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DEXAMETHASONE AND GRANULOCYTE COLONY-STIMULATING FACTOR CHANGE THE REGENERATIVE NEUROMA MORPHOLOGY

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Regenerative neuroma of the sciatic nerve is characterized by typical cellular composition and the Schwann cells and fibroblasts ratio. Dexamethasone or granulocyte colony-stimulating factor applied in the early stages of regenerative neuroma formation affects the final state of the stromal component of the neuroma and slightly increases Schwann cells in it. The combined use of dexamethasone and granulocyte colony-stimulating factor potentiates the effect of granulocyte colony-stimulating factor on increasing the number of Schwann cells in the regenerative neuroma and delays its involution. It leads to a noticeable change in the regenerative neuroma composition towards the neural component.

Key words: sciatic nerve, regenerative neuroma, nerve injury, fibroblast, Schwann cells.

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ДЕКСАМЕТАЗОН ТА ГРАНУЛОЦИТАРНИЙ КОЛОНІЄСТИМУЛЮЮЧИЙ ФАКТОР ЗМІНЮЮТЬ МОРФОЛОГІЮ РЕГЕНЕРАЦІЙНОЇ НЕВРОМИ

Регенераційна неврома сідничного нерву характеризується стереотипною кінетикою клітинного складу та співвідношенням вмісту клітин Шванна та фібробластів. Дексаметазон чи гранулоцитарний колонієстимулюючий фактор, застосовані на ранніх етапах формування регенераційної невроми, незначно впливають на кінцевий стан сполучнотканинного компоненту невроми та дещо збільшують представництво в ній шваннівських клітин. Сумісне застосування дексаметазону та гранулоцитарного колонієстимулюючого фактору призводить до потенціювання ефекту останнього щодо зростання у неврмі кількості клітин Шванна, а також затримує інволюцію невроми. Останнє призводить до помітного зсуву тканинного складу регенераційної невроми у бік нейрального компоненту.

Ключові слова: сідничний нерв, регенераційна неврома, ушкодження нерва, фібробласти, клітини Шванна.

The work is a fragment of the research project "To study the features of regenerative processes in the brain and nerve trunk during the modulation of the mesenchymal stem cells accumulation and differentiation ", state registration No. 0123U101051.

Peripheral nerve injury is a global medical problem due to the complexity of their treatment and the high level of disability (60–75 %). It determines the unabated interest of doctors and researchers in improving methods of treatment of such injuries [14].

An injured peripheral nerve is difficult to recover due to the degeneration of motor neurons, the lack of a survival environment for Schwann cells (SCs), and the relatively poor ability of nerve regeneration [12]. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) play prominent roles in supporting axonal regeneration during peripheral nerve repair [12]. Immune activation and release of immune mediators in the peripheral nervous system after injury have important effects on nerve degeneration and regeneration. Immune cells, including tissue basophils, macrophages and lymphocytes reside in peripheral nerves and/or are recruited to sites of nerve injury [4]. Secondary alteration after peripheral nerve injury, caused by an excessive immune response, increases Wallerian degeneration, which prevents the regeneration of peripheral nerves [4]. Thus, the impact on the regulatory components involved in the inflammatory reaction and the stimulation of their recovery with the exogenous growth factors (GFs) [8] during peripheral nerve injury may have great prospects in the development of effective strategies for the treatment of this pathology [4].

A large number of studies have been devoted to the effects of corticosteroids on peripheral nerve regeneration. However, their conclusions were ambiguous [2, 3]. It has been reported glucocorticoids improve regeneration by inhibiting NF-κB synthesis, reducing TNF-α expression, and transcapillary permeability as well [7, 5]. Moreover, previous studies have shown SCs express glucocorticoid receptors which promote Schwann cell proliferation, major myelin protein gene expression (P0) [7, 9, 10], reduce nerve swelling and perineural inflammation, protect cells from peroxidation, prevent the motor neurons death, reduce the rate of anterograde degeneration and accelerate nerve recovery [2], including functional

[5, 8], reduce fibrosis indicators [15]. Dexamethasone reduces the number of CD3-positive cells at the site of injury by inhibiting the recruitment and activation of T cells after nerve injury. Therefore, immunosuppression can contribute to neuroprotection by limiting the invasion of CD3-positive cells [4]. Dexamethasone treatment increases the expression of GAP-43, which is associated with the development and plasticity of the nervous system and whose level changes more than 100-fold from very low at rest to high levels during development or nerve damage [4]. Systemic dexamethasone administration in a dose of 5 mg/kg after axonotomy of the facial nerve and subsequent immediate neurorrhaphy showed a tendency to increase its functional recovery [11]. However, systemic dexamethasone administration in doses of 0.5, 1, and 10 mg/kg and local intraoperative use of dexamethasone in doses of 2 or 4 mg/ml did not lead to a significant functional improvement of nerve trunks compared to the control group [11]. Dexamethasone and vitamin B 12 contribute to the recovery of peripheral nerves after sciatic nerve injury in rats by increasing the expression of BDNF [13].

Currently, an important therapeutic strategy to stimulate nerve regeneration is to supplement treatment with exogenous growth factors (GFs) [8]. GFs activate the targets of various signaling pathways by binding to the corresponding receptors, thereby exerting their multiple effects on neurorestoration and tissue regeneration [8]. These positive effects are mainly manifested due to the enhancement of internal transduction capacity, regulation of interaction with molecular and mechanical signaling cascades after retrograde absorption mediated by receptors [6, 8]. The GFs family members, including nerve growth factor (NGF) and fibroblast growth factors (FGF), are secreted predominantly by SCs or neurons under physiological conditions [8].

The purpose of the study was to determine the changes in the regenerative neuroma morphology and the content of fibroblasts and Schwann cells in it after treatment with the use of dexamethasone , granulocyte colony-stimulating factor and their combination .

Materials and methods. A total of 168 adult male Wistar rats with an average body weight of 235 g (220–250 g) were used in this study. The experimental procedures were in accordance with all bioethical requirements of the European Convention for the Protection of Vertebrate Animals for Research and Other Scientific Purposes, Strasbourg, 18 March 1986. Permission of the Bogomolets National Medical University Bioethics Commission No. 128 dated 23.12.2019.

The operation was performed under intraperitoneal thiopental anesthesia (50 mg/kg). After removing the fur and treating the skin with an antiseptic solution, the skin and fascia on the back surface of the right thigh were cut. The muscles were bluntly disconnected and the sciatic nerve was cross-sected at the level of the middle of the femur. The wound was then closed in layers, the operative field is treated with an antiseptic solution.

The rats were randomly divided into four groups (n=42): group 1 (control, C) – 0,5 ml 0.9 % saline was injected subcutaneously from the 1st to the 5th day after the operation; group 2 – (Dex) dexamethasone (10 mg/kg) was injected subcutaneously at the same period; group 3 (GCSF) – granulocyte colony-stimulating factor (GCSF) (Granocyte® 34, Sanofi-Aventis, France) was injected subcutaneously (50 μg/kg) from the 1st to the 3rd day after the operation; group 4 – (GCSF+Dex) GCSF (50 μg/kg) was injected subcutaneously from the 1st to the 3rd day and dexamethasone (10 mg/kg) from the 1st to the 5th day after the operation. The injections were performed once daily based on rat weight.

The rats were sacrificed (by sodium thiopental 200 mg/kg) and the middle third of the sciatic nerve was immediately taken from each rat as an experimental sample on 1, 3, 7, 14, 28, and 56 (7 animals for each period) days after neurotomy. Obtained experimental samples were fixed in 10 % buffer formal saline (pH 7.4; 40^{o}C) for 48 hours, dehydrated, and embedded in paraffin. The 4-5 μm thickness sections were cut using a microtome and stained with hematoxylin and eosin.

Histological sections were examined and photographed using an Olympus B53 microscope with an SP180 digital camera (1224x920 RGB pixels, lighting mode – photo, standardized exposure). On x400 digital images (300x225 μm; 1224x920 RGB pixels, lighting mode – photo, standardized exposure) the regeneration neuroma morphology was examined, Fbs and SCs were counted and their ratio was calculated.

The obtained data were expressed as means ± SD and standard error of the mean. The exact Kolmogorov-Smirnov test showed that all data of experimental measurements do not contradict the normal distribution. Student's t-test was used to assess the intergroup differences significance; the values of $P<0.05$ were considered statistically significant.

Results of the study and their discussion. Our study has demonstrated that injured nerve restoration is carried out according to stereotyped kinetics in the animals of the C group (Fig. 1, 2). Thus, 1 day after surgery, there was hemorrhage and fibrinous accumulations between the segments of the injured nerve which had been penetrated by the lymphocytes, granulocytes, and macrophages. Single

mesenchymal-like cells, sometimes thin-walled blood vessels, could be observed near the ends of the nerve in the hemorrhage. After 3 days, young connective tissue rich in inflammatory cells and containing a significant number of thin-walled blood vessels and a fairly significant number of Fbs was found between the nerve segments. Directly near the stump of the nerve, mainly the central one, there were single cells that could be attributed to SCs. By the 7th day, the number of inflammatory cells in the RN decreased although the number of Fbs increased significantly. Also, the presence of cells with large oval nuclei with fine-grained chromatin, which could be verified as SCs, notably increased. After 14 days decrease in lymphocytes and macrophages was observed, and the number of oxyphilic connective tissue fibers increased. Generally, narrow, longitudinal bands of SCs were formed. Later (28 and 56 days after nerve injury), the RN progressively matured: the number of inflammatory cells and Fbs decreased. Connective tissue fibers formed thin bundles, mostly located along the nerve or at a small angle, rarely disordered. SCs formed different thickness bands, oriented mainly along the nerve`s length. The number of blood vessels decreased and their wall acquired a typical structure of the microcirculatory system elements.

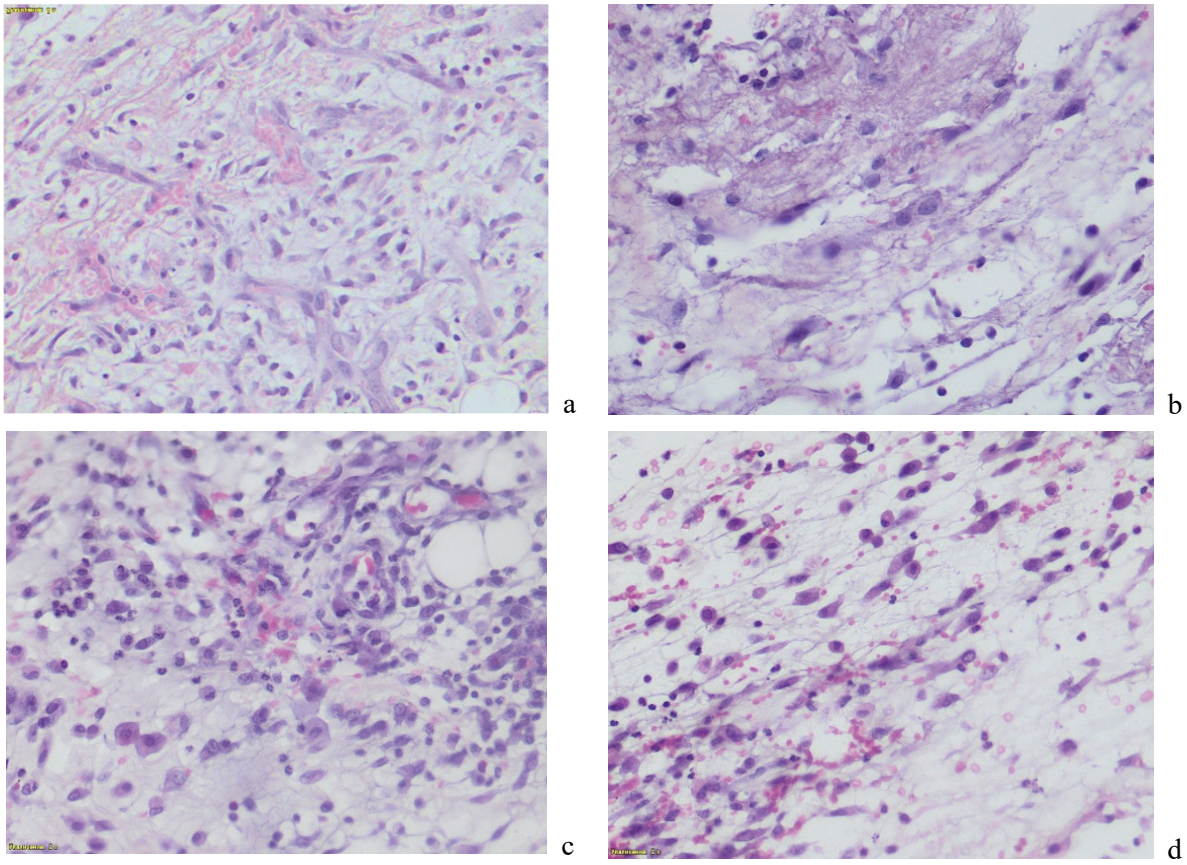

Fig. 1. Regenerative neuroma, 3rd day after neurotomy. Staining with hematoxylin and eosin, x 400; a – control; b – dexamethasone ; c – GCSF; d – Dex+GCSF.

Dex group (Fig. 1, 2), in comparison with the C after 1 day of the surgery observed an intense decrease in inflammation at the site of injury. Isolated mesenchymal-like cells and leukocytes penetrated into the masses of fibrin and erythrocytes between the segments of the injured nerve. In the 3 days was observed a few thin-walled vessels were growing in the mass filling the nerve defect, they were accompanied by single cells of irregular shape and lymphocytes. In this group in contrast to C, mesenchymal-like cells had large size, large rounded, light, and homogeneously colored nuclei. On the 7th day after the operation, RN with a small number of leukocytes and macrophages was formed. By this time, the content of the Fbs had markedly increased and a small number of thin collagen fibers had appeared. Large rounded cells with large, relatively light, and homogeneously colored nuclei continued to be detected. Sometimes they formed clusters. The moderate number of SCs began to be determined as well. On the 14th day, the increase of SCs in the RN is manifested. They could be complexed in longitudinally oriented bands. The number of Fbs considerably increased compared to the previous period of observation, but was smaller than in the C. As in the previous periods, large cells with a rounded, homogeneously colored nucleus were found in the RN, although in smaller numbers. On the 28th and 56th day, Bunger bands were already clearly visible in the newly formed part of the nerve, which looked denser than in the C and showed a distinct tendency to a longitudinal location. Compared to the C group, the number of Fbs

and collagen fibers was lower. The collagen fibers were located more longitudinally than in the C group. In general, on the 28th and 30th day of the sciatic nerve injury, the newly formed part of the nerve was only slightly thickened compared to the preexisting parts of the nerve.

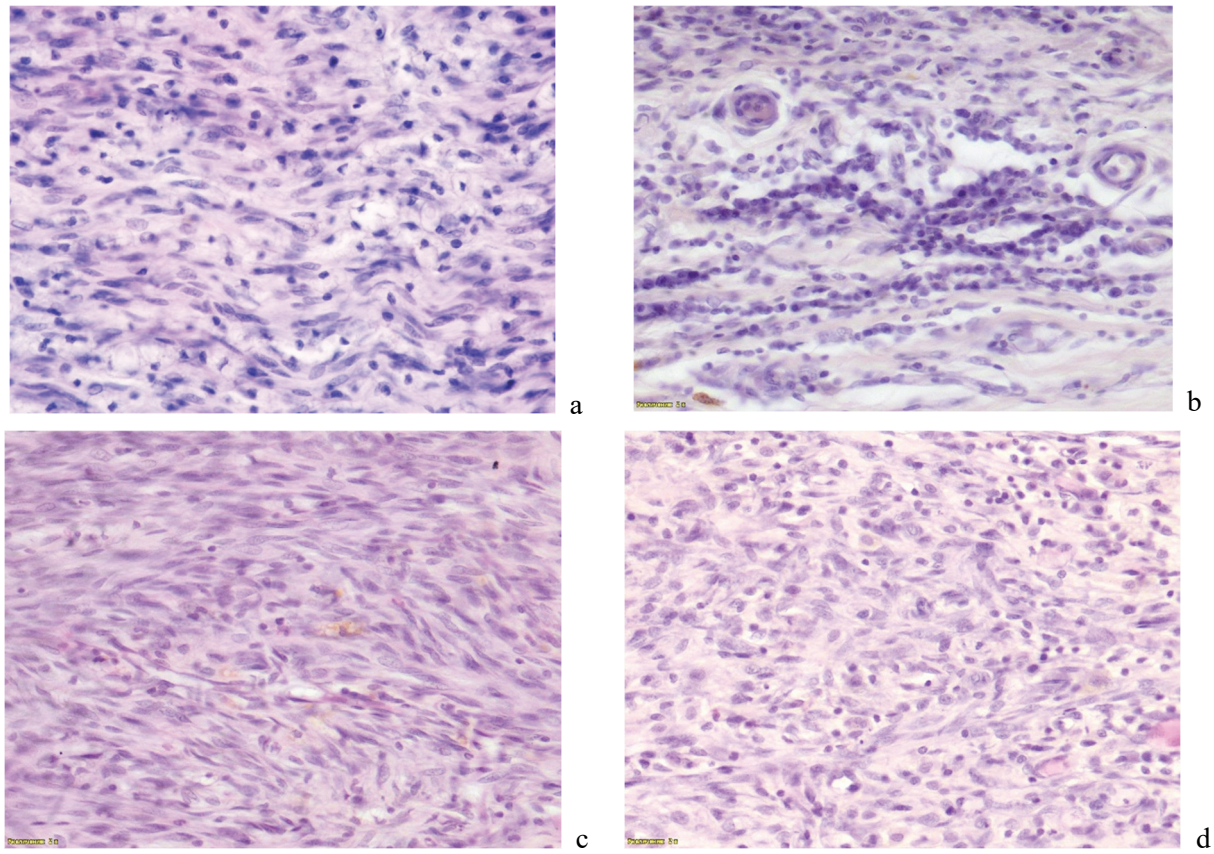

Fig. 2. Regenerative neuroma, 14th day after neurotomy. Staining with hematoxylin and eosin, x 400; a – control; b – dexamethasone; c – GCSF; d – Dex+GCSF.

GCSF 1 day after surgery led (Fig. 1, 2) to a moderate decrease in the inflammatory cells numbers at the site of injury and an increase of mesenchymal-like cells, often of a large size, that penetrated into the masses of fibrin and erythrocytes between the sciatic nerves segments. On the 3 days, there was a clearly visible increase of thin-walled newly formed blood capillaries. A significant number of polymorphic mesenchymal-like cells, they were often large in size and observed in the RN that was formed. On the 7th day, the cellularity of the RN was higher than in the C. The RN contained a substantial number of SCs and a relatively higher number of Fbs and large mesenchymal-like cells compared to the C. The leukocyte content on the contrary was lower. Up to 14 days, the number of Fbs and SCs in the RN continued to increase and the total number of them was greater than in the C. At the same time, collagen fibers were determined, but somewhat less than in the C. A small number of large polymorphic cells with large homogeneous nuclei continued to be observed. By this time, SCs formed clear Bügner bands. The further (28 and 56 days) the RN development was characterized by a slowdown in the growth of its cellularity, and then by a decrease. In addition, at the end of the observations, fewer Fbs were found in the RN than in the C, as well as a smaller volume of connective tissue fibers.

The simultaneous use of GCSF and Dex caused a sharp inflammation decrease at the site of injury. There was an active new formation of blood vessels that penetrated into the fibrinous tissue 1 and 3 days after nerve damage. They were accompanied by mesenchymal-like cells in a quantity that was generally greater than in the Dex group, but less than the GCSF group. Moreover, these cells were mostly irregular or process-like in shape, rather than rounded, which prevailed in the Dex group. Already after 3 days, a small number of SCs were detected near the ends of the crossed nerve. On the 7th day, the RN had a lower cellularity than during the action of GCSF, which brought it closer to the C. However, it contained more SCs. The number of Fbs and leukocytes was noticeably less than in the C. Quite often at this time, large cells of an irregular or rounded shape with a large homogeneously colored nucleus were found. Collagen fibers were present in a smaller number than in the C and had a predominantly longitudinal orientation. At this time, large cells with a large homogeneously colored nucleus continued to be found. After 28 and 56 days, the RN was characterized by a smaller number of Fbs and the volume of connective tissue fibers, well-visualized Bunger bands.

Quantitative assessment of Fbs content in the RN showed (Fig. 3, Fb) that in the C, the specific number of these cells increased, reaching a maximum between 14 and 28 days although then it decreased. Dex led to a clear decrease in the number of Fbs especially at the initial stages (1–14 days) of neuroma formation (during direct action (from 1 to 5 days). But at the end of the experiment, their number was practically as in the C. GCSF caused almost the reverse changes in the neuroma compared to Dex, but after 56 days the Fbs number was practically no different from the control values. The combined effect of Dex and GCSF also did not lead to a change in the amount of Fbs, although its level during the first month after nerve injury was significantly lower than in the C.

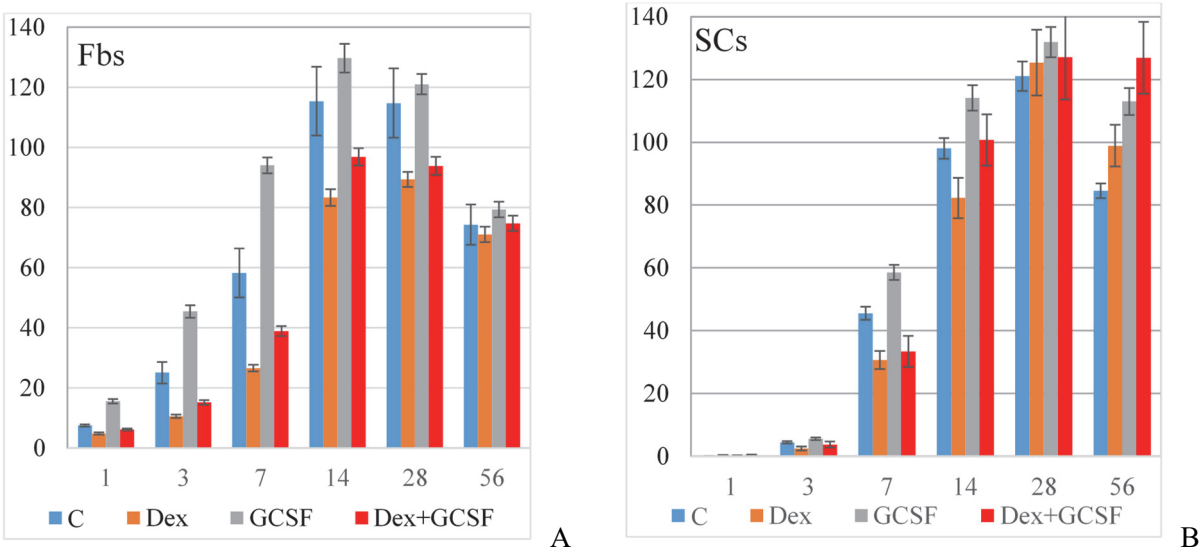

Fig. 3. The number of fibroblasts (Fbs, A) and Schwann cells (SCs, B) in damaged peripheral nerve neuroma (M±m). Rats were administered local subcutaneous injections with: Dex – dexamethasone, GCSF – granulocyte colony-stimulating factor, Dex+GCSF; C – control group. 1–56 days after sciatic nerve injury.

The SCs number in the RN (Fig. 3, SCs) on the 1st and 3rd day after sciatic nerve injury were very small, so it was not appropriate to estimate their number at these times. In the C, their number increased from the 3rd to the 28th day and then decreased. Dex led to a significant decrease SCs on the 7th and 14th day of the experiment, on the 28th it was almost the same as in the C although after 56 days it was significantly higher than in the C group. In the GCSF group, on the contrary, increased the SCs number in RN, but on the 28th day, their number was not statistically different from the control values. At the end of the experiment, the specific content of SCs in the GCSF group turned out to be significantly higher, compared to the C and to Dex. In the Dex+GCSF group on day 7, the number of SCs was at the same level as in the Dex group, but lower than in the C group. After 7, 14, and 28 days, their number was almost the same as in the C. After 56 days, the rats of this group (Dex+GCSF) did not observe a decrease in the specific amount of SCs in the RN, in contrast to other groups (Fig. 3, SCs).

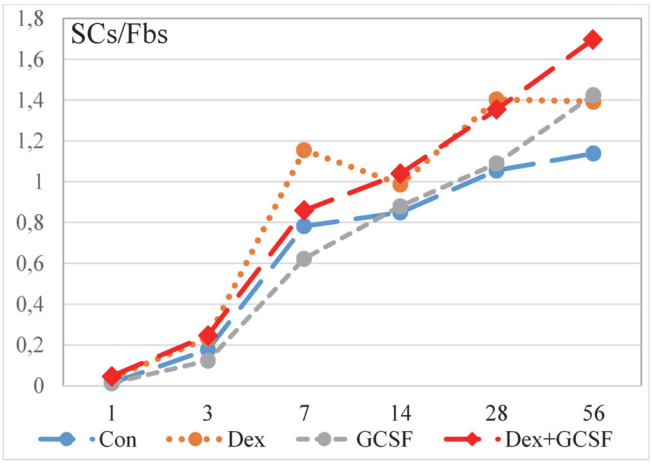

Fig. 4. The ratio of SCs/Fbs in the regenerating neuroma of rat sciatic nerve after subcutaneous administration of Dex – dexamethasone, GCSF – granulocyte colony-stimulating factor, Dex+GCSF; C – control. 1–56 – days after sciatic nerve injury.

The assessment of the ratio SCs/Fbs in the RN of rat sciatic nerve (Fig. 4) showed that in the control it increased uniformly during the entire period of observation. Dex led to a peak deviation of this proportion after 7 days of the experiment, and in the following it remained higher than in the C group. GCSF did not change SCs/Fbs during the first month of observation, but 56 days after neurotomy it shifted towards SCs. In the Dex+GCSF group for 1–28 days, the ratio of SCs/Fbs was the same as in group C, but even up to 56 days it grew and significantly increased towards SCs.

Thus, the conducted study showed that Dex and GCSF change the morphology of the RN, as well as the content of Fbs and SCs in it.

Dex expectedly reduced the inflammatory reaction and, as a result, reduced the accumulation of Fbs and the formation of connective tissue fibers [15]. More interesting was the fact that a certain decrease

SCs in regenerative neuromas in the first month after sciatic nerve injury is replaced by its increase in the second month. This gives reason to believe the inflammatory reaction and possibly the accumulation of mesenchymal-like cells at the site of injury is an SCs activation and mobilization factor. However, a distinct increase in ratio SCs/Fbs gives reason to believe that SCs are activated by Dex, which is consistent with data on the presence of corticosteroid receptors in them [7, 9, 10].

GCSF, being an activator of mesenchymal stem cells [1] increases Fbs in RN. At the same time, the content of SCs also increased, which led to an increase in the SCs/Fbs ratio at the end of the experiment. This gives reason to assume the existence in RN of several ways to increase SCs: due to the stimulating effect of mesenchymal cells, including their secretome [13], and direct GCSF stimulating effect on Schwann cells.

If Dex and GCSF separately, as a whole, increased or decreased the amount of SCs or Fbs in the RN, and slightly changed their ratio, then the use of Dex and GCSF together changed the ratio of the cellular composition of the RN. An increased amount of SCs and a decrease in the amount of Fbs leads to a change in the tissue composition of RN.

Conclusion

Regenerative neuroma sciatic nerve is characterized by typical cellular composition and the ratio of Schwann cells and fibroblasts. Dexamethasone (Dex) or granulocyte colony-stimulating factor (GCSF) applied in the early stages of regenerative neuroma formation affects the final state of the stromal component of the neuroma and slightly increases Schwann cells in it. The combined use of Dex and GCSF potentiates the effect of GCSF on increasing the number of Schwann cells in the regenerative neuroma and delays its involution. It leads to a noticeable change in the regenerative neuroma composition towards the neural component.

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