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ON THE PARTICIPATION OF EOSINOPHILS IN TISSUE RECOVERY AFTER A LOCAL COLD INJURY

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◆ The article studies the correlation of the content of peripheral blood and red bone marrow eosinophils with the level of secretion of fibroblast growth factor (FGF-21), insulin-like factor (IGF-1) and vasculoendothelial growth factor (VEGF-C) in blood serum during the formation of dermal collagen after local cold damage. Animals of the experimental group after the onset of narcotic sleep on the depilated skin of the back were simulated contact frostbite of the 3rd degree. On the 3rd, 7th, 14th and 21st day of the experiment, the concentrations of growth factors, % dermal collagen content, and also the content of eosinophils in peripheral blood and red bone marrow were determined in the blood serum. The research results showed that the development of eosinopenia after a local cold injury occurs due to the sequestration of eosinophils in the affected area. The presence of reactive changes after a local cold injury not only in peripheral blood, but also in the red bone marrow may indicate the participation of eosinophils in tissue repair processes after a local cold injury.

◆ **Keywords:** eosinophils; red bone marrow; peripheral blood; growth factors; cold injury.

ОБ УЧАСТИИ ЭОЗИНОФИЛОВ В ВОССТАНОВЛЕНИИ ТКАНЕЙ ПОСЛЕ ЛОКАЛЬНОЙ ХОЛОДОВОЙ ТРАВМЫ

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◆ Изучено соотношение содержания эозинофилов периферической крови и красного костного мозга и уровня фактора роста фибробластов (FGF-21), инсулиноподобного фактора (IGF-1) и васкулоэндотелиального фактора роста (VEGF-C) в сыворотке крови при формировании дермального коллагена после локального холодового повреждения. Животным опытной группы после наступления наркотического сна на депилированной коже спины моделировали контактное отморожение III степени. На 3, 7, 14 и 21-е сутки эксперимента в сыворотке крови определяли концентрацию факторов роста, содержание дермального коллагена, а также содержание эозинофилов в периферической крови и красном костном мозге. Результаты исследований показали, что эозинопения после локальной холодовой травмы развивается за счет секвестрации эозинофилов в зоне поражения. Реактивные изменения после локальной холодовой травмы не только в периферической крови, но и в красном костном мозге могут свидетельствовать об участии эозинофилов в процессах восстановления тканей после локальной холодовой травмы.

◆ **Ключевые слова:** эозинофилы; красный костный мозг; периферическая кровь; факторы роста; холодовая травма.

Introduction

Tissue recovery after thermal lesions, in particular cold injury, is one of the most relevant areas of regenerative medicine [1–6].

The activity of adhesion, migration, proliferation, and differentiation of cells in the affected tissue determines the result: the tissue will either recover and perform its functions or end by fibrosis [7–10]. In this regard, the study of the regulation mechanisms of collagen synthesis by fibroblasts is interesting.

As it is known, the structure of the collagen matrix determines the functional activity of fibroblasts: when the collagen matrix is fragmented, focal contacts between the fibroblasts and the collagen matrix are disrupted, which is observed on the first day after thermal damage. As a result, fibroblasts lose the ability to be in a stretched state, that is, a compulsory condition for their metabolic activity-synthesis and secretion [11, 12]. Simultaneously, growth factors can stimulate the proliferation and synthetic activity of fibroblasts in conditions of violation of the collagen matrix structure [3, 13–16].

There are almost no data on the participation of eosinophilic leukocytes in tissue recovery after thermal damage, namely, the presence of a link between eosinophils and collagen synthesis by fibroblasts or the level of growth factor secretion. Simultaneously, a large number of cytotoxic products and the increased content of which causes the formation of an expressed microbicidal potential against not only foreign substances but also surrounding tissues, which show that the functional capabilities of eosinophilic leukocytes go beyond the traditional understanding, in particular, the development of allergic diseases and anthelmintic immunity [17–21].

In this regard, it was interesting to compare the dynamics of peripheral blood and red bone marrow eosinophils and the level of secretion of fibroblast growth factor (FGF-21), insulin-like factor (IGF-1), and vasculoendothelial growth factor (VEGF-C) in dermal collagen formation after local cold damage.

Materials and methods

Local cold damage was made on mongrel male and female rats weighing 180–200 g, kept under the same conditions, with a standard food regime, in accordance with the rules of a laboratory practice (Decree of the Ministry of Health and Social Development of the Russian Federation dated August 23, 2010, No. 708n "On approval of the rules of a laboratory practice") and the provisions of the International Helsinki Convention on humane treatment to animals (1972).

A contact cold injury was performed according to the method proposed by Boyko et al. [22]: after the beginning of a narcotic sleep, a metal weight with a diameter of 2.5 cm, cooled in a liquid nitrogen, was applied to the depilated skin of the rat's back. As a result of such exposure, experimental animals developed local cold injury of the third degree.

Withdrawal from the experiment was made by overdosing the drug for anesthesia on days 3, 7, 14, and 21. Groups were formed for 20 animals to obtain statistically reliable results. The criterion for exclusion from the experiment was the addition of a secondary infection.

The control group consisted of mongrel rats of the same body weight, kept under the same conditions as the experimental group. The rats were decapitated following the principles of humanity in accordance with Annex No. 4 "About the procedure for euthanasia (killing) of an animal" for the rules for carrying out work using experimental animals (Annex to the Decree of the Ministry of Health of the USSR No. 755 dated August 12, 1977).

Method for determining the content of peripheral blood and red bone marrow eosinophils

Blood sampling for hematological examination was performed after thoracotomy by puncturing the heart cavity before removing the animal from the experiment. Red bone marrow sampling was performed in accordance with the method of E.I. Goldberg, which used a material directly taken from the proximal femur [23]. Blood and bone marrow smears after fixation were colored according to Romanovsky–Gimza, and the leukogram and myelogram were counted accordingly.

Method for determining the content of a dermal collagen

The dermis collagen content was determined in accordance with the method developed by us [24]. A piece of the afflicted skin was taken using punch scalpel no. 5. At the first stage, the skin was dried, preweighed, and frozen at –80°C in 1 mL of 0.9% isotonic sodium chloride solution. A piece

of tissue was dried in a freeze-dryer at a temperature of –46°C and a pressure of 0.040 mbar and prepared by cutting using a microtome blade into fragments no more than 3 mm thick.

At the second stage, the skin was prepared for enzymatic hydrolysis. The fragments obtained were placed in a 1.5-mL graduated Eppendorf tube filled with 1 mL of 15% aqueous sodium hydroxide solution for 24 h at a temperature of 18–20°C and then repeatedly washed with 1 mL distilled water at a temperature of 18–20°C. After reaching the target pH level of the supraventricular fluid 7.0, the supernatant was aspirated with a dispenser so that the amount of sediment in the test tube did not exceed 0.3 mL. The resulting material was frozen at –80°C, dried in a freeze-dryer, and weighed on analytical scales, determining the mass of material m1.

At the third stage, enzymatic hydrolysis of collagen was conducted. The resulting anhydrous precipitate was diluted in 900 mL of phosphate-buffered saline (pH 7.0), and 100 mL of collagenase solution (Russia) was added, which was prepared in advance by dissolving the lyophilized dried collagenase preparation in 10 mL of phosphate-buffered saline (pH 7.0). Then, the test tube with the contents was placed in the ES-20 shaker incubator (BioSan, Latvia) at a temperature of 37°C and intensively mixed for 2 h. After that, the test tube was centrifuged at 13,400 rpm for 5 min, and the supernatant fluid was aspirated with a dispenser. Distilled water (1 mL) was added, and the resulting material was homogenized. The procedure, including homogenizing by resuspending with a dispenser, centrifuging, aspirating supernatant fluid, and adding 1 mL water, was repeated five times. After that, the material was frozen at –80°C, freeze-dried, and weighed, determining the mass of material m_2. The mass difference between m_1 and m_2 was used to determine the mass of collagen contained in the tissue being studied. The percentage of collagen in the tissue was determined as the ratio of a certain mass of collagen to the mass of the tissue sample.

Thus, the collagen content was calculated using the following formula:

$$m_{\text{collagen}} = m_1 - m_2,$$

where m_1 is the skin mass before enzymatic hydrolysis (g) and m_2 is the skin mass after enzymatic hydrolysis (g).

The percentage of collagen content in the tissue was calculated using the following formula:

$$\% \text{ collagen content} = \text{mass of collagen / mass of tissue sample} \cdot 100\ \%.$$

Methods for determining growth factors

Blood samples were obtained during animal decapitation and then centrifuged at 15,000 rpm at 4°C for 10 min. Serum growth factors were determined using the Multiskan Fc immunoassay device (Thermo Fisher, USA): rat FGF-21 (enzyme-linked immunosorbent assay [ELISA], BioVendor, Czech Republic), IFG-1 (ELISA, Mediagnost, Germany), and rat VEGF-C (ELISA, Bender MedSystems, Austria).

Statistical processing of results was performed using SPSS 13.0 for Windows. The samples were described by calculating the median (*Md*) and interquartile interval (Q_{25}; Q_{75}). The probability of differences was estimated using the nonparametric Kolmogorov–Smirnov and Wilcoxon criteria. Correlation analysis was performed using the Kendall criterion (τ).

Research results

On the 3rd day after local cold injury (Fig. 1), reactive changes were noted in both the red bone marrow and peripheral blood. The essence of these changes was the tendency to increase the concentration of eosinophilic myelocytes (from 12.0% [10.0; 23.0] to 20.0% [13.0; 24.0]; $Z = 0.88$; $p = 0.41$) and metamyelocytes (from 20.0% [10.0; 21.0] to 24.0% [19.0; 30.0]; $Z = 1.28$; $p = 0.07$) against the background of a decrease in the content of eosinophils in the red bone marrow (from 6.0% [2.0; 9.0] to 2.0% [2.0; 5.0]; $Z = 1.16$; $p = 0.16$) and especially in the peripheral blood (from 2.0% [1.0; 4.0] to 0.0% [0.0; 1.0]; $Z = 1.98$; $p = 0.001$).

On day 7, the red bone marrow showed the opposite trend: a decrease in the concentration of eosinophilic myelocytes (up to 10.5% [9.0; 13.5]; $Z = 1.33$; $p = 0.05$) and metamyelocytes (up to 19.5% [12.0; 21.5]; $Z = 1.12$; $p = 0.16$), which was accompanied by a decrease in the eosinophil level in the red bone marrow (up to 1.5% [0.0; 5.75]), resulting in similar levels of eosinophils in bone marrow and peripheral blood ($W = –1.6$; $p = 0.1$; Fig. 1). On day 14, after local cold injury, the red bone marrow showed a weak tendency to increase

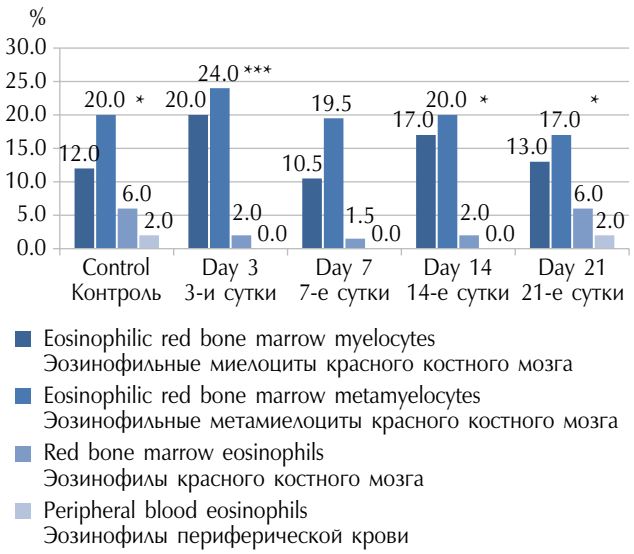

Fig. 1. The ratio of the content of eosinophils of red bone marrow and peripheral blood in rats after local cold damage (*Me*, %); $^{*}p < 0.05$; $^{***}p < 0.001$

Рис. 1. Соотношение содержания эозинофилов красного костного мозга и периферической крови у крыс после локальной холодовой травмы (*Me*, %); $^{*}p < 0{,}05$; $^{***}p < 0{,}001$.

the level of eosinophilic myelocytes (up to 17.0% [9.0; 23.0]; $Z = 0.92$; $p = 0.35$) and eosinophils (up to 2.0% [1.0; 3.0]; $Z = 0.47$; $p = 0.97$) without changes in their concentrations in the peripheral blood, which led to the restoration of differences in the eosinophil content in the red bone marrow and peripheral blood ($W = -2.03$; $p = 0.04$). Only on day 21 of the experiment, an increase in the eosinophil content was observed in the red bone marrow (up to 6.0% [4.5; 9.5]; $Z = 1.68$; $p = 0.007$) and peripheral blood (up to 2.0% [1.0; 3.0]; $Z = 1.26$; $p = 0.08$) against the background of a decrease in the concentration of eosinophilic myelocytes (up to 13.0% [9.5; 13.5]; $Z = 1.18$; $p = 0.12$) and metamyelocytes (up to 17.0% [16.0; 23.5]; $Z = 1.14$; $p = 0.14$).

In the first 3 days after cold damage, the proportion of dermal collagen decreased significantly (from 70.3% [69.5; 71.5] to 23.7% [21.3; 25.0]; $Z = 1.79$; $p = 0.003$; Fig. 2, *a*).

Starting from day 3, the value of the study indicator began to increase, reaching 38.8% (36.9; 40.1) by day 7 ($Z = 1.79$; $p = 0.003$), 52.1% (50.8; 59.8) by day 14 ($Z = 1.79$; $p = 0.003$), and 58.6% (56.5; 59.8) by day 21 ($Z = 1.79$; $p = 0.003$).

The decrease in FGF-21 concentration on day 3 after local cold injury (from 487.7 PG/mL [423.4; 610.6] to 209.6 PG/mL [69.0; 303.1]; $Z = 0.47$; $p = 0.84$) was replaced by an increase, reaching a peak value on day 7 (827.1 PG/mL [514.9; 1798.3]; $Z = 1.28$; $p = 0.07$; Fig. 2, *b*). After 7 days, a downward trend was formed again; as a result, the concentration of FGF-21 was the same as in the control group on day 21 (494.2 PG/mL [389.2; 749.4]; $Z = 0.44$; $p = 0.98$).

The concentration of IGF-1 in the first 3 days after local cold injury decreased from 6.0 PG/mL (2.3; 10.8) to 2.2 PG/mL ([1.5; 9.2]; $Z = 1.24$; $p = 0.09$; Fig. 2c). By day 7, its plasma content increased to 12.9 PG/mL ([4.0; 18.0]; $Z = 1.16$; $p = 0.13$). A statistically significant maximum value was registered on day 14 (up to 18.0 PG/mL [10.2; 21.0]; $Z = 1.92$; $p = 0.001$) and remained until the end of the experiment.

The content of VEGF-C in rat serum decreased on day 3 (from 417.2 PG/mL [113.8; 646.9] to 209.6 PG/mL [69.0; 303.1]; $Z = 1.07$; $p = 0.19$; Fig. 2, *d*). Then, its concentration increased to the level of the control group from days 7 (464.0 PG/mL [204.0; 648.0]; $Z = 0.57$; $p = 0.90$) to 14 (440.6 PG/mL [154.6; 719.5]; $Z = 0.51$; $p = 0.95$). After that, there was a sharp increase in concentration by day 21 (1684.8 PG/mL [614.8; 1899.4]; $Z = 1.80$; $p = 0.003$).

Discussion of results

Therefore, the essence of reactive changes is the formation of eosinophenia on day 3 after local cold injury: 0.0% (0.0; 1.0) versus 2.0% (1.0; 4.0; $Z = 1.98$; $p = 0.001$). Eosinopenia occurs in acute viral and bacterial infections as well as in exacerbation of chronic infectious diseases [25]. Given the possibility of increasing the secretion of corticosteroids and adrenocorticotropic hormone in response to cold damage, it can be assumed that the mechanism that causes eosinopenia is the full stop of the release of bone marrow eosinophils into the blood, which should be accompanied by an increase in the number of eosinophils in the red bone marrow [26]. According to the obtained data, only the content of eosinophilic myelocytes increases (from 12.0% [10.0; 23.0] to 20.0% [13.0; 24.0]; $Z = 0.88$; $p = 0.41$), which are capable of mitosis, as well as eosinophilic metamyelocytes (from 20.0% [10.0; 21.0] to 24.0% [19.0; 30.0]; $Z = 1.28$; $p = 0.07$), which cannot enter mitosis [27]. In the ratio of mature bone marrow eosinophils, the reverse reaction is observed: their content decreases (from 6.0% [2.0; 9.0]

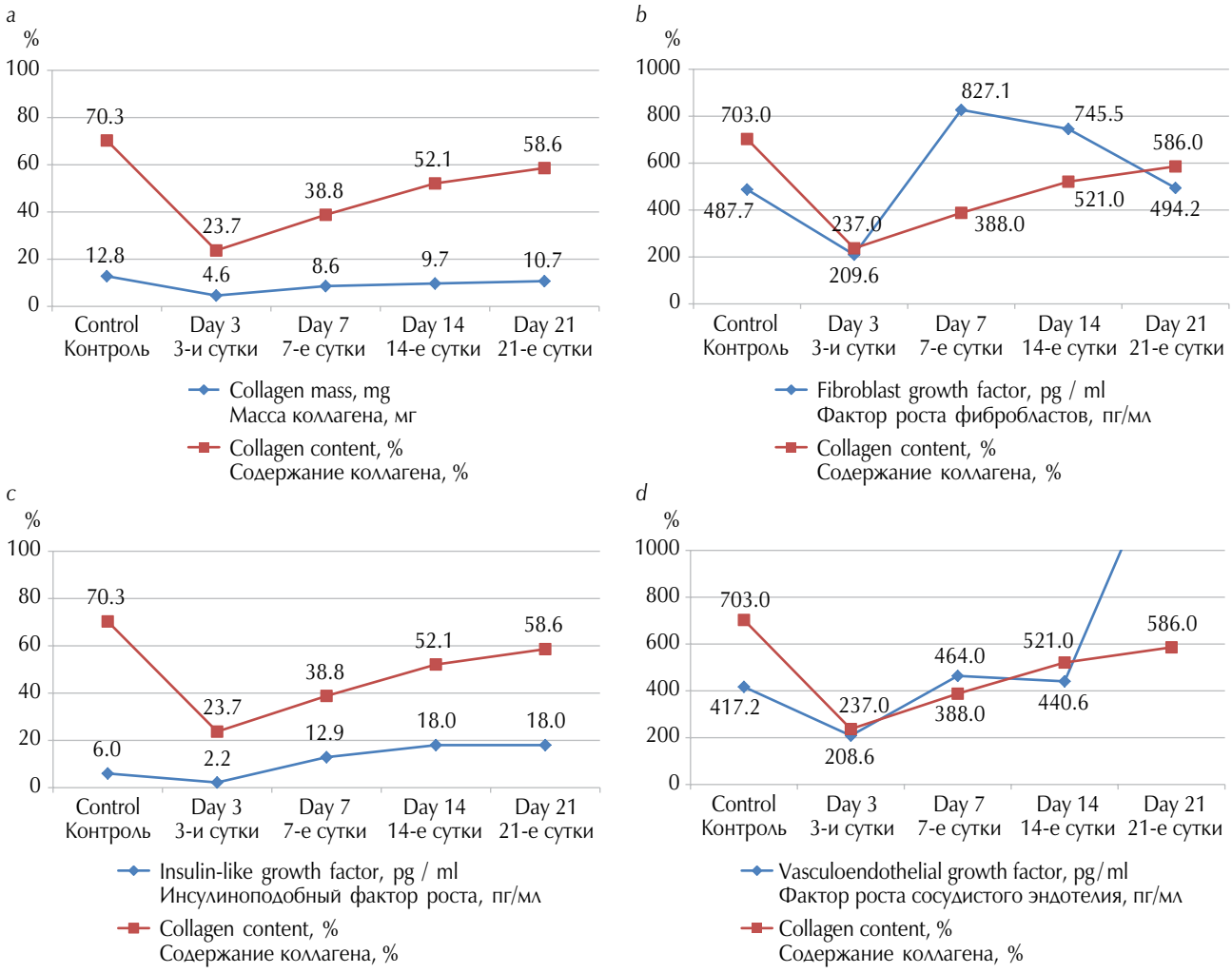

Fig. 2. The dynamics of the concentration of growth factors and collagen content

Рис. 2. Динамика концентрации факторов роста и содержания коллагена

to 2.0% [2.0; 5.0]; $Z = 1.16$; $p = 0.16$). Thus, it seems that with local cold damage, eosinophenia does not occur because of a delay in the release of bone marrow eosinophils into the blood. This confirms the development of eosinopenia in animals that have undergone adrenalectomy [25]. It is likely that eosinophilia after local cold injury develops because of eosinophil sequestration in the affected area. Eosinophil sequestration in tissues in allergic and rheumatic diseases and in tumors is known, but the difference from these conditions is the presence of eosinophilia and not eosinophilia after local cold injury [28].

It is interesting to study the dynamics of reactive changes after local cold injury. So eosinopenia appears itself already on day 3. On day 7 after local cold injury, the median content of bone marrow eosinophils reached a minimum value of 1.5% (0.0; 5.75), as a result of which the difference in the content of eosinophils in the red bone marrow and peripheral blood disappeared ($W = -1.6$; $p = 0.1$), which was a difference from the indicators in the previous and subsequent days of the experiment. A decrease in the concentration of bone marrow eosinophils was accompanied by a decrease in the eosinophilic myelocyte concentration to a minimum (10.5% [9.0; 13.5]). On day 14, the content of myelocytes and bone marrow eosinophils increased, and the difference in the concentration of eosinophils in the red bone marrow and peripheral blood was restored ($W = -2.03$; $p = 0.04$). Only on day 21 after local cold injury, the content of bone marrow and blood eosinophils was comparable with these indicators in the control group.

On day 3 of the third degree of cold injury, the epidermis is completely drained, and the papillary layer of the dermis is destroyed, and

there is a pronounced fibrinoid necrosis in the reticular dermis [1–5]. It is obvious that the low collagen content on day 3 was minimal because the collagen was destroyed, and new fibers were not synthesized. Considering the decrease in the proportion of a dermal collagen in the affected area, which is positively correlated with a decrease in the concentration of blood eosinophils ($\tau = 0.77$; $p = 0.04$), it can be assumed that the eosinophil sequestra tion on day 3 is also associated with the possible destruction of a damaged collagen by collagenase contained in the granules of mature eosinophils [29, 30].

By days 7–14 after local cold injury, an increase in collagen of the damaged area was recorded, which indicates the maximum synthetic activity of fibroblasts during this period. On day 7, the dermal collagen content was positively correlated with bone marrow eosinophils but not with blood eosinophils ($\tau = 0.73$; $p = 0.04$). Considering the presence of a direct correlation between the dermal collagen content and bone marrow eosinophils against the background of the disappearance of differences in the content of bone marrow and blood eosinophils, it seems that on day 7 after local cold damage, bone marrow eosinophils are already involved to control collagen formation.

The general trend of the study growth factors in the first 3 days after local cold damage is in reducing their concentration in the serum simultaneously with a decrease in the content of collagen in the dermis and the development of eosinopenia. On day 3, direct correlations were found between the eosinophilic myelocyte content and VEGF-C ($\tau = 0.0.62$; $p = 0.02$), as well as between blood eosinophil and FGF-21 ($\tau = 0.55$; $p = 0.04$).

After 3 days, the content of growth factors begins to increase, but the nature of their subsequent dynamics is different. Thus, the FGF-21 concentration reaches a peak value during the maximum synthetic activity of fibroblasts (from days 7 to 14) and positively correlates with the concentration of blood eosinophils ($\tau = 0.8$; $p = 0.04$) and returns to the control values on day 21. The level of IGF-1 in the serum reaches a peak value on day 14 and persists until day 21. The VEGF-C content reaches its maximum value only by day 21. Because the peak concentration of FGF-21 is observed from days 7 to 14, IGF-1 from days 14 to 21, and VEGF-C on day 21, it seems that the growth factors studied are consistently involved in the reparative process.

The maximum value of FGF-21 reaches in the period from 7 to 14 days, when the maximum synthetic activity of fibroblasts is observed, which is confirmed by an increase in the content of collagen in the dermis. Since the focal contacts between the collagen matrix and fibroblasts are disrupted in the first 3 days, it can be assumed that FGF-21 acts together with eosinophils as a stimulating factor that is necessary to enhance the synthetic function of fibroblasts.

The maximum IGF-1 secretion occurs on day 14 and continues until day 21. Probably, during this period, IGF-1 is more actively involved in regeneration in the absence of correlations with the content of eosinophilic leukocytes of the red bone marrow and blood.

The processes of angiogenesis, the formation of collateral blood circulation, and the restoration of oxygen supply to tissues begin to manifest themselves more actively after 21 days, when the concentration of VEGF-C becomes maximum, and during this period, it does not correlate with the level of eosinophils.

Therefore, reactive changes after local cold injury not only in the peripheral blood but also in the red bone marrow may indicate the participation of eosinophils in the processes of tissue recovery after local cold injury.

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