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Quantitative characteristics of mast cells in the course of wound healin in rats with chronic social stress

Makyeyeva L. V.[1,2], Aliyeva O. G.[1], Frolov O. K.[2]
ORCID: 0000-0002-3188-2638, 0000-0003-1287-674X
[1]Zaporizhzhia State Medical University, Ukraine
[2]Zaporizhzhia National University, Ukraine
lyudmylamakyeyeva@gmail.com

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Activation of sympathoadrenal and hypothalamic-pituitary systems under the influence of stress, accompanied by the release of neuromediators and neuropeptides, activation of mast cells producing cytokines, chemokines, etc., leads to the launch of a cascade of pathological processes which indicates that stress can lead to a violation of skin reparation. However, little is known about morphofunctional changes of skin cells under the influence of chronic social stress. The aim of this work was to assess changes in the number of mast cells in the rat skin after the influence of chronic social stress.

The research was performed on 20 Wistar male rats aged 12-13 months, weighting 390 -430 g: 1st group – control (n = 10); modelled chronic social stress to the 2nd group (n = 10) by the 3-week social isolation and prolonged psycho-emotional impact. Stress was confirmed in an open field test. A skin flap sized 1*1 cm was excised on the back in the interscapular region on the day of wounding, and days 1, 3, 7, 14, 30 of wound healing. Mast cells were counted in wound surface area sections, selectively stained with toluidine blue.

The wound healing process in the skin was characterized by a certain dynamic of mast cells' number, as key cellular regulators of inflammatory and regenerative processes. In intact skin of the experimental group revealed a ten times exaggeration of mast cells compared to the control. In the control group, the content of mast cells gradually increased over the next 2 weeks and decreased to the initial level on the 30th day. Initial high level of mast cells decreased by 86,8% in experimental rats already on day 1 with a gradual rise in the next 2 weeks and raised by 593% compared to control group at day 30 which is associated with migration of immune-dependent cells as a result of partial removal of the stress response.

Кількісна характеристика тучних клітин у процесі загоєння ран у щурів із хронічним соціальним стресом

Макєєва Л. В.[1,2], Алієва О. Г.[1], Фролов О. К.[2]
ORCID: 0000-0002-3188-2638, 0000-0003-1287-674X
[1] Запорізький державний медичний університет
[2] Запорізький національний університет
lyudmylamakyeyeva@gmail.com

Ключові слова: *порушення загоювання ран, стадія запалення, стадія проліферації, стадія ремоделювання, психо-емоційний стрес.*

Активація симпатоадреналової і гіпоталамо-гіпофізарної систем при стресі супроводжується вивільненням нейромедіаторів і нейропептидів, активацією тучних клітин, що продукують цитокіни, хемокіни та ін., призводить до запуску каскаду патологічних процесів, вказуючи, що стрес може призвести до порушення репарації шкіри. Однак у сучасних дослідженнях мало даних про морфофункціональні зміни клітин шкіри під впливом хронічного соціального стресу. Метою даної роботи була оцінка кількісних змін тучних клітин шкіри щурів після впливу хронічного соціального стресу.

Дослідження проводили на 20 щурах-самцях лінії Вістар вагою 390 -430 г., віком 12-13 міс.: 1-а група – контроль (n=10); тваринам 2-ї групи (n=10) моделювали стрес 3-тижневою соціальною ізоляцією і тривалим психоемоційним впливом. Стрес підтверджено тестом на відкритому полі. Висікали шкірний клапоть розміром 1*1 см на спині в міжлопатковій ділянці в день нанесення рани і на 1, 3, 7, 14, 30 добу загоєння ран. Тучні клітини підраховували в зрізах взятих області ранової поверхні, селективно забарвлених толуїдиновим синім.

Процес загоєння ран у шкірі характеризувався відповідними особливостями динаміки кількості тучних клітин, як регуляторів запальних та регенераторних процесів. В інтактних зразках експериментальної групи кількість тучних клітин була більшою в 10 р., ніж в контролі. Загальновідомі нормальні показники вмісту кількості тучних клітин у контрольній групі поступово збільшувались у наступні 2 тижні та зменшувались до вихідного рівня на 30 день загоєння ран. Початковий рівень тучних клітин у 2й групі знизився на 86,8% вже на 1 день з поступовим підйомом у наступні 2 тижні, проте він не досягав рівня контролю з подальшим підйомом на 593% порівняно з 1ю групою на 30 день, що пов'язано з міграційними процесами імунозалежних клітин як результат часткового зняття стресової реакції.

Introduction

Skin is the largest organ of the body that performs various homeostatic functions (barrier, thermoregulatory, sensory, excretory, etc.), including immunological ones – it is an organ of primary immune defence that prevents penetration of bacteria, viruses and other exogenous damaging factors. It is one of the most commonly damaged organs[1].

Among the most common risk factors for the development of various diseases, including dermatological pathology, one of the first places belongs to chronic social stress[2]. Long-term studies demonstrate that the activation of the sympathoadrenal and hypothalamic-pituitary systems under stress, accompanied by the release of neurotransmitters and neuropeptides, activation of mast cells producing cytokines, chemokines, prostaglandins, and leukotrienes, growth factors leads to the launch of a cascade of pathological processes[2, 3] indicating that stress can lead to impaired skin repair.

Wound healing is a cascade process that can be divided into three major phases: inflammation, proliferation and scar formation/remodeling[4]. The inflammatory phase begins at the time of initial damage and typically lasts a few days. This phase encompasses such critical elements: passive aggregation of platelets, the neutrophil influx, macrophage accumulation. Mast cells (MCs) or tissue basophils are innate immune cells abundant in the dermis, therefore MCs regulate acute wound inflammation and their activation is also

prominent early after injury[5]. MCs release many different pro-inflammatory mediators, causing hallmarks of inflammation like vasodilation, vascular permeability and activation/recruitment of circulating immune cells[6].

One of the main physiological functions of mast cells is the control and regulation of the state of loose connective tissue (LCT), including stimulation of angiogenesis, activation of the proliferative and synthetic activity of fibroblasts[7]. This function is enhanced during the reparative regeneration of the connective tissue environment during the entire healing process.

Studies indicate that activated tissue basophils produce cytokines and growth factors that support the proliferation and migration of several cell types in the skin and promote the development of the proliferative phase of healing. It is also clear that mast cell degranulation promotes scarring by stimulation of fibroblasts[8].

The aim of this work was to assess changes in the number of mast cells in the rat skin after the influence of chronic social stress.

Material and methods

The research was performed on 20 white male Wistar rats, aged 12-13 months, weighing 390 -430 g at the time of picking, and they were divided into 2 groups. The 1st group, control, comprised 10rats. We modelled chronic social stress on animals of the 2nd group, which were more susceptible to stress based on the open-field test results, (n = 10) by the three-week social isolation and prolonged psychoemotional impact[9]. Stress was confirmed in an open field test, which was performed by all animals before and after modelling chronic social stress. The behaviour of the animals was observed in an open field sized 80 x 80 cm, lined into squares 10 x 10 cm for 5 minutes. The field was illuminated with bright light (100 W) at a 1 m distance from the field surface. Animals were placed in the centre of the field and the number of urinations and defecations, freezings, latent time (s) of the first run from the central square, the number of crossed central and peripheral squares, time (s) and the number of groomings (long and short) and standings up on hind legs were recorded during 5 minutes of the test. After each animal, the surface of the open field was thoroughly washed with water and dried[9, 10].

For morphological assessment of the skin, a skin flap on the back in the interscapular region about 1cm*1cm in size was excised on the day of wounding and on days 1, 3, 7, 14, 30 of wound healing. Days 1 and 3 correspond to the inflammatory phase of wound healing, days 7 and 14 – proliferative and day 30 – scar formation/remodelling. Samples were excised so that both the wound healing site and undamaged tissue were in them.

Manipulations with animals were performed in compliance with regulated norms and rules for the treatment of laboratory animals: principles of bioethics, legislation and requirements following the provisions of the European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes (Strasbourg, France, 1986), The Law of Ukraine "On protection of animals from cruel treatment". All procedures performed were approved by the Bioethics Committee of Zaporizhzhia State Medical University.

Skin samples including the boundaries of the wound surface were fixed in 10% neutral formalin solution in a vessel with tinted glass, stored at room temperature for 3 days before histological experiments. Next, the skin was embedded into paraffin blocks according to standard histological methods, from which microtome serial sections with a thickness of 5 μm were made. Serial sections were made using a Thermo Scientific HM 325 microtome and stained by acidified toluidine blue staining. Morphometric studies were performed directly on histological specimens using a Carl Zeiss Primo Star microscope. We prepared microphotographs by PrimoStar iLED microscope and an Axio CamERc5s camera (ZEISS, Germany), that were analyzed by the ZEISS ZEN 2011 microscopy program (calculation of the dermis area). The results were expressed as the number of mast cells per 0,01 mm^2.

Statistical analysis and presentation of experimental results were performed using IBM SPSS Statistics version 20 (IBM corp., Armonk, NY, USA). Normality of quantitative indicators' distribution was checked by Kolmogorov-Smirnov single-sample test. Mann-Whitney test for independent samples with normal distribution was used for the open-field data. P-values less than 0,05 ($p < 0,05$) were considered statistically significant[11]. Each value is expressed as mean ± SEM. One-way ANOVA was used to test for overall differences in the extent of days of the wound healing process; where appropriate, Tukey's multiple comparison[12] was used to test for specific differences between control and experimental groups. A difference considered statistically significant at $p<0.05$.

Results

As follows from the data in the table, after modelling chronic social stress, the number of crossings of the open field in the centre decreased by 15,5% ($p<0,001$), and on the periphery by 53,1% ($p<0,001$); vertical mobility against the wall decreased by 40,2% ($p<0,001$) quantitatively and by 38,9% ($p<0,001$) over time and vertical mobility in the open space decreased by 68,7% ($p<0,001$) quantitatively and 55,5% ($p<0,001$) over time. There was a decrease in stereotypical acts of grooming – short by 68,3% ($p<0,01$), long by 69,2% ($p<0,01$). The number of defecations and urination increased by 10 times ($p<0,001$) compared with the control, and the number of freezing increased by 80,5% ($p<0,001$). Thus, we have identified increased anxiety in animals that have undergone chronic social stress.

Table 1 – Data of an open-field test after conduction of psycho-emotional stress

Group/ Indicator	Control	Experiment
Short grooming, sec	3,14 ± 0,34	1,98 ± 0,45**
Short grooming, pcs.	3,69 ± 0,24	1,17 ±0,11**
Long grooming, sec	4,83 ± 0,80	3,28 ±1,06**
Long grooming, pcs	1,07 ± 0,18	0,33 ±0,09**
Latent period, sec	0,02 ± 0,02	0,54 ±0,10**
Horizontal mobility, number of central squares	81,54 ± 2,66	68,90 ±3,01***
Horizontal mobility, number of peripheral squares	58,17 ± 3,57	27,31 ±2,12***
Vertical mobility against the wall, sec	26,46 ± 1,68	16,17 ±1,11***
Vertical mobility against the wall, pcs	14,88 ± 0,80	8,90 ±0,52***
Vertical mobility in open space, sec	16,51 ± 1,52	7,35 ±1,71***
Vertical mobility in open space, pcs	10,41 ± 0,99	3,26 ±0,79***
Number of urinations and defecations	0,14 ± 0,14	1,41 ±0,14***
Number of freezings	0,43 ± 0,15	2,20 ±0,57**

* – Differences compared to control group are significant at P < 0,05.
** – Differences compared to control group are significant at P < 0,01.
*** – Differences compared to control group are significant at P < 0,001.

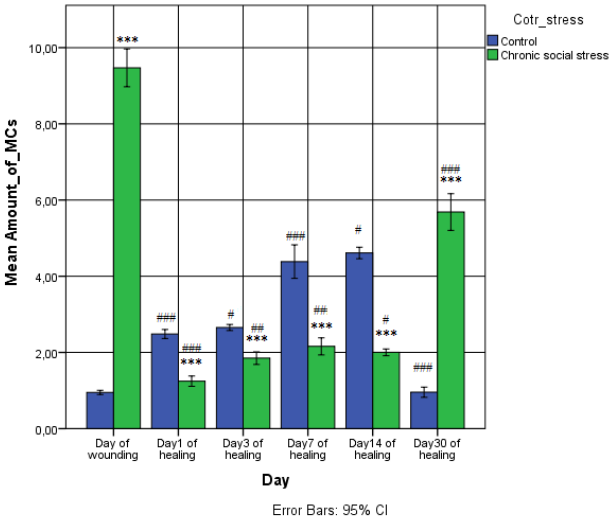

Fig 1. Time course of mast cells' infiltration in the dermis during skin wound healing in control rats and rats with chronic social stress. Results are expressed as the mean number of MCs per 0,01mm2 area, in the upper dermis for each time point after wounding in control and experimental groups.

*** – Differences compared to control group are significant at P < 0,001.

– Differences compared to previous day of wound healing are significant at P < 0,05.

– Differences compared to previous day of wound healing are significant at P < 0,01.

– Differences compared to previous day of wound healing are significant at P < 0,001.

On the day of the wounding in both groups, mast cells were evenly distributed in the dermis, predominantly surrounding blood vessels. In intact samples, the number of MCs was 0,95 ± 0,03 per 0,01 mm^2, which coincided with their physiological values, confirmed by other studies[13, 14], while in the experimental group it was 9,47 ± 0,23 per 0,01 mm^2 as a result of hormone-dependent inhibition of the proliferation of immunocompetent cells, in particular MCs, and their accumulation in immune-dependent organs (figure 1).

The process of wound healing in the skin was characterized by certain features of the number of mast cells' dynamics, as physiological regulators of loose connective tissue, inflammatory and regenerative processes during violation of homeostasis.

During the inflammatory phase (1st and 3rd day of wound healing), mast cells were noted in the tissues of both groups of animals in viable wound tissue. In the control group of animals on the 1st day of wound healing, the amount of MCs increased statistically significantly (P ≤ 0,001) to 2,48 ± 0,06 per 0,01 mm^2 and tended to increase on the 3rd day of wound healing and amounted to 2,65 ± 0, 04 to 0,01 mm^2. In the experimental group of animals on the 1st day of wound healing, the amount of MCs decreased sharply to 1,25 ± 0,06 per 0,01 mm^2, while it was 9,47 ± 0,23 per 0,01 mm^2 on the day of wounding and significantly differed from the control values (P ≤ 0,001). On the third day in the experimental group, there was a pronounced trend towards an increase in the number of MCs (1,85 ± 0,08 per 0,01 mm^2), but it still did not reach the control values. In both groups, there was a massive degranulation of mast cells which was observed in the dermis during the inflammatory stage of wound healing (Figure 2).

During the proliferative stage of wound healing (on the 7th and 14th day), mast cells were found in the fibrous areas of the wound in control and experimental animals. At this stage, on the 7th day of healing in control animals, the number of MCs increased significantly (P ≤ 0,001) compared with that in the inflammatory stage of wound healing and was 4,38 ± 0,20 per 0,01 mm^2, practically remaining at the same level during day 14 (4,61 ± 0,07 per 0,01 mm^2). In the group of experimental animals at this stage, a statistically significant (P ≤ 0,01) slight increase in the number of MCs was observed, followed by a slight statistically significant (P ≤ 0,05) decrease in

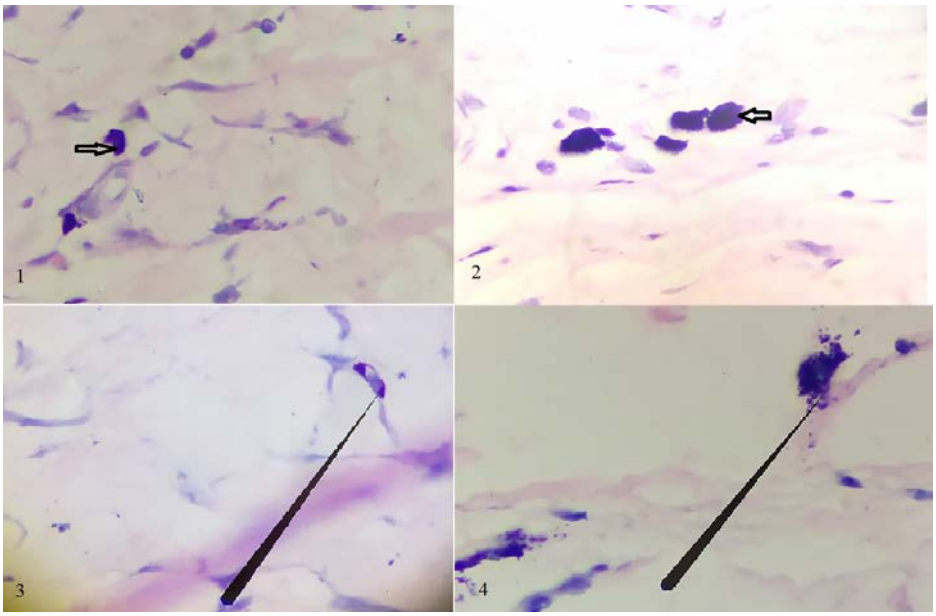

Fig. 2. Histological slides of rat skin stained by toluidine blue. 1, 3 – control animals at day of wounding and day 1 of wound healing respectively. 2, 4 – experimental animals with social chronic stress at the day of wounding and day 1 of wound healing respectively. Mast cells are pointed by arrows

the number of MCs (2,16 ± 0,3 and 2,00 ± 0,04 by 0,01 mm^2 on the 7th and 14th day of wound healing, respectively).

The number of mast cells in the control group of animals in the remodelling stage on the 30th day was almost equal to that on the day of the wounding – 0,96 ± 0,06 per 0,01 mm^2, but in animals with modelled social chronic stress it increased by 592,7% ($p \leq 0,001$) (5,68 ± 0,22 per 0,01 mm^2) in comparison with the control group on day 30, which is also associated with the migration processes of immune-dependent cells, as well as a result of the partial resolution of the stress response.

Discussion

The studied dynamics of the amount and physiological state of MCs in the skin coincides with modern literature data on their functional significance in controlling the structural and functional state of the skin as a polyfunctional organ and LCT in particular, a conductor of blood and lymphoid capillaries that provide homeostasis of the internal environment tissue[7, 15, 16]. Mast cells are similar in origin and set of biologically active substances to basophils, but differ from the latter in that they do not recirculate in the blood in the mature state, but are located along the LCT of blood capillaries, therefore they are ubiquitous. Functionally, MCs belong to cells of innate immunity and, as evidenced by modern studies, they are polyfunctional, actively interacting with both factors of innate immunity and other cells of innate immunity (macrophages, DCs, neutrophils), actively control the homeostatic state of the internal environment of the adjacent LCT, gradually releasing biologically active substances from granules: growth factors, vasoactive mediators, cytokines, enzymes[15]. These BAS components stimulate angiogenesis, capillary tone and permeability, control the cellular composition and structure of LCT, the main function of which is to nourish the cells of adjacent tissues.

In intact skin, mast cells were concentrated mainly in the area of blood vessels and evenly distributed in the dermis, in an amount that corresponds to data published by other researchers[13, 14].

A significant increase of MCs in the inflammatory stage of the wound healing process on the 1st and 3rd day confirms their role in initiating and maintaining this standard protective reaction of the organism. When the structure of tissues of the internal environment is disturbed by infectious and non-infectious agents, MCs' degranulation occurs simultaneously with the release of a large number of biologically active substances into the external space, including pro-inflammatory cytokines (IL-1, IL-8, IL-12, IL-18, IL-21, IL-23, TNF-α, etc.)[6, 16], which are either pre-formed and stored in granules or are synthesized de novo[15] and inducing the stages of the inflammatory reaction and further reparative regeneration (remodulation). A local increase in the number of MCs during inflammation occurs due to the migration of their precursors from the bloodstream, since mature differentiated granulocytes, including MCs lose their ability to divide via mitosis. A normally occurring vascular-cellular reaction during

inflammation promotes the migration of humoral and cellular homeostasis factors into the wound area, which contributes to further optimal wound healing. The continuing even greater significant increase of the number of MCs in the stage of proliferation (7-14 days of wound healing), compared with the inflammatory stage in control animals, once again demonstratively indicates their active participation in this stage of wound healing.

The fact that the number of mast cells on the 30th day in the control group of animals returns to the number of cells on the day of wounding, reflects the completion of the main reparative and remodeling processes.

The described dynamics of the number of MCs during regenerative processes in a skin wound reflects the modern idea of the primary function of the immune system in the morphogenetic control of the histogenesis of all tissues according to the genotype of a given organism, and, consequently, its participation in the regulation of structural and humoral homeostasis during physiological and reparative regeneration[17].

Thus, a significant increase in MC in control animals during the inflammatory stage indicates their participation as polyfunctional cells in the mobilizational integration of cellular and humoral factors of innate and adaptive immunity, and a further even greater increase in the number of MCs in control animals at the stage of active reparative tissue regeneration in the wound indicates even more of their participation at this stage.

Mast cells from a variety of receptors have leading ones, through which the cells of innate immunity, to which they belong, ensure the integration and activation of adaptive immunity cells (T and B-lymphocytes)[18]. Through a system of signalling pattern-recognizing receptors, the leading of which are Toll-like receptors, MCs provide the synthesis of costimulatory structures for T-lymphocytes and their humoral cytokine activation. Whereas it is indisputably known that the majority of newly formed T and B lymphocytes in the central organs of the immune system are moderately sensitized to cell autostructures and regulate the metabolic, proliferative and differentiation processes of tissue histogenesis during their physiological and reparative regenerations, i.e. morphogenetic reactions[19]. The latter fact is confirmed by our preliminary observations of increasing lymphocyte infiltration of the wound area, mainly at the stage of scar tissue formation.

The stress reaction dramatically changed the normal dynamics of MCs during the wound process and significantly affected the timing of its healing.

According to Selye's general ideas about stress, the dynamics of stress has stages of anxiety, resistance, and exhaustion. Its dynamics is under the control of the hypothalamic-pituitary-adrenal system, of which cortisol plays the leading role in the dynamics of immune cells, the effect of which is well studied. Thus, it inhibits proliferative reactions of cells and induces apoptosis in immunological organs, and inhibits their migration in the tissues of the internal environment with deposition in the tissues of predominantly parenchymal organs, which ensures their active participation in the stage of stress resolution. In this regard, the amplitudes of the number of cells at the stages of the wound process reflect the dynamics of the stress response. Thus, a multiple increase of the number of MCs in the intact skin of experimental animals can be explained by their deposition as a result of inhibition of migration under the influence of corticosteroids, in particular cortisol. Whereas their sharp decrease in the stage of inflammation (1-3 days of the wound process) and a significant lag in growth compared to control animals indicate their active apoptosis and ongoing inhibition of migration under the action of corticosteroids. Due to the lagging of a new wave of migration of mast cell precursors in the stage of inflammation, it reduces its effectiveness in mobilizing cellular and humoral factors for reparative processes at the subsequent stages of skin wound healing (7, 14 days). Therefore, the process of wound healing is stretched in time. One of the phenomena of the resolving stage of emotional stress, in which the level of cortisol in the internal environment decreases, in this experiment is an amplitude surge in the number of MCs on the 30th day. It occurs due to a new wave of migration of MC precursors from the bone marrow and other organs of the immune system, where they were also deposited. It should be noted that their number has already returned to the normal control group at this time due to the absence of a stressful state of animals.

Thus, we revealed the dynamics of the number of mast cells and their participation in the resolution of the wound healing process in the norm and under the influence of chronic social stress. In the latter case, we noted contrasting amplitude differences in the amount of MCs at the stages of wound healing in rat skin, increasing time parameters for restoring the structure and function of the skin, which reduces its general functional and immunological properties, requiring further study and correction with immunomodulatory drugs.

Conclusions

The wound healing process in the skin is characterized by a certain dynamic in the number of mast cells, as key cellular regulators of inflammatory and regenerative processes. The content of mast cells increases during the first 2 weeks and decreases by the end of 1 month.

Emotional stress has changed the dynamics of the amount of MCs during skin regeneration: with values many times higher than the control ones in intact

samples, before skin injury and samples taken at the end of 1 month of healing, with significantly inferior amplitude to control samples in the remaining phases of the wound process, which is a consequence of prolongation of the inflammatory and reparative response during wound healing.

Modelling the activity of mast cells during inflammation and regeneration in conditions complicated by stress or other factors is a promising target for the pharmacological regulation of key components in the mechanisms of skin regeneration.

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