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Immunohistochemical Assessment of the Possibility of Using a Number of Regenerative Technologies and High-Intensity Laser Radiation in Chronic Perineal Wounds Treatment

Conflict of interest: nothing to declare.
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Abstract

Purpose. Based on the assessment of the expression of the main collagens and matrix metalloproteinases, to establish the effectiveness of the experimental use of local cell therapy and high-intensity laser radiation of certain parameters in the treatment of chronic perineal wounds.
Materials and methods. After modeling a chronic wound of the perineum in animals of the first group A (n=24), the area of the chronic wound was exposed to defocused laser radiation with a wavelength of 1560 nm (0.1 s / 0.1 s, 5 W, light spot diameter 0.7 cm, distance 2 cm, total exposure 10 s). In the second group of animals B (n=24), a suspension of allogeneic adipose tissue mesenchymal stem cells (ATMSC) (1 ml, 500 thousand/ml) was injected into the edges and bottom of the wound. In the third group of animals B (n=24), 2 ml of allogeneic platelet- and leukocyte-rich plasma (L-PRP) was injected into the edges and bottom of the wound. In the fourth group G (n=24), 2 ml of physiological sodium chloride solution was injected once into the edges of the wound (placebo control). At various times after exposure, molecular biological markers in the tissues of laboratory animals were assessed using the immunohistochemical method using mono- and polyclonal antibodies.
Results. Immunohistochemical assessment of tissues with assessment of the level of expression of type I and III collagens at different times has established its multidirectional (multi-level) nature, depending on the type of therapeutic method used, the timing of the manifestation of the biological effect and the specifics of the individual reaction of laboratory animals to the therapeutic effect. At the same time, the use of all therapeutic technologies in dynamics reliably leads to a decrease in the activity of MMP-1 and MMP-9

production in comparison with the placebo control group, correlating with changes in the level of collagen expression, involution of inflammatory-degenerative processes in the wound and the level of microbial colonization of wounds. To a greater extent, this process is observed in the group of animals using laser radiation (at all times, starting from the 5th day of observation).

Conclusion. In view of the significant regenerative effect of the analyzed methods in relation to chronic perineal wounds identified in experimental studies, local use of laser radiation and cell therapy in clinical conditions should be considered appropriate.

Keywords: chronic wound, laser radiation, mesenchymal stem cells, platelet- and leukocyte-rich plasma, collagens, metalloproteinases

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Иммуногистохимическая оценка возможности применения ряда регенеративных технологий и высокоинтенсивного лазерного излучения в лечении хронических ран промежности

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Резюме

Цель. На основании оценки экспрессии основных коллагенов и матричных металлопротеиназ установить эффективность экспериментального использования при лечении хронических ран промежности локальной клеточной терапии и высокоинтенсивного лазерного излучения определенных параметров.

Материалы и методы. После моделирования хронической раны промежности у животных первой группы А (n=24) на область хронической раны осуществляли воздействие расфокусированным лазерным излучением длиной волны 1560 нм (0,1 с / 0,1 с, 5 Вт, диаметр светового пятна 0,7 см, дистанция 2 см, суммарная экспозиция 10 с). Во второй группе животных Б (n=24) в края и дно раны инъекционно вводили суспензию аллогенных мезенхимальных стволовых клеток жировой ткани (МСК ЖТ)

(1 мл, 500 тыс/мл). В третьей группе животных В (n=24) в края и дно раны вводили 2 мл аллогенной обогащенной тромбоцитами и лейкоцитами плазмы (L-PRP). В четвертой группе Г (n=24) в края раны однократно вводили 2 мл физиологического раствора поваренной соли (плацебо-контроль). В различные сроки после воздействия проведена оценка молекулярно-биологических маркеров в тканях лабораторных животных иммуногистохимическим методом с использованием моно- и поликлональных антител.

Результаты. Иммуногистохимическая оценка тканей с оценкой уровня экспрессии коллагенов I и III типа в различные сроки выявила его разнонаправленный (разноуровневый) характер, зависящий от вида применяемого лечебного метода, сроков проявления биологического эффекта и специфики индивидуальной реакции лабораторных животных на лечебное воздействие. При этом использование всех лечебных технологий в динамике достоверно приводит к снижению активности продукции MMP-1 и MMP-9 в сравнении с группой плацебо-контроля, коррелируя с изменениями уровня экспрессии коллагенов, инволюцией воспалительно-дегенеративных процессов в ране и уровнем микробной колонизации ран. В большей степени этот процесс наблюдается в группе животных с использованием лазерного излучения (во все сроки, начиная с 5-х суток наблюдения).

Заключение. Ввиду выявленного в экспериментальных исследованиях значимого регенераторного эффекта в отношении хронических ран промежности анализируемых методов следует считать целесообразным местное использование в клинических условиях лазерного излучения и клеточной терапии.

Ключевые слова: хроническая рана, лазерное излучение, мезенхимальные стволовые клетки, обогащенная тромбоцитами и лейкоцитами плазма, коллагены, металлопротеиназы

■ INTRODUCTION

Today, chronic wounds are a significant medical and social problem for society, significantly reducing the quality of life of patients, leaving a special imprint on medical activities and a huge financial burden on the economies of most countries of the world. In the USA alone, medical care is provided annually to 6.5 million patients with this pathology, with financial costs from the state budget for these purposes exceeding 25 billion dollars [1, 2]. In economically developed countries, the cost of treating this category of patients amounts to 2–4% of the total government spending on health care [3, 4]. Chronic wounds place a heavy burden on both patients and their families, with pain, infection, loss of function and significant financial costs, often leading to major surgery, amputation or sepsis. Only in 30–75% of patients such ulcers heal after 6 months of intensive treatment [5]. Today, this pathology is considered at the level of the most important medical and biological problems of humanity, such as population aging, obesity and diabetes mellitus. Chronic wounds are spreading throughout the world as a "silent epidemic", annually affecting the quality of life of more than 40 million people around the world, creating a serious threat to public health and the economy of most civilized countries of the world [4, 6–8].

Currently, a chronic wound is described as a tissue defect in a state of regeneration with a disruption in the chain of one or more phases of this process [9]. Most often, in a chronic wound, the regeneration process is disrupted at the stage of the inflammation and/or regeneration phase [10, 11]. Systemic factors leading to the development of a chronic wound are: old age, immunodeficiency conditions, diseases of the circulatory system with symptoms of heart failure, arterial hypertension, vasculitis, cachexia, polyneuropathy, stress, angiopathy, angiodysplasia, cancer, carbohydrate metabolism disorders (diabetes mellitus) , gout, antiphospholipid syndrome, collagenosis and other systemic (autoimmune) diseases [12–15]. Established local factors for the development of a chronic wound are: impaired arterial blood flow, impaired venous outflow, repeated tissue trauma, persistent infection, critical microbial colonization, local disruption of innervation, prolonged tissue edema, the presence of foreign bodies or fixed prostheses in the wound area, malignancy, continuous tension skin edges [2, 13, 14, 16].

A special place among the designated pathologies is occupied by chronic wounds of the perineum and sacrococcygeal region, characterized by a particular severity of the course and tolerance to many of the treatment methods used. The most important factors in their development are: constant mechanical traumatization of wounds and proximity to the natural excretory openings of the body with continuous or persistent contamination of the surface with pathogenic microflora (including anaerobic), as well as chemical components of urine and feces [2, 13]. In recent years, new data have been obtained indicating certain progress in understanding the cellular and molecular mechanisms of wound healing under normal conditions and in the presence of a number of diseases, as well as the causes leading to chronic transformation of wounds and the main factors contributing to this process.

■ PURPOSE OF THE STUDY

Based on the assessment of the expression of the main collagens and matrix metalloproteinases, to establish the effectiveness of the experimental use of local cell therapy and high-intensity laser radiation of certain parameters in the treatment of chronic perineal wounds.

■ MATERIALS AND METHODS

All experimental studies were carried out using white randombred rats of both sexes aged from 1 year to 2 years (Me=1.53 years, Q_{25}–Q_{75}=1–2) weighing from 210 to 286 g (M=254.4 ± 12.6 d) in the conditions of the pathophysiological, pathomorphological and immunological groups of the Research Laboratory of the state educational institution "Belarusian Medical Academy of Postgraduate Education". All studies were carried out in full accordance with modern principles and standards of bioethics [17, 18].

To simulate a chronic perineal wound under intramuscular anesthesia in the inguinal-femoral region at a distance of 1–1.5 cm from the anus, a microbial-fecal suspension was injected subcutaneously with a syringe at the rate of 0.5 ml per 200 g of animal weight. The suspension was prepared 10 minutes before the experiment from the contents of the animal's rectum (feces) by diluting it in a ratio of 1:4 with a physiological (0.9%) solution of table salt and subsequent filtration of the homogenate through 5 layers of medical gauze. After 3 days, an inflammatory infiltrate of 3.2±1.1 cm in diameter was formed in the injection area; by the 7th day, central softening of the infiltrate was

noted with the formation of a subcutaneous abscess in this area with an average size of 1.2±0.2 cm. During this period, under intramuscular anesthesia, an excision of the anterior wall of the abscess was performed with the transition of the incision line to the anal canal, resulting in the formation of a wound defect of the soft tissues of the anal-perianal area 1.5 ± 0.3 cm2 with putrefactive edges and bottom. On the 3rd, 7th and 14th days after opening the abscess, a microbial-fecal suspension was injected into the soft tissues of the edges of the wound defect from the side of the wound with an insulin syringe in combination with a medical gel for ultrasound of medium density "Mediagel" (Brookfield viscosity 23.0–27.0 Pa×s [corresponds to a viscosity of 12.0–14.0 Pa×s at a shear rate of (16.8±0.3) s-1]) in a 1:1 ratio with a total volume of 0.6 ml. After modeling the pathological process in all animals, the wound did not heal spontaneously within 30 days or more. The average time for complete spontaneous regeneration of a chronic wound was Me=39.7 (95% CI 32–44). Due to the lack of significant differences (in morphology, cytology, size) on the 21st and 30th days, in order to increase the efficiency of the experiment, it is the 21st day from the beginning of modeling the pathological process that is defined as the period of formation of a chronic wound in a laboratory animal and "readiness" wounds for experimental evaluation of the effectiveness of new therapeutic technologies.

Animals with a simulated pathological process were randomly divided into 4 groups. In animals of the first group A (n=24), the area of a chronic wound was exposed to defocused laser radiation with a wavelength of 1560 nm (0.1 s / 0.1 s, 5 W, light spot diameter 0.7 cm, distance 2 cm, total exposure 10 s). In most cases, laser exposure to the wound was carried out once using a medical device "Mediola Compact" (FOTEK, Republic of Belarus) using special devices for delivering laser energy to tissues. In the second group of animals Б (n=24), a suspension of allogeneic adipose tissue mesenchymal stem cells (ATMSC) (1 ml, 500 thousand/ml) was injected into the edges and bottom of the wound. ATMSC were injected into the wound area once. In the third group of animals В (n=24), 2 ml of allogeneic platelet- and leukocyte-rich plasma (L-PRP) was injected into the edges and bottom of the wound. L-PRP was injected into the edges of the wound once (usually at 4 points around the perimeter of the wound). In the fourth group Г (n=24), 2 ml of physiological sodium chloride solution was injected once into the edges of the wound (placebo control).

Assessment of the expression levels of molecular biological markers in the tissues of laboratory animals was carried out using the immunohistochemical method using mono- and polyclonal antibodies (Table 1) [19, 20]. Immunohistochemical (IHC) staining was carried out in accordance with the instructions of the antibody manufacturers and

Table 1
Characteristics of primary antibodies used in the study

Antibosy	Manufacturer's company	Type	Source	Dilution
Anti-Collagen I antibody	Abcam	Polyclonal	Polyclonal rabbit IgG	1 : 400
Collagen III Antibody	Thermo Fisher Scientific	Polyclonal	Polyclonal rabbit IgG	1 : 400
MMP-1 Antibody	Thermo Fisher Scientific	Monoclonal	Monoclonal murine IgG1	1 : 500
MMP-9 Antibody	R&D systems	Monoclonal	Monoclonal murine IgG	1 : 500

experimentally established titers. To do this, the sections were deparaffinized in xylene using 2 shifts of 10–15 minutes each. Next, the sections were rehydrated in alcohols of increasing concentration, using 2 shifts, 5 minutes each, followed by rinsing in distilled water. Unmasking of antigens was performed in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 10 minutes or in a water bath for approximately 40 minutes, after preheating the unmasking buffer. After completion of treatment, the preparations were cooled in solution at room temperature for at least 15–20 minutes. Endogenous peroxidase was blocked with 3% H_2O_2 for 20 minutes.

Incubation with primary antibodies was carried out in a humidified chamber for approximately 1 hour at room temperature or 24 hours at 12°C. The sections were incubated with the imaging system with a sufficient amount of HRP polymer conjugate pre-dropped for about 60 minutes at room temperature. The "Super PicTure Polimer Detection Kit Invitrogen Polimer Detection System (Invitrogen)" and "Expose Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (Abcam)" containing a complex of secondary antibodies and the chromogen diaminobenzidine (DAB) were used as imaging systems. Next, the chromogen diaminobenzidine (DAB) was applied to the sections at a concentration of 1 mg/ml with the addition of a 0.02% H_2O_2 solution. The incubation time was considered sufficient if the structures to be stained acquired a bright golden brown color while there was no background staining. After each procedure, sections were rinsed in several changes of phosphate-buffered saline (PBS). The preparations were placed in xylene for 1 minute, counterstained with Harris' hematoxylin, and embedded in Canada balsam. To control the activity of primary antibodies (excluding false-positive and false-negative results), one negative and one positive control staining were performed in each series. To obtain a negative control, instead of incubating with the primary antibody, sections were coated with 1% bovine serum albumin. For positive control, lung tissue (MMP-9) and skin (collagen I, III) were studied [19; 20].

The result of immunohistochemical (IHC) detection of collagen types I and III is presented in the form of homogeneous brown staining of the cytoplasm of smooth muscle cells, myofibroblasts, and extracellular matrix. Metalloproteinases are found in the cytoplasm of smooth muscle cells and fibroblasts in the form of uneven brown staining. The preparations were studied and microphotographs were taken using light microscopes Axio Imager (Zeiss, Germany) and DMLS (Leica, Germany). To carry out morphometry, micropreparations were photographed using a Leica DMLS microscope equipped with a JVC digital video camera (Japan) with a resolution of 7.0 MP at a magnification of 200 times and a minimum number of fields of view of 200. Digital images of non-overlapping fields of view with clear visualization of the nuclei and cytoplasm of cells were selected for analysis. Quantitative assessment of the expression of biomolecular markers was performed by analyzing a digital image using the "positive pixel count" algorithm and the morphometry program "Aperio Image Scope 12.1.0.5." [21]. In the process of software analysis of antibody expression, the intensity of brown color (DAB chromogen reaction products) was measured automatically by Aperio Image Scope and divided into 3 intensity levels and a negative reaction (red fields – pronounced expression, orange – moderately expressed, yellow – mild, blue and white color – lack of expression). To analyze the studied groups according to the nature of expression, positivity was calculated using the Aperio Image Scope program (in the positivity program interface), determined by the program using the standard algorithm for counting positive pixels "Positive Pixel Count v9".

The positivity index is the ratio of the number of positively stained pixels to the total number of pixels in the areas to be assessed; the expression index (EI) of biomolecular markers was calculated using the formula: EI=positivity ´ 100 (%).

To obtain a random sample of sections, a minimum of 10 series of sections were prepared from each paraffin block (20 sections in each series), every third section was selected from one series (a total of 10 microslides per animal). Morphometric analysis of histological preparations was carried out at a magnification of 200 in 20 randomly selected fields of view for each microslide (total 200 fields of view per rat), the area of the visual field was constant and amounted to 1268860.50 μm^2. Since the morphometric analysis data corresponded to a normal distribution, the mean EI value (M) ± standard deviation (S) was calculated for each rat based on 200 visual fields. Next, the EI indicators obtained for each subgroup of animals (n=6) were included in the statistical analysis (descriptive statistics). Thus, for each rat of the 6 rats in the subgroup, 200 visual fields were examined (10 microslides x 20 visual fields). In each field of view, the number of positive pixels and the total number of pixels were counted using the "positive pixel count" algorithm of the morph metric software Aperio Image Scope 12.1.0.5. Subsequently, the EI of the markers under study was calculated: 100 ´ (number of positive pixels / total number of pixels). The number of calculated indicators (EI) for each rat was ultimately 200, which was then averaged and one EI value was obtained, therefore, for each subgroup n=6 (average EI × number of rats in the subgroup).

When statistically processing the study results, the software package "Statistica 64 version 12" (2014) (StatSoft Inc., USA) was used. The non-normal nature of the distribution in the samples required the use of non-parametric methods to compare groups: for pairwise comparison of independent groups for one indicator, the Mann – Whitney U-test was used; when comparing three or more indicators, the Kruskal – Wallis H-test (Kruskal – Wallis ANOVA test); comparison of two dependent groups on one characteristic was carried out using the Wilcoxon test with Bonferroni correction, three or more – based on the Friedman method (Friedman ANOVA), to assess categorical data – the χ^2 test. Differences were considered significant at $p<0.05$.

■ RESULTS

Assessment of the dynamics of collagen type I and III expression in chronic perineal wounds in laboratory animals under various therapeutic options

When staining histological preparations with antibodies to type I collagen, a distinct brown staining of fibroblasts, myofibroblasts, elements of the extracellular matrix was determined, which was most pronounced in the stem cell treatment group on the 8th day, and in the control group of animals – on the 35th day of the experiment (Figures 1 b, d). Myoepithelial cells and fat cells showed light brown staining, which was most pronounced in the laser treatment group, and in the L-PRP treatment group – on the 12th day of the experiment (Figures 1 a, c). The sebaceous glands were stained unevenly.

When histological preparations were treated with antibodies to type III collagen, moderate light brown staining of fibroblasts, brown staining of myofibroblasts and extracellular matrix elements in the stem cell treatment group was determined, maximally expressed on the 8-9th day, and on the 10th day using L-PRP day of the experiment (Figures 2 b, c). Myoepithelial cells and fat cells showed faint light brown staining in the laser treatment group on day 11 and in the control group on day 35 (Figures 2 a, d), with weak and uneven staining of the sebaceous glands.

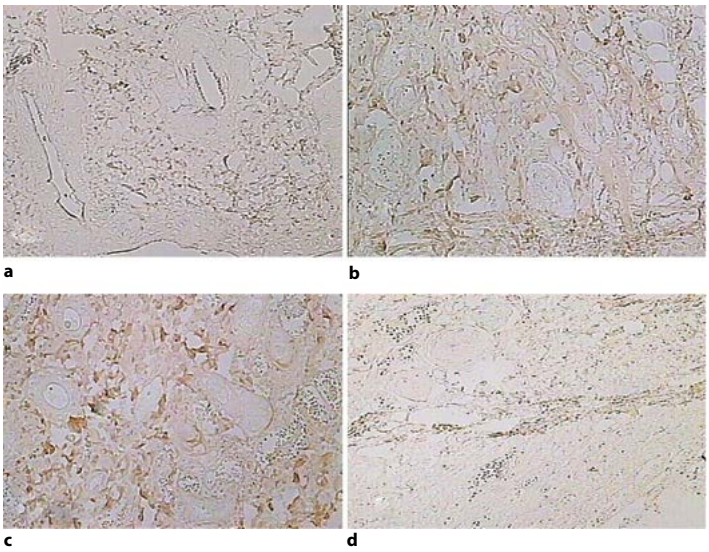

Fig. 1. Expression of type I collagen in the soft tissues of the perineum of rats. IHC staining with antibodies to collagen I, magnification ×200

Notes: a – expression of type I collagen in the laser treatment group on day 14; b – expression of type I collagen in the stem cell treatment group on day 12; c – expression of type I collagen in the L-PRP treatment group on day 12; d – expression of type I collagen in the control group on day 19.

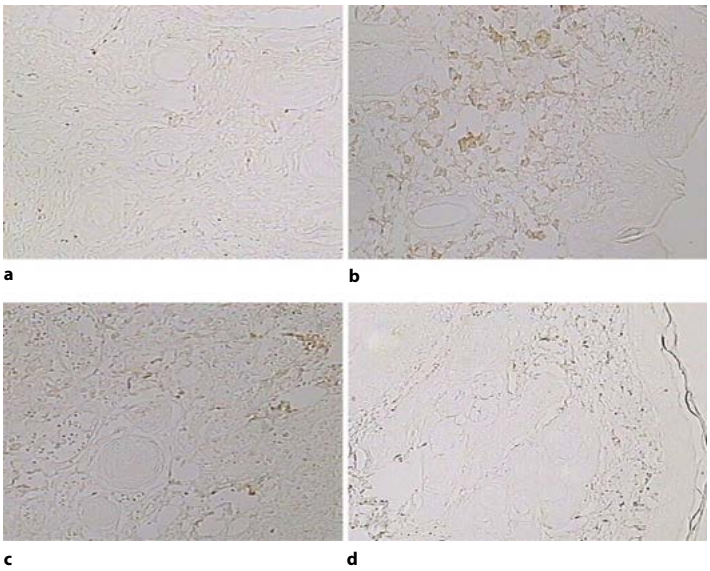

Fig. 2. Expression of type III collagen in the soft tissues of the perineum of rats. IHC staining with antibodies to collagen III, magnification ×200

Notes: a – expression of type III collagen in the laser treatment group on the 12th day; b – expression of type III collagen in the stem cell treatment group on the 12th day; c – expression of type III collagen in the L-PRP treatment group on day 12; d – expression of type III collagen in the control group on the 19th day of the experiment

The content of collagen I in group B (using L-PRP) on the 5th day of the experiment was significantly higher (p<0.05) compared to groups A [by 31.9%] and Б [by 36.5%], its expression index in group Б (use of ATMSC) was 2.49%, which was significantly lower compared to groups A (laser exposure) and Г (placebo control group). During the study, on the 5th day of the experiment, significant differences in the expression levels of type III collagen were established between groups B and A, B and Б, B and Г (p<0.05), with the maximum level of the expression index on the 5th day of the experiment detected in animals of group B (use of L-PRP) – 3.92%, minimal – 0.83% in group A (use of laser). Statistically significant differences in the indicators "ratio of type I collagen to type III collagen) were diagnosed between groups A and Б [by 46.5%], A and Г [by 57.9%] (p<0.0001), Б and B [by 58.1%], Б and Г [by 21.3%] (p<0.05). A significant increase in the level of expression of type I collagen compared to the production of type III collagen was noted in all analyzed groups of animals (p<0.05) (Table 2).

On the 8th day of the experiment, statistically significant differences in the expression levels of type I collagen were established between groups B and A, B and Г (p<0.001), while the EI of type I collagen in group B (use of L-PRP) exceeded the level of expression of this protein in comparison with group A (laser exposure) by 73.1% (3.72 times), and group Г (placebo control) by 70.2% (3.36 times). The expression of collagen I in group Б (use of ATMSC) was significantly higher (p<0.001) compared to groups A (laser exposure) [by 69.4%] and Г (placebo control) [by 66.1%]. Statistically significant differences in the expression levels of type III collagen were also revealed between groups Б and B, Б and Г (p<0.05). The maximum expression of collagen type III was detected in group Г (placebo control, p<0.05) – EI=2.41%, the minimum in group A (laser series, p<0.001) – EI=1.37%. During this observation period, statistically significant differences in the ratio of "type I collagen to type III collagen" were recorded between groups B and A, B and Г (p<0.0001), Б and A, Б and Г (p<0.0001). At the same time, there was an increase in the level of expression of type I collagen in relation to the expression of type III collagen in all analyzed groups (Table 3).

By the 12th day, the content of collagen I in the wound tissues in group B (use of L-PRP) was significantly higher compared to other comparison groups – by 13.2% compared to

Table 2
Comparative assessment of the expression levels of collagen types I and III depending on the type of exposure on the 5th day after the start of treatment (EI, %)*

Groups	Type I collagen		Type III collagen		Ratio of type I collagen to type III collagen	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
A (laser)	2,67 (1,94–5,17)	$p_{B–A, Б, Г}$<,05	0,83 (0,54–1,53)	$p_{B–A, Б, Г}$<0,05	4,82 (2,66–7,64)	$p_{B–Б, Г}$<0,0001 $p_{Б–A, Г}$<0,05
Б (ATMSC)	2,49 (1,69–3,12)		1,09 (1,05–1,69)		2,58 (1,44–4,71)	
B (L-PRP)	3,92 (3,00–6,72)		2,45 (1,91–2,93)		1,08 (0,78–1,60)	
Г (control)	2,74 (2,13–3,17)		1,41 (0,97–1,75)		2,03 (1,55–2,89)	

Note:* the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

Table 3
Comparative assessment of the expression levels of collagen types I and III depending on the type of exposure on the 8th day after the start of treatment (EI, %)*

Groups	Type I collagen		Type III collagen		Ratio of type I collagen to type III collagen	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	2,32 (0,99–4,12)	$p_{А–В, Г}$<0,001 $p_{Б–В, Г}$<0,001	1,37 (0,94–1,86)	$p_{А–Б, В}$<0,001 $p_{Б–В, Г}$<0,05	1,27 (0,41–2,36)	$p_{В–А, Г}$<0,0001 $p_{Б–А, Г}$<0,0001
Б (ATMSC)	8,63 (7,06–10,46)		2,19 (1,46–2,49)		4,43 (3,20–5,89)	
В (L-PRP)	7,59 (5,43–8,56)		1,74 (1,48–2,84)		5,17 (3,62–6,76)	
Г (control)	2,57 (1,74–3,42)		2,41 (1,10–3,25)		1,62 (0,95–2,11)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

group А (p<0.05), by 44.9% – compared to group Б (p<0.05) and 81.6% – compared to the placebo control group (p<0.05). Statistically significant differences in this indicator were also established between groups Б and А, Б and Г (p<0.05). Moreover, the expression of this protein in group Г (placebo control) was significantly (4.73 times) higher than in group А (laser series, p<0.05). During this observation period, there was a significant decrease in the level of expression of type III collagen in the wound tissues of the laser series of animals compared to groups В and Г – 1.98 and 1.91 times, respectively (p<0.05). The maximum expression of this protein was found in animals of the laser series А – EI=1.15% (p<0.0001), the minimum – in animals after using ATMSC – EI=0.57% (p<0.05). By the 12th day, a significantly lower level of the indicator "ratio of type I collagen to type III collagen" was revealed between groups А and Б (by 84.2%), А and В (by 77.2%), А and Г (by 79.1%) [p<0.0001]. In relation to the previous observation period, a varied change in the indicator "type I collagen: type III collagen" was noted in all presented comparison groups (Table 4).

Table 4
Comparative assessment of the expression levels of collagen types I and III depending on the type of exposure on the 12th day after the start of treatment (EI, %)*

Groups	Type I collagen		Type III collagen		Ratio of type I collagen to type III collagen	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})		Me (Q_{75}–Q_{95})	p
А (laser)	4,49 (3,92–4,80)	$p_{В–А, Б}$<0,05 $p_{Б–А, Г}$<0,05 $p_{А–Г}$<0,05	1,15 (0,81–1,50)	$p_{А–Б}$<0,0001 $p_{Б–В, Г}$<0,05	0,89 (0,54–1,65)	$p_{А–Б, В, Г}$<0,0001
Б (ATMSC)	2,85 (2,31–3,48)		0,57 (0,40–0,96)		5,64 (2,93–7,52)	
В (L-PRP)	5,17 (3,62–5,52)		1,13 (0,90–1,33)		3,90 (2,83–5,67)	
Г (control)	0,95 (0,58–1,54)		1,09 (0,84–1,50)		4,25 (2,84–6,31)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

On the 14th day of observation, the expression of collagen I in group B (use of L-PRP) was significantly higher than in series Б (use of ATMSC) and Г (placebo control) – 2.99 and 3.34 times, respectively (p<0.001). During this period, the level of expression of collagen I in the wounds of the laser series significantly exceeded in comparison with the control group of animals – by 61.6% (p<0.05). The maximum expression of type I collagen was detected in group B – median EI=7.62%, the minimum – in group Г – median IE=2.28% (p<0.001). During this period, the level of expression of type III collagen in wounds was significantly higher in group A (laser use) (by 60%) compared to control group Г (p<0.001). The maximum expression of type III collagen on the 14th day was observed in the wounds of animals in group B (use of L-PRP) – median EI=2.28%, the minimum in the placebo control group – median EI=0.44% (p<0.001). Statistically significant differences in the ratio of type I collagen to type III collagen were revealed between groups B and A (by %), B and Б (by %), B and Г (by %) [p<0.0001]. Compared with the previous level of expression of collagens I and III, multidirectional changes in indicators were noted in all groups presented (Table 5).

Despite the epithelialization of the absolute majority of wounds in the main series on the 19th day of observation, the level of expression of tissue type I and III collagens in the competent zone was assessed. At the same time, in laser group A of animals, the median expression of collagen I was significantly lower compared to series Б (using ATMSC) – by 51.4%, and with series B (using L-PRP) – by 64.8% (p<0.001). Statistically significant differences were determined between groups Б and B, with the level of collagen I in group Б being 27.5% lower than in group B (p<0.05). The maximum expression of type I collagen was detected in the L-PRP group (series B) – median IE=5.85%, the minimum in the laser group (A) – median EI=2.06% (p<0.001). There was a significant increase in the level of expression of collagen type III in groups B (by 51.1%) and C (by 48.9%) compared to series A (p<0.001). The maximum expression of this collagen on the 19th day was detected in the tissues of animals of group B (ATMSC use) – median IE=5.85%, the minimum in group A (laser use) – median EI=1.51% (p<0.001]). With regard to the indicator "ratio of type I collagen to type III collagen" no significant differences between the groups were established by this period. Compared with the previous levels of expression of collagens

Table 5
Comparative assessment of the expression levels of collagen types I and III depending on the type of exposure on the 14th day after the start of treatment (EI, %)*

Groups	Type I collagen		Type III collagen		Ratio of type I collagen to type III collagen	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})		Me (Q_{75}–Q_{95})	p
A (laser)	5,94 (5,20–7,37)	$p_{В–Б, Г}$<0,001 $p_{А–Г}$<0,05	0,89 (0,72–1,10)	$p_{В–А, Б, Г}$<0,05 $p_{Б–Г}$<0,001	3,51 (2,82–4,05)	$P_{В–А, Б, Г}$<0,0001
Б (ATMSC)	2,55 (1,16–8,39)		1,10 (0,98–1,29)		2,28 (0,90–6,51)	
B (L-PRP)	7,62 (7,03–8,44)		2,27 (2,00–2,53)		6,48 (4,78–8,71)	
Г (control)	2,28 (2,01–2,64)		0,44 (0,38–1,02)		4,94 (2,71–5,99)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

Table 6
Comparative assessment of the expression levels of collagen types I and III depending on the type of exposure on the 19th day after the start of treatment (EI, %)*

Groups	Type I collagen		Type III collagen		Ratio of type I collagen to type III collagen	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})		Me (Q_{75}–Q_{95})	p
А (laser)	2,06 (1,67–3,47)	$p_{А-Б, В}$<0,001 $p_{Б-В}$<0,05	1,51% (0,70–1,91)	$p_{А-Б, В}$<0,001	1,76 (1,07–3,85)	$p_{В-А, Б, Г}$>0,05
Б (ATMSC)	4,24 (3,57–4,47)		3,09% (2,24–4,11)		1,43 (0,89–2,20)	
В (L-PRP)	5,85 (5,43–6,35)		2,96% (2,54–3,26)		2,09 (1,73–2,31)	
Г (control)	2,18 (1,52–2,85)		2,96 (2,54–3,26)		2,01 (1,44–3,26)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

I and III, and the value of the "ratio of type I collagen to type III collagen," multidirectional changes in indicators were noted in all analyzed groups (Table 6).

Thus, the expression levels of type I and III collagens identified during experimental studies at different times were multidirectional (multi-level) in nature, depending on the

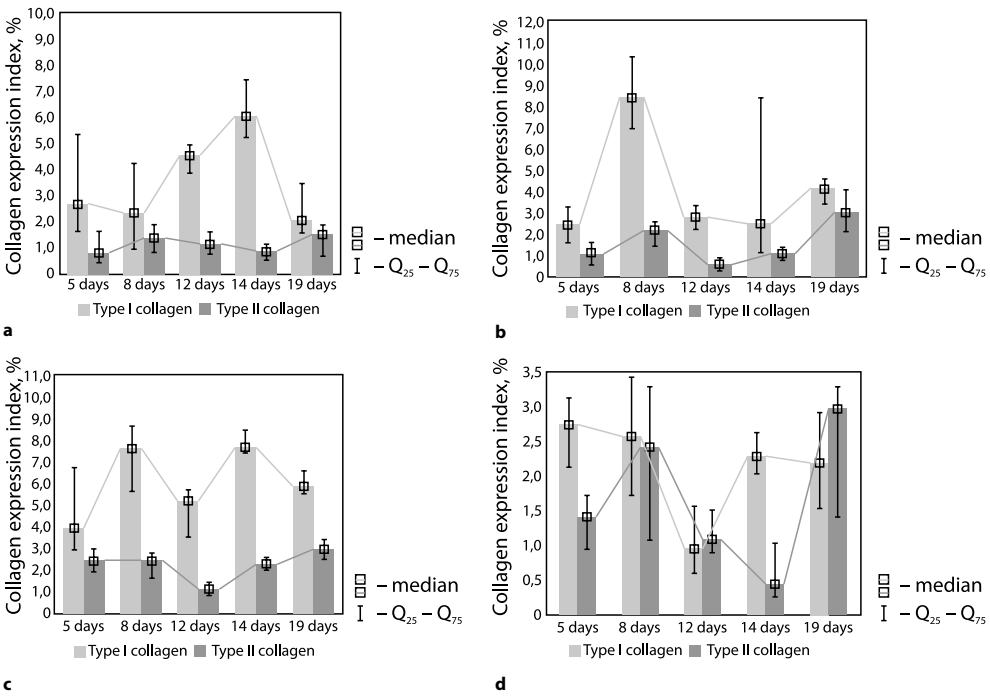

Fig. 3. Comparative assessment of the expression levels (EI) of type I and III collagen in chronic wounds of animals at various times after the application of individual therapeutic options and in the placebo control group

Notes: a – group A (use of laser); b – group B (use of ATMSC); c – group B (use of L-PRP); d – group G (placebo control).

nature of the applied treatment method, the timing of the manifestation of the biological effect and the specificity of the individual reaction of laboratory animals to the therapeutic effect (Figure 3).

Healing of acute and chronic wounds is a highly complex biological process that is largely regulated by the complex actions of several key classes of proteins, including growth factors, receptors, proteases, and extracellular matrix components. Molecular analysis of biopsies from chronic wounds reveals several types of proteins, the levels of which change dramatically compared to acutely healing wounds [22, 23]. Chronic wounds of the skin and soft tissue contain several types of collagens that perform not only a mechanical function (supporting cellular structures and tissue formation), but also ensure cell interaction, influencing various cellular processes (migration, proliferation, differentiation), as well as regeneration mechanisms connective tissue [24]. At the same time, collagen fibers regulate proliferation, differentiation, migration and apoptosis of cells by providing them with topographic, biochemical and mechanical signals [25, 26].

To date, information has been obtained on more than 28 types of collagens and more than 40 genes encoding them [24]. The main structural protein of skin and soft tissues (as well as bones, ligaments and other connective tissues) is type I collagen (it makes up 80–90% of total collagen), most often consisting of two α1 chains and one α2 chain. This is the most "ancient" collagen, which has the greatest strength and provides the basic biomechanical properties of the skin. Type III collagen (makes up 8 to 12% of collagen in the skin and soft tissues) has thinner fibers and intertwines with type I collagen fibers in the skin of an adult; most often it can be formed at the first stage of tissue regeneration after damage, being replaced by type I collagen fibers . Type III collagen fibers consist of three α1 chains and, together with the amorphous substance, form reticulin (reticular tissue), which is the basis for individual soft tissues. The collagen type III molecule is smaller in diameter compared to type I collagen, and in the presence of collagen types I and III in the same fibril, type III collagen regulates the fiber diameter. Type III collagen is a signaling molecule that promotes wound healing by participating in cell adhesion, migration, proliferation and differentiation through cell surface receptors (integrins). The regulatory role of type III collagen in the metabolism of type I collagen is known. With a deficiency of type III collagen, increased differentiation of fibroblasts into myofibroblasts is observed, wound contraction and abnormal fibrillogenesis of type I collagen are activated [24, 27].

The biochemical composition of type I and type III collagens does not differ significantly, but the presence of type III collagen, as well as changes in the "type I collagen / type III collagen" ratio during physiological and pathological processes can have a decisive effect on wound regeneration. It is type III collagen that is actively expressed during wound development and healing. Type III collagen can be co expressed with type I collagen fibers, forming heterotypic fibers, regulating the diameter of the fibrils, and affecting the mechanical properties of the skin and tissues in the damaged area [24, 28].

All changes in collagen metabolism in the wound identified during the experiment are consistent with the above data. At the same time, the use of all regenerative methods of influencing a chronic wound (main groups А, Б and В) had a significant effect on the synthesis of the main collagens in it, which was reflected in their effect on proliferation, differentiation, migration and apoptosis of cellular structures, and, ultimately account, on the nature and timing of wound healing. Thus, on the 5th day of the experiment, only in group В (use of L-PRP) the EI of type I collagen reached a level of 3.92% (Me, Q75–

Q95=3.92–4.80), exceeding by 30.1–36.5% level of similar proteins in wound tissues of the control series and groups A and Б ($p<0.05$). Also in the group using L-PRP at this period, the level of type III collagen was significantly higher compared to all other series ($p<0.05$). It is most likely that this process is associated with the activation of transcription of collagen synthesis genes by a whole group of growth factors contained in soluble platelet factors.

It is known from the literature that insulin-like growth factor-I (IGF-I) and transforming growth factor β1 (TGF-β1) are strong inducers of collagen biosynthesis [29]. IGF-I is most active in collagen synthesis, and TGF-β1 modulates other key components of the intercellular substance, such as fibrillar collagens [30]. In skin and soft tissue fibroblasts, TGF-β regulates collagen production and degradation to promote homeostasis by first binding to the TGF-β receptor type II (TβRII), phosphorylating receptor type I (TβRI). Thus, the formation of heteromeric Smad complexes is activated, moving into the nucleus and activating TGF-β target genes, including the genes of collagen, fibronectin, decorin and versican. Activation of matrix metalloprotease genes occurs when the activity of the Smad complex decreases, and matrix metalloprotease inhibitors occur when the activity increases [24, 31].

Probably, after the introduction of allogeneic ATMSC from adipose tissue (experimental group Б) in the early period (on the 5th day after administration), there is a period of cellular adaptation and engraftment with a not very high level of expression of growth factors, providing an insufficiently pronounced induction of the synthesis of collagen types I and III (EI 2.49% and 1.09%, respectively).

The low level of expression of type I and III collagens on the 5th day is also largely due to the degenerative-inflammatory changes in tissues that persist during this period and the fairly high microbial contamination of most wounds. This is largely due to the fact that the main inflammatory mediators (cytokines) – IL-1, TNF-α and interferon-γ – through the p50/p65 heterodimer of the NF-kB transcription factor – inhibit the transcription of genes of both chains of type I collagen [24, 32].

By the 8th day of the experiment, a significant progression of type I collagen synthesis was noted in the groups using cell therapy – 3.47 times in group Б (using ATMSC) [$p<0.001$] and 1.94 times in group В (using L-PRP) [$p<0.05$]. During this period, the level of expression of type III collagen remained at a fairly low level in all analyzed groups.

By the 12th day of the experiment, a decrease in the expression of type I collagen was noted in the analyzed groups Б, В and Г (by 3.02, 1.46 and 2.71 times, respectively), with the exception of group А (laser use), where EI increased 1.94 times ($p<0.05$). This was probably due to a significant decrease in the degree of degenerative-inflammatory reaction in the wound, associated with a significant decrease in its microbial contamination. Meanwhile, in all main groups of animals (А, Б and В), the IE of type I collagen during this period significantly exceeded the same indicator in the placebo control group (4.73, 3.0 and 5.44 times, respectively; $p<0.05$ ... $p<0.001$), which correlated with the degree of involution of degenerative and inflammatory phenomena in the wound, as well as the nature of its microbial contamination. In all main groups of experimental animals, by the 12th day, a slight decrease in the level of expression of type III collagen was recorded compared to the previous period, indicating a decrease in its regulatory role in the metabolism of type I collagen and increased differentiation of fibroblasts into myofibroblasts with activation of wound contraction and the appearance of atypical fibrillogenesis type I collagen [24, 27].

By the 14th day, in the wounds of animals of series A (laser application), B (L-PRP application) and Г (control), an increase in the expression of type I collagen was recorded (by 24.4%, 32.2% and 58.3%, respectively; $p<0.05$) compared with the previous observation period, indicating an incomplete active process of wound regeneration against the background of active transcription of collagen synthesis genes, as well as a significant regression of inflammatory phenomena and the degree of microbial contamination in the wound. The lack of significant dynamics in the level of expression of type I collagen in group Б (ATMSC use) at this time can be associated with complete epithelization of the wound surface, as well as possible "fatigue" of the implanted cells in terms of their production of the main growth factors (decreased paracrine effect). A low level of expression of type III collagen (EI=0.44%) persisted only in the placebo control group (Г), indicating the incompleteness of the regenerative process in this group of animals.

By the 19th day, all wound defects of the main series (A, Б and B) are covered with epithelium. A slight decrease in the expression of type I collagen was recorded in groups A, B and Г. And only in series B (using ATMSC) an increase in the synthesis of this protein was noted (by 39.9%), apparently associated with the residual paracrine effect of implanted stem cells . In series using therapeutic technologies (A, Б and B), the level of expression of type III collagen was lower than the level of expression of type I collagen, and only in the control group (Г) was an inverse relationship noted, indicating the incompleteness of the wound regeneration process.

The dynamics of the "type I collagen / type III collagen" ratio, established on the basis of IHC assessment of tissues in the area of chronic wounds after the use of regenerative treatment technologies and in the control group, is very indicative (Figure 4). According to a number of authors, this indicator more accurately determines the activity of regenerative processes in the wound than an isolated assessment of individual classes of collagens [22, 24, 27]. When analyzing this indicator in the tissues of a white rat, it was noted that

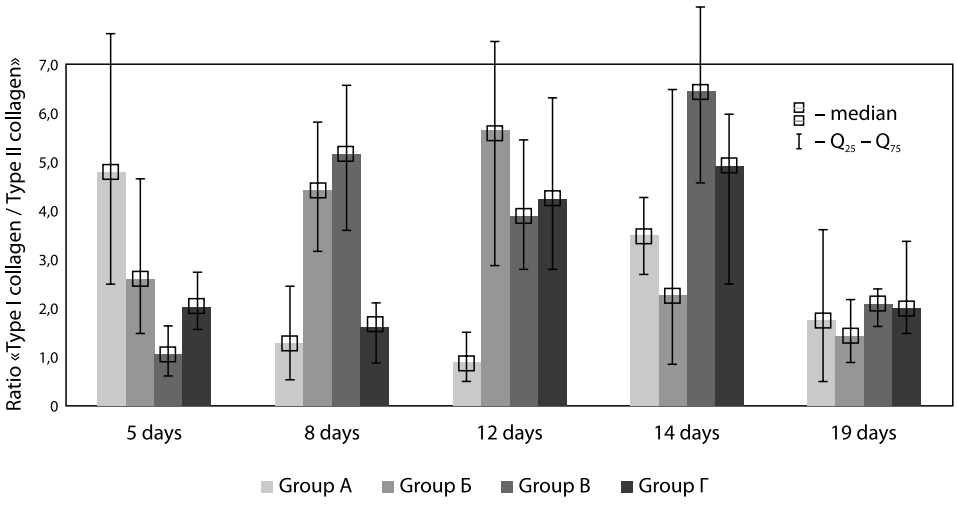

Fig. 4. Dynamics of the indicator "type I collagen / type III collagen" in chronic wounds of animals at different times after the application of certain therapeutic options and in the placebo control group

an increase in SCI-III above the level of 2.5 units. indicates an increase in the level of regenerative processes during the incomplete process of wound restoration. This process has a significant impact on the level of secretion in the tissues of inducers and inhibitors of collagen synthesis, as well as the severity of destructive-inflammatory processes in them. This indicator is at the level of 1.0–2.0 units. indicates the "stabilization" of the regenerative process with the restoration of the natural level of secretion of type I and III collagens.

Assessment of the dynamics of MMP-1 and MMP-9 expression in chronic perineal wounds in laboratory animals under various treatment options

Matrix metalloproteinases (MMP) are a family of extracellular zinc-dependent endopeptidases capable of degrading all types of extracellular matrix proteins (fibrillar and non-fibrillar collagen, proteoglycans, glycoproteins, denatured collagen, basement membranes and a number of cell surface proteins). They play a role in a number of physiological processes: tissue remodeling, reproduction, tissue resorption, angiogenesis, proliferation, migration and differentiation of cells, apoptosis, inhibition of tumor growth. Involved in the cleavage of membrane receptors, the release of apoptotic ligands such as FAS, as well as the activation and deactivation of chemokines and cytokines [33]. Multidirectional changes in MMP activity are observed in various pathological processes, incl. for trauma, acute and chronic inflammatory processes of organs and tissues. Literature data indicate dysregulation of the "MMP/TIMP" system (TIMP – tissue inhibitors of matrix proteinases) [34].

The ability of various serine proteases to initiate MMP activation leads to proteolysis of collagen, extracellular matrix and tissue regression. MMP-1 exhibits significant activity in the microenvironment of wounds, where proteinases are produced in excess by fibroblasts, macrophages and a number of granulation tissue cells [35]. The level of MMP-1 significantly increases in a number of pathological processes, incl. in conditions of chronic wounds of various origins. A clear relationship has been determined: high levels of most serine proteinases (kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase, chymase, etc.) correlate with increased levels of MMP-1. An increase in the level of proteinases activates the synthesis of MMP-1, and an increased level of metalloproteinase promotes an increase in the activity of proteinases, leading to activation of inflammation, degradation of the extracellular matrix, and reduction of the capillary network in the tissue [36].

MMP-9 also plays a role in maintaining inflammation. It destroys collagen, leading to the generation of collagen fragments (the latter serve as chemoattractants for neutrophils and inducers of increased production of MMP-9 by neutrophils), determining the development of a "vicious circle" of inflammation in tissues [37].

Studies conducted with IHC staining of histological preparations with antibodies to MMP-1 showed weak expression in the epithelium and upper layer of the dermis on the 8th day after the use of laser radiation (group A) and more pronounced expression in group B (use of L-PRP) on 12th day of the experiment, as well as in the control group G – on the 19th day of the experiment. In this case, the sebaceous glands were uniformly stained light brown, and the fibroblasts were dark brown (Figure 5).

IHC staining with antibodies to MMP-9 demonstrated unexpressed (weak) expression of this proteinase in the epithelium and upper layer of the dermis on day 8 in group A (after application of laser radiation). At the same time, moderate brown staining of fibroblasts and extracellular matrix was observed in all experimental groups. Invisible (weak) brown

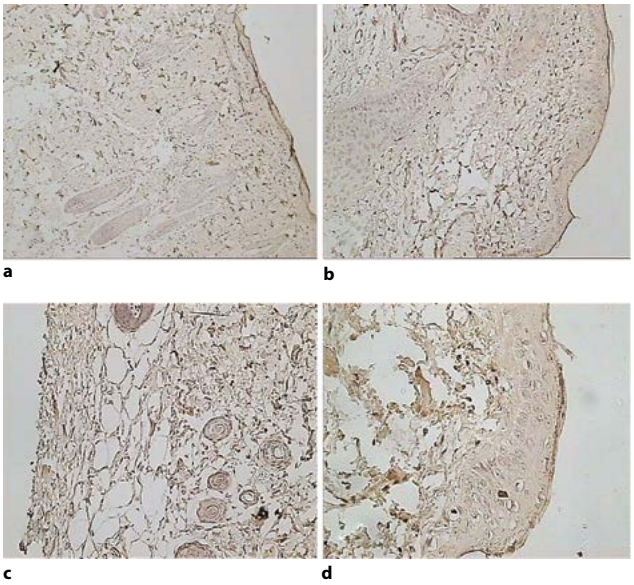

Fig. 5. MMP-1 expression in rat soft tissues. IHC staining with antibodies to MMP-9, magnification ×200

Notes: a – expression of MMP-1 in wound tissues in animals of group A (use of laser treatment of the wound) on the 8th day after exposure; b – expression of MMP-1 in wound tissues in animals of group B (administration of ATMSC) on the 12th day after exposure; c – expression of MMP-1 in wound tissues in animals of group B (use of L-PRP) on the 12th day after exposure; d – expression of MMP-1 in wound tissues in animals of group G (placebo control) on the 19th day after exposure.

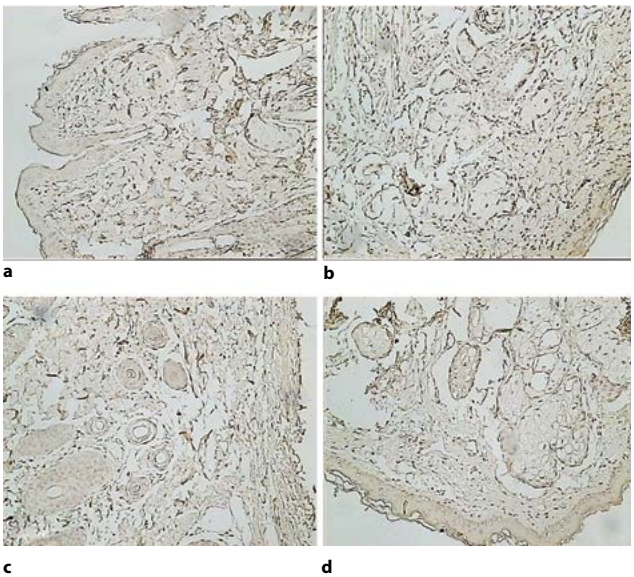

Fig. 6. MMP-9 expression in rat soft tissues. IHC staining with antibodies to MMP-9, magnification × 200

Notes: a – expression of MMP-9 in wound tissues in animals of group A (use of laser treatment of the wound) on the 8th day after exposure; b – expression of MMP-9 in wound tissues in animals of group B (administration of ATMSC) on the 8th day after exposure; c – expression of MMP-9 in wound tissues in animals of group B (use of L-PRP) on the 8th day after exposure; d – expression of MMP-9 in wound tissues in animals of group G (placebo control) on the 14th day after exposure.

staining, indicating a low level of MMP-9 expression, was observed in the sebaceous glands of animals in group Б (application of ATMSC) on the 8th day and in the control group Г on the 14th day of the experiment (Figure 6).

Analysis of the expression levels of MMP-1 and MMP-9 in chronic wounds of laboratory animals showed multidirectional and multi-level tissue reactions in groups using various methods of therapeutic treatment on the wound and in the control series. Thus, on the 5th day of the experiment, the lowest level of MMP-1 expression was noted in group A (laser use): it was 36.2% lower than in series Б using ATMSC; 33.5% lower than in series В (use of L-PRP), and 56.6% lower than in the control group ($p<0.05$). Also on the 5th, the lowest expression of MMP-9 was observed in the laser group (IE=4.78%). In other comparison groups (Б, В and Г) there were also high levels of MMP-9 expression: 5.40%, 5.21% and 6.54%, respectively, not significantly different from each other ($p>0.05$). This effectiveness of laser treatment on a chronic wound may be due to the effect of pronounced microbial decontamination and a decrease in overall infection of the tissue and wound surface, as well as the possible inactivation by laser radiation of a group of proteolytic enzymes (primarily serine proteases, on which the expression of MMPs depends) (Table 7).

On the 8th day of the experiment, the animals showed an increase in the expression of MMP-1 and MMP-9 ($p<0.05$), with the exception of the group using ATMSC (where the IE decreased insignificantly compared to the previous period – from 4.61 to 3. 39%). The same trend was registered on the part of MMP-9 (Table 8). These changes generally correspond to the dynamics of the expression of type I and III collagens, and are also largely associated with the level of microbial colonization of wounds and the degree of inflammatory changes in wound tissues.

By the 12th day, multidirectional dynamics of MMP-1 and MMP-9 indicators were noted in the wounds of the main series and control. Moreover, only in group Б there was a significant increase in the level of MMP-1 expression compared to the previous period – by 49.5% ($p<0.05$). An insignificant decrease in MMP-1 IE in this period was recorded in groups A (laser use), В (L-PRP use) and Г (placebo control) [$p>0.05$]. The phenomenon of the 12th day for series В using ATMSC, even despite the fact that during this period in 11% of animals in this group the wound surface was epithelialized, and the average area of the wound surface was minimal, amounting to Me=0.34 cm2 (Q25–Q75=0.22–0.47),

Table 7
Comparative assessment of the expression levels of MMP-1 and MMP- depending on the type of exposure on the 5th day after the start of treatment (EI, %)*

Groups	MMP-1		MMP-9	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	2,94 (1,57–4,36)	$p_{А–В}<0,05$ $p_{Б–Г}<0,05$ $p_{В–Г}<0,05$	4,78 (4,11–6,40)	p > 0,05
Б (ATMSC)	4,61 (2,33–6,76)		5,40 (4,14–6,48)	
В (L-PRP)	4,42 (3,87–5,86)		5,21 (4,37–6,51)	
Г (control)	6,77 (4,77–8,13)		6,54 (5,40–8,23)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

allows us to speak, on the one hand, about the persistence of degenerative-inflammatory processes in tissues "under the cover of the epithelium", and on the other hand, about the intensification in this period of the processes of proliferation and migration of keratinocytes in the regional wound area due to weakening of the contact between collagen and integrins [38, 39]. Also on the 12th day, the expression of MMP-9 in the wounds of animals of groups А, В and Г decreased – by 48.4%, 47.2% and 9.36%, respectively (Table 9). Considering that at the recovery stage, MMP-9 "cuts adhesion molecules, allowing a number of cells (including keratinocytes) to centripetally migrate to the wound area" [38, 39], the established fact of a slight increase in the expression of gelatinase В in the ATMSC group confirms the increase in the marginal epithelization of the wound detected at this time.

On the 14th day of observation in groups А, Б and В, there was a significant decrease in the expression of MMP-1 compared to the previous period (by 27.9%, 45.9% and 16.1%, respectively; $p<0.05$), indicating a significant reduction in degenerative, inflammatory and proteolytic processes in the wound (Table 10). In the control group Г, a fairly high level of MMP-1 expression remained during this period (Me=14.91, Q25–Q75=12.51–16.61), indicating a continued high destructive and inflammatory potential in wounds, which

Table 8
Comparative assessment of the expression levels of MMP-1 and MMP- depending on the type of exposure on the 8th day after the start of treatment (EI, %)*

Groups	MMP-1		MMP-9	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	5,95 (2,39–8,33)	$p_{А–Б}$<0,05 $p_{А–Г}$<0,05 $p_{Б–В}$<0,05 $p_{Б–Г}$<0,05	5,70 (4,70–7,25)	$p_{Б–В}$<0,05 $p_{А–Г}$<0,05 $p_{Б–Г}$<0,05
Б (ATMSC)	3,39 (2,34–5,35)		4,50 (2,80–5,56)	
В (L-PRP)	7,58 (6,60–9,45)		6,52 (5,49–7,79)	
Г (control)	9,68 (8,00–12,34)		7,91 (6,64–9,03)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

Table 9
Comparative assessment of the expression levels of MMP-1 and MMP- depending on the type of exposure on the 12th day after the start of treatment (IE, %)*

Groups	MMP-1		MMP-9	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	5,42 (4,58–6,67)	$p_{А–Г}$<0,05 $p_{Б–Г}$<0,05 $p_{В–Г}$<0,05	2,94 (0,97–4,12)	$p_{А–Б}$<0,05 $p_{А–Г}$<0,05 $p_{В–Г}$<0,05
Б (ATMSC)	6,71 (5,94–8,19)		5,13 (4,12–6,14)	
В (L-PRP)	7,06 (5,94–8,30)		3,44 (1,99–5,43)	
Г (control)	8,92 (6,60–10,08)		7,17 (6,14–8,57)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

Table 10
Comparative assessment of the expression levels of MMP-1 and MMP- depending on the type of exposure on the 14th day after the start of treatment (EI, %)*

Groups	MMP-1		MMP-9	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	3,91 (3,21–4,97)	$p_{А–В}$<0,05 $p_{А–г}$<0,05 $p_{Б–г}$<0,05 $p_{В–г}$<0,05	5,82 (5,26–7,13)	$p_{А–г}$<0,05 $p_{Б–г}$<0,05 $p_{В–г}$<0,05
Б (ATMSC	3,63 (2,39–6,96)		6,09 (4,52–8,34)	
В (L-PRP)	5,92 (3,54–9,24)		7,49 (5,21–8,91)	
Г (control)	14,91 (12,51–16,61)		9,11 (7,70–12,07)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

Table 11
Comparative assessment of the expression levels of MMP-1 and MMP- depending on the type of exposure on the 19th day after the start of treatment (EI, %)*

Groups	MMP-1		MMP-9	
	Me (Q_{75}–Q_{95})	p	Me (Q_{75}–Q_{95})	p
А (laser)	3,72 (1,76–4,44)	$p_{А–Б}$<0,05 $p_{А–г}$<0,05 $p_{В–г}$<0,05	2,69 (1,97–4,03)	$p_{А–Б}$<0,05 $p_{А–В}$<0,05 $p_{А–г}$<0,05
Б (ATMSC)	5,92 (4,97–7,07)		5,08 (4,72–6,25)	
В (L-PRP)	3,66 (1,88–6,44)		4,48 (2,36–6,96)	
Г (control)	10,00 (8,94–12,66)		5,82 (4,88–6,33)	

Note: * the assessment was carried out at the light-optical level over 200 fields of view of the preparation for each animal.

corresponded to the identified morphological and cytological changes and correlated with the expression levels of the main types of collagens and their ratio. The expression of MMP-9 by the 12th day increased in all series (to varying degrees), indicating inhibition of the processes of active proteolysis of collagen and glycosaminoglycans with the utilization of damaged components of the extracellular matrix in the area of an epithelized or persistent chronic wound [40].

By the 19th day of the experiment, the majority of animals of the main groups (A-B) showed complete epithelization of the wound defect. At the same time, stabilization of the expression levels of metalloproteinases type 1 and 9 was noted in the tissues of the regeneration zone (Table 11). MMP-1 expression indices in these series varied from 3.66% (group B) to 3.72% (group A) and 5.92% (group Б). Only in the control series (Г) in the presence of wound defects, the median EI of MMP-1 was 10.0% (8.94–12.66), significantly exceeding the expression levels of the main groups ($p<0.05$).

Assessing the intensity of MMP-9 production, it should be noted that the lowest level of expression of this enzyme was recorded on the 19th day in tissues after laser exposure to the wound – 2.69% (1.9–4.03). This was significantly lower than in the control group, as well as in series Б and B. By the 19th day of observation, the expression of MMP-9

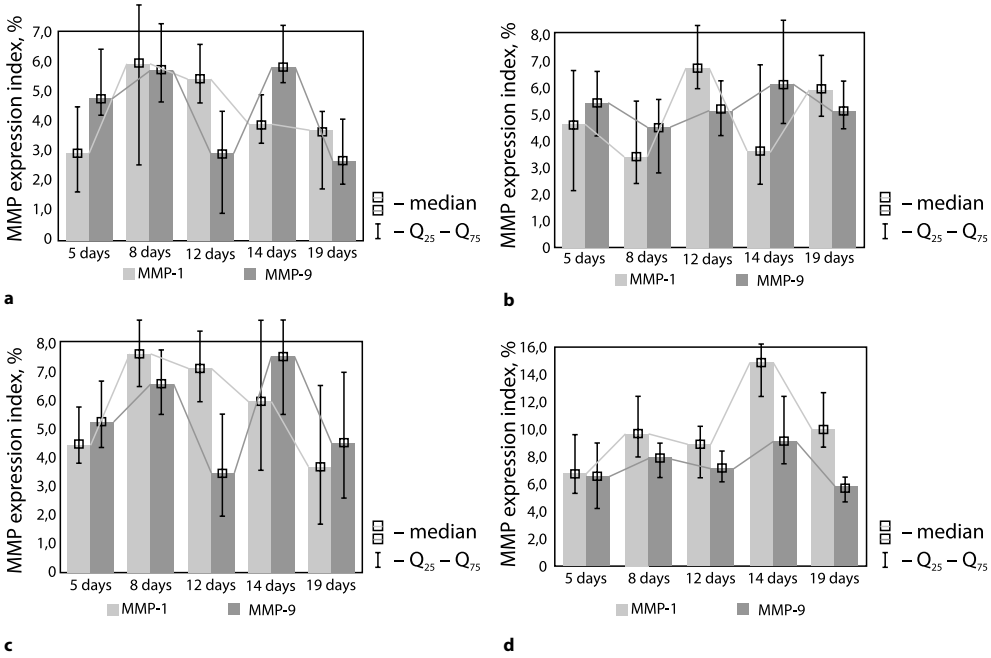

Fig. 7. Dynamics of expression of MMP-1 and MMP-9 in the tissues of chronic wounds after various types of therapeutic effects

Notes: a – group A (use of laser wound treatment); b – group B (administration of ATMSC); c – group B (use of L-PRP); d – group G (placebo control).

decreased in all series (to varying degrees), indicating suppression of the processes of active proteolysis of collagen and glucoseaminoglycans with the utilization of damaged components of the extracellular matrix in the area of an epithelialized or persistent chronic wound [40].

An assessment of the dynamics of the expression of MMP-1 and MMP-9 in wounds after the use of various therapeutic technologies and a control series showed multidirectional effectiveness of the use of regenerative methods of treatment in the main series of the experiment (Figure 7).

■ DISCUSSION

After the introduction of allogeneic ATMSC (experimental group Б), in the early period there is a period of cellular adaptation and engraftment with a not very high level of expression of growth factors, providing a weak induction of the synthesis of collagen types I and III (EI 2.49% and 1.09%, respectively). This is largely due to persistent degenerative-inflammatory changes in tissues and fairly high microbial contamination of most wounds, as well as inhibition of the transcription of genes of both chains of type I collagen by the main inflammatory mediators (cytokines). By the 8th day of the experiment, a significant progression of type I collagen synthesis was recorded in the groups using cell therapy (ATMSC and L-PRP groups).

By the 12th day of the experiment, there is a slight decrease in the expression of type I collagen in groups Б, В and Г (by 3.02, 1.46 and 2.71 times, respectively), with the exception of group А (laser use), where EI increased 1.94 times ($p<0.05$), which is associated with a significant decrease in the degree of degenerative-inflammatory reaction in the wound, associated with a significant decrease in the level of its microbial contamination. During this period, in all main groups of animals, the index of type I collagen expression significantly exceeded the same indicator in the placebo control group (4.73, 3.0 and 5.44 times, respectively; $p<0.05$ ... $p<0.001$), which correlated with the degree of involution of degenerative and inflammatory phenomena in the wound, as well as the nature of its microbial contamination.

By the 14th day, a new wave of increased expression of type I collagen is recorded in the wounds of animals of the main series, indicating an incomplete active process of wound regeneration against the background of active transcription of collagen synthesis genes, as well as a significant regression of inflammatory phenomena and the degree of microbial contamination in the wound. The lack of significant dynamics in the level of expression of type I collagen in group Б (ATMSC use) at this time can be associated with complete epithelization of the wound surface, as well as possible "fatigue" of the implanted cells in terms of their production of the main growth factors (decreased paracrine effect). At the same time, the low level of expression of type III collagen (EI=0.44%) during this period remained only in the placebo control group, indicating the incompleteness of the regenerative process in this group of animals.

After the wound defects are closed by the epithelium, by the 19th day in animals of the main series (А, Б and В), a slight decrease in the expression of type I collagen is recorded. And only in series В (using ATMSC) is there an increase in the synthesis of this protein (by 39.9%), associated with the prolonged paracrine effect of implanted stem cells. Moreover, in series using therapeutic technologies (А, Б and В), the level of expression of type III collagen during this period is lower than the level of expression of type I collagen. In the control group (Г), an inverse relationship was registered, indicating the incompleteness of the wound regeneration process. The dynamics of the "type I collagen / type III collagen" ratio largely follows the trends established for the expression of type I collagen.

Under conditions of a simulated chronic perineal wound in laboratory animals, a high level of expression of matrix metalloproteinases (primarily MMP-1) is recorded, which initiates in the tissues and microenvironment of the wound excessive production of serine proteinases (kallikrein, trypsin, neutrophil elastase) by fibroblasts, macrophages and a number of granulation tissue cells , cathepsin G, tryptase, chymase, etc.). In turn, an increase in the level of proteinases activates the synthesis of metalloproteinases, the increased level of which contributes to an increase in the activity of serine proteinases, leading to activation of inflammation, degradation of the extracellular matrix, and reduction of the capillary network in tissues [36].

The established high level of MMP-9 expression in the tissues of a simulated chronic perineal wound emphasizes its negative role in maintaining the inflammatory process. MMP-9, destroying collagen, leads to the generation of collagen fragments (the latter serve as chemoattractants for neutrophils and inducers of increased production of MMP-9 by neutrophils), determining the development and progression of the "vicious circle" of inflammation in tissues [37].

Thus, the use of regenerative technologies in the treatment of an experimental chronic wound (exposure to high-intensity laser radiation with a wavelength of 1560 nm, injection of allogeneic ATMSC and L-PRP, makes it possible to reduce the median time of epithelization of a wound defect by 1.84–2.5 times in comparison with the group placebo control (Mann – Whitney U Test Z=5.641231, p=0.0000001).

The mechanisms of this wound-healing effect are different in the main groups. For series A, they are largely associated with the bactericidal effect of high-intensity laser radiation of certain parameters on the wound, as well as the formation of a peptide-coagulation film on the wound surface, which subsequently prevents exogenous infection of tissues in this area and promotes wound regeneration in conditions of insignificant (limited) microbial contamination. Also likely factors for the accelerated regeneration of a chronic wound after laser exposure are: inactivation of the pool of proteolytic enzymes by laser light, as well as "aftereffects" as a result of treatment of pathologically altered tissues with laser light (electromagnetic and ultrasonic waves, cellular oscillation, changes in the antioxidant and electrostatic properties of tissues, and etc.). For series Б and В, accelerated wound healing is associated with a powerful paracrine effect, caused by multiple and prolonged effects on the marginal zone and tissue in the area of the wound bottom of a large group of growth factors, cytokines, inducers of collagen synthesis, stimulating, first of all, marginal epithelialization due to the activation of progenerative cells of the border zone and enhancing the centripetal movement of epithelial cells (epithelial blasts). At the same time, longer functioning of implanted ATSM in wound tissues provides a more powerful and long-lasting paracrine effect, leading to an unreliable decrease in the median time of epithelization of the wound defect compared to the L-PRP group (Mann – Whitney U Test Z=1.165010, p=0. 244016) and a significant reduction (by 26.3%) – compared with animals of the laser series (Mann – Whitney U Test Z=3.92804, p=0.000086).

■ CONCLUSIONS

1. All methods of therapeutic effects on a chronic wound evaluated in the experiment (in animals of group A – using defocused laser radiation with a wavelength of 1560 nm, group Б – with injection into the bottom and edges of the wound of a suspension of allogeneic mesenchymal stem cells of adipose tissue 500 thousand / ml and group В – with injection into the bottom and edges of the wound of allogeneic plasma enriched with platelets and leukocytes) should rightfully be classified as regenerative technologies, since they significantly and reliably accelerate its healing, reducing the median time of epithelization of the wound defect in comparison with the placebo group – control by 1.84–2.5 times (Wilcoxon Z=4.285714, p=0.000018).
2. Immunohistochemical assessment of tissues with assessment of the level of expression of type I and III collagens at different times has established its multidirectional (multi-level) nature, depending on the type of therapeutic method used, the timing of the manifestation of the biological effect and the specificity of the individual reaction of laboratory animals to the therapeutic effect. All changes in collagen metabolism in a chronic wound identified during the experiment using regenerative technologies and the control series largely correlate with the morphological and cytological changes identified. The use of all regenerative methods of action on a chronic wound (groups A, Б and В) has a significant effect on the synthesis of basic collagens in it, which is reflected in their effect on proliferation, differentiation, migration and apoptosis

of cellular structures, and, ultimately, on nature and timing of wound healing. Thus, already on the 5th day of the experiment in group B (use of L-PRP), the expression index of type I collagen exceeded by 30.1–36.5% the level of similar proteins in the tissues of wounds of the control series and groups A and Б ($p<0.05$), also when using L-PRP in this period, the level of type III collagen was significantly higher compared to all other series ($p<0.05$), which indicated the activation of transcription of collagen synthesis genes by a whole group of growth factors contained in soluble platelet factors.

3. Analysis of the expression levels of MMP-1 and MMP-9 in chronic wounds of laboratory animals demonstrates multidirectional and multi-level tissue reactions in groups using various methods of therapeutic treatment on the wound and in the control series. At the same time, the use of all therapeutic technologies in dynamics reliably leads to a decrease in the activity of their products in comparison with the placebo control group, correlating with changes in the level of collagen expression, the involution of inflammatory-degenerative processes in the wound and the level of microbial colonization of wounds. To a greater extent, this process is observed in the group of animals using laser radiation (at all times, starting from the 5th day of observation). This effectiveness of laser treatment on a chronic wound may be due to the effect of pronounced microbial decontamination and a decrease in overall infection of the tissue and wound surface, as well as the possible inactivation by laser radiation of a group of proteolytic enzymes (primarily serine proteases, on which the expression of MMPs depends).
4. In view of the significant regenerative effect revealed in experimental studies in relation to chronic wounds of the perineum, it should be considered appropriate to use locally in clinical conditions defocused laser radiation with a wavelength of 1560 nm (0.1 s / 0.1 s, 5 W, light spot diameter 0.7 cm, distance 2 cm, exposure from 3 to 5 seconds per 1 field) with possible combined injection into the edges and bottom of the wound of allogeneic adipose tissue mesenchymal stem cells or platelet- and leukocyte-rich plasma. Taking into account the established high level of MMP-1 (and, accordingly, serine proteinases) in a chronic wound, the complex of therapeutic measures should also be supplemented with local or systemic use of proteolysis inhibitors.

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