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PERIPHERAL BLOOD LEUKOCYTE DIFFERENTIAL DYNAMICS DURING CUTANEOUS EXCISIONAL WOUND HEALING IN RATS FOLLOWING CHRONIC SOCIAL STRESS

Makyeyeva L.V. ✉, Frolov O.K., Aliyeva O.G., Tavrog M.L., Pototska O.I., Hromokovska T.S.

Peripheral blood leukocyte differential dynamics during cutaneous excisional wound healing in rats following chronic social stress.

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ABSTRACT. Background. Blood reflects the functional state of the organism and the morphogenetic function of the immune system in ensuring structural and humoral homeostasis during physiological and pathological changes. Chronic social stress is a common systemic factor that disrupts immune regulation and delays skin repair. **Objective.** To assess changes in the peripheral blood leukocyte differential in rats during cutaneous excisional wound healing accompanied by chronic social stress. **Methods.** 48 male Wistar rats were divided into a control group of stress-resistant rats and an experimental group of stress-prone rats exposed to twenty-one days of chronic social stress verified by the open field test. Full-thickness excisional skin wounds were made, and tail vein blood smears were collected at wounding and on the 1, 3, 7, and 14 days, stained by Pappenheim, and analyzed microscopically. **Results.** Eosinophils and basophils varied minimally in blood as they act mainly as tissue-resident cells in the dermis. Systemic recirculating leukocytes—neutrophils, monocytes, and lymphocytes—showed stage-dependent dynamics. In control rats, segmented neutrophils peaked on day one, monocytes peaked on day three, and lymphocytes dropped on day one due to migration into the wound before normalizing by day 7. In stressed rats, baseline neutrophils and monocytes were elevated while lymphocytes were reduced; after wounding, elevated neutrophil and monocyte counts persisted through days 7 and 14 alongside sustained lymphopenia. **Conclusion.** Peripheral blood dynamics of segmented neutrophils, monocytes, and especially lymphocytes are informative indicators of reparative regeneration, showing that chronic social stress prolongs inflammation and delays tissue remodeling.

Key words: peripheral blood, leukocyte differential, cutaneous wound, reparative regeneration, chronic social stress, lymphocytes, neutrophils, monocytes.

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Introduction

Blood reflects the functional state of the organism and the morphogenetic function of the immune system to ensure homeostasis during its physiological and pathological changes [1-3]. One of the most widespread and cheapest immunological methods that objectively reflect the state of the immune system, immune response, and lymphogenesis in the examined organism is the leukocyte formula [1]. Shifts in circulating white blood cells reflect the intensity of bone marrow hematopoiesis and the directional migration of leukocytes into damaged tissues [4, 5].

Cutaneous wound healing comprises overlapping phases of acute inflammation, cellular proliferation, and remodeling [6-8]. Following wounding, segmented neutrophils rapidly migrate into the wound bed to clear microorganisms and detritus [6, 7], while infiltrating monocytes differentiate into macrophages that switch destructive inflammation to reparative proliferation [8, 9]. Lymphocytes with macrophages and dermal cells regulate cell differentiation and remodeling [1, 2, 7].

One of the systemic factors known to affect immune cells is stress [10, 11]. More than three-quarters of adults report symptoms of stress, including headache, tiredness, or sleeping problems [12], which makes this problem relevant. Chronic social stress (CSS) activates the hypothalamic-pituitary-adrenal axis and sympathetic nervous system, stimulating stress-induced myelopoiesis, suppressing lymphopoiesis, and disrupting skin homeostasis [10, 13-17]. Therefore, this immunological analysis can be studied as a possible indicator of chronic social stress.

Objective

The aim of this work was to assess changes in the peripheral blood leukocyte formula after the influence of chronic social stress in rats during wound healing.

Materials and methods

The study was conducted on 48 male Wistar rats aged 12–13 months and weighing 210–230 g, kept under standard vivarium conditions in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 18.03.1986) [18] and the Law of Ukraine No. 3447-IV [19], as well as the International Guiding Principles for Biomedical Research Involving Animals [20], the Rules for conducting work with experimental animals [21], and Order of the Ministry of Health of Ukraine No. 281 of 01.11.2000 [22]. Based on behavioral screening in the Open Field test [23, 24], animals were divided into: control group (n = 20), in which a full-thickness excisional skin wound [25, 26], and experimental group (n = 28, 7 animals per time point), in which a similar excisional wound was inflicted after the influence of chronic social stress (CSS) [15]. To model CSS for 21 days, each rat was housed individually surrounded by 4 transparent cages containing aggressive dominant conspecific males, ensuring continuous visual, auditory, and olfactory threat without physical trauma [15, 27-34].

Peripheral blood was obtained from the tails of rats [35] and stabilized by heparin on the day of wounding and on days 1, 3, 7, and 14 of wound healing in both groups of animals. Blood smears were stained by Pappenheim and the leukocyte formula was calculated in accordance with standard methodology [1]. Statistical analysis was performed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) [36]. Normality of quantitative indicator distributions was checked by the Kolmogorov-Smirnov single-sample test [37]. The values in the tables are presented in the form $\bar{x} \pm SD$, where $\bar{x}$ is the arithmetic mean and SD is the standard deviation. P-values less than 0.05 ($p \leq 0.05$) were considered statistically significant (Student's T-test) [38].

Results and discussion

The factors determining changes in the relative indicators of peripheral blood leukocytes across the stages of cutaneous excisional wound healing in rats (Table 1) are: the intensity of their hematopoiesis, the migratory properties of a specific cell population, as well as their tissue functional properties [1, 2, 7].

Analyzing the changes in the amplitudes of the studied blood cells, one can notice that certain types of leukocytes, such as eosinophils and basophils, fluctuate within insignificant limits during the observation period (eosinophils from 0.45 ± 0.08% to 1.81 ± 0.18% in the control group and from 0.49 ± 0.14% to 1.58 ± 0.24% in the experimental group; basophils from 0.59 ± 0.29% to 0.85 ± 0.31% and from 0.43 ± 0.20% to 0.63 ± 0.24%, respectively) and regardless of the examination group, whereas other populations (lymphocytes, neutrophils, and monocytes) demonstrate a definite significant dynamics, and the reason for these differences, in our opinion, lies in the difference in the functional tropism of the analyzed leukocyte populations.

Thus, our studies [16, 17], confirmed by other authors [6, 7, 39], have shown that eosinophils and basophils are located along the course of blood vessels in the loose fibrous connective tissue with the main task of controlling the physiological constancy of the internal environment; therefore, their cellular progeny after the proliferation of hematopoietic progenitors, bypassing the recirculating environment, transit into the skin tissues. This explains their single frequency in peripheral blood and the rapid emergency replenishment of cellular expenditure during physiological or reparative regeneration, which followed from our studies [16, 17], in which the quantitative amplitudes of these cells during wound healing fluctuated significantly across the examined groups and stages of the reparative process.

Table 1

The leukocyte blood formula of laboratory rats (control) and in rats, experienced chronic social stress during wound healing, $\bar{x} \pm SD$

Group of laboratory rats	Day of wound healing	Neutrophils, %		Lymphocytes, %	Monocytes, %	Eosinophils, %	Basophils, %
		banded	segmented				
Control	wounding	3.69 ± 0.47	28.88 ± 1.32	62.51 ± 3.25	3.42 ± 0.35	1.72 ± 0.15	0.59 ± 0.29
	1	6.12 ± 0.75***	51.24 ± 2.35***	37.40 ± 1.95***	4.18 ± 0.42**	0.45 ± 0.08***	0.61 ± 0.25
	3	4.23 ± 0.54***	30.97 ± 1.48***	52.63 ± 2.74***	8.37 ± 0.85***	1.08 ± 0.12***	0.72 ± 0.28
	7	3.37 ± 0.43*	28.19 ± 1.29**	61.82 ± 3.12***	3.96 ± 0.40***	1.81 ± 0.18***	0.85 ± 0.31
	14	3.56 ± 0.45	28.39 ± 1.35	62.03 ± 3.18	3.67 ± 0.38	1.74 ± 0.16	0.61 ± 0.26
Experimental	wounding	4.29 ± 1.08	36.19 ± 4.82##	49.43 ± 5.61###	6.43 ± 0.37###	0.67 ± 0.30###	0.43 ± 0.20
	1	7.53 ± 0.92***#	54.04 ± 3.85***	31.05 ± 3.48***##	6.24 ± 0.45###	0.66 ± 0.19#	0.48 ± 0.21
	3	4.12 ± 0.68***	45.52 ± 3.42**###	36.78 ± 3.95*###	12.46 ± 0.94***###	0.49 ± 0.14###	0.63 ± 0.24
	7	3.59 ± 0.55	43.47 ± 3.18###	43.87 ± 4.12*###	7.53 ± 0.58***###	1.02 ± 0.21***###	0.52 ± 0.22
	14	3.62 ± 0.52	32.71 ± 2.45***##	55.70 ± 4.35***#	5.91 ± 0.46***###	1.58 ± 0.24**	0.48 ± 0.19

Notes: * – statistically significant changes compared to the previous wound healing period ($p \leq 0.05$); ** – statistically significant changes compared to the previous wound healing period ($p \leq 0.01$); *** – statistically significant changes compared to the previous wound healing period ($p \leq 0.001$); # – statistically significant changes compared to the control group at the corresponding time point ($p \leq 0.05$); ## – statistically significant changes compared to the control group at the corresponding time point ($p \leq 0.01$); ### – statistically significant changes compared to the control group at the corresponding time point ($p \leq 0.001$).

Thus, eosinophils and mast cells can be attributed to tissue forms of immune cells. At the same time, basophils/mast cells as the main immunological cells of the skin ensure structural and humoral control and regulation of trophic loose connective tissue, in which blood and lymphatic capillaries are located, and in the case of a violation of structural homeostasis, they induce an inflammatory reaction and its reparative completion [6, 16, 17]. Eosinophils are inflammatory cells, but with other functional features – they regulate the strength of inflammatory reactions and remodeling processes through limiting biologically active substances from mast cells in the granules exocytosed by them into the intercellular space [7, 39]. Therefore, outside of cases of complicated cutaneous wound healing in rats by the type of secondary bacterial contamination, as well as allergic and oncogenic cases, the indicators of basophils in peripheral blood (0.59 ± 0.29% to 0.85 ± 0.31% in control and 0.43 ± 0.20% to 0.63 ± 0.24% in the experimental group) are of low informativeness, compared with their comparison with mast cells in the tissues of the skin and the wound itself [16, 17].

The blood indicators of the other group of immune cells, which we called systemic for their ability to recirculate and migrate into organs – neutrophils, monocytes, lymphocytes – proved to be adequate to the reparative situation.

Thus, in the group of control animals, for which the infliction of an excisional skin wound is an acute stress mobilizing the protective reactions of the organism, the dynamics of the systemic group of immune cells demonstrated their physiologically adequate dynamics at all periods of healing. Thus, in the control group before wounding, all systemic immunological blood indicators differed among themselves according to their functional properties (banded neutrophils – 3.69 ± 0.47%, segmented neutrophils – 28.88 ± 1.32%, lymphocytes – 62.51 ± 3.25%, monocytes – 3.42 ± 0.35%) and were comparable with the data of other authors [1, 4, 5].

After wounding, the quantitative indicators of segmented neutrophils sharply increased from the initial amount of 28.88 ± 1.32% to 51.24 ± 2.35% ($p \leq 0.001$), along with banded neutrophils increasing from 3.69 ± 0.47% to 6.12 ± 0.75% ($p \leq 0.001$), as was to be expected from the first line of defense of motile cells migrationally filling the wound space, forming with other blood factors a protective barrier against the expansion of microorganisms [6, 7]. On

the 1st day of inflammation it was maximal and decreased significantly by its end on the 3rd day to 30.97 ± 1.48% ($p \leq 0.001$) for segmented neutrophils and 4.23 ± 0.54% ($p \leq 0.001$) for banded, which indicates sufficient clearance of detritus in the wound and its preparation for subsequent successful reparative processes, which was confirmed by the indicators of neutrophils in the early period (day 7: segmented – 28.19 ± 1.29%, $p \leq 0.01$; banded – 3.37 ± 0.43%, $p \leq 0.05$) and late period of proliferation (day 14: segmented – 28.39 ± 1.35%; banded – 3.56 ± 0.45%).

A noticeable rise in quantitative ratios was also observed among monocytes in the blood during the acute period (from 3.42 ± 0.35% before wounding to 4.18 ± 0.42%, $p \leq 0.01$ on the 1st day and to a representative 8.37 ± 0.85%, $p \leq 0.001$ on the 3rd day), when the significance of monocytes/macrophages increases particularly [8, 9]. Together with mast cells and lymphocytes, they switch the destructive processes of the acute phase to a constructive one, shifting the cellular and cytokine profile from the Th1 cytotoxic type to the Th2 reparative type [7-9], which was maintained in the subsequent period of the proliferative process, already contributing to a decrease in the migratory activity of monocytes for replenishing tissue macrophages on day 7 (3.96 ± 0.40%, $p \leq 0.001$) and day 14 (3.67 ± 0.38%).

There is evidence that monocytes/macrophages in skin tissues control the number of cells and their mass during histogenesis [8, 9], whereas lymphocytes in reparative situations as the main cells of the immune system subpopulationally and clonally regulate phenotype-determined differentiation and remodeling processes [1, 2, 15]. The intensive migratory dynamics of lymphocytes points to these data. Thus, from 62.51 ± 3.25% before wounding, they maximally decreased on the 1st day of the acute period to 37.40 ± 1.95% ($p \leq 0.001$) due to intensive migration into the shock organ, and by the 3rd day, when reparative processes began to resolve acute inflammation, their count restoratively increased to 52.63 ± 2.74% ($p \leq 0.001$) and returned to the initial level at all remaining periods of observation (61.82 ± 3.12%, $p \leq 0.001$ on day 7 and 62.03 ± 3.18% on day 14). This indicates that in the subsequent proliferative periods of wound healing, the tension of migratory processes of lymphocytes decreased significantly and their count returned to the initial indicator, which testifies to their sufficiency for the completion of reparative processes.

Onto the systemic inflammation of the skin in stressed animals, confirmed by our studies [15-17], destructive processes upon the infliction of an excisional wound were superimposed, which aggravated dysregulatory processes in the skin. This was reflected in the indicators of the systemic level of immune cells. In the stressed group of animals, even before wounding, a relative increase in segmented neutrophils to 36.19 ± 4.82% compared to 28.88 ± 1.32% in the control ($p \leq 0.01$) was noticeable, with a sharp increase to 54.04 ± 3.85% ($p \leq 0.001$) on the 1st day of healing. During this period, the number of immature (banded) neutrophils also increased from 4.29 ± 1.08% to 7.53 ± 0.92% ($p \leq 0.001$; $p \leq 0.05$ vs. control). This high level of segmented neutrophils remained high both on the 3rd day of the inflammatory period (45.52 ± 3.42%, $p \leq 0.01$ vs. day 1; $p \leq 0.001$ vs. control) and at the beginning of the proliferative period on the 7th day of the experiment (43.47 ± 3.18%, $p \leq 0.001$ vs. control), decreasing only on day 14 to 32.71 ± 2.45% ($p \leq 0.001$ vs. day 7; $p \leq 0.01$ vs. control). Dynamics of neutrophils indicates that despite tense myelopoiesis and tense migration into the wound, it nevertheless testifies to the chronicization of its healing [7, 13]. Thus, after the influence of CSS, the accumulation of detritus at the beginning of the inflammatory period prolonged the inflammatory period by more than a week, delaying the development of other reparative processes as well.

Thus, the elevated indicators of monocytes even before wounding (6.43 ± 0.37% vs. 3.42 ± 0.35% in control, $p \leq 0.001$) remained high on the 1st day of healing (6.24 ± 0.45%, $p \leq 0.001$ vs. control) and continued to increase on the 3rd decisive day of reparation (12.46 ± 0.94%, $p \leq 0.001$ vs. day 1; $p \leq 0.001$ vs. control), when the destructive consequences of skin traumatization should be resolved with switching them to morphogenetic proliferative and differentiating processes of remodeling, remaining elevated on day 7 (7.53 ± 0.58%, $p \leq 0.001$ vs. control) and day 14 (5.91 ± 0.46%, $p \leq 0.001$ vs. control). The presence of memory for a decrease in the phagocytic and regulatory cytokine function of macrophages in the wound has been established by researchers [10, 13, 40]. It persisted for more than a week, even outside the influence of stress hormones (corticosteroids and catecholamines) [13, 14, 40]. Therefore, the indicators of lymphocytes, whose function is closely connected with macrophages, are expectedly reduced. Their indicators in stressed animals did not reach the initial level of control animals (49.43 ± 5.61% and 62.51 ± 3.25%, respectively, $p \leq 0.001$) and remained sharply reduced at all periods of observation (31.05 ± 3.48%, $p \leq 0.01$ vs. control on day 1 and 36.78 ± 3.95%, $p \leq 0.001$ vs. control on day 3), only with a tendency to approach the control level by the beginning of the proliferative period on day 7 (43.87 ± 4.12%, $p \leq 0.001$ vs. control) and its end on day 14 (55.70 ± 4.35%, $p \leq 0.05$ vs. control). These indicators of lymphocytes clearly show that when they are suppressed, the development of granulation tissue decreases, as well as its reorganization into definitive tissues by means of remodeling [1, 2, 15]. These reduced indicators clearly point to their close cooperation with cellular and humoral factors of innate immunity: with the insufficiency of preparatory and reparative processes at the previous stages of wound healing, for example, clearance of detritus and development of granulation tissue, the populational and clone-spe-

cific regulation of lymphocytes in remodeling processes is delayed.

Conclusion

Peripheral blood indicators of tissue forms of eosinophils and basophils are of low informativeness in wound processes compared to their assessment in skin tissues. Monitoring the effectiveness of reparative processes is indicative when evaluating the dynamics of segmented neutrophils, monocytes/macrophages, and especially lymphocytes as the main cells of the peripheral blood of rats.

Prospects for further research

Investigating lymphocyte subpopulations and macrophage phenotypes in regenerating skin under chronic social stress.

Information on conflict of interest

There are no potential or apparent conflicts of interest related to this manuscript at the time of publication, and are not anticipated.

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Макєєва Л.В., Фролов О.К., Алієва О.Г., Таврог М.Л., Потоцька О.І., Громоковська Т.С. Динаміка лейкоцитарної формули периферичної крові під час загоєння різаної рани шкіри у щурів після впливу хронічного соціального стресу.

РЕФЕРАТ. Актуальність. Кров відображає функціональний стан організму та морфогенетичну функцію імунної системи у підтриманні структурного і гуморального гомеостазу за фізіологічних та патологічних змін. Хронічний соціальний стрес є поширеним системним чинником, що порушує імунну регуляцію та сповільнює загоєння шкіри. **Мета.** Оцінити зміни лейкоцитарної формули периферичної крові на

тлі хронічного соціального стресу у щурів під час загоєння різаної рани шкіри. **Методи.** Сорок вісім самців щурів лінії Вістар поділили на контрольну групу стресостійких тварин і дослідну групу схильних до стресу тварин після двадцяти однієї доби хронічного соціального стресу, підтвердженого у тесті відкритого поля. Після нанесення повношарової різаної рани шкіри мазки крові з хвостової вени брали у день нанесення рани та на 1, 3 7 і 14 добу, фарбували за Паппенгеймом та аналізували мікроскопічно. **Результати.** Частки еозинофілів і базофілів у крові коливалися незначно, оскільки вони діють переважно як тканинні клітини дерми. Системні рециркулюючі лейкоцити — нейтрофіли, моноцити та лімфоцити — виявили стадійну динаміку. У контролі частка сегментоядерних нейтрофілів була максимальною у 1 добу, моноцитів — на 3 добу, а лімфоцитів — знижувалася у 1 добу через міграцію в рану з відновленням до сьомої доби. У стресованих щурів до поранення вміст нейтрофілів і моноцитів був підвищеним, а лімфоцитів — зниженим; після поранення високі показники нейтрофілів і моноцитів трималися до 7 та 14 доби на тлі стійкої лімфопенії. **Підсумок.** Динаміка сегментоядерних нейтрофілів, моноцитів та особливо лімфоцитів крові є інформативним показником репарації, що свідчить про хронізацію запалення і затримку ремоделювання рани при хронічному соціальному стресі.

Ключові слова: периферична кров, лейкоцитарна формула, різана рана шкіри, репаративна регенерація, хронічний соціальний стрес, лімфоцити, нейтрофіли, моноцити.

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