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PHOTODYNAMIC THERAPY OF PURULENT WOUNDS IN EXPERIMENTAL DIABETES MELLITUS

Abstract. *The aim* of the study was to study the effectiveness of the method of photodynamic therapy of purulent wounds in conditions of experimental diabetes mellitus.

Materials and methods. Experimental studies were carried out on 30 mature male Wistar rats, which were injected with diabetogenic cytotoxin streptozotocin to induce type 2 diabetes mellitus, and also simulated a purulent wound. Various combinations of topical treatments have been used for treatment. A comparative analysis of the use of various methods of treating purulent wounds was carried out using the following methods: clinical, bacteriological, planimetric, histological, and statistical data processing.

Results and discussion. In the course of clinical observation, it was found that a day after the modeling of infected wounds, the general condition of the animals could be assessed as moderate: the animals were lethargic, inactive, some of them had sanious discharge from the nose. Wounds in all animals had signs of suppuration: accumulation of liquid fibrinous-hemorrhagic exudate was noted in the cavity of the ring, in some animals there was no exudate. in two experimental groups (B and C), PDT sessions were performed according to the plan; in the control group (A), the wounds were treated with chlorhexidine solution. During the experiment, it was shown that the proposed method for the treatment of purulent wounds against the background of diabetes mellitus makes it possible to optimize the wound process, reduce the time for the appearance of granulations and the filling of mature granulation wounds. The use of photodynamic therapy contributed to the rapid cleansing of wounds, the appearance and marginal epithelization. Microbiological examination testified to the rapid decontamination of wounds.

C*onclusions.* The use of the photosensitizer 5-ALA in the complex of wound treatment under conditions of photoactivation enhances the antibacterial, anti-inflammatory and pro-regenerative effects; This opens up the possibility of a promising use of this complex for antibacterial photodynamic therapy as a new method for the treatment of infectious and inflammatory diseases of the skin and soft tissues in diabetes mellitus.

Keywords: *photodynamic therapy, diabetes mellitus, purulent wounds.*

Introduction

The treatment of purulent wounds, especially in diabetes mellitus (DM), is one of the oldest, but still relevant, problems of surgical clinics [1, 2]. The widespread use of antibiotics that have a mutagenic effect on the microflora causes a change in the etiological structure of a purulent infection, as well as the biological properties of a microbial cell with the appearance of antibiotic-resistant strains of microorganisms [1, 3]. The consequence of this was a decrease in the effectiveness of antibiotic therapy (ABT) and the traditionally prescribed local treatment of wounds against the background of a growing allergization of the population, the tendency of patients with DM to form biofilms, as well as the immunobiological resistance of the macroorganism [4, 5, 6]. These factors determine the deterioration of the results of treatment of patients with a surgical profile due to an increase in the duration of treatment and the number of adverse outcomes [2, 6].

The aim of the study was to study the effectiveness of the method of photodynamic therapy of purulent wounds in conditions of experimental diabetes mellitus.

Materials and methods

Experimental studies were carried out on 30 sexually mature male Wistar rats weighing 250–300 g, obtained from the vivarium of experimental animals of KhNU named V.N. Karazin (Kharkiv). The animals were kept under standard vivarium conditions with 12 hours of daylight, air temperature 20-25°C, air humidity 50-55%. All manipulations with animals were carried out in accordance with

the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Strasbourg, 1986) and the resolution of the IV National Congress on Bioethics (Kyiv, 2010). In order to exclude seasonal fluctuations in the studied indicators, all experiments were carried out in the autumn-winter period. The sampling of material for research was carried out in the morning. Euthanasia of animals was carried out with an overdose of anesthesia.

In order to induce type 2 diabetes, the administration of the diabetogenic cytotoxin streptozotocin (STZ) was used, which is a toxic compound from the group of nitrosourea derivatives associated at the C2 position with D-glucose, which selectively penetrates into pancreatic beta cells via the GLUT-2 transporter. The STZ powder was dissolved in an opaque glass container and injected into the experimental animal intraperitoneally. Type 2 DM was diagnosed 1 week after STZ administration by performing an oral glucose tolerance test, during which fasting glycemia levels were measured in animals and after 30, 60, 90, and 120 minutes. after intragastric administration of a 40% glucose solution at a dose of 3 g/kg, and a glycemia level of 9.0 to 14.0 mmol/l.

As an experimental model of wounds, a model of a full-layer planar wound was used (L. I. Slutsky, 1969, A. B. Shekhteretal., 2005). Under general anesthetization with propofol (3 mg per kg of animal body weight), a concentric (about 8–10 mm in diameter) skin flap with subcutaneous tissue up to the proper fascia was excised on the previously depilated back skin in the interscapular region. A sleeve-shaped ring was introduced into the formed defect, thus, wounds of a standard size, with an area of 300 mm^2, were obtained in all animals.

To model an infected wound, the own fascia and the muscles of the bottom of the wound were injured with a Kocher clamp, and 0.5 ml of a suspension of a suspension of a microbial culture was applied to its surface, limited by a ring. A cellophane film was fixed along the upper cut of the cylindrical part of the ring to prevent the wound from drying out and external contamination. To infect wounds, we used a suspension of a daily associated microbial culture containing 106 microbial bodies per 1 ml. The association contained pathogenic pathogens of different groups in equal proportions: gram-positive bacteria – *Staphylococcus aureus* S7 (clinical strain); gram-negative rods – *Escherichia coli* ATCC 8739 (standard strain used for testing antimicrobial activity); *Pseudomonas aeruginosa.*

On the 4th day after the modeling of the wound and its infection (the next day after the last irradiation), fixing rings were removed from the wounds. Further wound healing proceeded by secondary intention.

Animals were divided into three groups of 10 animals each: control group (A) (animals whose infected wounds were treated with 0.05% Chlorhexidine solution and covered with hydrogel dressings) and experimental (B) (wounds were treated using one session of photodynamic therapy (PDT)) and **C** (animals underwent two PDT sessions).

Methodology and procedure for conducting PDT. Under general anesthesia, the protective film was removed from the surface of the ring. If exudate was present in the ring cavity, it was removed with a sterile swab, after which Levuderm gel (6% 5-aminolevulinic acid phosphate gel, 5-ALA) was applied to the wound surface, the wound surface was covered with a napkin, and the animals were placed in a dark box. Photo treatment of the wounds was started 15 min later. PDT sessions of wounds were performed using photonic matrices with a wavelength of 661 nm and an output power of 0.1-2.0 W as a source.

The bottom of the wound from a distance of 1–2 cm from the surface was irradiated with a defocused beam (the diameter of the irradiation spot was about 0.5–0.8 cm, with a power density of 1 W/cm^2) for 1 min. After the end of irradiation, the surface of the ring was again covered with a film. The procedure was repeated on the third day after the wound modeling. On the 4th day after the modeling of the wound defect and its infection (the next day after the last PDT session), the Teflon restrictive rings were removed from the wounds and a smear was taken for bacteriological examination.

A comparative analysis of the use of various methods for the treatment of purulent wounds was carried out using the following methods: clinical (general condition of the animals, the nature of the inflammatory reaction, the state of the walls and bottom of the wound, the timing of the appearance of granulations, the nature of the granulation tissue, the timing and activity of epithelization), bacteriological, planimetric, histological. Clinical and planimetric data of the course of the wound process were recorded daily from the first day of exposure. Tissue sampling for histological examination was carried out on the 1st, 3rd, 5th days of treatment in 6 animals from each group.

Statistical processing was carried out using the standard MS Office for Windows software package. To objectify the descriptive characteristics of the state of the granulation tissue, morphometric studies were performed - measuring the thickness of the granulation tissue layer at several points in each of the fields of view (at least 5) of photographic images obtained by viewing micropreparations. To determine the possibility of using parametric methods of analysis, we first evaluated the distribution of the values of the studied trait using the Kolmogorov-Smirnov one-sample test. According to the results of this test, the distribution of granulation tissue

thickness values in the animals of the studied sample is statistically significantly different from the normal one. Therefore, two approaches were used for further analysis: 1) determination of the means and boundaries of 95% confidence intervals for the means; 2) testing of statistical hypotheses. To test the statistical hypothesis about the absence of differences between the compared groups in terms of the thickness of the granulation tissue layer, the following were used: the Kruskal-Wallis test, which confirmed the presence of statistically significant differences between the groups and the possibility of further pairwise group comparisons, and the Mann-Whitney test to compare the values of the studied trait in pairs of groups.

Results

One of the main and significant criteria for evaluating the effectiveness of the treatment of infected soft tissue wounds is a microbiological study. When studying the microbial contamination of wounds before the start of treatment in each experimental group, it was found that the concentration of Staphylococcus aureus averaged $(3.25\pm0.05)\times10^6$ CFU, and *Escherichia coli* and *Pseudomonas aeruginosa* — $(4.20\pm0.05)\times10^6$ CFU. Microbial contamination was measured twice at each stage of the study: before treatment, after the first PDT session, before the second session, and at the end of the second session.

Quantitative indicators of microbial contamination of wounds are presented in table 1.

As shown in table 1, 24 hours after modeling before the first PDT session, the initial contamination of wounds in all groups of animals was the same and amounted to Coccal flora: $(3.00\pm0.05)\times10^6$ – $(3.80\pm0.05)\times10^6$ and for Gram-negative rods $(2.80\pm0.05)\times10^6$ – $(5.00\pm0.05)\times10^6$.

In the control group, the primary treatment of wounds with a solution of chlorhexidine led to a decrease in the number of gram-negative rods to $(4.20\pm0.05)\times10^4$, but did not significantly affect the presence of staphylococci. Re-treatment of wounds with chlorhexidine also did not lead to a decrease in the level of contamination of both coccal and gram-negative flora compared to the initial (before the start of the 2nd treatment) level. It should be noted that the mortality rate in this group is 33.3% (2 animals died).

In group B (after the 1st PDT session), the contamination with staphylococci decreased by 1.5 times. With respect to gram-negative rods, the use of LEVUDERM as a photosensitizer led to a significant reduction in bacterial contamination: in crops, the number of germinated colonies was less than 10 Colony Forming Unit/ml (lg Colony Forming Unit/ml =1). The same data were obtained after the 2nd PDT session, which allows us to speak about the selectivity of this type of photochemical treatment in relation to gram-negative flora.

In the course of the experiment, we found the selective antimicrobial activity of LEVUDERM against gram-negative bacteria. In animals of group B (2 sessions of PDT), when comparing the level of total wound contamination using a paired Student's t-test to logarithmic values of Colony Forming Unit/ml (lg Colony Forming Unit/ml) with subsequent Holm-Bonferroni correction, a significant decrease in bacterial contamination of wounds was obtained: after the 2nd PDT session, the coccal flora was practically not sown from the surface of the wounds: the number of Colony Forming Unit/ml was < $(1.0\pm0.05)\times10^1$ (lg Colony Forming Unit/ml =1). Gram-negative microorganisms were not sown.

In the course of clinical observation, it was found that a day after the modeling of infected wounds, the general condition of the animals could be assessed as moderate: the animals were lethargic, inactive, some of them had sanious discharge from the nose. Wounds in all animals had signs of suppuration: accumulation of liquid fibrinous-hemorrhagic exudate was noted in the cavity of the ring, in some animals there was no exudate. The bottom of the wound over the entire surface was covered with a thin layer of fibrin, thickening at the edges of the wound. The underlying tissues were edematous and had a bluish tinge. The perifocal reaction was mod-

Table 1

Bacterial contamination of wounds during local treatment

Research term	Group of animals	Relation to processing	Gram positive cocci		Gram negative rods	
			Colony Forming Unit/ml (M±m)	lg Colony Forming Unit/ml	Colony Forming Unit/ml (M±m)	lg Colony Forming Unit/ml
24 hours after modeling	A	Before	3,10±0,05*10^6	6,4913	5,00±0,05*10^6	6,6989
		After	3,00±0,05*10^6	6,4771	4,20±0,05*10^4	4,6232
	B	Before	3,10±0,05*10^6	6,4913	5,00±0,05*10^6	6,6989
		After	2,40±0,05*10^3	3,3802	<1,0±0,05*10^1	1
72 hours after modeling	A	Before	5,00±0,05*10^3	3,6989	3,9±0,05*10^3	3,544
		After	3,00±0,05*10^3	3,4771	3,7±0,05*10^3	3,5682
	B	Before	3,60±0,05*10^3	4,5563	3,50±0,05*10^3	3,544
		After	4,2±0,05*10^3	3,6232	<1,0±0,05*10^1	1
	C	Before	3,30±0,05*10^4	4,5185	3,70±0,05*10^4	4,5682
		After	<1,0±0,05*10^1	1	2,90±0,05*10^4	4,4623

erately expressed: the skin margins were slightly swollen, no dense infiltration was observed, and the hyperemia was insignificant.

During these terms of the study, the first PDT session was performed in two experimental groups (B and C), in the control group, the wounds were treated with a solution of chlorhexidine. One animal of this group A died. In one animal of group B, single punctate petechiae were observed in the bottom of the wound.

On the next day after the first PDT session (2nd day after modeling), the general condition of the animals of group A deteriorated, one animal died.

On the 3rd day, the general condition of the surviving animals of the experimental groups could be assessed as satisfactory: they became more active. Only 1 animal in group B retained nasal discharge. In the control group, the general condition of the animals remained moderate. Another animal died, which showed signs of diarrhea.

During these periods, local differences in the state of wounds became noticeable. In animals of groups B and C, exudation decreased. In animals of group B, only in 3 observations in the cavity of the ring, a liquid cloudy serous exudate was present in a small amount (about 0.5 ml). The bottom of the wounds was filled with a layer of fibrin, thicker and looser along the edges of the wound. Puffiness of the skin edges of the wounds noticeably decreased, hyperemia disappeared. In group A, exudate was still present in all wounds in a sufficient amount (more than 0.5 ml), while in 2 animals it had a clearly purulent character: it was cloudy, mucus-like, with an unpleasant odor; in 3 other animals, the exudate was more liquid, fibrinous-hemorrhagic. Swelling of the skin around the wounds increased slightly.

During these observation periods, the 2nd PDT session was performed in group B, and in the control group, the wounds were treated with chlorhexidine solution.

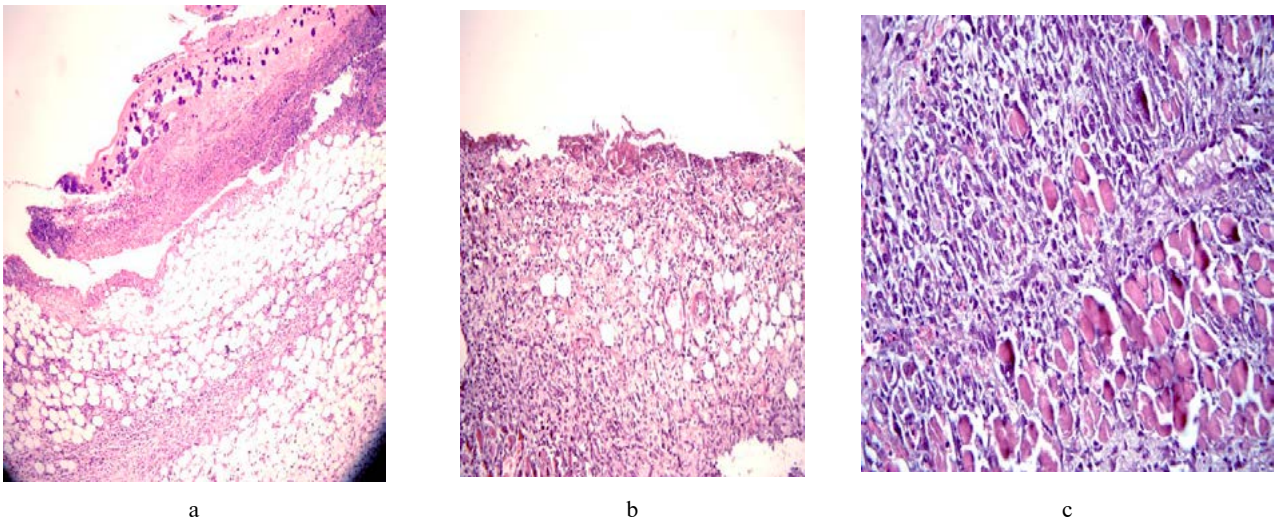
On the 4th day after the induction of the experiment and 2 sessions of PDT, the general condition of the animals of group B improved markedly. During this period, the animals were withdrawn from the experiment in order to remove wound tissues for histological examination.

Group A (control). The bottom of the wound is lined with a fibrinous-leukocyte layer containing neutrophils and lymphocytes, as well as inclusions of necrotic detritus. In some animals, the exudate layer is relatively thick and numerous large colonies of microbes are visible in it (Fig. 1.a). Under it, an immature granulation tissue is found (Fig. 1.b, 1.c).

Group B. The wound surface is covered with a fibrinous-leukocyte layer. Below it is a layer of hemorrhagic exudate with a large number of erythrocytes. Even deeper is immature granulation tissue, consisting of newly formed capillaries and randomly located fibroblasts. It contains a large number of cells of the inflammatory infiltrate (neutrophils, lymphocytes and macrophages), as well as accumulations of red blood cells (Fig. 2.a).

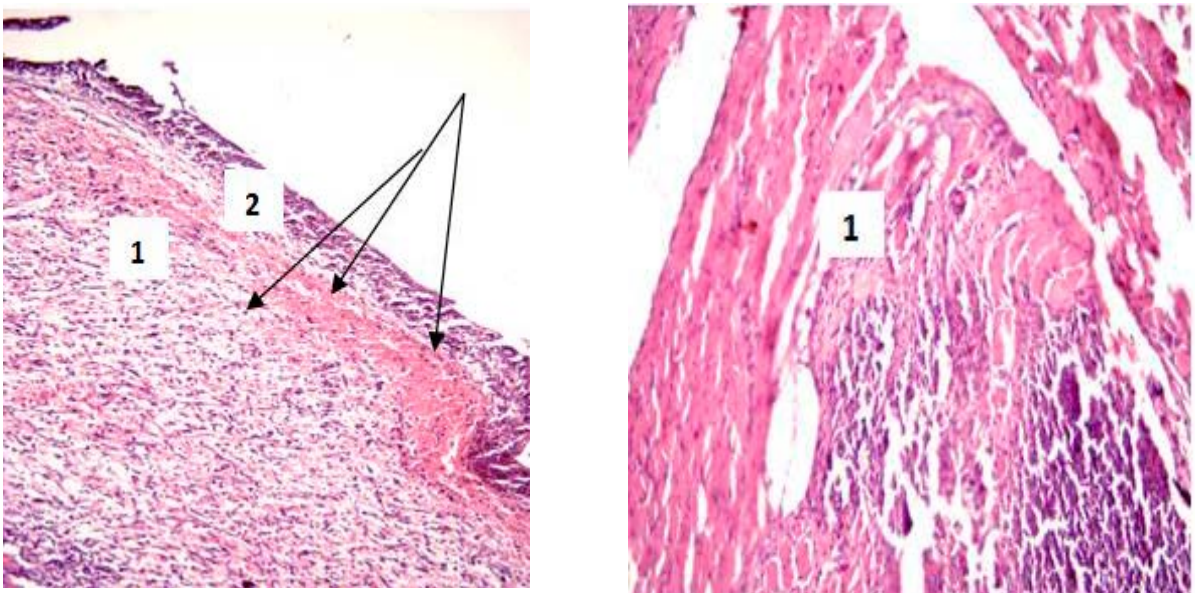
As in the control, there is a pronounced inflammatory infiltration with a large number of decaying neutrophils and partial necrosis of muscle fibers (Fig. 2.b).

Group C. In all cases, a relatively wide fibrinous-leukocyte layer is visible on the wound surface, consisting of loose fibrin, neutrophils (partially destroyed) and lymphocytes. Unlike the above groups, the layer of fibrinous-leukocyte exudate does not have a hemorrhagic character.



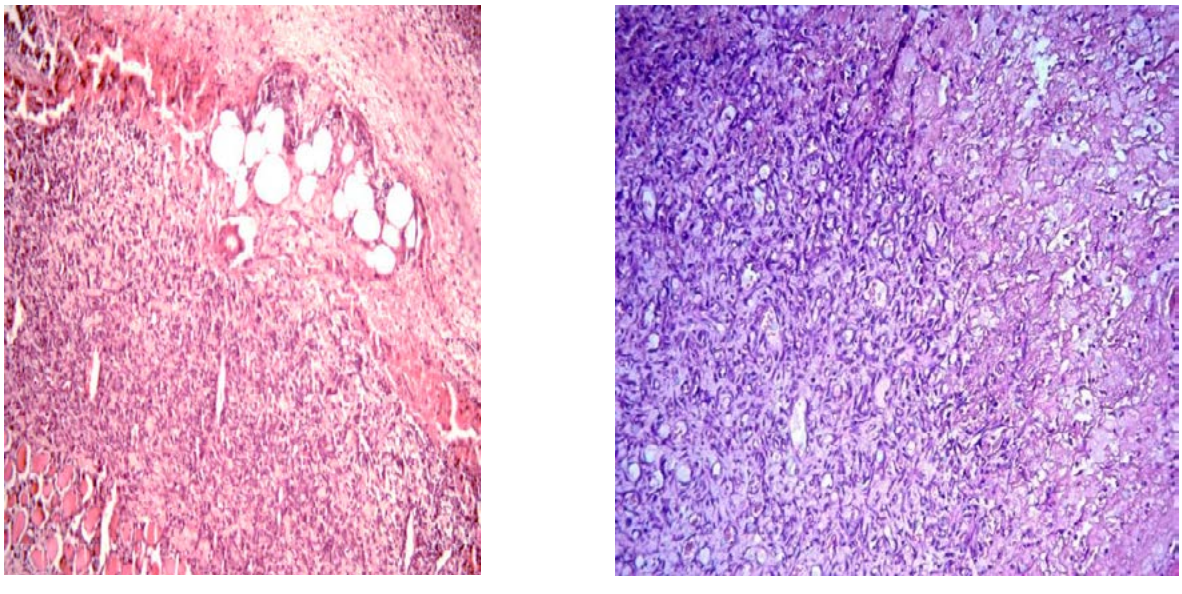
a b c

Fig. 1. Histological examination of the wounds of animals of group A: a) a thick layer of fibrinous-leukocyte exudate with numerous colonies of microbes; granulation tissue develops within adipose tissue; b) on the surface of the wound - a fibrinous leukocyte layer, under which an immature granulation tissue with remaining fat cells is visible; c) neutrophilic, macrophageal and lymphocytic infiltration of the muscle layer, destruction of muscle fibers. Staining with hematoxylin and eosin, ×200



a b

Fig. 2. Histological examination of the wounds of group B animals: a) immature granulation tissue (1) covered with a fibrinous-leukocyte layer (2), between them there are accumulations of erythrocytes (shown by arrows); b) inflammatory infiltration of muscle tissue, in the area of infiltration — necrosis of muscle fibers (1). Staining with hematoxylin and eosin, ×400



a b

Fig. 3. Histological examination of the wounds of group C animals: a) part of the fibrinous-leukocyte layer, under it — an island of adipose tissue and a wide layer of relatively mature granulation tissue; b) pronounced growth of granulation tissue with fibroblast proliferation and enhanced neoangiogenesis. Staining with hematoxylin and eosin, ×200 magnification

The underlying layer of granulation tissue occupies the entire wound surface. It is wider than in all other groups. The granulation tissue itself is mature. Fibroblasts predominate in it, although there is still lymphomacrophage infiltration with a very small admixture of neutrophilic leukocytes (Fig. 3.a). Part of the capillaries acquires a vertical orientation (Fig. 3.b).

The morphological signs of regeneration revealed during the histological examination of wound tissues, which develop under the influence of various external factors, make it possible to assess the activity of wound healing, as well as the role of these factors, either aggravating or, on the contrary, stimulating its course. The most important morphological feature that characterizes the degree of activity of reparative-regenerative processes during wound healing is the state of granulation tissue: the degree of its maturity, the prevalence of the area of the wound surface and the thickness of its layer.

In table 2 presents information about the thickness of the layer of granulation tissue in the wounds of animals of various subgroups.

As can be seen from the data in table. 2, in the control group the thickness of the granulation tissue

Table 2

Distribution of granulation tissue layer thickness values in the studied groups

Groups	Medium	Confidence interval95%		Standard deviation	MIN value	MAX value	Sandard error
		Lower boundsa	Upper bound				
A	79,50	58,70	100,20	44,40	20,00	200,00	9,90
B	150,20	109,90	190,50	86,10	58,00	420,00	19,30
C	506,30	442,20	570,40	136,90	300,00	800,00	30,60

layer was the smallest (79.5±44.4 μm). In the study groups, the thickness of the granulation tissue was statistically significantly greater than in group A. In group B, the layer of granulation tissue was statistically significantly thicker than in all other groups.

Discussion

Thus, the proposed method for the treatment of purulent wounds against the background of DM makes it possible to optimize the wound process, reduce the time for the appearance of granulations and the filling of mature granulation wounds. The use of PDT contributed to the rapid cleansing of wounds, the appearance and marginal epithelization, which corresponds to the data of the development of this topic [5, 6]. Microbiological examination indicated rapid decontamination of wounds, which eliminates the need for antibiotic therapy. It should be noted the selectivity of this type of photochemical exposure in relation to gram-negative flora, which dictates the need to select the frequency of sessions. PDT with 5-ALA promotes an increase in type I collagen fibers in photodamaged skin, and an increase in type I and III procollagen in photoaged skin has also been noted [7]. The literature also describes a significant increase in the amount of procollagen types I and III, collagen precursors, which reflected an increased synthesis of skin collagen, as well as an increase in immunoreactivity (TGF-β and TβRII) and a decrease in the level of MMP-9, which indicates the anti-inflammatory effect of PDT with 5- ALA [8, 9, 10], which is indirectly confirmed by our histological studies.

Conclusions

The use of the photosensitizer 5-ALA in the complex of wound treatment under conditions of photoactivation enhances the antibacterial, anti-inflammatory and pro-regenerative effects; This opens up the possibility of a promising use of this complex for antibacterial photodynamic therapy as a new method for the treatment of infectious and inflammatory diseases of the skin and soft tissues in diabetes mellitus.

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ФОТОДИНАМІЧНА ТЕРАПІЯ ГНІЙНИХ РАН ПРИ ЕКСПЕРИМЕНТАЛЬНОМУ ЦУКРОВОМУ ДІАБЕТІ

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С. М. Граматюк,
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Реферат. *Мета дослідження* — вивчення ефективності методики фотодинамічної терапії гнійних ран за умови експериментального цукрового діабету.

Матеріали та методи. Експериментальні дослідження проведені на 30 статевозрілих щурах-самцях лінії Вістар, яким з метою індукції цукрового діабету 2 типу застосовували введення діабетогенного цитотоксину — стрептозотоцину, а також моделювали гнійну рану. Для лікування використовували різноманітні комбінації місцевого лікування. Порівняльний аналіз застосування різних методів лікування гнійних ран проводили за допомогою наступних методів: клінічних, бактеріологічних, планіметричних, гістологічних та статистичної обробки даних.

Результати та обговорення. У ході клінічного спостереження встановлено, що через добу після моделювання інфікованих ран загальний стан тварин можна було оцінити як середньотяжкий: тварини мляві, малорухливі, у них відзначалися сукровичні виділення з носа. Рани у всіх тварин мали ознаки нагноєння: у порожнині кільця відзначалося скупчення рідкого фібринозно-геморагічного ексудату, у частини тварин ексудат був відсутній. У двох дослідних групах (Б та В) були проведені сеанси ФДТ відповідно до плану, у контрольній групі (А) рани оброблені розчином хлоргексидину. В ході експерименту показано, що запропонований спосіб лікування гнійних ран на тлі цукрового діабету дозволяє оптимізувати рановий процес, скоротити час появи грануляцій та заповнення ран зрілими грануляціями. Застосування фотодинамічної терапії сприяло швидкому очищенню ран, появі крайової епітелізації. Мікробіологічне дослідження свідчило про швидку деконтамінацію ран.

Висновки. Застосування фотосенсибілізатора 5-амінолевулінової кислоти у комплексі обробки ран в умовах фотоактивації посилює антибактеріальну, протизапальну та прорегенеративну дію; це відкриває перспективу можливості використання цього комплексу для антибактеріальної фотодинамічної терапії як нового методу лікування інфекційно-запальних захворювань шкіри та м'яких тканин при цукровому діабеті.

Ключові слова: *фотодинамічна терапія, цукровий діабет, гнійні рани.*