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INDICATORS OF NEUTROPHIL FUNCTIONAL ACTIVITY AS PREDICTORS OF SKIN GRAFTING RESULTS

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Цель. Оценить возможность использования лабораторных тестов оценки функциональной активности полиморфноядерных лейкоцитов (ПЯЛ) крови для прогнозирования исхода аутодермопластики (АДП) у пациентов с хроническими ранами (ХР).

Материал и методы. У 148 пациентов с хроническими ранами, госпитализированных для проведения аутодермопластики (АДП), произведен анализ функциональной активности нейтрофилов периферической крови. Поглотительную способность нейтрофилов определяли в реакции фагоцитоза с оценкой фагоцитарного индекса (ФИ) и фагоцитарного числа (ФЧ). Кислород-продуцирующую активность нейтрофилов оценивали в реакции базального (НСТб) и стимулированного S.aureus (НСТст) восстановления нитросинего тетразолия. Формирование нейтрофильных внеклеточных ловушек (NET) в крови оценивали до (спонтанный уровень; NETб) и после инкубации нейтрофилов со стимулятором (стимулированный уровень; NETст). В связи с различиями в течение послеоперационного периода АДП пациенты были разделены на 2 группы. Группу 1 составили 126 пациентов с благоприятным исходом АДП, группу 2 – 22 пациента, у которых АДП осложнилась лизисом аутодермотрансплантата.

Результаты. С помощью метода логистической регрессии и построения ROC-кривых установлена информативность НСТб, NETб, NETст для прогнозирования исхода АДП у пациентов с хроническими ранами. Оптимальным пороговыми значением для НСТб является 15% (86,5% чувствительность и 93% специфичность), для NETб – 7% (97% чувствительность и 95,2% специфичность), для NETст – 15% (86,4% чувствительность и 90% специфичность). При дооперационных значениях НСТб, NETб, NETст ниже пороговых прогнозируется отторжение аутодермотрансплантата, а при значениях НСТб, NETб, NETст выше пороговых прогнозируется приживление лоскута с вероятностью до 92%.

Заключение. Показатели функциональной активности нейтрофилов (НСТб, NETб, NETст) являются информативными предикторами исхода АДП у пациентов с хроническими ранами, а также рекомендуются для осуществления контроля эффективности дополнительной предоперационной подготовки при планировании АДП.

Ключевые слова: хроническая рана, аутодермопластика, НСТ-тест, нейтрофильные внеклеточные ловушки, прогноз аутодермопластики, эффективность, логическая регрессия

Objectives. To estimate the possibility of using the laboratory tests for evaluating the functional activity of the blood polymorphonuclear leukocytes (PNL) to predict skin grafting (SG) results in patients with chronic wounds (CW).

Methods. The analysis of the functional activity of peripheral blood neutrophils was carried out in patients with chronic wounds (n=148) requiring skin grafting (SG). Ingestion capacity of neutrophils was determined in the phagocytosis reaction with the evaluation of phagocytic index (PhI) and phagocytic number (PhN). Oxygen-producing activity of neutrophils was estimated in response of the spontaneous NBT-test (NBTsp) and S. aureus-stimulated (NBTst) nitroblue tetrazolium restoration. Activity of neutrophil extracellular traps (NET) in the blood were quantified prior (spontaneous; NETsp) and after neutrophils incubation with the stimulator (stimulated; NETst). Taking into account the differences of autodermoplasty outcomes within the incisional period all the patients were divided into 2 groups: group 1 (n=126) – favorable results of skin grafting and group 2 (n=22) – SG was complicated by lysis of autodermograft.

Results. With the usage of logistic regression and ROC-curves methods the informativeness of NBTsp, NETsp, NETst for flap viability prognosis was established in patients with chronic wounds. The optimal cut-off value for NBTsp is 15% (86,5% sensitivity and 93% specificity), NETsp – 7% (97% sensitivity and 95,2% specificity), NETst – 15% (86,4% sensitivity and 90% specificity). At preoperative values of NBT, NETsp, NETst below cut-off, rejection of the autodermotransplant is considered to be predicted, and at NBT, NETsp, NETst above cut-off values, analysis demonstrates engraftment rates with a probability up to 92%.

Conclusion. The values of functional activity of neutrophils (NBTsp, NETsp, NETst) are considered to be the informative predictors of the final outcome in patients with chronic wounds and they are also recommended for monitoring the efficiency of additional preoperative preparation in SG planning.

Keywords: chronic wound, skin grafting, NBT-test, neutrophils extracellular traps, skin grafting prognosis, efficiency, logical regression

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Indicators of Neutrophil Functional Activity as Predictors of Skin Grafting Results
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Introduction

Chronic wounds (CW) are characterized by a prolonged inflammation supported by the constant presence of a large number of polymorphonuclear leukocytes (PNL).

Activated polymorphonuclear leukocytes create a microenvironment of high prooxidant activity in chronic wound (CW) due to the production of reactive oxygen species (ROS) [1]. The mechanism of such activation of PNL is called a "respiratory brust" and is caused by the activation of cytoplasmic NADPH-oxidase, which catalyzes the regeneration of oxygen molecule to the superoxide radical anion [2]. ROS are superoxide anion, hydroxyl radical, singlet oxygen, hydrogen peroxide, PNL are excreted in the process of phagocytosis and destruction of a microbial agent [3, 4]. Other potential targets for ROS produced by activated PNL are the endothelial cells and fibroblasts of a wound [5]. The significant role of ROS in the pathogenesis of long-term non-healing wounds is shown in various studies in vitro and in vivo [3].

Current local dressings enable to eliminate disturbances in the microcirculatory channel and promote its rapid purification, but chronic wounds often remain in the inflammatory stage [6]. In such cases, the most effective method in the complex treatment of patients is thought to be a plastic surgery involving replacing with the autologous skin the lost dermal layer. [7]. Skin grafting (SG) is considered to be one of the widespread method for closing cutaneous wound defects of different etiology.

Although SG is a technically demanding procedure to perform; an operation even of impeccable quality doesn't guarantee success.

Currently, most of researchers confess that taking into account only the clinical state of the wound in determining the optimal time to perform SG is thought to be insufficient. Due to the specificity of pathogenesis, the registration of a complete set of clinical criteria of readiness for SG in cases of CW is not always possible [8, 9, 10].

Therefore, the problem of interpretation of the clinical evaluation results of a wound arises in clinical medicine prior to conducting SG. Timely registration of local and systemic changes is essential for preparing the wound bed to heal efficiently or detecting its infringement so as for assessment the efficacy of wound healing.

The search for specific pathogenetically grounded criteria determining the effective preparation of CW for a plastic closure and the forecast of the outcomes of its performance is considerted to be a mandatory requirement [10].

Objectives. To estimate the possibility of using laboratory tests for evaluating the functional activity of the blood polymorphonuclear leukocytes (PNL) to predict skin grafting results in patients with chronic wounds.

Methods

In the clinical laboratory medicine department of the SE "Republican Scientific and Practical Center for Radiation Medicine and Human Ecology" the functional activity of PNL of the peripheral venous blood was evaluated in patients (n=148) with different types of CW. Patients were treated in the burn department of SME " Gomel City Clinical Hospital N1" (2011-2016).

In 40% of cases (n=59), wound injuries in patients were caused by accidental mechanical trauma that occur at home or work, by thermal or chemical burns. In 35% (n=52) of patients, chronic wound defects were formed against the previous primary purulent-inflammatory diseases of skin and soft tissues: necrotizing erysipelas, phlegmon or abscesses of soft tissues with the skin necrosis. In 30 patients (20%), CW were defined as trophic ulcers formed on the lower limb stump after amputation and wearing the prosthesis, and formed at the site of the fistula in osteomyelitis. In some cases, the etiology of trophic ulcers was chronic venous insufficiency or thrombophlebitis in anamnesis. In 7 patients (5%) CW was in the form of bedsores due to prolonged immobilization of a patient.

The history of the wounds was from 4 weeks to a year or more, the wound area was not more than 5% of the total body surface, which is defined as a local wound process. Most often (60%, n=89), wound injuries were located on the lower extremities shin, thigh, buttocks, foot, followed by lesions of the upper limbs (forearm, shoulder, hand) (25%, n=37) and the trunk (back, anterior abdominal wall, lumbar region, thoracic region, sacrum) (15%, n=22).

For wound closure (after the preoperative preparation) there were used results SG split perforated skin flap (0,3-0,4 mm thick). Evaluation of the readiness of the wound surface to SG was carried out according to the approved clinical visual criteria: absence of signs of inflammation, absence of pronounced exudation and purulent discharge from the wound, high adhesiveness of the wound, presence of marginal epithelization, characteristic appearance of granulations (mature fine-grained red or bright pink) [11]. The patients, mentioned above, had clinical signs of the readiness of a wound to the surgical skin restoring.

All patients had no accompanying diseases affecting the result of this study – diabetes mellitus, immunodeficiencies, malignant tumors, sepsis,

acute infections. Also, the criterion for exclusion from the study was the intake of drugs affected the immune status of patients such as glucocorticoids, cytostatics, immunosuppressants.

Depending on the outcome of the postoperative period and study purpose, the patients were divided into 2 groups. In patients of the 1st group (n=126), surgical intervention was successful – the flap was fixed on the 2nd-3rd days with a complete engraftment which was defined as the 5th-9th consecutive days.

In patients of the 2nd group (n=22) the flap rejection occurred in the incisional period of SG – on the 2nd-3rd days its paleness and lack of fixation followed by the regression of the autodermotransplant by the 5-8 days had been observed.

The group of patients was formed on the basis of informed consent, the study was approved by the local ethical committees of the SE "Republican Scientific and Practical Center of Radiation Medicine and Human Ecology", EE "Gomel State Medical University".

Prior to performing SG, the functional activity of PNL was evaluated by the tests in the PNL absorption activity, in the reaction of phagocytosis, superoxide anion-producing function of PNL in the reaction of the NBT test, the formation of neutrophil extracellular traps (NET) [12, 13]. The material for the study consisted of leukocytes isolated from the heparinized venous blood, incubated for 30 minutes at 37°C, and then centrifuged at 1,000 rpm for 10 min at 250 g.

The number of PNL in the suspension was adjusted to the concentration of 5×10^6 cells / ml by diluting with a 0,9% NaCl solution. The viability of cells according to the trypan blue exclusion test was not less than 95%.

For the phagocytosis reaction, a suspension of heat-killed S. aureus (strain ATCC 25923) was used in a titer of 10^8 CFU/ml, followed by the preparation of stained smears and evaluation of the phagocytic index (PhI – percentage of PNL that absorbed 2 or more microbial particles) and the phagocytic number (PhN – the average number of absorbed microbial cells per one PNL).

Oxygen-producing activity of PNL was evaluated in the reaction of spontaneous (NBTsp) and stimulated with S.aureus (NBTst) restoration of nitroblue tetrazoliumfollowed by counting of formazan-positive cells.

The formation of NET in the blood was assessed before (basal level; NETsp) and after incubation of PNL in the presence of soluble products of S. aureus (stimulated level; NETst) by a modified method of I.I. Dolgushin, which consists in increasing the incubation time from 30 minutes to 150 minutes at 37°C. To induct the formation of NET, soluble S. aureus products were used.

To assess the results of performed tests of the functional activity of PNL, microscopy of the stained preparations was performed using immersion increase (×1000); the percentage of cells was taken into consideration, counting at least 200 cells in the preparation [12, 13].

The results were statistically processed. To verify the hypothesis of normal distribution of quantitative indicators, the Kolmogorov-Smirnov test was used. Since the distribution of the studied indicators differed from the normal one, nonparametric methods of statistics were used. Distribution characteristics were expressed as a median and 25 and 75 quartiles: Me (25%, 75%), and also the value of 95% confidence interval (CI±95%) was presented.

To compare one common sequence of independent samples values the Mann-Whitney U test was used (Z values).

To assess the clinical informativeness of the indices of the PNL functional activity, the logistic regression method was used to predict the outcome of the SG.

The estimation of the predictive value of predictors and the choice of their cut-off values were carried out using the construction of ROC-curves with the calculation of the diagnostic specificity and sensitivity of the tests. Differences were considered significant at $p<0,05$.

Results

The indices of PNL functional activity in patients of groups 1, 2 before SG treatment differed significantly. Values of NBTsp, NBTst, NETsp, NETst in the patients of the 1st group were higher than those in the 2nd group (Table 1).

Taking into account the revealed significant differences in the functional activity of the blood PNL in patients with different results of SG (engraftment/rejection of the autodermotransplant), in the next stage the clinical informative values of the parameters using the logical regression method were assessed, which makes it possible to calculate the probability of occurrence of an event depending on the values of independent variables-predictors. The in dicators functional activity of PNL were used as independent variables and the expected event was considered the outcome of the SG operation – engraftment of the flap or its rejection. When calculating, a step-by-step method was used to decide which of the variables are most significant in terms of forecasting the SG outcome. In the course of the analysis, the variables statistically significant in terms of the prognosis of the SG outcome were revealed: NBTsp, NETsp, NETst (Table 2).

With the integrated use of selected predictors,

Table 1

Functional activity of PNL in patients with different outcome of the operation of plastic wound closure

Indicator	Group 1 (n=126)		Group 2 (n=22)		Significance (Mann-Whitney U test) Z, p-level
	Me (25%; 75%)	CI (±95%)	Me (25%; 75%)	CI (±95%)	
NBTsp	22 (18; 26)	21,2-23,6	10,5 (9; 14)*	9,39-13,06	6,45; p<0,001
NBTst	56 (48; 63,5)	53,5-56,9	50,5 (45;56)*	44,8-54,19	2,27; p=0,02
PhI	77 (69; 80)	72,9-75,9	73 (70; 77)	68,9-75,2	1,45; p=0,14
PhN	7 (6; 9)	6,8-7,5	8 (6; 10)	6,7-9,0	-1,29; p=0,19
NETsp	11 (9; 14)	11,2-12,8	4 (2; 6)*	3,0-5,0	7,19; p<0,001
NETst	21 (17; 27)	21,0-22,8	11 (8; 15)*	9,4-13,2	6,65; p<0,001

Note: * – significant intergroup differences are marked.

Table 2

The significance level of the indicators differences

Indicator	Value	The significance level of the indicators differences between the groups
NBTsp	45,647*	0,000
NBTst	3,340	0,065
PhI	1,527	0,217
PhN	2,267	0,132
NETsp	44,992*	0,000
NETst	48,567*	0,000

Note: * – indicators, significant for prognosis.

the information content of the model increased significantly. Thus, out of 126 studied patients in Group 1 (a good outcome of the operation), the proportion of correct predictions was 97,6% (123 patients). For 22 patients of the 2nd group (rejection of the transplanted flap), the outcome of the SG was correctly recognized in 19 cases (86,3%). The share of correct prognoses for the sampling in general is 92%. To further characteristics of the informative value of predictors, the estimation of the measure of the model-specificity according to the value of the Nadelkerkes R-square (ideally the indicator tends to 1) used, which indicates that part of dispersion that can be explained by logistic regression. In this case, the measure of certainty increased with the addition of each predictor (NBTsp, NETsp, NETst) and when they combined from the value 0,6657, it maximally approached to 1, comprising 0,827, which reflected an increase in the informative value of the model. The quality of the approximation of the regression model is estimated using the similarity function, the measure of which is the negative doubled value of the logarithm of this function (-2LL). Decreasing the value from 55,143 in step 1 (NETst) up to 30,465 in step 3 (NBTsp, NETsp, NETst) means improving the quality of the model (Table 3).

According to the results of logistic analysis, the indicators of NBTsp, NETsp, NETst in patients with CW are thought to be used as predictors of

Table 3

Clinical informative value of predictors their individual and combined use

Step	Outcomes predicted in groups			R^2 Nadelkerkes	-2LL
	True results	False results	Percentage of true values, (%)		
Step 1 (NETst)				0,657	55,143
Group 1 (n=126)	122	4	96,8		
Group 2 (n=22)	14	8	63,3		
Total percentage value			80,05		
Step 2 (NETsp, NETst)				0,785	36,965
Group 1 (n=126)	121	5	96,0		
Group 2 (n=22)	16	6	72,7		
Total percentage value			84,35		
Step 3 (NBTsp, NETsp, NETst)				0,827	30,465
Group 1 (n=126)	123	3	97,6		
Group 2 (n=22)	19	3	86,3		
Total percentage value			92		

Note: * – true results: the number of correct SG prognoses; False results: the number of incorrect SG prognoses; Step: consecutive increase in the number of applied predictors (from one to three); -2LL: -2 logarithmic likelihood

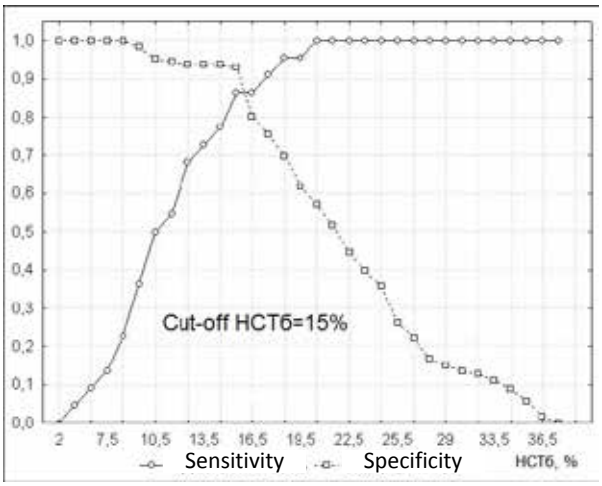


Fig. 1. The balance point between sensitivity and specificity and the cut-off value of NBTsp.
The Y axis represents the sensitivity and specificity values, expressed in %/100. On the X axis, the values of NBTsp in %. Cut-off value of NBTsp – a point of intersection of curves (balance between sensitivity and specificity)

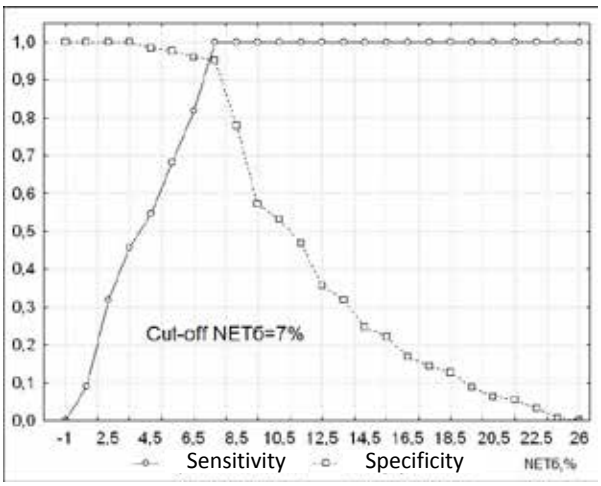
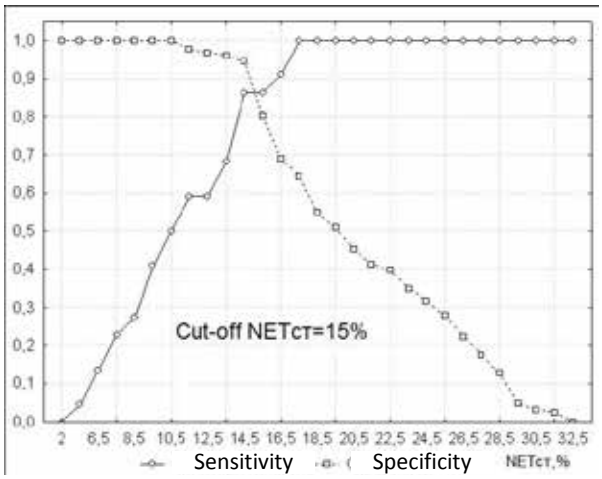


Fig. 2. The balance point between sensitivity and specificity and the cut-off value of NETsp.
The Y axis represents the sensitivity and specificity values, expressed in %/100. On the X axis, the values of NETsp in %. Cut-off value of NETsp – a point of intersection of curves (balance between sensitivity and specificity)

the outcome of the SG operation. To determine the cut-off values of the selected indicators and to assess their sensitivity and specificity in terms of the prognosis of the SG result, the construction of ROC curves was performed. Next, we determined the cut-off values of the predictors of the SG outcomes on the basis of the point of the optimal balance of specificity and sensitivity of the applied test. It was found that the optimal cut-off values for NBTsp are 15% (86,5% sensitivity and 93% specificity), NETsp – 7% (97% sensitivity and 95,2% specificity), NETst – 15% (86,4% sensitivity and 90% specificity) (Fig. 1, 2, 3).

In the case of pre-operative values of NBTsp, NETsp, NETst below the cut-off ones, rejection of the autodermotransplant is predicted, and for the values of NBTsp, NETsp, NETst above the cut-off values prior to the operation, the engraftment of the flap is predicted. Thus, the conducted analysis showed the probability of using the indicators of NBTsp, NETsp, NETst as the predictors of the outcome of the SG operation in patients with CW. Complex application of these indicators allows predicting the risk of acute skin flap rejection with a probability of up to 92%, and, consequently, planning correctly the terms of surgery and the need for additional pre-surgical preparation of patients.

Fig. 3. The balance point between sensitivity and specificity and the threshold value of NETst.
The Y axis represents the sensitivity and specificity values, expressed in %/100. On the X axis, the values of NETsp in %. Threshold value of NETsp – a point of intersection of curves (balance between sensitivity and specificity)



The retrospective analysis was conducted at the stage of assessing the clinical informative value of the PNL functional activity indicators and revealing the predictors of the SG outcome. After establishing the possibility of using these markers to predict the risk of acute skin flap rejection, the effectiveness of their application on specific clinical examples was evaluated. The following clinical cases are demonstrated with the consent of the patients.

Clinical case 1. A 59-year-old female patient was hospitalized with a diagnosis of a post-traumatic granulating wound of the left foot (Fig. 4 A). The wound was obtained at home as a result of a mechanical trauma (the impact of a solid object), wound duration-related to healing rates was 3 months. The patient underwent preoperative preparation using ultrasound debridement therapy. On a day of the planned SG, a clinical evaluation of the wound was made for the readiness to perform plastic closure (Fig. 4 B). Before the operation of SG, the patient received the heparinized venous blood (5 ml), followed by the evaluation of the

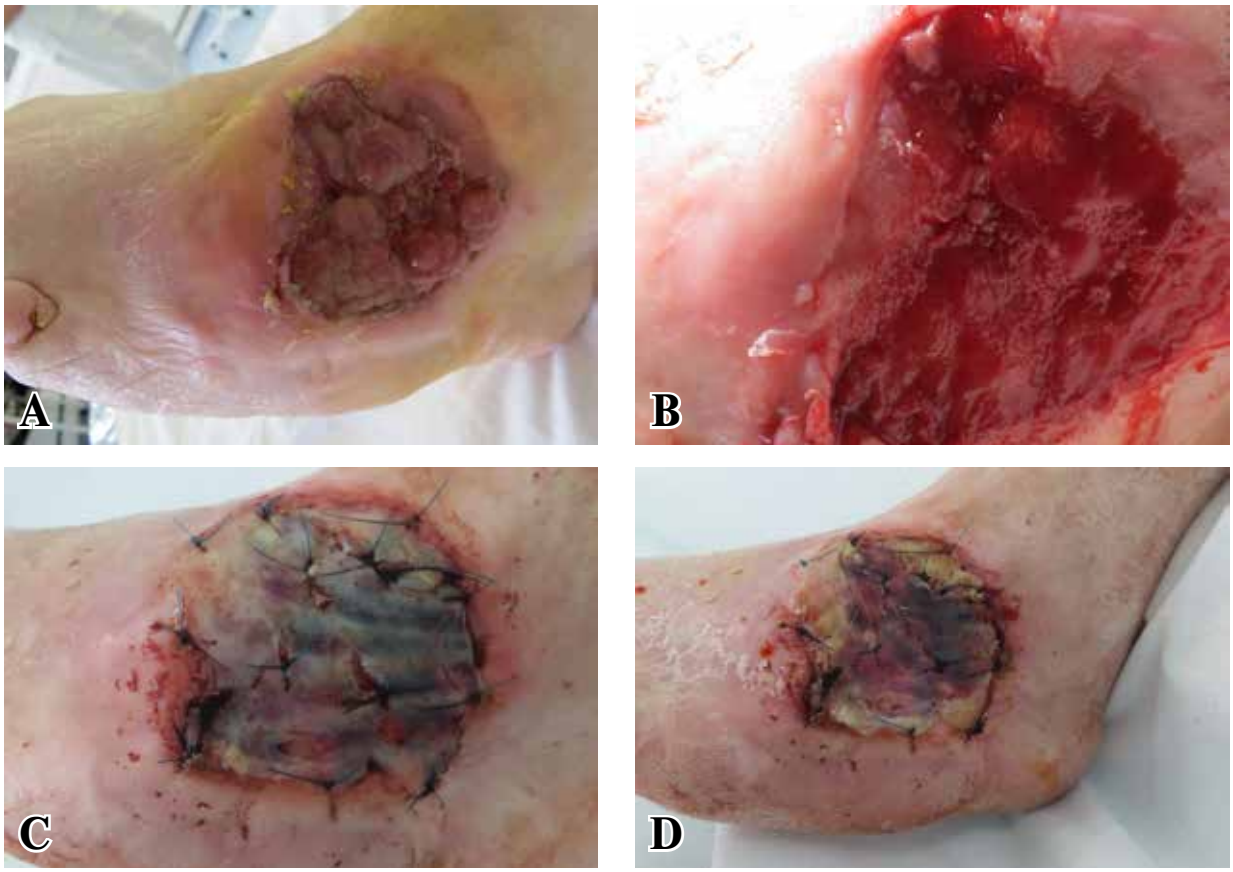


Рис. 4. Dynamics of the wound state in a patient with a complicated course of the postoperative period of ADP. A – CW appearance on hospitalization; B – wound appearance before SG (correspondence to clinical signs of readiness); C – the state of the transplanted skin flap on the 2nd day after SG; D – Signs of regression of the autodermotransplant (6th day)

parameters of the functional activity of the PNL (NBTsp, NETsp, NETst) The following results were obtained: NBTsp=8%, NETsp=4%, NETst = 10%, serving a basis for predicting the unfavorable outcomes SG. In the incisional period the signs of flap instability (Pallort, absence of fixation) with a complete rejection on the 6th day after the operation (Fig. 4 C, D) were observed. This required the physiotherapy treatment provision, the prescription of vascular drugs, the application of special wound dressings for additional wound preparation, and performance of the repeated SG.

Clinical case 2. A 57-year-old female patient was hospitalized with a diagnosis of the post-traumatic granulating wound of the right femur (Fig. 5 A). The wound was obtained at home as a result of a thermal injuries caused by extreme hot water temperatures; the wound period was 60 days. The patient underwent preoperative preparation using ultrasound debridement procedure (Fig. 5 B). On a day of the planned SG, the evaluation of the wound was made for its readiness of performing plastic closure. Before SG, the patient received the heparinized venous blood (5 ml), followed by the evaluation of the parameters of the functional activity of PNL. The following results were obtained: NBTsp = 27%, NETsp = 14%, NETst = 26%, which was the basis for predicting the successful outcome of SG. The transplanted skin flap was fixed for 2-4 days after the operation with full engraftment on the 10th-13th day after the operation (Fig. 5 C, D).

Discussion

According to the opinion of different authors, the effectiveness of SG in the complex treatment of patients with CW can reach 98%, when a number of conditions are followed as by the recipient wound (absence of signs of infection, necrosis, appearance of mature granulations and marginal epithelialization), as by the split flap [14, 15]. However, according to other data, even when complete clinical readiness for SG is achieved, the percentage of complications of the plastic wound closure such as instability of the graft, as well as the development of necrosis and lysis of the transplanted flap, ranges from 4 to 30% [16].

Recent researches in the field of biochemistry, pathophysiology, immunology explain the

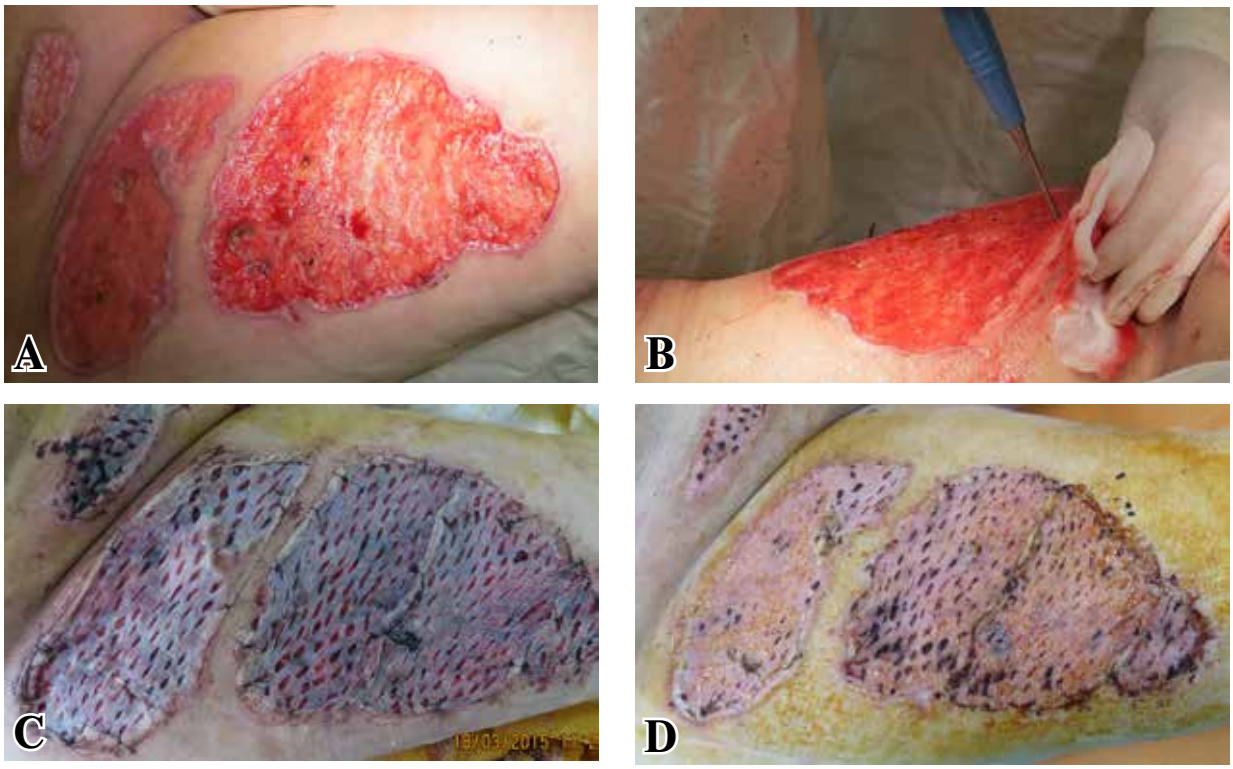


Fig. 5. Dynamics of wound state in a patient with a successful ADP result. A – CW appearance on hospitalization; B – wound appearance after its preparing and before ADP (correspondence to clinical signs of readiness); C – the state of the transplanted skin flap on the 2nd day after ADP; D – complete engraftment of the flap (13th day after ADP)

new mechanisms of prolonged pathological inflammation and chronic wound healing, the imbalance of immune mechanisms is considered to be one of them. In the process of carrying out their basic functions to eliminate the microbial factor and to cleanse the wound, the PNL synthesize a large number of ROS. Phospholipids of cell membranes and cellular organelles are the main substrate of free radical processes and lipid peroxidation occurs when ROS interact directly with them.

ROS are involved in all stages of wound healing, such as migration, adhesion, proliferation, neovascularization and remodeling. ROS in the early stages of wound healing provide the growth of connective tissue, reinforcement the accumulation of macrophages in the wound area, activation the proliferation of fibroblasts and the synthesis of collagen. At the same time, ROS and the products of their action serve as an element of protection against foreign objects, since they possess by an antibacterial effect, providing the effector function of PNL. ROS provide resorption of necrotic tissue in the process of wound purification due to denaturation and proteolysis of the products of necrosis.

To fight infection and to debride the wound, PNL consume a large amount of oxygen to produce ROS. In this regard, untimely closure of the wound causes the state of hypoxia [1, 5]. As a result, PNL will not be able to synthesize completely ROS and the fight with infection will become ineffective, causing complications of plastic wound closure.

Taking into account that PNL as the main initiators of free radical oxidation and producers of ROS, assessment of their status is thought to be a valuable diagnostic study in monitoring treatment of CW and assessing the wound healing, including predicting the outcome of plastic wound closure.

Conclusions

1. To predict the outcome of SG (skin flap engraftment/rejection) in patients with CW to use the indicators of the functional activity of PNL (NBTsp, NETsp, NETst) is recommended taking into account changes in their values relative to the cut-off ones. The optimal cut-off values are: NBTsp 15% (86.5% sensitivity and 93% specificity), NETsp – 7% (97% sensitivity and 95.2% specificity), NETst – 15% (86.4% sensitivity and 90% specificity).

In the case of pre-operative values of NBTsp, NETsp, NETst below the cut-off, the autoreceptor rejection is considered to be predicted, and at NBTsp, NETsp, NETst values above the cut-off prior to surgery, analysis demonstrates engraftment rates with a probability up to 92%.

2. The determination of the indicators of PNL functional activity (NBTsp, NETsp, NETst) is highly recommended for monitoring the effectiveness of additional preoperative preparation in the SG planning.

Clinical cases are demonstrated with the consent of patients

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