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Features of neuroglia at the epicenter of spinal cord contusion injury and at distant areas in mini-pigs

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Abstract

Aim. To determine the delayed (after 2 months) effect of spinal cord injury (SCI) in the lower thoracic region in the mini-pigs on the morphologic state of macro- and microglia in nearby and remote caudal areas.

Materials and methods. Sexually mature female Vietnamese pot-bellied pigs were randomly divided into two groups: SCI (n = 3) and intact (n = 3). Dosed contusion SCI was modelled at the level of the Th8–Th9 vertebrae, and transverse cryostat sections of the caudal segment adjacent to the epicenter of injury and the lumbar thickening (L4–S2) were examined 2 months later. The expression of astrocyte markers (glial fibrillary acidic protein, GFAP) and microglial markers (ionized calcium-binding adapter molecule 1, Iba1) was assessed as the relative immunopositive area occupied by cells. When counting the number of oligodendroglial cells (oligodendrocyte transcription factor 2, Olig2), the presence of nuclei detectable with 4',6-diamidino-2-phenylindole (DAPI) was taken into account.

Results. After SCI, an increase in the relative areas occupied by GFAP-positive astrocytes and Iba1-positive microglia and a decrease in Olig2-positive oligodendrocytes were detected in both the lesion area and lumbar thickening. In both regions, 2 months after SCI, the proportion of astrocytes was not significantly different in the anterior horns and doubled in the posterior horns. Microglia cells with SCI were 2.5 times more in the anterior horns of both regions and in the posterior horns of the lumbar thickening, while the presence of microglia increased slightly (1.2 times) in the posterior horns in the SCI region. The number of oligodendrocytes decreased in the area of the epicenter of SCI in the anterior and posterior horns by 1.5–1.75 times, and in the lumbar thickening more significantly: the number decreased by 2.5 times in the anterior horn and 5.5 times in the posterior horn.

Conclusion. The results of the study revealed a similar pattern of macro- and microglial cell distribution both in the SCI region and in remote areas. The obtained data testify to the necessity to take into account the state of the areas of nervous tissue remote from the epicenter of SCI when stimulating neuroregeneration in such patients.

Keywords: Vietnamese pot-bellied pig; spinal cord contusion injury; neuroglia of the thoracic region; neuroglia of the lumbar thickening; immunofluorescent analysis of neuroglia

MeSH terms:
SPINAL CORD INJURIES – PATHOLOGY
SPINAL CORD INJURIES – PHYSIOPATHOLOGY
BLAST INJURIES – PATHOLOGY
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Ethics statements. The study complies with the standards of the EU Directive for the Protection of the Vertebrate Animals used for Experimental and other Scientific Purposes. All manipulations with animals were approved by the Local Bioethics Committee of the Kazan State Medical University, No. 5 of 26.05.2020.
Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request. Data and statistical methods used in the article were examined by a professional biostatistician on the Sechenov Medical Journal editorial staff.
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Характеристика нейроглии в эпицентре и в удаленной от травмы области при контузионном повреждении спинного мозга у мини-свиньи

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Аннотация

Цель. Установить отсроченное (спустя 2 месяца) влияние травмы спинного мозга (ТСМ) в нижнегрудном отделе у мини-свиньи на морфологическое состояние макро- и микроглии в близлежащих и отдаленных каудальных участках.
Материалы и методы. Половозрелых самок вьетнамской вислобрюхой свиньи случайным образом разделяли на две группы: с ТСМ (n = 3) и интактную (n = 3). Дозированную контузионную ТСМ моделировали на уровне Th8–Th9 позвонков, через 2 месяца исследовали поперечные криостатные срезы каудального сегмента, прилегающего к эпицентру травмы, и поясничного утолщения (L4–S2). Экспрессию маркеров астроцитов (глиальный фибриллярный кислый белок, glial fibrillary acidic protein, GFAP) и микроглии (ионизированная кальций-связывающая адаптерная молекула 1, ionized calcium-binding adapter molecule 1, Iba1) оценивали как относительную иммунопозитивную площадь, занимаемую клетками. При подсчете количества олигодендроглиальных клеток (фактор транскрипции олигодендроцитов 2, oligodendrocyte transcription factor 2, Olig2) учитывали наличие ядер, выявляемых при помощи 4',6-диамидино-2-фенилиндола (4',6-diamidino-2-phenylindole, DAPI).
Результаты. После ТСМ выявлено увеличение относительных площадей, занимаемых GFAP-позитивными астроцитами и Iba1-позитивными клетками микроглии, а также уменьшение Olig2-позитивных олигодендроцитов как в области повреждения, так и в поясничном утолщении. В обеих областях спустя 2 месяца после ТСМ доля астроцитов в передних рогах существенно не отличалась, а в задних рогах увеличивалась вдвое. Клетки микроглии занимали площадь в 2,5 раза больше в передних рогах обеих областей и в задних рогах поясничного утолщения, в задних рогах в области ТСМ присутствие микроглии увеличилось незначительно (в 1,2 раза). Количество олигодендроцитов уменьшилось в области эпицентра ТСМ в передних и задних рогах в 1,5–1,75 раза, в поясничном утолщении более значимо – в 2,5 раза в передних и в 5,5 раза в задних рогах.
Заключение. Результаты исследования обнаружили схожую картину распределения клеток макро- и микроглии как в области ТСМ, так и в удаленных участках. Полученные данные свидетельствуют о необходимости учитывать состояние удаленных от эпицентра ТСМ участков нервной ткани при стимулировании нейрорегенерации у таких пациентов.

Ключевые слова: вьетнамская вислобрюхая свинья; контузионная травма спинного мозга; нейроглия грудного отдела; нейроглия поясничного утолщения; иммунофлуоресцентный анализ нейроглии

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КОНТУЗИИ – ПАТОФИЗИОЛОГИЯ
НЕВРОГЛИЯ – ПАТОЛОГИЯ
ИММУНОФЛЮОРЕСЦЕНТНЫЙ АНАЛИЗ

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Соответствие принципам этики. Исследование проведено с соблюдением положений Европейской конвенции о защите позвоночных животных, которые используются для экспериментальных и других научных целей. Все манипуляции с животными проведены в соответствии с разрешением Локального этического комитета ФГБОУ ВО «Казанский государственный медицинский университет» Минздрава России (№5-26.05.2020).
Доступ к данным исследования. Данные, подтверждающие выводы этого исследования, можно получить у авторов по обоснованному запросу.
Данные и статистические методы, представленные в статье, прошли статистическое рецензирование редактором журнала – сертифицированным специалистом по биостатистике.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.
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Abbreviations:
SCI – spinal cord injury
DAPI – 4',6-diamidino-2-phenylindole
GFAP – glial fibrillary acidic protein
Iba1 – ionized calcium-binding adapter molecule 1
IL-10 – interleukin-10
Olig2 – oligodendrocyte transcription factor 2
PBS – phosphate buffered saline

According to the World Health Organization, 250,000 to 500,000 cases of spinal cord injury (SCI)[1] are registered annually worldwide. The injury results in the impairment of motor, sensory, and autonomic functions of the body, with differing severity [1].

Post-SCI pathological dynamics include massive death of spinal cord cells, tearing of nervous fibres, bleeding, and ischemic and inflammatory damage; as a result, cavities and cysts may be formed [2]. Unfortunately, there is still no efficient treatment for patients with severe SCI [3], which is mainly due to a lack of understanding of the cell machinery in spinal cord injury.

Natural post-SCI regeneration is currently considered to involve a partial recovery (remodelling) of the spinal cord around the epicentre of the injury [2, 4]. Meanwhile, at the epicentre, there is a cluster of endogenous proliferating fibroblasts, pericytes, endothelial cells, and leukocytes. At the same time, both nerve and glial cells launch their regeneration.

[1] World Health Organization. Spinal cord injury. https://www.who.int/news-room/fact-sheets/detail/spinal-cord-injury (access date: 13.12.2022).

From days 1–2 and until approximately days 7–10 after SCI, the proliferation of astrocytes is quite active, and the cells move to the boundaries of the lesion [5] to constitute a thick borderline [6]. Such a glial scar is well-limited and consists of type IV collagen, extracellular matrix, and chondroitin sulfate proteoglycans [7]. Thus, any expansion of inflammation from the epicentre of SCI is avoided.

Outer scar borders comprise astrocytes, which contact reactive progenitor cells of oligodendroglia expressing chondroitin sulfate proteoglycan 4/NG2 (neural/glial antigen 2, neural glial antigen 2). This protein assists in blocking axonal growth through the injury epicentre [8]. Generally, reactive nervous tissue constitutes a vast layer around the glial scar [4]. This reactive tissue includes practically all the structural elements of relatively intact and functioning nervous tissue, such as activated glia with astrocytes, microglia, and oligodendrogliocyte precursors. The number of the latter tends to decrease gradually far from the epicentre. Hypertrophic reactive astrocytes interact with neurons, probably improving neuronal survival [2] and contributing to local axonal growth and synaptic formation [9].

Microglia have drawn more attention than other elements of reactive nervous tissue. Two phenotypes are typical of these cells: pro-inflammatory (M1) and anti-inflammatory pro-regenerative (M2). The balance between them is crucial for the response to injury and further spinal cord structural regeneration [10]. After SCI, activated M1 antigen-presenting microglia express molecules of MHC class II and launch numerous immune reactions [11]. In acute and subacute SCI models, M1 macrophages express higher levels of chondroitin sulfate proteoglycans to support the microenvironment [12], while M2 cells produce greater levels of interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and arginase-1 to stimulate post-SCI regeneration [4]. For instance, M2 microglia induce the differentiation of oligodendroglia via the IL-10 machinery [13].

The study aims to elucidate the impact of lower thoracic SCI on micro- and macroglia distribution at the caudal segment both near the epicentre and at distant areas (lumbar enlargement of the spinal cord) in mini-pigs.

MATERIALS AND METHODS

We used mature 4-month-old female Vietnamese Pot-bellied pigs with body masses of 20–25 kg. Every animal was kept alone in a corral with 12-hour periods of daytime and nighttime for two weeks before the intervention. The pigs had free access to water and feed.

For the surgery, we randomized animals to two groups: intact animals (n = 3) and mini-pigs with contusive SCI (n = 3). We performed premedication by intramuscular administration of 1.5 ml of 2% xylazine solution (0.1 ml/kg), 1.5 ml of tiletamine and zolazepam solution (0.1 ml /kg), and ceftriaxone (1 g/5 ml). Then, we anaesthetized pigs via inhalation (Minor Vet Optima, Zoo med, USA) with a mixture of 2.0–2.5% isoflurane (Laboratorios Karizoo, S.A., Spain) and oxygen. We modelled contusive SCI according to an earlier protocol [14]. After D8-D9 laminectomy, we fixed a metal cylinder (impactor) 1 mm beyond the surface of the spinal cord. Standardized contusive injury was achieved via a weight drop of 50 g cargo from 50 cm onto the spinal cord covered with dura mater.

We removed spinal cord samples from the mini-pigs 2 months after the neurotrauma modelling. We injected animals with zolazepam 100 (Zoletil ® 100) at 0.5 ml IM with a gradual increase in inhaled isoflurane up to 5.0 vol.%. For the immunofluorescence assay, we took 5 mm portions of the caudal part of the spinal cord at 5 mm from the injury epicentre and portions from the lumbar enlargement (L4-S2).

We also took the same regions of the spinal cord from the intact animals. The samples obtained were postfixed in 4% paraformaldehyde solution (Sigma, USA) at 4 °C for 12 hours and incubated in 30% sucrose solution (Sigma, USA) for cryoprotection. We cut the samples with a Microm HM 560 cryostat to obtain free-floating 20 μm cross sections of the spinal cord. Then, we washed the sections with phosphate-buffered saline (PBS) with 1% Triton X-100 3 times for 5 minutes. We blocked nonspecific primary antibody binding with PBS containing 1% Triton X-100 and 5% donkey serum for 1 hour at room temperature.

We detected astrocytes with primary antibodies against glial fibrillary acidic protein (GFAP). For oligodendroglia, we employed antibodies against oligodendrocyte transcription factor 2 (Olig2), and for microglia, we employed antibodies against ionized calcium-binding adapter molecule 1 (Iba1) (Table 1). The first stage of the reaction was carried out at a temperature of 4 °C for 12 hours. After washing in PBS, the slides were incubated for 2 hours with secondary antibodies (Table 1).

To visualize the cell nuclei, we additionally stained the sections with a solution of 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma, USA). Digital images of spinal cord structures were obtained with a Carl Zeiss AxioScope A1 microscope (Carl Zeiss, Germany) and analysed in a square of 0.05 mm^2 by ImageJ software (NIH, USA). We also stained some spinal cord sections with secondary antibodies only to provide negative controls of the immunospecificity. We

examined the grey matter: anterior horns – VII, VIII, IX plates; posterior horns – I, II, III, IV plates (according to Rexed). The expression of astrocyte (GFAP) and microglial (Iba1) markers was counted as the ratio between immunopositive and total areas. The number of oligodendroglial cells (Olig2+) was calculated by DAPI-stained nuclei.

Statistical analysis

Descriptive statistics are presented as medians of the 1st and 3rd quartiles. We used the Kruskal–Wallis test to compare the values of quantitative parameters between the groups. For multiple comparisons, we used the Dunn test, and the differences were considered statistically significant at $p<0.05$. Statistical analysis of the obtained data was carried out with the help of the statistical software environment R 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Immunofluorescence assay of the area occupied by astrocytes (GFAP-positive cells) at the anterior horns of the spinal cord near the injury epicentre showed no significant difference between animals after SCI and intact animals (Fig. 1A): 25.11 (21.35; 28.34)% vs. 22.59 (19.94; 25.66)%, $p > 0.05$. In the posterior horns of the spinal cord in mini-pigs after SCI, the GFAP-immunopositive area was larger than that in intact animals: 12.53 (11.69; 13.73%) vs 6.23 (4.36; 8.73)%, $p = 0.0173$ (Fig. 1A).

The number of astrocytes at the lumbar enlargement of the posterior horns was increased in animals after SCI compared with intact animals: 19.87 (15.68; 23.70)% vs. 10.10 (9.17; 12.10)%; $p = 0.002$. At the anterior horns, these values did not differ significantly between experimental and intact animals with 23.54 (17.83; 29.59)% and 20.89 (16.18; 25.88)%, respectively; $p > 0.05$ (Fig. 1B).

The number of oligodendrocytes (Olig2-positive cells) at the anterior and posterior horns near the epicentre of injury in the experimental group was significantly reduced: 20.2 (20.0; 21.2) and 24.5 (22.25; 25), respectively, compared with the intact group: 34.0 (34.0; 40.4) and 43 (40.75; 45.5), respectively; $p = 0.0002$ and $p < 0.0001$ (Fig. 2A).

At the lumbar enlargement, the number of Olig2-positive cells in intact mini-pigs reached 12 (11; 13) at the anterior horn and 16.5 (16; 20.75) at the posterior horn. At the same time, a smaller number of Olig2-positive cells (5 (4; 6) and 3 (2; 4.25), respectively) was found in mini-pigs after SCI ($p < 0.0001$ and $p = 0.0001$) (Fig. 2B).

An increase in microglia (the area of Iba1-positive sites) was detected at the anterior and posterior horns near the injury epicentre in animals with SCI: 24.31 (22.10; 27.19)% and 30.37 (29.29; 32.43)%, respectively, compared with intact mini-pigs: 9.87 (8.87; 10.26)% and 26.10 (23.89; 28.09)%, respectively; $p = 0.0053$ and $p = 0.0014$ (Fig. 3A).

The Iba1-positive area occupied by microglia at the lumbar enlargement was greater for both anterior and posterior horns in mini-pigs with SCI: 25.41 (20.50; 26.25)%, $p = 0.0001$ and 22.19 (14.29; 23.53)%, respectively; $p = 0.0001$; compared with intact animals: 9.80 (7.35; 10.61)% and 8.12 (7.58; 8.79)%, respectively (Fig. 3B).

DISCUSSION

The results of our study showed that the same posttraumatic reaction occurred 2 months after SCI in mini-pigs, both in the caudal part of the spinal cord near the injury epicentre and at distant areas of the lumbar region. The phenomena manifested by an increase in astrocytes (GFAP-positive cells) and microglia (Iba1-positive cells), which are responsible for phagocytosis; we also found a simultaneous decrease in the number of oligodendrocytes (Olig2-positive cells), which provided the myelination of nerve fibres [2].

The data obtained indicate chronic dynamics in the pathology extending to the lumbar spinal cord (distant from the epicentre of SCI). Literature sources also refer to SCI causing neuroinflammation not only at the

Table 1. **Primary and secondary antibodies used to label astrocytes, oligodendrocytes, and microglia in mini-pig spinal cord sections**

Таблица 1. **Первичные и вторичные антитела, использованные для идентификации астроцитов, олигодендроцитов и клеток микроглии в срезах спинного мозга мини-свиней**

Markers / Маркеры	Origin / Происхождение	Dilution / Разведение	Manufacturer / Производитель
GFAP (monoclonal / моноклональные)[a]	Mouse / Мышь	1:200	Santa Cruz (Cat#sc-33673)
Iba1 (monoclonal / моноклональные)[a]	Rabbit / Кролик	1:150	Abcam (Cat # ab178847)
Olig2 (monoclonal / моноклональные)[a]	Rabbit / Кролик	1:100	Abcam (Cat # ab220796)
Anti-mouse IgG conjugated with Alexa 488[b]	Donkey / Осел	1:200	Invitrogen (Cat#A-21202)
Anti-rabbit IgG conjugated with Alexa 647[b]	Donkey / Осел	1:200	Invitrogen (Cat#A-31573)

Note: [a] – primary antibodies; [b] – secondary antibodies.
Примечание: [a] – первичные антитела; [b] – вторичные антитела.

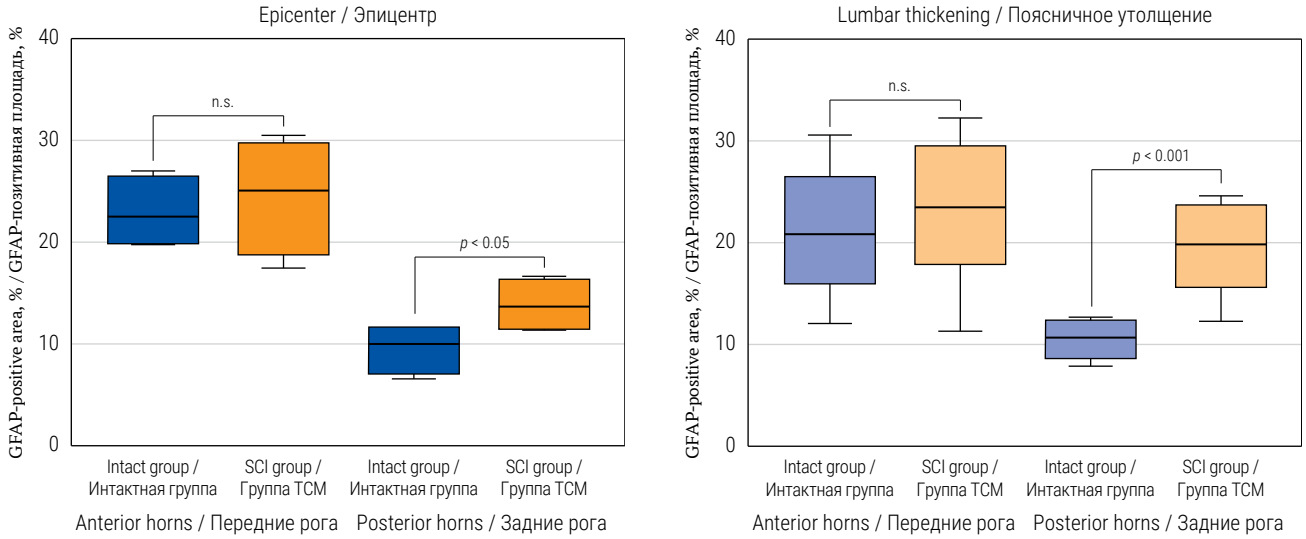

FIG. 1. Quantitative analysis of GFAP-positive areas in the spinal cord of mini-pigs 2 months after SCI modelling.
РИС. 1. Количественный анализ GFAP-позитивных площадей в спинном мозге мини-свиней через 2 месяца после моделирования ТСМ.

Note: n.s. – not significant; SCI – spinal cord injury.
Примечание: n.s. – not significant, не значимо; ТСМ – травма спинного мозга.

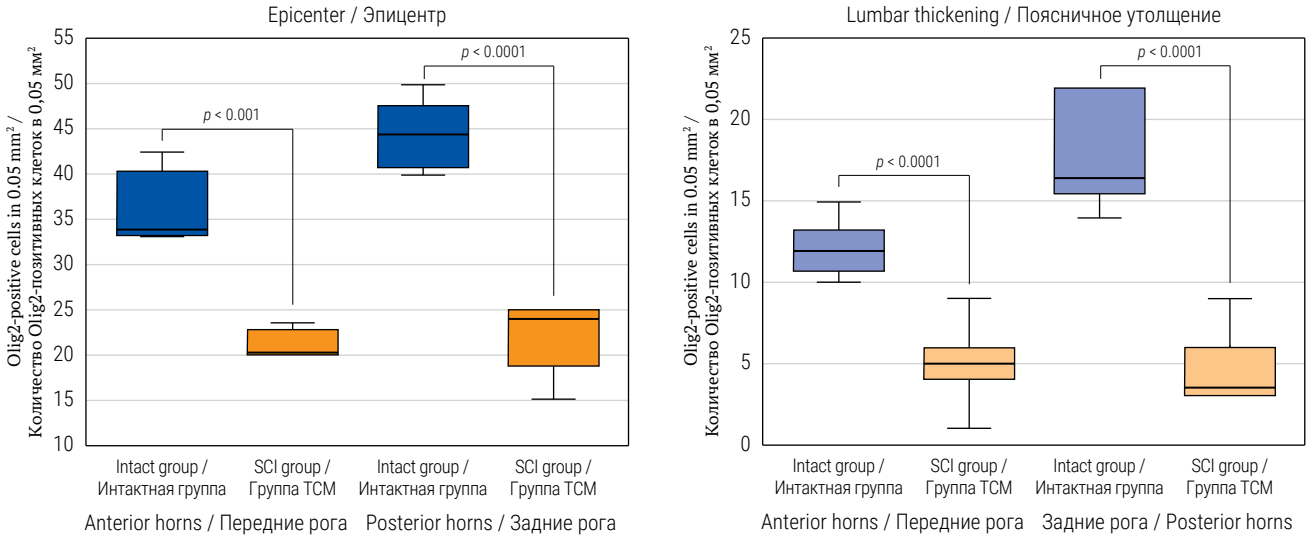

FIG. 2. Quantitative analysis of Olig2-positive cells in the spinal cord of mini-pigs 2 months after SCI modelling.
РИС. 2. Количественный анализ Olig2-позитивных клеток в спинном мозге мини-свиней через 2 месяца после моделирования ТСМ.

Note: n.s. – not significant; SCI – spinal cord injury.
Примечание: n.s. – not significant, не значимо; ТСМ – травма спинного мозга.

epicentre of the injury [2] but also at more distant areas in both (cranial and caudal) directions [11].

We emphasize that SCI usually provokes a long-term negative effect on everyday activity with physical and psychological harm [15]. The disability of patients with SCI is a significant socioeconomic problem not only for patients and their families but also for governments. Limited cell regeneration of the central nervous system is a key problem requiring novel approaches for SCI treatment. Experimental methods of posttraumatic morphofunctional regeneration of the spinal cord are initially being developed in models of SCI in animals. However, one cannot simply transfer these methods to clinical practice without any modifications. We chose mini-pigs because their anatomy is similar to humans, especially the structure of their nervous system and their physiological and biochemical

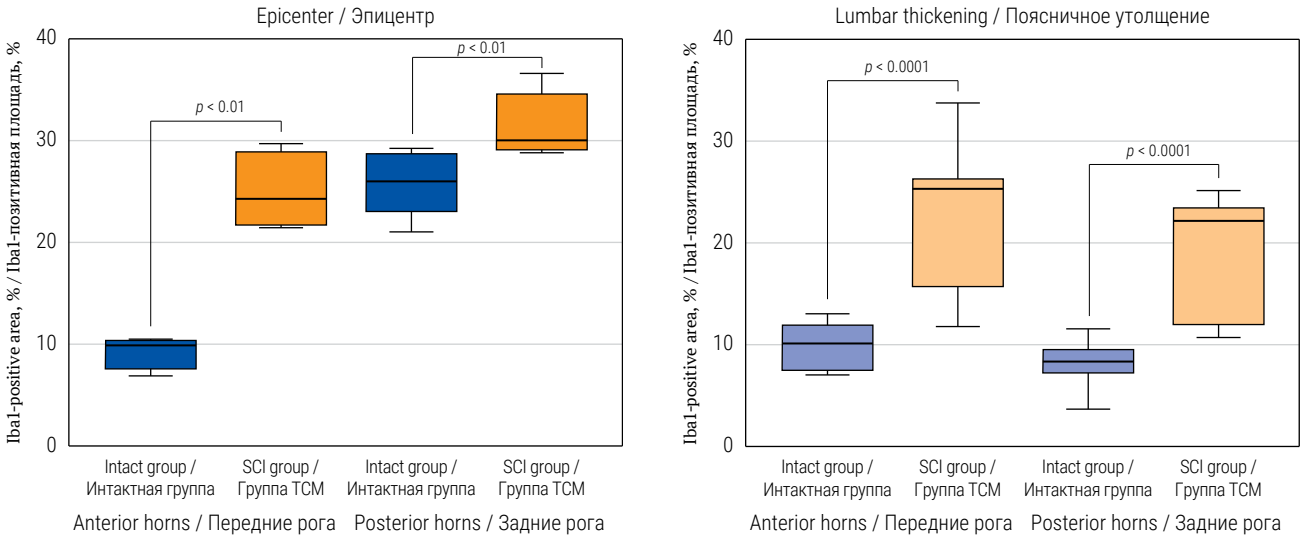

FIG. 3. Quantitative analysis of Iba1-positive areas in the spinal cord of mini-pigs 2 months after SCI modelling.
РИС. 3. Количественный анализ Iba1-позитивных клеток в спинном мозге мини-свиней через 2 месяца после моделирования ТСМ.

Note: n.s. – not significant; SCI – spinal cord injury.
Примечание: n.s. – not significant, не значимо; ТСМ – травма спинного мозга.

characteristics. Thus, mini-pigs are considered to be the optimal animals for preclinical studies [15].

Nevertheless, spinal cord remodelling after SCI is multifaceted [11]. In the epicentre, a focus of primary damage is formed with further activation of macroglial cells and inflammatory infiltration by neutrophils, macrophages, and resident microglia [16]. Astrocytes and microglia assist in the degeneration of impaired nerve fibres located at the SCI epicentre or passing through it, and they also promote the reciprocal activation of neurons [11]. As a result, the nerve fibres of the descending and ascending pathways are destroyed, and the links between the cerebral and spinal neurons of the brain are interrupted. Therefore, the inflammatory response for the site of injury is well known [17] but not for distant areas [11]. At the same time, a comprehensive understanding is crucial for developing new treatment options.

Today, options such as gene therapy (increasing the survival rate of spinal cord cells, stimulating axonal growth, inhibiting neuroinflammation, and preventing the formation of astroglial scars) and electrostimulation (promoting neuroplasticity and integration of efferent and afferent signals, thereby contributing to the functional regeneration of the spinal cord) tend to resolve the problem of pathogenetic and pathomorphological damage in the subacute and chronic phases of SCI. Recent studies have drawn attention to the prevention of pathology in the spinal cord not only directly near the injury epicentre but also at distant areas.

Limitations of the study

Most of the works devoted to the study of pathogenetic and pathomorphological processes in SCI are focus mainly on the epicentre of primary damage. In this study, we present data on the reaction of macro- and microglia in the caudal segments of the spinal cord after contusion injury in the lower thoracic region in mini-pigs. However, the pathological process also involves neighbouring cranial segments. In this regard, additional studies of restructured neuroglia above the injury epicentre of damage are needed.

Perspectives for further research

Remodelling of neuroglial cells is one of the immediate consequences of SCI. In our model, the study of spinal injury dynamics in mini-pigs should include the examination of molecular restructuring of the extracellular matrix, neuroinflammation, degeneration and regeneration of nerve conductors, reorganization of the trans-traumatic neuronal network, and especially the issues of damage to the blood–spinal cord barrier and its further restoration.

CONCLUSION

The results of our study demonstrate the same patterns of macro- and microglia distribution both in intact nervous tissue caudally adjacent to the epicentre of SCI and distant areas of lumbar enlargement. Remodelling of neuroglia at distant areas in SCI is an important task that should be mentioned in further development of neuroregenerative methods for patients with SCI.

AUTHOR CONTRIBUTIONS

Ravil R. Garifulin, Andrey A. Izmailov, Vage A. Markosyan, Irina S. Minyazeva – conducting a research and data collection. Ravil R. Garifulin, Andrey A. Izmailov – data curation and analysis. Victor V. Valiullin, Rustem R. Islamov – writing and editing the draft of the article. Rustem R. Islamov – work leader, conceptualization, supervision. All authors approved the final version of the publication.

ВКЛАД АВТОРОВ

Р.Р. Гарифулин, А.А. Измайлов, В.А. Маркосян, И.С. Минязева провели исследование и собрали материал. Р.Р. Гарифулин, А.А. Измайлов проанализировали результаты. В.В. Валиуллин, Р.Р. Исламов написали и отредактировали текст рукописи. Р.Р. Исламов – руководитель работы, разработал идею исследования, сформулировал цели и задачи и осуществлял руководство работой. Все авторы утвердили окончательную версию публикации.

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