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# Морфологическая и иммуногистохимическая характеристика интенсивности репаративных процессов в мягких тканях нижних конечностей у лиц с нейропатической и нейроишемической формами синдрома диабетической стопы

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***Цель.*** *Оценить морфологические и иммуногистохимические параметры формирования грануляционной ткани у пациентов с синдромом диабетической стопы.*

***Материалы и методы.*** *Проанализированы гистологические (световая микроскопия) и иммуногистохимические (CD68, остеопонтин, MMP-9, TIMP-1) показатели репаративных процессов мягких тканей нижних конечностей у пациентов с сахарным диабетом. Обследованы 63 пациента с синдромом диабетической стопы после хирургической обработки.*

***Результаты.*** *После хирургической обработки в ранах отмечался выраженный отек, плохо организованный экстрацеллюлярный матрикс (ЭЦМ), низкое содержание фибробластоподобных клеток и выраженная воспалительная инфильтрация, выявлено наличие молодой грануляционной ткани. По результатам иммуногистохимического (ИГХ) исследования у всех обследованных больных отмечалось умеренное количество макрофагов (иммунопозитивных с антителами к CD68) и интенсивная окраска матриксной металлопротеазы-9 (MMP-9), слабое окрашивание тканевого ингибитора металлопротеаз-1 (TIMP-1) и остеопонтина (OPN).*

***Заключение.*** *Согласно полученным данным гистологического и иммуногистохимического исследований, репаративные процессы в мягких тканях нижних конечностей у обследованных лиц с сахарным диабетом замедлены.*

***Ключевые слова****: сахарный диабет; хронические раны; репарация; иммуногистохимические маркеры; гистология*

## The morphological characteristic of tissue repair in patients with neuropathic and neuroischemic forms of diabetic foot syndrome

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***Aim****. To evaluate the morphological and immunohistochemical features of granulation tissue formation in patients with diabetic foot syndrome.*

***Materials and methods****. We analysed the histological (light microscopy) and immunohistochemical (CD31, CD68, osteopontin, MMP-9 and TIMP-1) features of tissue repair processes in patients with diabetes mellitus. The study involved 63 patients with diabetic foot syndrome after surgical debridement.*

***Results****. We found severe intercellular oedema, poorly organised extracellular matrix, small amounts of fibroblast-like cells and expressed inflammatory infiltration, along with the presence of young granulation tissue. According to the results of the immunohistochemical studies, there were a moderate number of macrophages (immunopositive with antibodies to CD68), intense staining of MMP-9 and weak staining of TIMP-1 and osteopontin.*

***Conclusion****. According to the findings of the histological and immunohistochemical studies, tissue repair processes in patients with diabetes mellitus are decelerated.*

***Key words****: diabetes mellitus; chronic wounds; repair; immunohistochemistry; histology; repair markers*

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Diabetes mellitus (DM) is an extremely important medical and social problem. In 2014, the International Diabetes Federation published data showing that the number of people with DM worldwide had reached 382 million [1]. One of the serious complications of the disease is diabetic foot syndrome (DFS). Its most typical manifestation is development of chronic wounds (trophic ulcers) of the lower limbs,

leading to a decrease in the patient's quality of life and significant treatment costs [2]. Chronic wounds of the lower extremities of various origins are found in 30%–80% of persons with DM, which is due to slow repair processes in the presence of impaired carbohydrate metabolism.

Normal wound healing involves a series of successive biological and molecular processes that begins with the migration and proliferation of cells in the area of the injury and the restructuring of the extracellular matrix (ECM) and ends with the re-establishment of newly formed tissue and blood vessels. Efficient repair is impossible without timely and accurate cell responses, including the activation of keratinocytes, fibroblasts, endothelial cells, macrophages and platelets [3], which synthesise various growth factors and cytokines that regulate the process of wound healing that concludes with epithelialisation [4]. There are five phases of normal wound healing: coagulation, inflammation, proliferation, migration (including ECM restructuring) and remodelling [3, 5]. During the coagulation and inflammation phases, under conditions of hypoxia, the following processes occur: platelet aggregation and release of fragments of fibrinogen and other proinflammatory mediators, fibrin clot formation, release of growth factors and cytokines, chemotaxis and wound healing. These processes involve platelets, neutrophils, macrophages and monocytes. The next phase, proliferation, lasts several days and involves migration of keratinocytes and interactions between matrix metalloproteinases (MMPs), integrins and cytokines, resulting in the migration of activated cells and ECM products as well as the initiation of neoangiogenesis. The most important cells during this phase are keratinocytes, fibroblasts and endothelial cells. During the next, quite long, phase (up to several months), remodelling occurs, which includes scar formation and ECM degradation, followed by contraction and resistance to extension. The most important cells in this phase are myofibroblasts.

In acute wounds, these are parallel processes, but this feature is absent in chronic wounds. A wound is considered chronic if it does not heal within a period of time that is considered normal for the skin defects of the specific type and localisation. In the healing process of an acute wound, there is a clear chronological order of events; in a chronic wound, such balance is absent. Chronic wounds of diabetic or other genesis (pressure ulcers or venous and ischemic ulcers) are often stuck in one phase of healing or another; thus, the healing process is slowed down [6]. It is believed that in the presence of long-lasting and poorly controlled DM, the local expression of growth factors is reduced because of the development of advanced micro- and macrovascular complications [7].

Currently, there are numerous studies on the role of cytokines and growth factors in wound healing in patients with diabetes. However, there is no uniform assessment of the role of these molecules in tissue repair. Hyperglycaemia leads to the disruption of immune responses, which greatly increases the risk of secondary infection. Protein glycation alters their structure and function, and this plays an important role in the development of microvascular complications of DM [7]. According to the experimental studies, a high concentration of glucose in cell culture inhibits the proliferation of human fibroblasts, bovine endothelial cells and mice primary skin keratinocytes [8]. Fibroblasts lose their sensitivity to stimulation by growth factors, and endothelial cells and human macrophages start to produce higher quantities of MMP [8].

In diabetic conditions, chronic wound defects remain in a phase of inflammation for a longer period of time than conditions of normal carbohydrate metabolism, and there is a decreased activity of inflammatory cells and slow restructuring of ECM. The above problems contribute to the transformation of an acute injury into chronic [6]. In diabetic patients, the number of inflammatory cells is reduced, there is a lack of growth and migration of epidermal cells and there is luminal narrowing and occlusion of capillary blood vessels at the bottom of the wound [9,10]. Impaired carbohydrate metabolism is accompanied by the sluggish phagocytic activity of leucocytes, which results in changes in neutrophil and macrophage migration into the wound. All together, these deficiencies increase the risk of wound infection in patients with DM [6]. Numerous studies demonstrated decreased activity or low rates of growth factor production [3, 4, 11], vascularisation defects [3], decreased macrophage activity [11] and accumulation of collagen, including impaired barrier function of the epidermis and deterioration of granulation tissue quality [3] in patients with diabetes.

One condition of normal wound healing is adequate tissue oxygenation, which is provided by the developing vasculature. Vascular endothelial growth factor (VEGF) stimulates neoangiogenesis, thereby enhancing tissue oxygenation (pO2) [11]. Some studies have shown that the number of blood vessels, detected by staining with antibodies to CD31 and by histological methods, was higher in patients with diabetes whose wound defects were epithelialised in a shorter period of time than in a group of patients with non-healing trophic ulcers of the lower extremities [11].

Macrophages play an important role in the healing process. They are one of the key participants of cellular repair, since they are involved in wound cleansing and produce a number of cytokines and growth factors (VEGF, platelet-derived growth factor [PDGF]) and affect the content of ECM and its remodelling [10, 11]. Some studies demonstrated a gradual decrease in the number of macrophages on day 5 after injury in experimental animals with diabetes compared with the control group [12]. An important role in repair is played by cell matrix protein osteopontin, which regulates cell adhesion and migration and plays a role in the formation of granulation tissue. PDGF, produced by macrophages, stimulates the synthesis of osteopontin by fibroblasts [13]. Osteopontin is important for the formation of collagen, macrophage activation and inhibition of enzymes that destroy the ECM, such as MMP [13]. It plays a key role in the interaction between osteoblasts and osteoclasts in a bone

remodelling [13]. MMPs are also involved in the reparative processes. They play a role in cell migration, growth factor production, and ECM rebuilding. MMP-9 destroys type 4 and 7 collagen chains, which are major components of the basal membrane. MMP-10 (stromelysin-2) degrades non-collagen matrix components [14]. During the healing process, fibroblasts and myofibroblasts produce tissue inhibitors of metalloproteinases (TIMP), which decelerate the action of MMP and suppress its activity [14]. High levels of MMP-9 in the wound fluid indicates inflammation and is a marker of poor wound healing in diabetes [15]. Other studies suggest the use of the ratio of MMP-9/TIMP-1 as a predictor of wound healing in the DFS [16]. Furthermore,

It was shown that the ratio of MMP-9/TIMP-1 in the exudate decreased during the successful treatment of wound defects in patients with type 2 DM, in contrast to the group of poorly healing trophic ulcers, where this ratio was high [16].

Thus, the process of wound healing in patients with DM is extremely complex and not fully understood. Understanding the mechanisms of repair will help in finding ways to treat patients with diabetes and will significantly improve their therapeutic prognosis.

## Aim

The purpose of this study was to evaluate the morphological characteristics of the reparative processes in the soft tissues of the lower extremities in patients with neuropathic and neuroischaemic forms of DFS.

## Materials and methods

The study included 63 patients (21 women and 42 men) with neuropathic and neuroischaemic forms (after revascularisation and without critical ischaemia) of DFS who underwent surgical treatment of the wound defect. After the surgical treatment, tissue samples were taken for biopsy, followed by histological and immunohistochemical (IHC) studies. Table 1 shows the clinical characteristics of the examined patients.

IQR, interquartile range; DM, diabetes mellitus; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate.

All laboratory tests were performed according to standard methods of diagnostic laboratories at Endocrinology Research Centre, Moscow, Russian Federation (Ilyin A.V., Head of department).

The area of the wound was determined by delineating the contours of the wound through the transparent film (Opsite Flexigrid, Smith & Nephew), followed by a calculation of the area inside the contour. For transcutaneous oximetry, Radiometer (Denmark) equipment was used. Sensors were positioned at a distance of 0.5–1 cm from the edges of the wound. Morphological studies were performed at the Laboratory of Histology and Immunohistochemistry jointly with Criobank group FGBI Endocrinology Research Center (Department Head prof. A.J. Abrosimov). Biopsy material was analysed using histological and IHC techniques. Biopsy samples (0.5 mL) were taken from the central area of the wound bed. The material was fixed in 10% formalin solution and then embedded in paraffin in the usual manner. Serial sections (3–5 μm thick) were deparaffinised in the standard way and stained with hematoxylin and eosin. Histological evaluation of the severity of oedema and ECM was performed using semi-quantitative scoring as follows: +, poorly expressed; ++, moderately expressed; +++, strongly expressed; and ++++, very strongly expressed.

Granulation tissue maturity was evaluated by determining the area of necrosis, number of blood vessels, severity of inflammatory infiltrate and cell composition (polymorphonuclear leucocytes,

Table 1

Clinical characteristics and laboratory test results of patients

| Characteristic | Number of patients (n = 63) or median (IQR) |
|---|---|
| Age, years | 60 (51, 66) |
| DM type | |
| 1 | 11 |
| 2 | 52 |
| Duration of DM, years | 15 (10, 20) |
| HbA1c, % | 8.7 (7.6, 10) |
| Cholesterol, total, mmol/L | 4.3 (3.4, 5.2) |
| HDL, mmol/L | 0.7 (0.6, 1.0) |
| LDL, mmol/L | 2.4 (1.9, 3.2) |
| Triglycerides, mmol/L | 1.5 (1.2, 2.2) |
| Haemoglobin, g/L | 120 (114, 132) |
| Leucocytes, 109/L | 7.6 (6.3, 9.8) |
| Total serum protein, g/L | 72 (69, 75) |
| ESR, mm/h | 61 (39, 75) |
| GFR, mL/min/1.73 m2 | 85 (65, 102) |
| Wound area, cm2 | 19.1 (15, 34.2) |
| Wound depth, cm | 3.1 (2.2, 4.8) |
| Wagner stages, number of patients | |
| II | 33 (52%) |
| III | 30 (48%) |
| Foot soft tissue oxygenation, mmHg | 45 (38, 51) |
| Diabetic neuropathy | |
| Absent | 3 |
| Present | 60 |
| Diabetic nephropathy | |
| Absent | 19 |
| Microalbuminuria | 24 |
| Proteinuria | 12 |
| End-stage renal disease | 8 |
| Diabetic retinopathy | |
| Absent | 12 |
| Non-proliferative | 30 |
| Pre-proliferative | 6 |
| Proliferative | 15 |

lymphocytes, macrophages and fibroblasts). Granulation tissue development was subdivided into three types: young, intermediate, and mature. Young granulation tissue is rich in cells and thin-walled blood vessels in the connective tissue, with a large number of lymphocyte-like, undifferentiated connective tissue cells, leucocytes, plasma cells and mast cells between blood vessels. In intermediate (transitional) granulation tissue, differentiation of cells, fibrous structures and blood vessels continues. The number of haematogenous cells decreases, and the number of fibroblasts increases. Because of the production of collagen by fibroblasts, first argyrophilic and then collagen fibrils develop in the intercellular space. As the fibroblasts mature, the amount of collagen fibrils increases and they form collagen fibres. Mature granulation tissue is fibrous connective tissue.

The phases of inflammation were determined according to Kuzin (1990), with phase 1 characterized by a predominance of neutrophilic granulocytes, monocytes and macrophages; phase 2 characterised by regeneration and maturation of the granulation tissue, which is formed in separate pockets on the bottom of the wound and characterised by intense capillary neovascularisation, and large numbers of fibroblasts and development of collagen and elastin fibres; and phase 3 characterised by scar development and epithelisation, with granulation tissue being converted into a mature fibrous tissue with fibrocytes and coarse collagen fibres.

For IHC studies, the slides were processed on a Leica BOND-MAX immunostainer. Antibodies to TIMP-1, MMP-9, macrophage marker CD68 and osteopontin (Dako, Glostrup, Denmark) were used in standard dilutions and with positive controls. Expression of cytoplasmic markers (TIMP-1, MMP-9 and osteopontin) was assessed semi-quantitatively: +, only individual cells stained (<30% of the cells); ++, 30%–60% of cells stained; +++, 60%–90% of cells stained and ++++, >90% of cells stained.

The degree of expression of CD68 (a marker of macrophages and myeloid histiocytic cells) was estimated semi-quantitatively from the total number of immunopositive cells and varied in the following ranges: 5%–10%, 10%–15%, 15%–20%, 20%–30% and 30%–40% of cells stained with specific antibodies. Photomicrographs were taken with a DFC450 C camera (Leica, Germany).

The study protocol was approved by the Ethics Committee of Endocrinology Research Centre (28 November 2012; Minutes No. 18). All patients signed an informed consent.

Statistical analysis was performed using Statistica software version 7.0 (StatSoft). Statistical parameters used for analysis were median, interquartile range and Spearman's rank correlation coefficient.

## Results

The paper presents for the first time a comprehensive assessment of the level of intensity of repair processes in the soft tissues of the lower limbs in patients with DFS using chosen morphological techniques.

Biopsy material was taken from the bottom of the wound defects after surgery but before local therapy treatment during the study. The biopsy material was subjected to histological and histochemical studies.

All wounds were characterised by marked intercellular oedema with a median severity of 3 (3, 4); poorly organised ECM with a median value of 2 (1, 2); low content of fibroblast-like cells and significant inflammatory infiltration and the presence of young granulation tissue with a median value of 1 (1; 2). Despite the fact that in many patients wound healing process was protracted and resistant to local treatment, surgical treatment allowed a transformation from a chronic to an acute process. The majority of patients studied had wounds in inflammation phase with a median value of 1 (1, 2). Figure 1 shows a typical histological picture of the biopsy material from a wound defect before local treatment.

**IHC features of wound healing process in patients with DFS**

IHC studies demonstrated the presence of a moderate amount of macrophages (immunopositive to CD68 antibodies), intensive staining for MMP-9 and weak staining for TIMP-1 and osteopontin in all patients (Table 2).

Despite the long history of diabetes in the examined patients, there was no correlation (Rs = -0.01) between the duration of the disease, the value of glycated haemoglobin (HbA1c) and the intensity of the reparative processes in the soft tissues of the lower limbs.

Figures 2–5 show the results of IHC studies of wound biopsies after the surgical treatment.

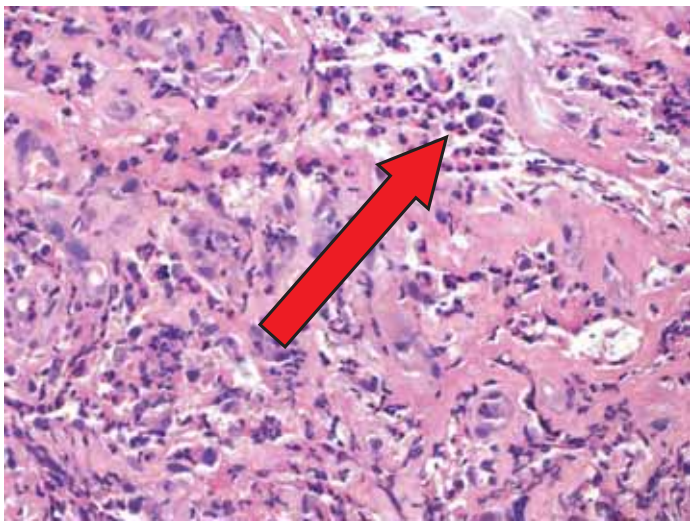

Fig. 1. Typical histology of biopsy tissue from the wound defect before treatment (stain: hematoxylin–eosin, ×400). The arrow indicates the inflammatory infiltrate and the surface necrotic layer, which consists mostly of leucocytes and lymphoid cells at different stages of maturity. Marked intercellular oedema and developing granulation tissue are seen.

## Discussion

Chronic wound defects of the lower extremities in diabetic patients are becoming increasingly common due, in particular, to the growing incidence of DM. Healing processes are slowed down when carbohydrate metabolism is impaired, but the pathophysiological basis of this fact is not fully understood. The purpose of this study was to evaluate the morphological and IHC repair indicators in patients with DM.

This paper presents a comprehensive study of 63 patients with DFS, aged 32–78 years old, who were admitted to the Department of the Diabetic Foot at FGBI Endocrinology Center, Ministry of Health, Russian Federation, between September 2012 and December 2014. All patients underwent surgical treatment of the wound defect, antibacterial therapy and reduction of load on the affected limb. During in-patient treatment, all patients underwent correction of dietary therapy with the goal of achieving individual targets for glycaemia indicators [17].

Clinical examination and laboratory test results showed that the median HbAqc levels in all patients was 8.7% (7.6, 10); thus, the carbohydrate metabolism of the patients was unsatisfactory. Christman et al. [18] demonstrated that the rate of lower-extremity wound healing in patients with diabetes is directly correlated to the level of HbA1c. With HbA1c ≤7%, the healing rate was 0.197 cm2/day; if HbA1c was 7%–8%, wound defects decreased in size at the rate 0.157 cm2/day; in patients with HbA1c ≥8% the rate of healing was 0.028 cm2/day.

Most patients did not have diabetic nephropathy or were at the stage of microalbuminuria, which means that protein deficiency as a factor unfavourable for tissue

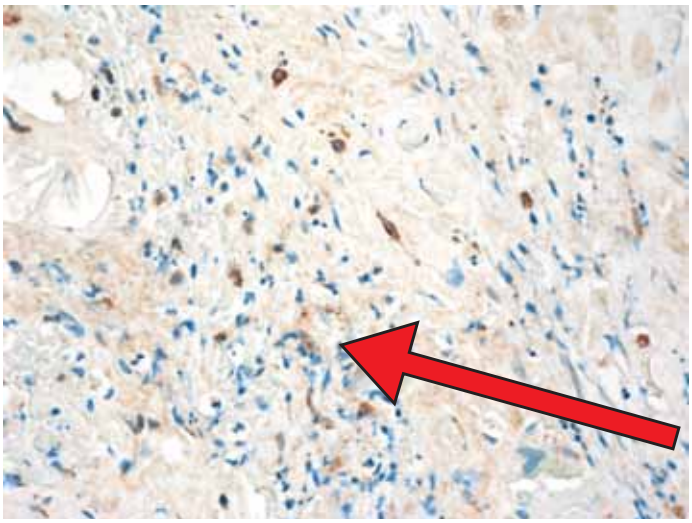

Fig. 2. Immunohistochemical photomicrograph of the wound defect biopsy before treatment showing staining of macrophages with antibodies to CD68 (×200). The arrow indicates a moderate amount of stained macrophages.

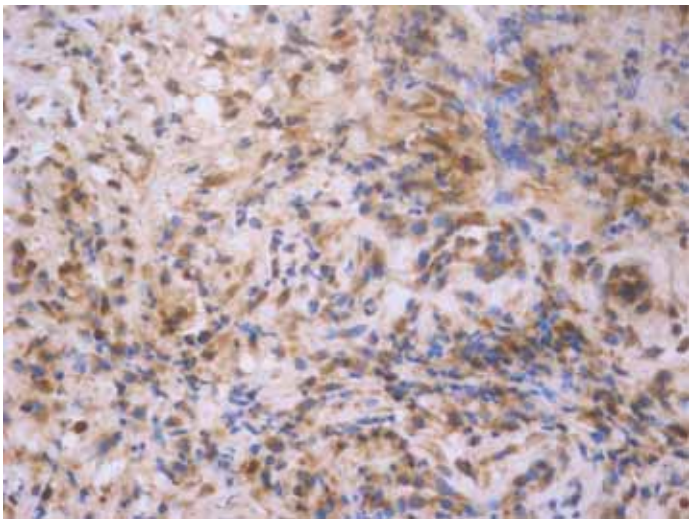

Fig. 4. Extracellular matrix staining pattern with antibodies to matrix metalloproteinase-9 (×400). The marked expression indicates an increased proteolytic activity.

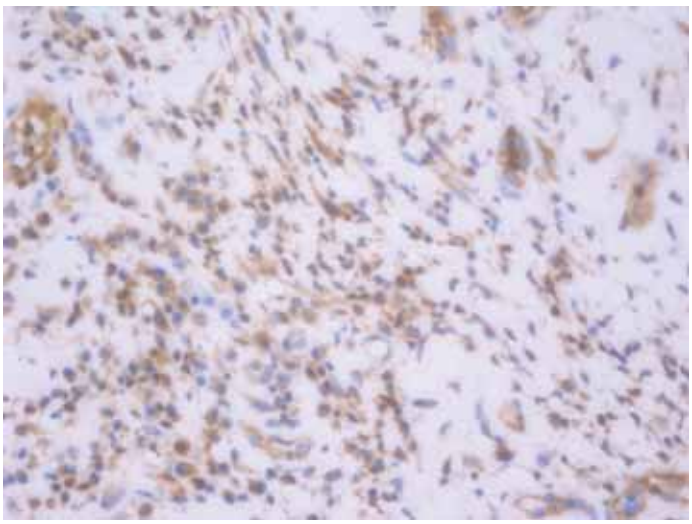

Fig. 3. Immunohistochemical photomicrograph of the wound biopsy before treatment showing staining of fibroblasts with antibodies to osteopontin (×400).

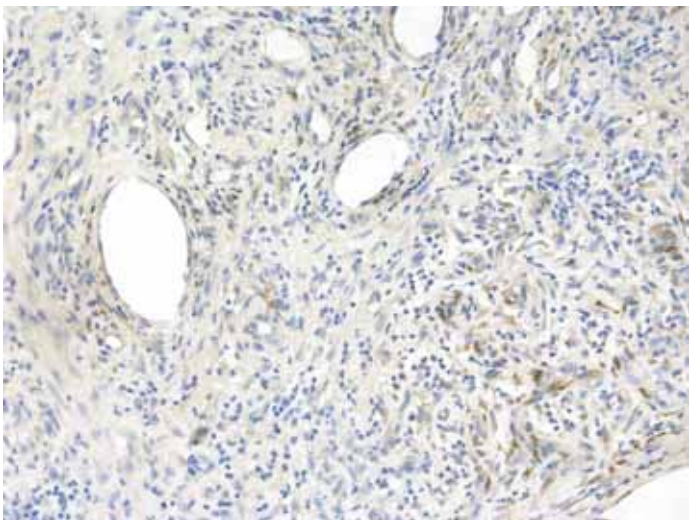

Fig. 5. Extracellular matrix (ECM) staining pattern with antibodies to tissue inhibitor of metalloproteinases-1 (×400) before treatment showing weak ECM staining.

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Table 2

Expression of repair process markers according to immunohistochemical studies

| Marker | Median (IQR) |
|---|---|
| Amount of macrophages; staining with antibodies to CD68 | 10 (10, 20) |
| OPN | 3 (2, 3) |
| MMP-9 | 3 (3, 4) |
| TIMP-1 | 1 (1, 3) |

IQR, interquartile range; OPN, osteopontin; MMP matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

healing was absent, which was confirmed by laboratory tests.

The majority of patients had diabetic polyneuropathy. Despite the fact that in a small number of patients, there was no decrease of peripheral sensitivity, hyperkeratosis and dyshidrosis were present, which allowed us to interpret the above symptoms as the initial manifestation of a reduction of the peripheral innervation.

The histological examination of wound defect biopsy samples taken before local treatment showed that all patients had oedema, poorly organised ECM, low content of fibroblast-like cells and a significant inflammatory infiltrate. All wounds were in the inflammation phase of healing. These findings are consistent with those of Boulton et al. who demonstrated that diabetic wounds have a long inflammatory phase, decreased activity of inflammatory cells and delayed ECM reorganisation. These disturbances prevent healing and foster the transition of the wound from the acute to the chronic form [6].

According to Baltziz [8], the interaction between glycated collagen, ECM and fibroblasts or endothelial cells prevents cell adhesion, proliferation and reduction of the wound defect size. IHC studies also demonstrated the slowing of reparative processes in the soft tissues of the lower extremities in patients with DM. In the present study, we found that the level of osteopontin expression was reduced in the majority of patients. This is consistent with the literature, which refers to the decrease in the expression of osteopontin in patients with impaired glucose metabolism [13]. It was suggested that one of the reasons for the reduced rate of reparative processes in patients with DM is a decrease in osteopontin expression in the wound bed, which manifests as deficient migration of immunocompetent cells, accumulation of decomposition products, decreased number of endothelial cells, slow neoangiogenesis and disorganisation of ECM [13].

Moderate-intensity staining for macrophage marker CD68 was noted in all patients. In experiments on laboratory models, it was established that in the presence of DM, it takes longer for macrophages to accumulate in the wound, which may be associated with increased levels of pro-inflammatory cytokines and proteases and reduced levels of growth factors. Similar results were obtained in patients with diabetes [8].

The increased MMP-9 expression and weak staining of TIMP-1 observed following surgical treatment indicate high proteolytic activity in wounds, and according to some studies, result in slower collagen reorganisation and is predictive of poor prognosis in wound healing. For example, Liu et al. [15] noted that high MMP-9 levels in the wound fluid is indicative of inflammation and is a marker of poor wound healing in patients with DM. Other researchers suggested using the ratio of MMP-9/TIMP-1 as a predictor of wound healing in patients with DFS [16]. Although there was no control group in the present study, our results are consistent with published data.

Thus, on the basis of these data, we can assert that in patients with DM, reparative processes in soft tissues are slowed down, but currently, the amount of data on the long-term results of treatment is insufficient, and further research is required.

## Conclusions

1. According to the histological study, patients with DFS had marked intercellular oedema, poorly organised ECM and wounds in the phase of inflammation and formation of granulation tissue.
2. Patients with chronic wounds of the lower extremities and DM have increased proteolytic activity of granulation tissue, which manifested itself in the increased MMP-9 expression and decreased TIMP-1 expression, according to the IHC results.
3. We observed moderate quantities of macrophages (see results of the staining with antibodies to CD68) and moderate expression of osteopontin, indicating that the wounds were staying in the initial phases of wound healing, which was confirmed histologically.

## Funding information and conflict of interest

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The authors declare the absence of explicit and potential conflicts of interest associated with the publication of this paper.

The contribution of each author: E.L. Zaitseva — collection and processing of material, data analysis and writing of the text; Doronina L.P. — collection of material; Molchkov R.V. — a histological study; Voronkova I.A. — immunohistochemical studies; Tokmakova A.Y. — the concept and design of the study.

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