilled water and the nuclei were stained with Mayer's hematoxylin for 2-3 minutes. After that, the slides were dehydrated in a battery consisting of distilled water, alcohols of increasing concentration (70%, 80%, 95%, absolute alcohol) and xylene. After that, the sections were covered with coverslips using Bio-Mount synthetic medium (Bio Optica, Italy).

Positive and negative controls were used. Samples without incubation with primary antibodies was used as negative controls. A positive control for each antibody was selected according to the antibody manufacturer's recommendations. Semi-quantitative assessment of the intensity of expression of markers was carried out in points for collagens and % staining of wound stromal cells for VEGF and SMA.

Bacteriological assay

The assessment of the microbial species and the degree of dissemination of pressure ulcers was carried out by analyzing the bacteriological culture. Patients of the experimental and control groups underwent bacteriological culture. The study of seeding was performed at the initial examination, after 2 weeks and on the 28th day after the end of therapy by LTP discharges. Wound secretions were collected before wound treatment by spreading on a swab. The material immersed in the Ames medium with coal was delivered to the bacteriological laboratory within 1 hour. The material was inoculated on the following nutrient media: Uriselect, blood agar, ENDO medium, agar, Saburo, MSA. For inoculation on the medium, the swab-loop method was used. The result was evaluated after 24 hours. When the microorganism grew, a pure culture was isolated from the dish, transferring to another dish with nutrient medium. At this stage, the CFU was calculated. Initially, various colonies of pathogenic microorganisms, as well as their typical associations, were seeded from the wound exudate and the surface of the pressure ulcers. The sensitivity of isolated bacterial strains to antibiotics was also determined.

Statistic

Data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). The analysis of the dynamics of indicators was carried out on the basis of nonparametric Friedman test. The statistical significance of different values for binary and nominal indicators was determined using the χ^2-Pearson's chisquared test in the case of independent samples. Student's t-test was used to compare quantitative samples. Fisher's test was used to compare nonparametric data. The result was considered statistically significant according to the $p < 0.05$ criterion.

Results

Fitting of parameters to low-temperature plasma generation

Oscillograms of current and voltage in the electrical circuit “AE – Electrolyte – NE” at different current frequencies are shown in Figure 4. Analysis of the oscillograms shows that with an increase of frequency current becomes more symmetrical. At the same time increasing of current frequency decreased constant component of current, which responses for electrolysis and destructive processes in tissues.

Constant component of current disappear at current frequency of 6.78 MHz and more. From frequencies of 13.56 MHz, the shape of the current and the nature of the LTP discharges practically do not change, however dosing of generator by energy became significantly more difficult. The error of no more than 9% was showed to dosing of the energy in the range of real loads at a frequency of 6.78 MHz during subsequent testing of the LTP generator. These results indicated a fairly high repeatability of LTP generation condition. Summarizing the above a frequency of 6.78 MHz was chosen to LTP generation in next experiments.

From the point of view of a possible mechanism of activation of reparative processes, an increase in the frequency of the ion current

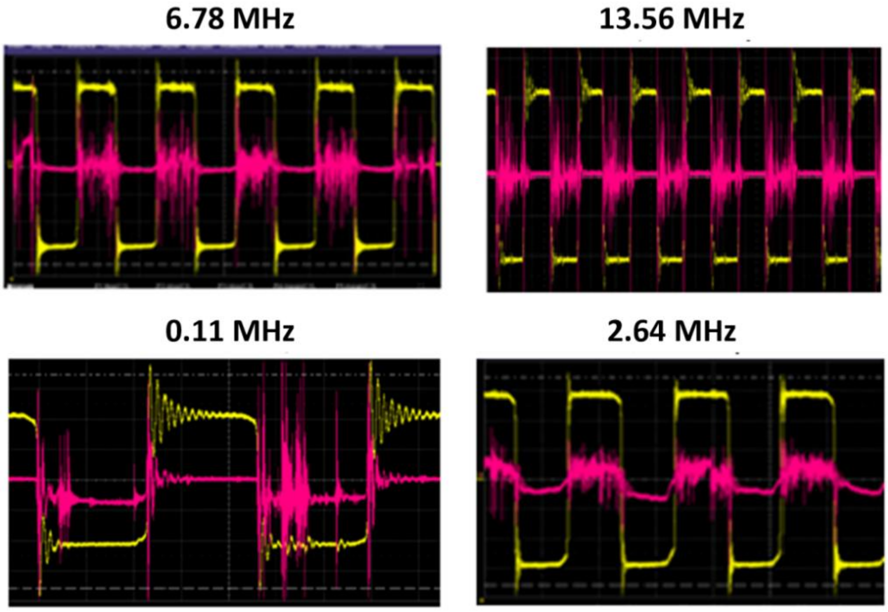


Fig. 4. Characteristics of electric current (red line) and voltage (yellow line) at different current frequencies in electrical circuit «AE – Electrolyte – NO»

above 10 MHz ceases to influence the formation of a transfer wave of calcium ions involved in ion transport inside the cell [25–29] and begins to block the transport of calcium ions [29–31]. For this reason, it is also advisable to use a frequency of 6.78 MHz.

Activation of ion transport into the cells is considered as mechanisms of activation of tissues regeneration. Calcium plays a key role in regulation of ion transport in a cell. An increase of ion current frequency over 10 MHz ceases its influence on generation of a transfer wave of calcium ions and consequently ion transport in cells (Chattopadhyay, 2000; Chubinskiy-Nadezhdin *et al.*, 2017; Weiger *et al.*, 2002; Zavodnik, 2016). Furthermore ion current frequency over 10 MHz inhibits calcium transport in a cell (Chattopadhyay, 2000; Lin, 2012; Pall, 2013) Therefore it is also advisable to use a frequency of 6.78 MHz.

Clinical study

At the start of treatment, all pressure ulcers had the appearance of open long-term non-healing ulcerative-necrotic defects with a moderately severe inflammatory reaction and obvious stagnation of reparative processes, including fibrin plaque, necrosis foci, rare loci of flaccid granulations, edematous rigid edges, exudation, etc.

The dynamics of the bedsore condition in the experimental and control clinical groups is shown in Table 1. Examples of photographs were shown in Figures 5 and 6.

The obtained results indicate that reparative processes activation by LTP discharges promotes accelerated necrolysis, growth and maturation of granulation tissue in the decubitus ulcers. Cohort analysis of reparative processes was performed (Table 1). According to the key macroscopic parameters, it can be seen that the experimental group was ahead of the control group by about 6–7 days ($p < 0.05$) in rate of wounds healing. The most pronounced intergroup differences in the duration of necrolysis (more than 8 days). In addition, activation of reparative processes by LTP discharges favored a decrease in wound exudation and relief of perifocal inflammation at an earlier time.

Histological assay

The results of a histological study of tissue samples from the edges of bedsores from experimental and control groups are shown in Figures 7 and 8 respectively. Initially, in the prep-

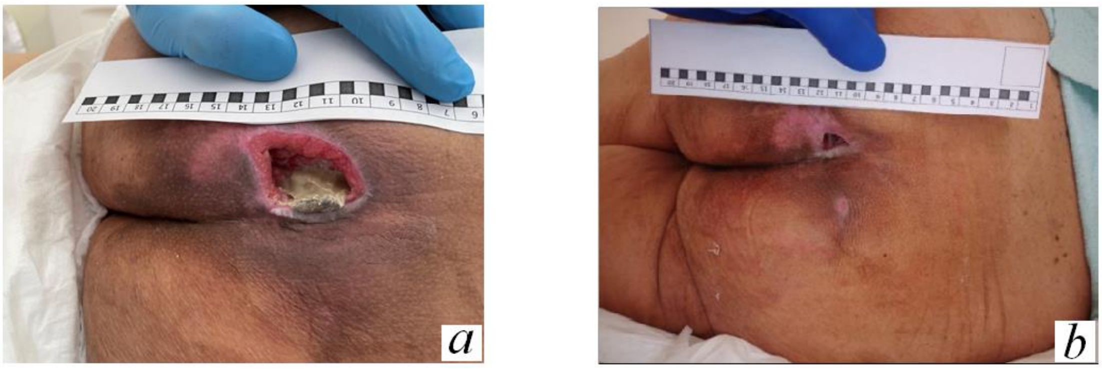


Fig. 5. The state of the bedsore in the experimental group: (a) before treatment, (b) on the 30th day

Fig. 6. The state of the bedsore in the control group: (a) before treatment, (b) on the 30th day

Table 1

Characteristics of wound healing process in studied groups

Characteristic	**Experimental group (n = 38)**	**Control group (n = 29)**	***p*-value**
Complete cleansing times (M ± m), days	11.2 ± 0.5	19.8 ± 0.01	<0.05
Time of appearance of the first granulations in the wound (M ± m), days	12.4 ± 0.2	19.0 ± 0.4	<0.05
Filling of pressure ulcers with granulation tissue 100% (M ± m), days	32.4 ± 1.0	39.4 ± 0.1	<0.05
Beginning of epithelization (M ± m), days	27.4 ± 0.8	34.0 ± 0.6	<0.05
Cupping of paravulnar inflammation (M ± m), days	28.9 ± 0.3	34.7 ± 0.1	<0.05
Epithelialization rate, test of L.N. Popova (1942)	2.8 ± 0.2%	2.0 ± 0.5%	<0.05
Bates-Jensen scores (19792) on days 14/21/28, M	31/26/24	32/30/27	-
Duration of treatment, days, Me [C_{25};C_{75}]	36 [30; 53]	44 [37; 63]	<0.05

Note: p-value between experimental group and control groups, Student's t-test for lines 1–7; Fisher's test for line 8.

arations of the edge of the bedsore in patients of the experimental (Fig. 7) and control (Fig. 8) groups was observed a zone of fibrinoid-necrotic changes with an underlying layer of slightly pronounced granulation tissue, edema of the interstitial tissue, scattered infiltration of polymorphic nuclear leukocytes with impurity of eosinophils. Newly formed vessels of the capillary type were not observed.

On the 14th day noticeable results of reparation processes activation were observed in in the decubital ulcer zone in experimental group. Active neoangiogenesis in the form of fragments of granulation tissue was observed in the zone of fibrinoid-necrotic changes (Fig. 7, white arrows). Elements of polymorphonuclear leukocytes of the interstitial tissue was observed. In the patient of the control group, the histological picture of scrapings remained practically unchanged. Proliferation of fibroblasts was expressed extremely poorly. Massive fibrinoid-necrotic changes persisted.

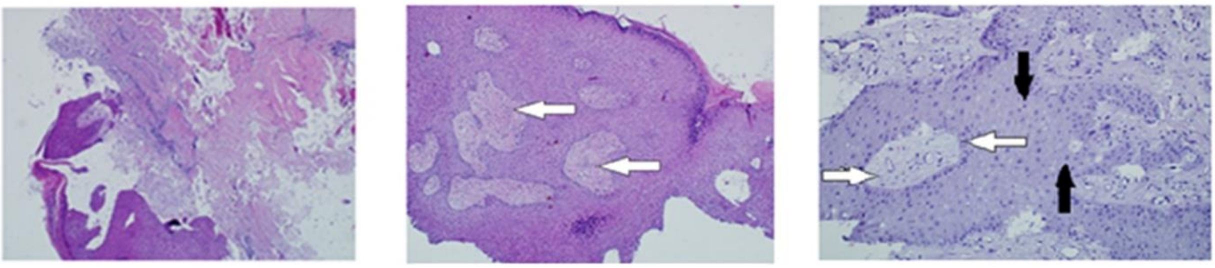
On the 28th day of treatment in experimental group the histological picture of edges and bottom of the decubital ulcer indicates that areas of maturing granulation tissue and mature connective tissue predominate in the study area. Pronounced regenerative changes in the integumentary stratified squamous epithelium with acanthosis and increased capillary neoangiogenesis are observed (Fig. 7, white arrows). A maturing granulation tissue is observed, replacing the foci of fibrinoid-necrotic changes with mature connective tissue (Fig. 7, black arrows). In the control group the morphological picture slowly and gradually passed into the structuring phase. Granulations were visible, but more sluggish and meager than in the experimental group. Weak marginal epithelialization was noted, mainly of a small-focal nature, zones of necrosis remained.

Immunohistochemistry assay

The dynamics of the markers of reparative processes was studied in 3 patients of the main group (7.8% of the number of patients) of experimental group. Collagen types 1 and 3, vascular endothelial growth factor (VEGF), smooth muscle actin (SMA) is a marker expressed by vascular smooth muscle elements and myofibroblasts were chosen. The rise in the level of the above indicators is considered as a reliable criterion for the activation of reparative processes. An analysis of wound reparation markers showed that before treatment, type 1 and 3 collagens from tissues of bottom and edges of the pressure ulcer were found in the extracellular matrix only in the form of minor deposits of amorphous structures without the formation of fibers. Minimal expression of VEGF in the endothelium of a few vessels (15% of wound stromal cells), as well as SMA (15% of wound stromal cells) (Table 2) was detected.

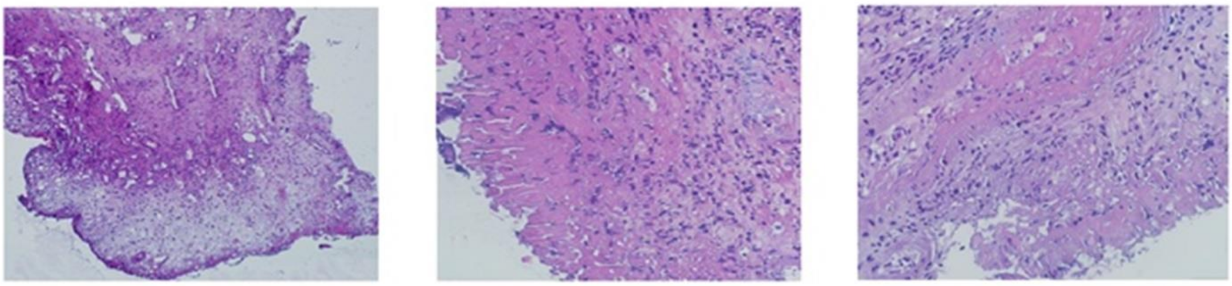
On the 14th day of complex treatment using LTP technology, a moderate increase in the amount of type 1 and 3 collagens (up to 4 points each) was detected in the bottom and edges of the pressure ulcer. These proteins formed clear fibrous structures in the extracellular matrix. At the same time, a higher content of collagen cells was noted in the edges of the pressure ulcer compared to the bottom (Table 2). In the bottom and edges of the pressure ulcer, an increase in the expression of the VEGF marker was noted compared to the initial stage to a moderate level, especially in the endothelium of capillary-type vessels, the number of which also increased (30% of wound stromal cells). The amount of the SMA also increased compared to the pre-treatment stage (60% wound stromal cells). The highest expression of the marker was noted at this intermediate stage.

On the 30th day of complex treatment, a moderate increase in the amount of collagens (up to 6 points each) is detected in the bottom and edges of the pressure ulcer, which also form clear fibrous structures in the extracellular matrix. A markedly higher content of collagens in the edges of the bedsore compared to the bottom. The level of collagen content is comparable to the 14th day of treatment. The expression of the VEGF marker in the endothelium of the vessels of the bottom and edges of the pressure ulcer is higher compared to the 14th day of the start of treatment (70% of wound stromal cells). The number of capillaries also increased. The dynamics of SMA marker expression by day 30 is also positive compared to the starting point,



day 1 magnification 1:300 | day 14 magnification 1:400 | day 28 magnification 1:400

Fig. 7. Histology of the edge of the decubital ulcer of the patient of experimental group



day 1 magnification 1:300 | day 14 magnification 1:400 | day 28 magnification 1:400

Fig. 8. Histology of the edge of the decubital ulcer of the patient of control group

Table 2

Average values of expression of reparative processes marker in pressure ulcer tissue before and after treatment

Group	Collagen type 1 (points)	Collagen type 3 (points)	VEGF (% positive stromal cells)	SMA (% positive stromal cells)
Experimental group before treatment	0	0	15.0 ± 0.5	10.0 ± 1.0
Experimental group after 14 days treatment	4.0 ± 0.3*	4 ± 0.3*	30.0 ± 1,5*	60.0 ± 2.0*
Experimental group after 30 days treatment	6.0 ± 0.5*	6 ± 0.5*	70.0 ± 2,0*	30.0 ± 1.2*
Control group	0	0	12.0 ± 0.5	15.0 ± 1.0*

Note: * — $p < 0.05$ vs experimental group before treatment, Fisher's test for collagens type 1 and 3, Student's t-test for VEGF and SMA.

but somewhat worse when compared to the intermediate time point (day 14 of therapy) (30% of wound stromal cells).

Bacteriological assay

We also evaluated the microbial species composition of the wound infection in decubitus ulcers. The data are presented in Table 3. Klebsiella pneumoniae, Proteus mirabillis, Pseudomonas aeruginosa resistant to most antibacterial drugs were observed in samples from pressure ulcer of some patients before treatment. Antimicrobial therapy was carried out purposefully according to the results of the analysis of crops for sensitivity from the main focus of infection.

Table 3

Species of pathogenic microorganisms detected in isolates from decubitus ulcers of experimental group patients

Species of microorganism	Frequency of occurrence, %		
	initial	14 days	28 days
Acinetobacter baumannii	5.1%	4.8%	5.0%
Acinetobacter baumannii/calcoaceticus комплекс	2.5%	2.0%	2.0%
Citrobacter farmeri	2.5%	3.5%	3.1%
Citrobacter freundii	2.5%	2.4%	2.8%
Corynebacterium amycolatum/striatum	2.6%	2.1%	2.9%
Enterococcus faecalis	6.8%	7.1%	5.6%
Enterococcus faecium	3.4%	4.3%	4.2%
Escherichia coli	5.1%	1.9%	6.8%
Klebsiella ozaenae	2.6%	3.0%	2.0%
Klebsiella pneumoniae	15.3%	14.8%	16.0%
Proteus mirabillis	18.8%	17.6%	18.0%
Providencia stuartii	3.4%	2.9%	3.1%
Pseudomonas aeruginosa	17.0%	16%	17.0%
Serratia marcescens	1.7%	2.0%	2.1%
Staphylococcus aureus	4.2%	7.4%	5.1%
Staphylococcus epidermidis	3.0%	3.5%	1.9%
Staphylococcus haemolyticus	2.6%	2.5%	2.0%
Candida ps.	0.9%	2.5%	0.4%

The species composition of bacteria in the pressure ulcer did not change during therapy with LTP discharges (Table 3). No significant relationship was found between the qualitative composition of the bacterial flora of pressure ulcer and LTP treatment at different times. This is explained by the fact that all patients included in the study were initially in CCI, which required expensive and long-term maintenance of vital functions (ventilation, bladder catheterization, gastrostomy, etc.). The severity of the patients' condition is due to the severe course of both the underlying disease and chronic infection (pneumonia, uroinfection, chronic colitis). The presence of these factors leads to the inevitable and regular contamination of bedsores with drug resistant nosocomial microorganisms. Thus, the qualitative indicators of the microbial species composition of the bedsore wound in patients in the CCI did not change significantly when using the technology of LTP discharges. At the same time, the indicator of bacterial contamination of decubitus ulcers in both studied groups was approximately the same and varied within 10^5–10^7 microbial bodies per 1 g of tissue, on average 6.2×10^6 ($p > 0.05$).

Discussion

LTP was applied in various fields such as agriculture, food industry, bioengineering, exhaust gas cleaning, soil and wastewater treatment (Danilejko *et al*., 2021; Konchekov *et al*., 2022; Zheng *et al*., 2019). LTP has shown itself well in the inactivation of microorganisms in both biofilm and planktonic forms (Belov *et al*., 2022a; Ehlbeck et al., 2011; Mukhachev *et al*., 2013; Sun, 1999). There are data on the using of LTP for the stopping of bleeding, healing of wounds and burns (Koshelev *et al*., 2006), as well as for tasks of regenerative medicine to increase the biocompatibility of the material and improve the efficiency of drug delivery. Depending on the conditions and time of exposure LTP is able to stimulate the differentiation of different types of cells (preosteoblasts, fibroblasts, immune cells, stem cells, etc.) (Belosludtsev *et al*., 2019; Xu *et al*., 2016). In present

Table 4

The level of microbial contamination of pressure ulcer

Species	**CFU/ml (microbial bodies per 1 g of wound exudate tissue)**					
	day 1		**day 14**		**day 28**	
	LTP treated	**Control**	**LTP treated**	**Control**	**LTP treated**	**Control**
Klebsiella pneumoniae	Me 12×10^5 [9×10^5; 9.8×10^7]	Me 2×10^6 [11×10^5; 9.3×10^7]	Me 7×10^5 [1.6×10^5; 11×10^5]	Me 4×10^6 [$3.8\cdot10^5$; $16\cdot10^6$]	Me 7.5×10^4 [6.25×10^4; 8×10^4]	Me 5.3×10^5 [2.6×10^4; 5.8×10^6]
Proteus mirabillis	Me 17×10^6 [5×10^6; 56×10^6]	Me 10×10^6 [5×10^6; 44×10^6]	Me $3.6\cdot10^6$ [$1.05\cdot10^6$; $9\cdot10^6$]	Me $8.8\cdot10^6$ [$5\cdot10^6$; $24\cdot10^6$]	Me 5.5×10^5 [1.9×10^5; 6×10^5]	Me 18×10^5 [12×10^5; 22×10^6]
Pseudomona aeruginosa	Me 6.5×10^5 [2.3×10^5; 13.8×10^5]	Me 7.1×10^5 [2.0×10^5; 14.2×10^5]	Me $3.9\cdot10^5$ [2.5×10^5; 7.5×10^5]	Me $7.0\cdot10^5$ [$3.7\cdot10^5$; 12.5×10^5]	Me 6.7×10^4 [5.3×10^4; 33×10^4]	Me 6.8×10^5 [2.8×10^5; 10.8×10^5]

study the effectiveness of LTP discharges in the complex treatment of pressure ulcers in patients with CCI of various etiologies was evaluated. It was found that the process of regeneration in the skin and muscle tissues of the bedsore zone proceeds most efficiently when using a current with a frequency of 6.78 MHz compared with terapy without LTP treatment. The optimal exposure parameters for needle electrodes with a diameter of 0.30 ± 0.05 mm and a length of 3.0–6.0 mm were power 5.0 ± 1.5 W, exposure time 2.0-3.0 s. In patients who received LTP therapy was found an accelerated normalization of the microvasculature, a more pronounced growth and maturation of granulation tissue with the formation of type 1 and 3 collagen fibers both in the bottom area and in the edges of the bedsore defect (Tables 1 and 2).

A pronounced activation was observed in the period from 14 to 21 days. The effectiveness of LTP therapy in terms of the rate of wound healing ranged from 14 to 16%, depending on the etiology of the decubitus wound. It has been established that this treatment also stimulates neongiogenesis with VEGF expression by the vascular endothelium, which is most pronounced after 30 days, as well as the accumulation of myofibroblastic elements (SMA) in tissues, which appear in the greatest amount 2 weeks after the start of combined local therapy. In general, the described changes correspond to the patterns of the regeneration process in the experimental group obtained by histological examination. It was also found that local therapy using the LTP technology is accompanied by a significant decrease of the bacterial contamination of pressure sores (Table 4).

Comparing our results with the literature data, we propose that the effect of LTP on biological structures is based on the synergy of strong electric fields and electromagnetic radiation (Vasilieva, 2015). The action of these factors can lead to the generation of reactive oxygen species (ROS) such as hydroxyl radical, superoxide, perhydroxyl and oxide anions, as well as hydrogen peroxide and ozone, or reactive nitrogen species (nitrogen oxide radicals, nitrate and nitrite anions, peroxynitrite, nitric, nitrous and peroxynitrous acids), which directly affect the properties of tissues and cells of living organisms (Ashurov *et al*., 2020; Vasilieva, 2015). This may also be associated with ROS-induced initiation of intracellular signaling cascades, including those involving subcellular organelles (primarily mitochondria), which play a crucial role in the processes of cell differentiation and proliferation, as well as trigger the mechanisms of cell death through apoptosis and necrosis (Weiger *et al*., 2002). In addition, there is reason to believe that LTP discharges are a source of short nanosecond pulses of electric field strength, which are capable of causing electroporation of the cytoplasmic membrane of cells in the affected area (Rems &Miklavčič,

2016; Wittkampf *et al*., 2018). This is evidenced by data according to which the local voltage gradient in the LTP discharge region can reach values of 10^5–10^6 V/m (Chang &Reese, 1990; Zheng *et al*., 2019). The probability of pore formation in cell membrane is an additional factor contributing to the activation of ion transport in tissue cells in the affected area. The combination of these phenomena may underlie the induced activation of reparative processes in the tissues of the decubitus wound. It is also important to note that no cardiac arrhythmias were detected during the cold plasma activation of RP in persons with cardiac pathology during ECG monitoring. It expands the scope of LTP treatment in cardiovascular diseases therapy. In addition, the use of LTP did not require additional anesthesia, and the use of disposable needle electrodes (injection needles) removed the issues associated with sterilization of the working part of the equipment.

Conclusion

The obtained results of clinical study allow us to conclude that the complex treatment of pressure ulcers using the method of activating reparative processes in tissues with cold plasma discharges initiated by high-frequency current is promising. LTP treatment induced a significant acceleration of regenerative processes in the pressure ulcers, reducing the period of inpatient treatment. The effectiveness of LTP therapy in terms of the rate of wound healing ranged from 14 to 16% depending on the etiology of the decubitus wound. The increase of regeneration markers expression and a decrease in bacterial contamination of wounds were also shown after LTP treatment.

Contribution

Concept and research preparation – S.A.M., Y.A.A.; research planning – S.A.M.; research conducting – B.S.V., D.Y.K., B.S.V., K.E.A., A.E.L., S.A.V., B.I.V., S.U.Z., L.V.I. and E.A.B.; writing and drafting of article – D.Y.K., B.S.V., O.E.G., S.D.A., A.M.E.; project administration – A.M.E. and Y.A.A. All authors have read and agreed with the published version of the manuscript.

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