ROLE OF MAST CELLS IN SKIN REGENERATION AFTER THERMAL BURN TREATED WITH MELATONIN-ENRICHED DERMAL FILM

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The development of novel local therapies for thermal burns (TB) and their pathogenetic rationale are a pressing challenge. Melatonin (MT) is an endogenous factor of hemostasis regulation with pleiotropic potential. The aim of this study was to assess some parameters of tissue regeneration, the functional state of mast cells and the levels of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) in the experimentally induced TB treated with the original MT-enriched dermal film (DF). A second-degree burn (3.5% of the total body surface area) was modelled by exposing a patch of skin to hot water. Applications of 12 cm^2 DF enriched with 5 mg/g MT were performed every day for 5 days. The following parameters were calculated: the wound area, the rate of wound epithelization, the number of MC in the wound, the intensity of degranulation, and the levels of MMP-9 and VEGF expression. Over the course of treatment, the absolute wound area shrank by 35%, its epithelization rate increased, the number of MC rose, their functional state changed, and the expression of MMP-9 and VEGF increased. A negative correlation was established between the wound area and the expression of MMP-9 and VEGF, as well as between the wound area and the degranulation coefficient. Applications of MT-enriched DF resulted in the reduction of the wound area, higher epithelization rate, an increase in the total MC count and degranulation intensity on days 5 and 10; it also led to a reduction in the total MC count and a loss in degranulation intensity on day 20 (166.87 (154.95; 178.78) un/mm^2 vs. 464.84 (452.92; 476.76) un/mm^2) in the group of intact animals), an increase in MMP-9 expression on day 5 (14.20 (11.30; 18.10) vs. 3.30 (2.20; 4.40) in the intact group), an increase in VEGF expression on days 5 and 10 (33.00 (30.20; 34.90) vs 25.40 (22.20; 29.30) in the intact group), and a reduction in MMP-9 expression on days 10 and 20 after thermal injury.

Keywords: thermal burn, melatonin, dermal film, mast cells, regeneration, VEGF, MMP-9

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Compliance with ethical standards: the study was approved by the Ethics Committee of South-Ural State Medical University (Protocol № 10 dated November 15, 2019). The study was conducted at a standard vivarium in strict compliance with guidelines on the care and euthanasia of laboratory animals outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS № 123 , March 18, 1986, Strasburg), the EU Commission Recommendation 2007/526/EC on Guidelines for the Accommodation and Care of Animals used for Experimental and other Scientific Purposes (June 18, 2007), and the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes (September 22, 2010).

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РОЛЬ ТУЧНЫХ КЛЕТОК В РЕПАРАЦИИ КОЖИ ПОСЛЕ ТЕРМИЧЕСКОЙ ТРАВМЫ ПРИ ПРИМЕНЕНИИ ДЕРМАЛЬНОЙ ПЛЕНКИ С МЕЛАТОНИНОМ

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Разработка и патогенетическое обоснование применения новых средств для локальной терапии термической травмы (ТТ) — одна из актуальных проблем медицины. Мелатонин (МТ) — эндогенный фактор регуляции гомеостаза с плейотропным потенциалом. Целью работы было оценить показатели репарации, функциональное состояние тучных клеток (ТК), содержание матриксной металлопротеиназы-9 (ММР-9) и фактора роста сосудистого эндотелия (VEGF) в очаге повреждения кожи в динамике экспериментальной ТТ в условиях применения оригинальной дермальной пленки (ДП) с МТ. ТТ степени IIIA площадью 3,5% моделировали погружением участка кожи в кипящую воду. ДП площадью 12 $см^2$ с МТ в концентрации 5 мг/г наносили ежедневно в течение пяти суток. Вычисляли площадь раны и скорость ее эпителизации, в ожоговой ране определяли число ТК и интенсивность дегрануляции, экспрессию ММР-9 и VEGF. В динамике ТТ абсолютная площадь ожоговой раны уменьшается на 35%, увеличивается скорость ее эпителизации, в очаге ТТ увеличивается число ТК, изменяется их функциональная характеристика; увеличивается экспрессия ММР-9 и VEGF. Выявлена обратная связь площади ожоговой раны с экспрессией ММР-9 и VEGF, коэффициентом дегрануляции ТК. Применение МТ при ТТ ведет к уменьшению площади ожоговой раны, увеличению скорости ее эпителизации, увеличению в ожоговой ране общего числа ТК и дегрануляции на 5-е и 10-е сутки, снижению общего числа и дегрануляции ТК на 20-е сутки ТТ (166,87 (154,95; 178;78) ед./$мм^2$; в контроле — 464,84 (452,92; 476,76) ед./$мм^2$), увеличению экспрессии ММР-9 на 5-е сутки (14,20 (11,30; 18,10); в контроле — 3,30 (2,20; 4,40)), экспрессии VEGF на 5-е и 10-е сутки (33,00 (30,20; 34,90); в контроле — 25,40 (22,20; 29,30)), снижению экспрессии ММР-9 на 10-е и 20-е сутки ТТ.

Ключевые слова: термическая травма, мелатонин, дермальная пленка, тучные клетки, репарация, VEGF, ММР-9

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Thermal burns are often associated with high mortality and temporary disability. According to WHO, about 11 million people seek medical care for burns every year; many of them sustain a temporary disability. It is estimated that 200,000 people die from burns annually [1]. Despite significant advances in the therapy of burns (skin grafting, stem cell therapy, etc.), slow wound healing, infection and scarring still pose a serious therapeutic challenge as they result in prolonged hospital stay, disfigurement, reduced quality of life, and emotional distress [2]. This can be largely explained by the complexity and diversity of pathogenetic mechanisms underlying the response to heat exposure and the progression and outcomes of thermal burns (TB). Burn researchers hold the opinion that knowledge of TB pathophysiology is essential for finding ways to effectively stop burn wound progression and develop pathogenetically informed methods for burn healing [3]. Neutrophils, macrophages, various subpopulations of lymphocytes, changes in the cytokine profile, oxidative stress, and events of the acute phase response play the key role in the pathogenesis of TB and determine the intensity and success of wound healing [4]. Mast cells (MC), which are abundant in the skin, are among the first responders to TB. They are resident inflammatory cells containing secretory granules filled with preformed and de novo mediators that participate in vascular and exudative responses, pain signaling, fibroblast proliferation, collagen synthesis, and scar remodeling. Regulation of MC function in TB holds promise for novel therapeutic approaches [5].

Apart from protecting the wound from infection and trauma, modern wound dressings promote healing and keep the wound adequately moist. They are fabricated as hydrogels, textile-based gel dressings, hydrocolloids, polyurethane foam, calcium alginate fibers, etc. [6]. Most dressing materials are produced by non-Russian companies, so the development of an original dermal film (DF) for burn care may be high on the Russian research agenda. Dressings for TB contain antiseptics, hemostatic agents, antioxidants, healing-promoting agents, stimulators of tissue regeneration, etc. The Russian State Registry of Medicinal Products has a few entries on medicated films: nitroglycerin films for buccal administration, sildenafil orodispersible films and ocular inserts with taurine. The search for novel therapeutic approaches to TB treatment has a special focus on endogenous regulators of homeostasis [7, 8]. There is theoretical evidence that melatonin (MT) may have a therapeutic potential for TB. MT is mostly known for regulating the sleep-wake cycle and neuronal excitation, as well as synchronizing circadian rhythms and physiological functions [9]. In contemporary science, MT is considered an endogenous factor with multifaceted effects, including antioxidant, pro/anti-inflammatory, immunomodulatory, and antiapoptotic effects, and a regulator of cell proliferation and differentiation, which makes MT an attractive candidate therapeutic agent [10]. The literature on novel MT-enriched wound dressings is scarce; the mechanism of local MT effects on TB has not been studied yet, and there are no topical MT-enriched formulations for the therapy of burns on the Russian pharmaceutical market [11, 12]. Mammalian skin has its own melatoninergic system [13]. MT receptors have been detected not only in keratinocytes and fibroblasts, hair follicles and melanocytes but also in MC, suggesting MT involvement in the regulation of the functional activity of MC, especially in mitigating vascular and exudative responses and stimulating tissue regeneration after TB [14]. Besides, skin cells, including MC, are capable of producing and secreting MT. However, the mechanism of possible MT effects on MC involvement in skin regeneration after TB is not fully clear and cannot be associated with the secretion of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) by mast cells. The aim of this study was to assess some parameters of tissue regeneration, the functional state of mast cells and the levels of MMP-9 and VEGF in the experimentally induced thermal burn treated with an original MT-enriched dermal film.

METHODS

The experiment was conducted in 70 male Wistar rats weighing 200–240 g. The animals were randomized in 4 groups: group 1 (n = 7) — intact animals; group 2 (n = 21) — animals with TB treated with aseptic dressings (TI + AD); group 3 (n = 21) — animals with TB treated with DF and aseptic dressings (TB + DF); group 4 (n = 21) — animals with TB treated with MT-enriched DF and aseptic dressings (TB + MT DF). Aseptic dressings and DF were changed every day for 20 days after TB.

Thermal burns are most commonly caused by hot liquids and flames; in two thirds of the patients, less than 10% of the total body surface area is involved [15]. So, to model a second-degree burn (ICD-10 codification, corresponds to a IIIA degree burn according to the classification proposed at the 27th All-Soviet Congress of Surgeons held in 1960) involving 3.5% of the total animal body surface, the area of skin between the shoulder blades was exposed to distilled water at 98–99 °C for 12 s. Burn depth was verified morphologically. The animals were anesthetized with 20 mg/kg tiletamine-zolazepam. The DF matrix and the MT-enriched DF (12 cm^2) were applied immediately after inducing TB (groups 3 and 4, respectively). The matrix was fixed with an aseptic dressing. The dressing was changed every day for 5 days. In our preliminary experiment, we designed a sodium carboxymethylcellulose film (sodium poly-1,4-β-O-carboxymethyl-D-pyranosyl-D-glycopyranose) enriched with 5 mg/g MT. The matrix was evaluated for its pharmacological and fabrication characteristics: organoleptic properties (appearance, color, transparency, elasticity, presence of impurities, microcracks), adhesiveness, tensile strength, and thickness [16]. In group 3, we used an MT-free DF matrix similar in size and properties to the MT-enriched DF.

The wound area was measured by digital planimetry on days 5, 10 and 20 after TB using a Nikon Coolpix S2800 camera (Nikon; China) and Microsoft Office Visio software (Microsoft; USA). The rate of wound epithelization (VS) was calculated by the formula: VS = S – Sn / t, where S is the wound area before treatment (the area at previous measurement); Sn is the area at subsequent measurement; t is days between measurements. The wound area at subsequent measurements was calculated as % of the initial wound area and expressed as % / day.

On days 5, 10 and 20 after TB, the burned skin and a small amount of healthy skin at the wound margin were excised for pathomorphological and immunohistochemical examinations. Microscopy was performed using a DMRXA microscope (Leika; Germany) and ImageScope M software (Germany) at ×100 and ×400 magnifications.

After staining the tissue sections with toluidine blue (pH = 2,0) (Biovitrum; Russia), a total mast cell count was performed. Mast cells were typed by the degree of degranulation: 1st degree (1–2 granules visualized outside the cell), 2nd degree (3–10 granules outside the cell), 3rd degree (over 10 granules outside the cell); mast cells of each degranulation type were counted. Additionally, the average MC brightness was assessed. The degranulation coefficient was calculated as the ratio of degranulated MC to the total number of mast cells in 10 random fields of view.

The abundance of MMP-9 and VEGF in the wound was determined immunohistochemically using MMP-9 rabbit anti-rat polyclonal antibodies (catalog item PAA553Ra01, Cloud-Clone Corp.; China), VEGF rabbit anti-rat polyclonal antibodies (catalog item PAA143Ra01, Cloud-Clone Corp., China) and a Rabbit Specific HRP/DAB Detection IHC Kit (Abcam; Latvia). Diaminobenzidine was used as a chromogenic substrate to visualize the polymer complex. We calculated the relative surface area of MMP-9-positive and VEGF-positive structures; the integral indicator of MMP-9 and VEGF content was calculated as the product of the relative surface area of the positively stained structures and the intensity of staining. The result was presented in arbitrary units (un/mm^2).

The obtained data were processed in IBM SPSS Statistics 19 (SPSS: An IBM Company; USA). The results were presented as a median (Me) and quartiles (Q_1; Q_3). The significance of differences was assessed using the Kruskal–Wallis, Mann–Whitney *U* and Wald–Wolfowitz runs tests. Correlations between the studied parameters were tested using the Spearman's correlation coefficient (R). The Bonferroni-adjusted significance threshold was $p < 0.01$.

RESULTS

In group 2, there was a reduction in the absolute wound area on day 10, compared to day 5 of the experiment; the rate of epithelization had increased by day 10, and the relative wound area had decreased (Table 1). On day 20, there was a reduction in the absolute wound area, compared to days 5 and 10; the relative wound area on day 20 was also smaller than on day 5. Thus, by day 20 the rate of epithelization had increased in comparison with day 5, and the wound reduction area had grown in comparison with days 5 and 10. The median wound area measured on day 20 was 35% smaller than on day 5.

To investigate the role of MC in the pathophysiology of wound healing, we measured their abundance and activity from the intensity of degranulation in the burn (Table 2). On day 5 the median total MC count in the wound in group 2 was 43% higher than in the group of intact animals; this may be explained by active basophile migration from the bloodstream to the wound site. The number of degranulated MC was more than 3 times higher in group 2 than in the group of intact animals; of them the number of MC in the 1st degree of degranulation was 3 times higher, the number of MC in the 2nd degree of degranulation was 4 times higher, and the number of MC in the 3rd degree of degranulation was 10 times higher. The median value of the degranulation coefficient was 3.5 times higher than in the group of intact animals. Notably, many MC (mostly those in the 3rd degree of degranulation) had a "ghostly" appearance due to the loss or release of their granules. This was reflected in the significant loss of average MC brightness on day 5 of the experiment. On day 10, the rise in the total MC count was even more pronounced than on day 5: the median total number of MC cells in the wound in group 2 was 72% higher than in the group of intact animals. On day 10, the number of MC in the 1st and 2nd degrees of degranulation was 3 times higher than in the intact group; the number of MC in the 3rd degree of degranulation had risen tenfold in comparison with the intact group. The median value of the degranulation coefficient now was 2.5 times higher than in the group of intact animals. The average MC brightness measured on day 10 was 2 times lower than in the intact group. By day 20, the total MC count in group 2 had reached its peak and was 1.7 times higher than in the intact group; the number of degranulated MC, MC in the 1st and 3rd degrees of degranulation and the degranulation coefficient were significantly higher than in the group of intact animals, and the average MC brightness was lower. The highest total MC count, the highest number of degranulated MC and the highest number of MC in the 1st degree of degranulation were observed on day 20; the number of MC in the 2nd degree of degranulation peaked on day 5, and the number of MC in the 3rd degree of degranulation reached its maximum on days 5 and 10. The highest value of the degranulation coefficient was observed on day 5; the lowest MC brightness was observed on days 5 and 10. Summing up, the MC count peaked on day 20 after inducing the burn, and degranulation was the most pronounced on days 5 and 10 of the experiment.

Figure shows the dynamics of MMP-9 and VEGF expression in the wound throughout the experiment. On days 5, 10 and 20 after TB, VEGF expression was significantly elevated in comparison with the intact group. MMP-9 expression was significantly elevated on days 10 and 20. On day 10, VEGF expression in the wound was higher than on day 5; on day 20 it was lower than on day 10. MMP-9 expression on days 10 and

Table 1. Parameters of tissue regeneration after experimentally induced thermal burn treated with MT-enriched DF (Me (Q_{25}; Q_{75}))

Parameter	Group 2 (TB + AD)			Group 3 (TB + DF)			Group 4 (TB + MT DF)		
	Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)	Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)	Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)
Absolute wound area, cm^2	11,66	9,48	7,59	11,59	9,4	7,29	10,33	8,34	5,54
	(11,50; 11,94)	(9,28; 9,93)	(7,23; 7,84)	(11,00; 11,99)	(9,22; 9,81)	(7,01; 7,52)	(10,17; 10,56)	(8,19; 8,51)	(5,24; 5,88)
		*	* **				#	#	#
Relative wound area, %	3,34	3,17	2,99	3,31	3,16	2,81	3,36	3,02	1,98
	(3,25; 3,39)	(3,10; 3,29)	(2,94-3,12)	(3,22; 3,42)	(3,10; 3,28)	(2,74-3,09)	(3,23; 3,42)	(2,91; 3,13)	(1,87; 2,23)
			*					#	#
Epithelization rate, % /day	0,89	1,9	2,26	0,91	2,01	3,06	1,33	6,57	14,3
	(0,86; 0,89)	(1,88; 1,95)	(2,14; 2,55)	(0,85; 0,92)	(1,93; 2,05)	(2,73; 3,15)	(1,29; 1,35)	(5,92; 6,93)	(13,38; 15,17)
		*	* **				#	#	#
Reduction in wound area, %	2,61	3,68	11,49	2,6	3,71	13,58	9,8	16,1	19,98
	(2,59; 2,64)	(3,53; 4,23)	(11,43; 11,64)	(2,58; 2,64)	(3,63; 4,31)	(12,93; 14,01)	(9,64; 10,08)	(14,62; 17,73)	(19,30; 20,38)
		*	* **				#	#	#

Note: * — differences are significant ($p < 0.01$) for comparisons with group 2 on day 5; ** — with group 2 on day 10; # — with group 3 on the specified day.

Table 2. Functional characteristics of mast cells in the burn wound treated with MT-enriched DF (Me (Q_{25}; Q_{75}))

Parameter	Group 1 Intact animals (*n* = 7)	Group 2 (TB + AD)			Group 3 (TB + DF)			Group 4 (TB + MT DF)		
		Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)	Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)	Day 5 (*n* = 7)	Day 10 (*n* = 7)	Day 20 (*n* = 7)
Total mast cell count, un/mm²	166,87	238,38	286,05	441	226,46	286,06	464,84	250,3	369,49	166,87
	(160,91; 172,82)	(226,46; 238.38) *	(274,14; 286,05) *	(441,00; 452,92) * # ##	(202,62; 262,22)	(274,14; 297,97)	(452,92; 476,76)	(250,30; 250,30)	(357,57; 381,41)	(154,95; 178;78)
					*	*	*	* &	* &	&
Number of degranulated mast cells, un/mm²	35,76	178,78	166,87	214,54	178,78	166,87	226,46	202,62	190,7	83,43
	(35,76; 41,72)	(166,87; 178,78) *	(154,95; 178,78) *	(202,62; 214,54) * # ##	(154,95; 190,70)	(166,87; 178,78)	(226,46; 250,30)	(190,70; 214,54)	(178,78; 202,62)	(83,43; 83,43)
					*	*	*	* &	* &	* &
Number of mast cells in the 1st degree of degranulation, un/mm²	23,84	83,43	59,59	178,78	83,43	59,6	178,78	143,03	154,95	59,6
	(11,92; 29,79)	(71,51; 83,43)	(47,68; 71,51)	(178,78; 178,78) * # ##	(71,51; 95,35)	(59,60; 71,51)	(178,78; 202,62)	(143,03; 154,95)	(131,11; 154,95)	(59,60; 59,60)
		*	* #		*	*	*	* &	* &	* &
Number of mast cells in the 2nd degree of degranulation, un/mm²	11,92	47,68	35,76	11,92	47,68	47,68	13,84	35,76	23,84	13,84
	(0; 17,88)	(35,76; 47,68)	(35,76; 47,68)	(11,92; 11,92)	(35,76; 47,68)	(23,84; 59,60)	(13,84; 13,84)	(35,76; 59,60)	(23,84; 39,20)	(11,92; 15,76)
		*	* #	# ##	*	*		*	* &	
Number of mast cells in the 3rd degree of degranulation, un/mm²	5,96	59,59	59,59	11,92	59,6	59,6	23,84	11,92	11,92	11,92
	(0; 11,92)	(47,68; 59,59)	(59,59; 83,43)	(11,92; 11,92)	(35,76; 59,60)	(47,68; 71,51)	(11,92; 23,84)	(11,92; 23,84)	(0,00; 11,92)	(0,00; 11,92)
		*	*	# ##	*	*	*	* &	* &	* &
Degranulation coefficient, au	0,22	0,75	0,59	0,49	0,76	0,6	0,5	0,81	0,5	0,5
	(0,21; 0,26)	(0,73; 0,76)	(0,56; 0,63)	(0,49; 0,49)	(0,71; 0,77)	(0,56; 0,61)	(0,48; 0,53)	(0,81; 0,86)	(0,48; 0,53)	(0,47; 0,54)
		*	* #	* # ##	*	*	*	* &	* &	*
Average mast cell brightness, au	79,51	57,14	33,83	37,69	57,38	38	36,38	51,36	38,13	37,87
	(73,14; 83,76)	(51,36; 64,76)	(33,67; 39,22)	(37,69; 39,33)	(50,0; 58,42)	(33,26; 39,57)	(34,36; 37,9599)	(48,50; 59; 32)	(33,83; 40,78)	(32,50; 39,33)
		*	* #	* #	*	*	*	*	*	*

Note: * — differences are significant ($p < 0.01$) for comparisons with group 1, # — with group 2 on day 5, ## — with group 2 on day 10, & — with group 3 on the specified day.

20 was higher than on day 5. Thus, VEGF expression reached its peak on day 10, whereas MMP-9 expression, on days 10 and 20. MC can be a source of MMP-9 and VEGF, factors that promote effective tissue regeneration in the wound. In this study, we established a correlation between the degranulation coefficient of MC and the expression of MMP-9 and VEGF in the burn wound (Table. 3). On day 10, MMP-9 expression in the wound was directly moderately correlated with the value of the degranulation coefficient. On day 20, we detected a direct weak correlation between VEGF expression in the wound and the degranulation coefficient. We think that MC activity and the expression of MMP-9 and VEGF in the wound play a role in wound healing and systemic response to TB. While analyzing possible correlations between the absolute wound area and the degranulation coefficient, we established a weak negative correlation on day 5 and a moderate negative correlation on days 10 and 20 between these 2 variables. The absolute wound area was negatively correlated with MMP-9 expression on day 10 (the correlation was weak) and also negatively correlated with MMP-9 expression on day 20 (the correlation was moderate; Table 4). On day 10, a weak negative correlation was observed between the absolute wound area and VEGF expression. No significant correlations were established between the absolute wound area and VEGF expression on days 5 and 20 and between the absolute wound area and MMP-9 expression on day 5.

The effect of local application of MT-enriched DF (group 4) was assessed and compared with the effect of MT-free DF in group 3 over the course of 5 days. MT causes a significant reduction in the absolute wound area on days 5, 10 and 20 and in the relative wound area on days 10 and 20 of the experiment (Table 1). On days 5, 10 and 20, the rate of epithelization and the reduction in the wound size in group 4 were increased in comparison with group 3. On day 5, the median value of the absolute wound area in group 4 was 12.2% smaller than in group 3; peak values of the absolute wound area were observed on day 20 when it was 31.6% smaller than in group 4 and the median rate of epithelization was 4.7-fold lower than in the DF group. On days 10 and 20 in comparison with day 5 and on day 20 in comparison with day 10, the absolute wound area was significantly reduced; the rate of epithelization and the rate of wound size reduction were significantly increased ($p < 0.01$). Notably, from day 5 to day 20 the absolute wound area decreased by 46% vs 37% in the group treated with MT-free DF.

MT incorporated in DF changes the abundance and functional activity of MC in the experimentally induced burn wound (Table 2). On day 5, the total count of MC in the wound was significantly higher in group 4 than in group 3 (no MT). Besides, on day 5 the total number of degranulated MC and the number of MC in the 1st degree of degranulation was increased in comparison with group 3; the degranulation coefficient in group 4 was also higher on day 5, and the number of MC in the 2nd and 3rd degrees of degranulation was lower; the average MC brightness did not differ between the two groups on day 5. On day 10, there was a significant increase in the total MC count in the wound and a rise in the

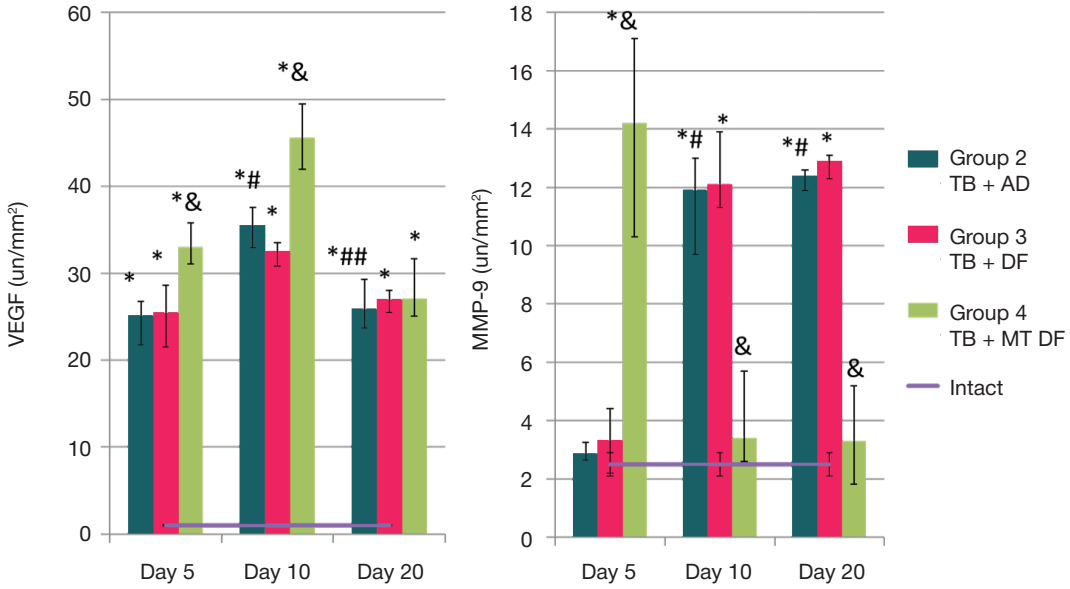

Figure. Effects of melatonin-enriched dermal film on the immunohistochemical parameters of burn wound tissue (Me (Q_{25}; Q_{75})). * — differences are significant ($p < 0.01$) for comparisons with group 1; # — with group 2 on day 5; ## — with group 2 on day 10; & — with group 3 on the specified day

number of degranulated MC and the number of MC in the 1st degree of degranulation in group 4, but the number of MC in the 2nd and 3rd degrees of degranulation was lower, and the degranulation coefficient had fallen, in comparison with group 3. Finally, on day 20 the total MC count and the number of MC in the 1st and 3rd degrees of degranulation were significantly decreased. So, local applications of MT-enriched DF result in the increased number and degranulation of MC on days 5 and 10 after thermal injury; by day 20, the abundance and activity of MC shifts towards reduction. The degranulation coefficient and average MC brightness, which are the integral indicators of MC activity, were significantly higher and lower, respectively, than in the group of intact animals at all time points of the experiment.

In group 4 (treated with MT-enriched DF), MMP-9 and VEGF expression was significantly elevated on day 5; by day 10, MMP-9 expression had fallen significantly, whereas VEGF expression continued to increase. On day 20, MMP-9 expression was decreasing, while VEGF expression showed no significant changes. VEGF expression was significantly higher ($p < 0.01$) on day 10 than on day 5; on day 20, it was lower than on days 5 and 10. MMP-9 expression was lower ($p < 0.01$) on days 10 and 20 than on day 5. Notably, VEGF expression on days 5, 10 and 20 and MMP-9 expression on day 5 were significantly higher in group 4 than in the group of intact animals. MMP-9 expression on days 10 and 20 did not differ significantly from that in the group of intact animals.

DISCUSSION

We think that the detected dynamics of tissue regeneration parameters in the burn wound are associated with the participation of MC in the key events of wound healing. In the first 5 days after thermal injury the process is dominated by secondary alteration vascular, exudative and leukocyte responses, MC degranulation and release of preformed mediators, like histamine, TNFα, IL1β, IL6, etc., leading to arterial and venous hyperemia, exudation and phagocyte activation. Protease-4 released by MC acts as a chemoattractant for leukocytes in the inflammation phase. Later, on days 10–15, mast cells start to secrete the keratinocyte growth factor and VEGF, activating fibroblasts and collagen synthesis, which is often excessive and leads to hypertrophic scarring. Increased collagen synthesis is associated, among other things, with the activation of the TGFβ1/Smads signaling pathway by chymase secreted by MC and fibroblast proliferation [17]. Serotonin secreted into the wound primarily by MC inhibits apoptosis and improves survival of fibroblasts and keratinocytes, thereby participating in the regulation of wound healing; inhibitors of endogenous serotonin release reduce the rate of epithelization [18].

The intensity of MMP-9 expression in the burn wound and serum levels of MMP-9 are risk markers for a systemic inflammatory response syndrome (SIRS) and predictors of poor outcomes in patients with burns [19]. Neutrophils activated by GM-CSF, IL8, TNFα and other cytokines, as well as some other cells, including MC, are the major source of MMP-9 at the early stages after thermal injury. MMP-9 expression reflects the abundance and activity of neutrophils and macrophages in the wound. MMP-9 is involved in the degradation of the extracellular matrix (especially collagen types IV and V), increases vascular permeability in the wound, participates in neutrophil chemotaxis, activation and deactivation of autacoids, and thus creates the right conditions for tissue regeneration and successful epithelization. MMP-9 can be inhibited by alpha1-antichymotrypsin, a proteinase-1 inhibitor [20]. MMP-9 plays a special role in the dynamics of chronic wounds: overexpression of MMP-9 results in the degradation of the extracellular matrix, inhibition of growth factors and tissue regeneration. According to some reports, the knockout of the MMP-9-encoding gene or MMP-9 inhibition accelerates wound healing [21]. As a rule,

Table 3. Correlations between the degranulation coefficient of mast cells and immunohistochemical parameters of the experimentally induced thermal wound

Parameter	Day 5 ($n = 7$)	Day 10 ($n = 7$)	Day 20 ($n = 7$)
VEGF, un/mm²	0,26	**0,37**	0,31
MMP-9, un/mm²	0,23	**0,64**	**0,37**

Note: The table features Spearman's correlation coefficient. Significant correlations ($p < 0.05$) are shown in semibold.

Table 4. Correlations between the absolute burn wound area and VEGF/MMP-9 expression and between the absolute burn wound area and the degranulation coefficient

Parameter	Day 5 (n = 7)	Day 10 (n = 7)	Day 20 (n = 7)
VEGF, un/mm^2	– 0,13	**– 0,37**	0,17
MMP-9, un/mm^2	– 0,31	**– 0,47**	**– 0,54**
Degranulation coefficient, au	**– 0,37**	**– 0,51**	**– 0,64**

Note: The table features Spearman's correlation coefficient. Significant correlations ($p < 0.05$) are shown in semibold.

low MMP-9 activity in the acute wound is associated with its inhibition by alpha1-antichymotrypsin.

Members of the VEGF family (VEGF-A, VEGF-B, VEGF-C, VEGF-D) in general and VEGF-A in particular are produced in the wound by keratinocytes, mast cells, macrophages, monocytes, and activated fibroblasts. VEGF-A receptors have been detected on endothelial cells, fibroblasts and other cells [22]. GM-CSF stimulates VEGF production in the wound and directly increases keratinocyte and fibroblast activity [23]. VEGF participates in the regulation of angiogenesis, collagen synthesis and production of other connective tissue components; it is also involved in scar formation. Inhibition of VEGF prevents excessive scarring and formation of hypertrophic and keloid scars after TB, thus holding promise for the therapy of burns. However, the mechanism underlying these VEGF effects is not fully clear and may be associated with direct activation of fibroblast migration, fibroblast proliferation and collagen synthesis and/or indirect activation of endothelial cells, neutrophils, macrophages, and mast cells accompanied by the production of profibrogenic mediators (cytokines, growth factors) [24]. Specifically, the therapeutic effect of interferon alpha2b in patients with hypertrophic and keloid scars is associated with mitigation of VEGF effects and angiogenesis. It is reported that bevacizumab, a humanized anti-VEGF antibody, exerts a positive effect against hypertrophic scarring [25]. In this regard, the correlations established in our study are very illustrative, including the negative correlation between the wound area and the abundance of MMP-9 and VEGF in the wound, the direct correlation between the wound area and the degranulation coefficient, and the direct correlation between the degranulation coefficient and the levels of MMP-9 and VEGF in the wound. Therefore, it can be hypothesized that the wound area after thermal injury shrinks as MMP-9 and VEGF levels grow; in turn, MMP-9 and VEGF levels in the wound increase as MC undergo degranulation.

We believe that the therapeutic effects of MT-enriched DF can explained by the pleiotropic properties of MT. The antioxidant effect of MT can curb the progression of secondary injury expansion after TB, which occurs due to the activation and recruitment of neutrophils, monocytes and lymphocytes to the affected site. Previously, we demonstrated that MT-enriched DF limits the growing levels of lipid peroxidation products and proteins in the burn wound and thus accelerates healing [26]. Modulation of the inflammatory response by MT may be associated with a reduction in secondary alteration, mitigation of vascular-exudative responses, suppression of cytokine and autacoid production, thus leading to changes in the wound and the acute phase response. MT-enriched DF limits the death of leukocytes in an experimentally induced TB [27]. MT may exert direct effects on the synthesis and activity of factors involved in tissue regeneration. In the experiment in vitro conducted on the endothelial cells of cerebral vessels, MT has been shown to reduce the permeability IL1β-activated cells by inhibiting MMP-9 [28]. MT has the ability to directly bind MMP-9. Specifically, MT binds excessive MMP-9 in COVID-19-mediated immune response and squamous-cell carcinoma of the oral cavity [29]. MT downregulates overexpression and reduces excessive activity of MMP-9 by regulating the NOTCH3/NF-κB and TLR4/NF-κB signaling pathways [30]. In the experiments *in vitro* involving the serum of rats with experimentally induced thermal burns, MT has been shown to reduce the permeability of endothelial cells due to MMP-9 inhibition and the antioxidant activity [31]. MT is protective against MC damage by chemical agents and may affect the secretory activity of MC [32]. Besides, MT effects on MC may vary depending on the type of receptor MT interacts with (M1 or M2). Thus, a complex effect of locally applied MT on the expression of MMP-9 in the burn wound may be associated with protection of MC against injurious factors in the wound on day 5; in turn, the increased secretion of MMP-9 by MC may ensure more effective and rapid removal of cell detritus from the wound, preparing tissue for regeneration. On days 10 and 20, the decline in MMP-9 expression in the wound may be associated with direct or indirect inhibition and reduced synthesis of this proteinase following exposure to MT; this halts tissue destruction in the wound, activates cell proliferation and differentiation driven by growth factors and results in rapid tissue regeneration. Therefore, stimulation of VEGF expression in the wound on days 5 and 10 by the local application of MT has a rationale.

The reports on MT effects on VEGF synthesis and expression are controversial. On the one hand, MT inhibits VEGF synthesis by SH-SY5Y human neuroblastoma cells, prostate cancer cells and other malignancies, exerting an antiangiogenic effect and inhibiting tumor growth [33]. This phenomenon is associated with the inhibition of STAT3 and HIF-1α stabilization and the genes they control, including VEGF and the VEGFR2 receptor [34]. According to other studies, MT does not change VEGF expression in ischemic lesions [35]. However, recent data suggest that multifunctional regulatory MT effects on angiogenesis are dose-dependent and determined by the initial tissue condition. MT directly or indirectly enhances angiogenesis during fractured bone healing, skin healing after mechanical or chemical damage, in experimentally induced gastric ulcers and myocardial or cerebral ischemia [33]. MT boosts the angiogenic potential of mesenchymal stem cells in the wound due to Erk1/2-mediated VEGF synthesis [36]. Besides, MT can stimulate production of platelet-derived growth factor, which is a known angiogenesis stimulator, and confers resistance to ischemic stress.

CONCLUSIONS

In our study, the absolute area of the experimentally induced burn shrank by 35% from day 5 to day 20, the wound epithelization rate and the total MC count in the wound increased, and the functional activity of MC changed: the cells lost their granularity and underwent significant degranulation. MMP-9 and VEGF expression in the wound was elevated. A negative correlation was established between the wound area and the expression of MMP-9 and VEGF; the wound area and the degranulation coefficient were also negatively correlated. MMP-9 and VEGF expression in the wound increased as the degranulation of mast cells intensified. MT-enriched DF caused a reduction in the wound area observed on days 5, 10 and

20 after thermal injury and increased the wound epithelization rate. The medicated film increased the total MC count in the wound and stimulated MC degranulation on days 5 and 10. On day 20, there was a reduction in the total MC count in the wound and a decline in degranulation. The application of MT-enriched DF resulted in elevated MMP-9 expression on day 5 and elevated VEGF expression on days 5 and 10; MMP-9 expression on days 10 and 20 was reduced. The obtained results expand our knowledge of the contribution of changes in MC activity and the dynamics of MMP-9 and VEGF expression to the pathogenesis of TB. They create a basis for further clinical studies into the role of MC in skin burns and help to identify diagnostic markers, predictors of complications and markers of treatment efficacy among their secretory products. Established at the preclinical stage, the stimulatory effect of MT-enriched DF on tissue regeneration is associated with changes in the abundance and activity of mast cells and MMP-9 and VEGF expression in the wound. It paves the way for the study of mechanisms underlying the effects and efficacy of MT in the clinical setting in patients with TB. Considering that wound healing mechanisms, regardless of their nature, act in alliance, we believe that our findings suggesting a stimulatory effect of MT-enriched DF on tissue regeneration due to changes in mast cells activity and in the abundance of MMP-9 and VEGF in the wound can be extrapolated to wounds of other etiologies.

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