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Dynamics Of IL-10 Level In Mice Skin Wounds Under The Bactericidal Influence Of Non-Thermal Plasma.

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ABSTRACT

The serious problem of current day is the processes involved in wound healing and chronic wounds treatment. The best investing strategyin treatment of chronic wounds is the bactericidal effect of non-thermal atmospheric pressure plasma. The paper presents experimental results of full-skin wounds healing in mice on the background of immune suppression when treating the wound surface with non-thermal plasma. A comprehensive analysis of changes in the wound surface area, bacterial contamination and IL-10 level under the influence of non-thermal plasma revealed signs of accelerated re-epithelialization and stimulation of epithelial cell proliferation, indicating activation of the healing process.

Keywords: cytokines, IL-10, skin wounds micro biota, wound healing, non-thermal atmospheric pressure plasma.

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INTRODUCTION

The process of tissue regeneration begins immediately after their defeat. Restoration of tissue integrity is a simple linear process in which growth factors induce cell proliferation, which leads to the integration of dynamic changes in which soluble mediators, blood cells, production of extracellular matrix and parenchymal cell proliferation are involved [1].

The processes occurring during wound healing can be divided into the following partially overlapping stages: inflammatory reaction; cell proliferation and synthesis of elements constituting the extracellular matrix; and the last period, called recovery (remodeling) [2].

Stage of inflammation Cell reaction at the stage of inflammation is characterized by an influx of leukocytes into the wound area. This is a very fast process that is accompanied by key signs of inflammation, such as swelling and erythema at the site of the lesion. The main purpose of this phase is to clean the wound from pathogenic microorganisms and foreign material, localize the damage and restrain the further spread of the inflammatory process. In the damage zone (in the area of inflammation), neutrophils appear, attracted by chemoattractant and the waste products of bacteria. They phagocytize decay products of tissue, bacteria and damaged matrix. Mast cells that release histamine are activated. This causes the expansion of capillaries and an increase in the permeability of their walls, as a result - local edema and redness. Vascular dilation occurs in the wound tissues, which contributes to the accumulation of neutrophils and monocytes.

The complex interaction of cytokines also helps regulate this phase, culminating in the transition of monocytes into macrophages, which are considered to be the main regulator of the inflammation phase [3]. Macrophages play an important role in wound healing, contributing to the release of lysosomal enzymes and reactive oxygen species, as well as facilitate the purification of cellular debris (destroyed cells). Macrophages secrete various growth factors that activate keratinocytes, fibroblasts and endothelial cells. It is believed that this stage begins within the first 24 hours and can last up to two days.

Stage of proliferation the purpose of the proliferative stage is to reduce the area of the affected tissue and fibroplasia (formation of connective tissue), to establish a viable epithelial barrier for the activation of keratinocytes. This stage is responsible for closing the affected tissue, which includes angiogenesis, fibroplasia and re-epithelialization. Endothelial cells enter a phase of rapid growth, and angiogenesis occurs inside the granulation tissue, creating a rich vascular network. These processes begin in the microenvironment of the lesion during the first 48 hours and can continue until the 14th day after the start of the lesion.

Stage recovery this phase begins in two to three weeks from the moment of injury and may last for one year or more. At this final stage, an attempt is made to restore the structure of the damaged tissue, and granulation tissue is gradually reconstructed, forming scar tissue. As soon as the lesion surface is covered with a monolayer of keratinocytes, its epidermal migration ceases, and the new stratified epidermis with the underlying basal lamina is restored from the wound boundaries to its inner part. When the wound is closed, type III collagen undergoes degradation and the synthesis of type I collagen by fibroblasts increases. Throughout the remodeling, there is a decrease in the amount of hyaluronic and fibronectic acids [1]. The number of fibroblasts and macrophages decreases, their metabolic activity decreases, capillary growth stops.

To understand the mechanism of tissue repair in the final stage, we should mention some factors of the immune system, such as B and T-lymphocytes. Morphologically, T-lymphocytes are divided into functional populations: CD4 (auxiliary T-lymphocytes) and CD8 (suppressor / cytotoxic T-lymphocytes). T-CD4 cells are characterized based on their cytokine production profiles, such as the Th1 subpopulation producing IL-2 and IFN-gamma; Th2, which produces IL-4, IL-5 and IL-10; Th17, which is characterized by the formation of IL-17 [4].

An important factor in wound healing is the presence of oxygen in the tissues surrounding the damaged area, which interacts with numerous cytokines, contributes to the process of cell proliferation and acts as an effect or for activating the neutrophilic respiratory burst. It has been established that for the normal wound healing process it is necessary that the oxygen partial pressure (voltage) in the tissues is 20 mmHg. In difficult-to-heal wounds, the oxygen tension is significantly lower — 5 mm Hg. [3]. In addition, with low oxygen tension, an increased content of cellular debris was observed, which promotes the growth of microorganisms

in the wound. Thus, special care should be taken with wounds resulting from diseases of peripheral vessels, in particular with diabetic ulcer.

Along with the oxygen tension, during wound healing, it is necessary to take into account the level of supply of damaged tissues with nutrients, especially proteins [3]. However, it is also important to have other factors, such as vitamins A, C, zinc, etc.

Difficult healing wounds the complex regulation of many factors contributing to proper wound healing, it is not surprising that chronic wounds are quite common.

After an acute wound, such as an injury with tissue damage, surgery, or even a bite, the healing scheme given above takes effect. However, the outcome will depend on the nature of the acute wound — its location, size, depth, and type. So, in the presence of aggravating factors, such as chronic diseases, a non-healing wound can form. Thus, a non-healing wound can be considered as a wound, the healing of which for some reason deviated from the previously described natural physiological course of events. Reasons for the development of non-healing wounds can be very diverse factors: impaired blood supply to tissues (peripheral vascular disease), changes in immune status (for example, immune suppression or acquired immune deficiency), metabolic diseases (such as diabetes), medication or previous local tissue damage (for example, radiation therapy). External factors, such as prolonged pressure, temperature and humidity, also play an important role in the wound healing process.

Non-healing wounds in healthy people are usually associated with the presence of a chronic disease in a patient, ranging from diabetes to cancer. One of the main problems associated with the treatment of dangerous chronic wounds in diabetes, occurring in 15% of patients [5]. A wide range of factors are believed to affect all phases of wound healing, and proper glycemic control can have a significant effect on the rate of wound healing in a diabetic patient.

Wound treatment methods. The most common prevention of wound healing is to prevent the development of a bacterial infection, with antimicrobial drugs prescribed empirically. Since bacteria are a normal part of the micro biota of the skin, a threshold concentration was determined, which amounts to 10^5CFU of bacteria. This concentration is considered borderline between colonization and clinically significant. Conducting a clinical diagnosis to identify pathogens from the surface of the wound is a prerequisite for the successful treatment of an infected wound.

A distinctive feature of incurable wounds is chronic inflammation caused by bio films of microorganisms. In most cases, Staphylococcus aureus isolates in chronic wounds, including methicillin-resistant S. aureus (MRSA) in 20-50% of cases. At the same time, the prevalence of MRSA strains in medical institutions is increasing and now amounts to 70% in most clinics around the world. Infections with MRSA strains significantly contribute to the morbidity and mortality of patients.

Bacteriological studies of the deeper layers of wound tissue are somewhat more controversial. Despite the fact that the deeper layers of tissues have better sensitivity and specificity in terms of isolation of the pathogen in an infected wound, isolates from different parts of the same wound showed that they have different levels of contamination by different microorganisms [6].

There are many approaches to the treatment and prevention of wound infections. For example, silver has been used as an aid in treating wounds for more than 2000 years. A great deal of experience has been gained in the use of iodine-containing compounds. Numerous antibiotic preparations for the treatment of wounds have also been developed. Although these drugs remain popular, clinical evidence suggests that the benefits of the widespread use of antibiotic ointments are highly questionable and the only real indication for the use of antibiotics is an infected wound, with a clinically confirmed pathogen. Numerous studies indicate that the uncontrolled use of antibiotic ointments may lead to the development of resistance to antibiotics and contact dermatitis [7]. The global trend in the use of antibiotic ointments in dermatology is that the use of antibiotics should be reserved for cases such as impetigo or an infected wound, and not for general prophylaxis [8].

In addition, methods such as skin grafting, vacuum treatment and using a pressure chamber or laser are used to treat wounds.

Despite the abundance of methods for treating wounds, new concepts and strategies are needed to combat possible complications such as wound inflammation and to improve the treatment of chronic wounds. One of these promising strategies is the use of physical non-thermal atmospheric pressure plasma (NTAPP) [9].

Non-thermal atmospheric pressure plasma

Physical plasma is considered as the fourth state of matter and is determined as fully or partially ionized gas. Irwin Langmuir (1928) was the first to name the "plasma" of ionized gas. In a plasma, electrons, positive and negative ions, neutral atoms and neutral or charged molecules can be identified. Plasma is also characterized by temperature, various types of radiation (for example, UVB) and electric fields. Plasma can be seen in everyday life, such as lightning during a thunderstorm, northern lights, neon lights, or plasma displays.

Plasma can be thermal or non-thermal. Thermal plasma is almost completely ionized, and non-thermal plasma is only partially ionized. For artificially creating plasma, you can add energy to a gas, such as air, argon, or helium, igniting them at low or atmospheric pressure.

Plasma has been used for quite some time in various fields: in engineering and industry, in the construction of vehicles and in metallurgy.

At present, NTAPP with a temperature of about 30-40°C is used in medicine and biology; such plasma is used to treat living cells, tissues, and other heat-sensitive material. The new direction "Plasma Medicine", combining plasma physics with the science of life and medicine is developing rapidly [10]. New plasma sources and devices have been introduced for various applications.

Sources of plasma for the study of cells and tissues

For biomedical purposes, at least three different principles have been developed for creating non-thermal plasma at atmospheric pressure [11]:

- Plasma jet
- Corona plasma sources
- Sources of dielectric barrier discharge plasma (DBD)

It has been found that non-thermal atmospheric plasma very effectively inactivates various microorganisms and is capable of destroying bacterial bio films. Even patients with multidrug resistance and wound pathogens are sensitive to NTAPP. A complete inactivation of various bacteria, including MRSA, using NTAPP is described. All this led to the hypothesis that plasma could be an alternative solution for the antiseptic treatment of chronic infected wounds or disinfection of surgical instruments and catheters [11].

The first positive results of wound healing in experimental animals are demonstrated. Recently, NTAPP has been used to treat skin diseases such as itching, atopic eczema, and psoriasis [11].

However, the mechanisms of action of NTAPP on wound healing are still at the research stage.

The action of plasma the action of plasma has been extensively studied in vitro using various types of cells. Wound-related cells, such as keratinocytes, fibroblasts, epithelial and endothelial cells, as well as inflammatory cells, especially chronic infected wounds Studies were performed either with cell lines or with primary cells. For plasma processing of the material, the researchers mainly used two fundamentally different plasma sources: plasma jets and plasma discharge sources of the dielectric barrier.

Currently, there is no standardization of plasma sources in the world regarding technical data. Only in Germany published "General requirements for plasma sources in medicine" (DIN SPEC 91315, 2014), which were presented at the 5th International Conference on Plasma Medicine (ICPM5) Mann et al. (2014).

The studies are guided by generally accepted biological (microorganism inactivation, cytotoxicity and chemical detection in liquids) and physical (temperature, heat capacity, optical emission spectrometry, UV irradiation, gas release and leakage current) criteria for characterizing the NTAPP.

Today it is the main criteria for assessing plasma sources for use in therapeutic practice.

The lack of generally accepted assessment standards makes it very difficult to compare the published results of various laboratories regarding plasma treatment times and plasma doses, which cause a stimulating or lethal effect on cells or tissues. However, despite the use of different cell types or different plasma sources, the following general patterns of the action of NTAPP depending on time and dose of exposure were observed in all studies [11]:

- Changes in the plasma membrane,
- Induction of intracellular reactive oxygen radicals,
- Impact on mitochondria,
- Induction of apoptosis and necrosis with a decrease in cell viability and cell death,
- Increase or decrease in cell proliferation,
- Increase or decrease cell migration,
- Violation of the structure of DNA with cell cycle arrest.

All these effects depend not only on the plasma treatment time, but also on the process gas (ambient air, argon, helium), the treatment regimen (direct, indirect), the time of the study after plasma exposure, cell type and cell culture quality - suspension (immune cells) or a monolayer of adherent cells (for example, keratinocytes, fibroblasts).

The results allowed us to make the following conclusion [11]: short plasma treatment time intervals / low plasma doses have a stimulating effect (increased proliferation and migration, induction of DNA recovery), and long-term / high doses cause lethal effects (cell death by apoptosis, arrest of proliferation, DNA damage, cell cycle arrest).

The first option for the treatment of NTAPP can be used to improve wound healing, the latter for the treatment of cancer cells.

The aim of our study is to determine the level of contamination of mouse wound and to determine the dynamics of IL-10 level in mice wound exposed non-thermal atmospheric pressure plasma.

MATERIALS AND METHODS

Non-thermal atmospheric pressure plasma generator

The installation scheme of the generator of non-thermal atmospheric pressure plasma on the basis of a helium jet is described in our previous work [12]

Animals in the experiment, white mongrel male mice aged 7–8 weeks, weighing 20–22 grams (laboratory animal nursery «Rapppolovo», Russia) were used. Animals were kept in individual cages on a balanced diet and free water regime. Each animal was weighed before and on the following days of the experiment.

From the area of the surgical field on the back between the shoulder blades, the hair was removed with a depilatory cream (Eveline Cosmetics, Poland). The cream was applied twice for 6 minutes, then rinsed with a cotton swab moistened with water [13].

After anesthesia, zolitil (50 mg / kg) mice were inflicted with a full-skin wound. The operative field was treated with alcohol. Using a Dermo-punch 3 mm skin biopsy stylet (Sterylab, Italy), a full-skin wound of 3 mm in diameter between the shoulder blades was applied, including a layer of panniculus carnosus, which is deep down to the superficial fascia of the muscles. The excised skin was removed with tweezers and scissors [13]. The day of the wound was considered the beginning of the experiment and was designated as “0 day”.

Experimental procedures were carried out in accordance with all modern standards of the Ethics Committee and the requirements of bioethical norms for working with experimental animals [14].

The healing rate of the wound surface was determined by measuring the area of wounds. Throughout the experiment, the wounds were photographed daily. The obtained images were transferred to a computer, calibrated and measured the area of the wound lesion using the Scion Image program (NIH, USA). The results were expressed as a percentage of the original area.

After the operation, the animals were divided into groups: 1. control (free wound healing), and 3 experienced: 2. wound healing during plasma treatment; 3. wound healing on the background of immune suppression (hydrocortisone); 4 wound healing on the background of immune suppression (hydrocortisone) and plasma treatment. There were 5 mice in each group. To obtain a complicated course of the wound process and immune suppression in mice, the 3rd and 4th group of mice were injected with hydrocortisone acetate (Gideon Richter, Hungary) at a dose of 25 mg / kg intramuscularly daily for 7 days of the experiment, for the first time 24 hours before inflicting wounds. Wounds in experimental group 2 and 4 were treated daily with cold plasma for three days.

The selection of material for microbiological seeding and determining the level of IL-10 was performed on the 3rd and 7th day of the experiment. For this, animals were euthanized with zolitil (625 mg / kg) intramuscularly in the thigh area. A section of healthy skin with a wound area in the center was isolated using a Dermo-punch 5 mm skin biopsy stylet. The number of experiments was 3n.

Bacteriological studies the obtained tissue sample was homogenized on a D-160 manual homogenizer, after which it was sown on a dense nutrient medium — Muller-Hinton agar (Hi Media, India). Crops were cultivated at a temperature of 37°C for 18-24h. The results of sowing were evaluated visually by the number of colony forming units (CFU).

Determination of IL-10 level the level of IL-10 was determined using a standard ELISA method using reagents from Sigma-Aldrich (USA).

RESULTS AND DISCUSSION

Wound area

The results of measuring the area of the wound surface showed that with the same initial area of the wound lesion, on the 3rd day there was no statistically significant difference in the area of the wound surface. 7 days after the start of the experiment in mice treated with non-thermal plasma (group 2), the area of the wound surface was significantly less than in the control group. So in the control group, the wound area decreased by 18% relative to the initial area of the wound lesion, whereas in the experimental group 2 (plasma treatment) over the same period of time, the wound surface area decreased by 36%. In experimental group 3 (hydrocortisone), the area of the wound surface increased by 20%, and in group 4 (hydrocortisone and plasma) decreased by 25%.

The findings suggest that with a similar initial area of the wound lesion, the healing process in non-thermal plasma treated wounds was much faster than in the wounds of the control group, despite the presence of immune suppression in mice.

Bacteriological examination

The bacteriological data showed that microorganisms at a concentration of 10^4 CFU/cm^2 were isolated from the wounds of the control group of mice on the 3rd day of the experiment. On the 7th day, the concentration of bacteria in the wounds of this group of mice was reduced by 2 orders of magnitude. Similar data were obtained from group 4 (hydrocortisone + plasma). In the wounds of the experimental group 2 (plasma), after treatment with non-thermal plasma, on the 3rd day 10^2 CFU/cm^2 of bacteria were detected by the 7th day from the beginning of the experiment, no bacteria were detected in the wounds of this group of mice. In the 3rd group (hydrocortisone), immune suppression resulted in increased CFU indices compared to

the control, on the 3rd day 10^7 CFU/cm^2 was detected, and by the 7th day this index decreased only by 3 orders. Studies have shown a pronounced bactericidal effect exerted by cold plasma on the wounds of mice with complicated wound healing.

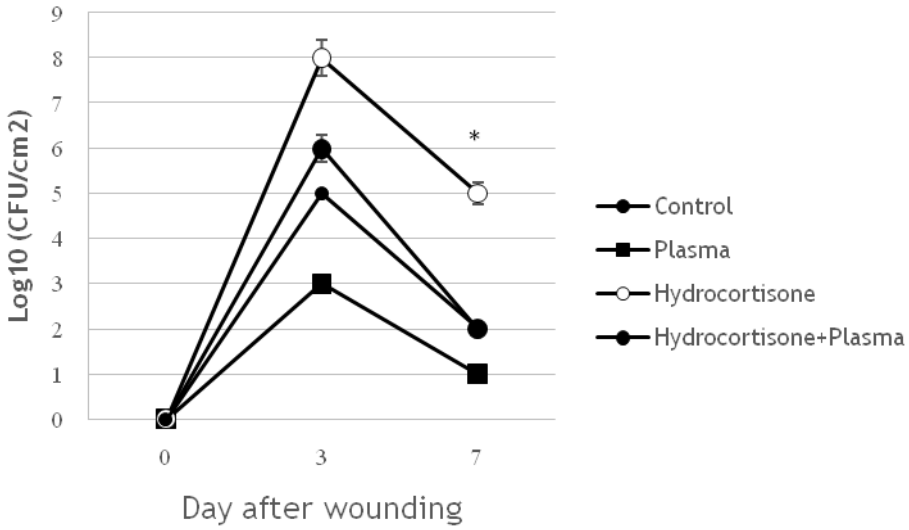

Figure 1: Viable bacterial counts (CFU, colony-forming units) in mice wound of different experimental groups.* - p<0.05 relative to other groups on the seventh day

IL-10 level

Interleukin-10 (IL-10), along with IL-4, IL-5, IL-13 and IL-25, is among the pro-inflammatory cytokines. Determining the level of IL-10 in wound tissues showed that on the 3rd day from the beginning of the experiment, 62.6 pg/mg of protein were detected in the control group, and in the experimental group 3 (hydrocortisone) the level of IL-10 was higher and amounted to 71.2 pg/mg. In groups 2 and 4, the level of IL-10 was 18.3 and 26.0 pg/mg, respectively. Further, on day 7, IL-10 was detected in the wound tissues of the control group in the amount of 50.1 pg/mg protein, and in group 2 - 69.3 pg/mg, whereas in groups 2 and 4 (after exposure to non-thermal plasma) it was found IL-10 in the amount of 8.5 and 12.9 pg/mg protein. Thus, on the seventh day, the level of IL-10 remains elevated in the 3rd experimental group (hydrocortisone) and in the control groups, which indicates that the inflammatory reaction continues in the wound tissues; however, this process is much less active in plasma-treated mice and on the 7th day, the indices decreased by 2-4 times.

When assessing the level of IL-10 in serum, no statistically significant differences were found when comparing the data.

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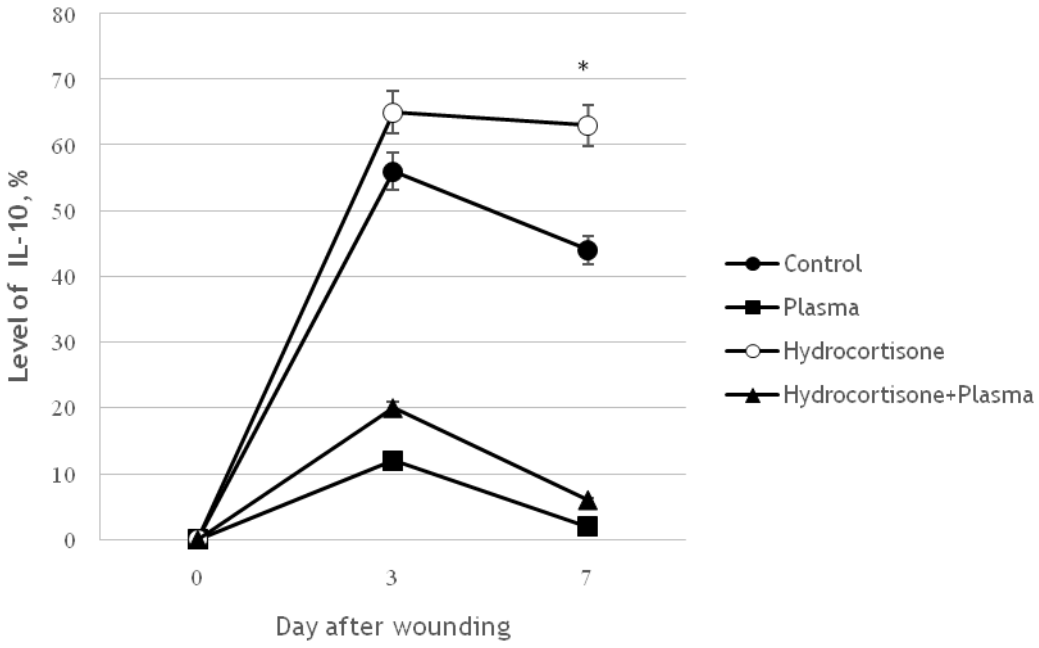

Figure 2: Dynamics of IL-10 level in mice wound of different experimental groups. * - p<0.05 relative to other groups on the seventh day

CONCLUSION

It is considered that IL-10 is one of the main inhibitors of the synthesis of pro-inflammatory cytokines, inhibits the activity of macrophages and excessive growth of the endothelium. Its producers can be monocytes, macrophages, activated T-helper cells. IL-10 inhibits the production of IFN-γ by T-lymphocytes, the production of all pro-inflammatory cytokines by macrophages, the expression of TNF and IL-12 receptors. In this case, an excess of IL-10 leads to a decrease in anti-infective protection and the development of chronic infections [15]. Possible mechanisms of observed reduced (compared to control) levels of IL-10 in mice whose wounds were treated with non-thermal plasma may be associated with accelerated re-epithelialization of plasma-treated wounds. It can be assumed that cold plasma stimulates the proliferation of epithelial cells and thereby accelerates the healing process, which correlates with literature data [11].

The results obtained in the analysis of the effects of non-thermal plasma on wound healing in mice with a complicated wound process indicate a wound-healing and bactericidal effect of plasma. For the first time, data were obtained on the reaction of the immune system to the effect of non-thermal plasma in vivo. A decrease in the level of IL-10 was found in plasma-treated wounds by a factor of 2,4 compared with untreated wounds on the 7th day from the start of the experiment. Further research in this area will help investigate the mechanism of plasma action on animal tissue, which will certainly help to address the problem of treating difficult-to-heal wounds.

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