Chapter

# The Experimental Bioengineering of Complete Spinal Cord Injury in Adult Rats

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## Abstract

The chapter is devoted to the research of experimental complete mechanical spinal injury in adult rats and attempts at bioengineering restoration of the structure and function of the spinal cord using a protein-polysaccharide construct that includes bovine collagen, highly purified crab chitosan ascorbate, nanostructuring additives in the form of sodium chondroitin sulfate, sodium hyaluronate, heparin sulfate in the presence of complete nutrient medium DMEM, neural supplement N2, conditioned nutrient medium, obtained about brain cell mouse embryos and mouse embryonic stem cells, retinoic acid, and mouse neural progenitor cells derived from embryonic stem cells. After a complete intersection of the spinal cord at the level of the ninth thoracic vertebra, the authors directly implanted a collagen-chitosan construct into the gap between the ends of the spinal cord. Analysis of the recovery of motor and sensory and vegetative functions of the spinal cord for 20 weeks after surgery using the cytological immune-fluorescent method showed a high viability of the transplanted neural cell precursors during the entire observation period and the early emergence of activity of mediators of nerve signal transmission in the implantation zone of the structure with accompaniment active dynamics of reducing neurodeficiency.

**Keywords:** collagen-chitosan scaffold, complete intersection of the spinal cord, mouse neuronal progenitor cells, adult rats, neurological deficiency, neurotransmitters, immunofluorescence analysis

## 1. Introduction

Solid biodegradable constructs as an implant in the spinal cord based on proteins and polysaccharides are able to create favorable conditions for the adaptation, proliferation, differentiation, and sprouting of the precursors of neural cells placed in the implant. At the same time, direct transplantation of the structure together with the precursors of neural cells substantially eliminates weight loss during cell transfer and the apoptosis reaction and increases the survival time of cells in new conditions, and the polysaccharide components of the matrix not only play the role of shielding the protein base from the immune system, and also provide a high function cell transfer system to the central and peripheral ends of the central nervous system. The problem of spinal injury remains acute without addressing the issues of effectively eliminating neuro-inflammation in the area of injury and

creating conditions for the formation of pathways and sprouting axons under the conditions of glial scar formation.

### 1.1 Embryonic stem cells in the correction of spinal cord injury

A significant improvement of the neurological deficit was achieved by Bottai et al. by intravenous administration of undifferentiated ESCs at a dose of 1 million cells 2 hours after injury [1]. The protective role of multipotent cells was demonstrated during the development of an inflammatory reaction, accompanied by a decrease in the intensity of infiltration of the affected area with elements of microglia. The presence of hESCs in the spinal cord injury zone in adult rats or mini-pigs for 4.5 months induces the formation of embryoid bodies with expression of specific (hNUMA, HO14, hNSE, and hSYN) and nonspecific (DCX, MAP2, CHAT, GFAP, and ACP) antigens. Research results of Yang et al., in a rat spinal cord contusion injury model using green fluorescent protein-labeled pig embryonic stem cells (pES/GFP (+)), indicate a decrease in expression of green fluorescent protein within 3 months, and positively stained cells for neural are detected in the transplant area of specific antigens: anti-NFL, anti-/MBP, anti-SYP, and anti-Tuj 1 [2]. The use of undifferentiated cells significantly reduces the efficiency of functional restoration of the hind limbs in rats, compared to the use of neural progenitors derived from ESCs. The value of indicators of the neurological status on the BBB scale was 15.20 ± 1.43 points on the 24th week of the posttransplant period.

### 1.2 Neural stem cells and their precursors

Under certain conditions, these cells are able to differentiate into neurons, astrocytes, and oligodendrocytes. Neuronal progenitors and precursors of oligodendrocytes showed positive dynamics in reducing neuro-deficiency in spinal cord injury models. The use of two cell populations with neurogenic and gliogenic activity leads to the restoration of locomotor functions and stimulates angiogenesis, the growth of axons, and their re-myelination [3–11]. A monolayer of neural precursors (NPs) from ESCs is obtained by a combination of diffusion of nutrients and growth factors; it includes extracellular matrices such as laminin, poly-d-lysine, and matrigel [12]. A three-dimensional matrix with the precursors of neuronal cells or oligodendrocytes creates the conditions for the formation of intercellular contacts, ensures survival, differentiation, and proliferation [13, 14]. Cultivation of neural precursors on a three-dimensional substrate forms oligodendrocyte precursors with the expression of Nestin and PAX6 [15, 16]. At the same time, NP cultivation conditions and their micro-environment are of key importance [17].

### 1.3 Neurotrophic support in the area of spinal cord injury

Re-myelination of damaged axons and improvement of their conductivity are possible with transplantations of neural progenitors in the experiment [18, 19]. The effect of transplantation is enhanced by trophic support of neurons and immune modulation [20–23]. Transplantation of spinal cord precursors of neuronal cells to the zone of spinal cord injury is accompanied by activation of axon growth, their locomotion, and registration of G-CSF, BDNF, CNTF, FGF, EGF, and NT-3 growth factors in cells [24–27]. Consequently, neurotrophic support is a prerequisite for the regeneration of specialized nervous tissue when it is lost. The presence of neurospheres of exogenous growth factors such as EGF, bFGF, and CNTF in the environment creates conditions for the long-term cultivation of viable precursors of neurons, differentiation into mature neurons, and successful transfer to the area of

spinal injury [28]. Spinal cord regeneration with the help of neural cell progenitors indicates the important role of stem cells under conditions of intensive neurotrophic support by NGF, BDNF, CNTF, GDNF, and IGF-1 molecules [24, 29].

### 1.4 Use of degradable biopolymer substrates as implants in the damaged spinal cord

Modern technologies for obtaining biomaterials with unique consumer properties make them the most important link in the task of reconstruction of spinal cord injury. Neuroprotection from re-inflammatory response is possible, provided that the growth of neurites is artificially oriented with the inclusion of neurotrophic support in this system. Studies confirm that the creation of such conditions leads to the restoration of lost tissue, enhancement of axonal growth and active sprinting to the head and caudal segments of the damaged area, suppression of astrocytosis, and the appearance of inter-synaptic connections.

The strategy of reconstruction of the damaged spinal cord includes the use of prepared cell mass in solid or liquid, biodegradable, highly compatible 3D matrices with favorable conditions for its cultivation, proliferation, and differentiation. Such constructions allow direct transfer of cellular material to the area of spinal cord injury.

Matrigel, laminin, type-1-collagen, fibronectin, chitosan, glycosaminoglycans, poly-d-lysine, and fibrin are used in experimental practice. The final results of the studies do not allow asserting one of the mentioned polymers as the best sample, despite the use of combined materials with a neurotrophic microenvironment [30].

The differentiation of hESC in contact with neuronal progenitors occurred more actively when laminin was used [31]. In other conditions of cultivation of neuronal precursors, the efficiency of using Matrigel is confirmed [32, 33]. A number of authors obtained satisfactory results when using a combination of collagen IV, laminin, and poly-L-lysine [12, 16, 34]. Such nonstandardized approaches to the creation of culture conditions do not exclude the risk of immune reactions.

### 1.5 Materials based on chitosan to obtain neural substrates

Chitosan, a linear copolymer consisting of N-acetylglucosamine and N-glucosamine, when in contact with the wound surface biodegrades without causing toxic reactions from surrounding somatic cells and is capable of screening protein molecules from the immune system during biodegradation. The number of scientific publications on the use of chitosan polymers as the basis of the matrix for implantation in the spinal cord in trauma is immeasurably small compared to the use of other biopolymers in spinal injury models. The strategy for using chitosan polymers for spinal cord reconstruction is developed by Freier et al. [35] and Nomura et al. [36]. The advantages of liquid and solid substrates are known for cell mass transplantation and neurotrophic support in the spinal cord in trauma. However, sponge structures are capable of covering extensive defects in the damage zone. An important aspect of the technology is the formation of oriented microchannels in the matrix for the directed growth of axons and, thus, enhanced sprouting of cells in the graft area and beyond. The polycationic properties of chitosan ensure constant contact of cells with a biopolymer, which enhances cell migration and axon regeneration [37] and lengthens and thickens neurites [35], and in low concentrations, the biopolymer is able to shield stem and progenitor cells [38].

In 2008, experimental transplantation of neural cell precursors as part of a chitosan microchannel biomaterial was performed first after complete transection of the spinal cord. The use of NSPS in the spinal cord of Sprague-Dawley rats as a base of a highly de-acetylated chitosan hydrochloride micro-channel substrate confirmed

that massive cellular transitional bridges containing a high number of differentiated cells are formed in the spinal cord within 100 days after the surgical intervention. Cell-free channel matrices also formed transition zones, but the thickness of the transitions was significantly less [39]. If precultured neural progenitor cells (NSPCs) are placed in the micro-channels of the chitosan substrate, in the damaged rat spinal cord, one can demonstrate very high cell survival for 5 weeks and their differentiation not only into neurons but also astrocytes and oligodendrocytes [40]. High cell survival and differentiation of not only neurons but also glial cells in chitosan extramedullary channels and in the spinal cord after transplantation and active sprouting from the central to the caudal segment of the spinal cord with the formation of active transitional brain tissue are accompanied by significant restoration of the motor and sensory functions of the hind limbs [41]. The inflammatory process in the area of spinal cord injury stimulates the synthesis of type IV collagen in endothelial cells, fibroblasts, and Schwann cells, which leads to the formation of a massive glial scar. Productive inflammation in the connective tissue inhibits the growth of axons [42–44]. Exogenous suppression of the synthesis of collagen type IV prolongs axonal growth. The introduction of type I collagen into the system enhances the growth of axons and neural connections between the central and peripheral zone of injury [45, 46]. It is convincingly shown that the presence of stem cells in the composition of the polymer chitosan in the area of spinal cord injury improves cell viability and stimulates their differentiation and the expression of neurotrophic factors. As a result, functional neurodeficiency is reduced. Prolonged presence of chitosan in the brain tissue and its biodegradation do not stimulate the immune response [47, 48].

Preliminary studies have resulted in 4 variants of the neural matrix that can support long-term human ESC and animals (rats) in complete medium and translate them into a state of differentiation with reception on the 5th day of cells with neuronal markers exposed on its membrane (neuro-filament, MBP, and GFAP) [49]. The approaches proposed in this work to the reconstruction of the spinal cord in experimental complicated spinal injury (total mechanical spinal cord transection) are based on modern biodegradable polysaccharide matrices containing the necessary microenvironment, including the products of growth and differentiation of stem cells and neuronal cells and neuronal precursor cells for the reconstruction of spinal cord to restore its motor and sensory functions. Consumer properties of the proposed cellular matrix showed competitive advantages due to the lack of matrices satisfying the task. They are as follows: high biocompatibility, biodegradability, nontoxicity, the system of information transfer, the creation of a strict orientation of the tissues in tissue engineering implant thanks to a rigid linear structure of chitosan, the regulation of collagen synthesis, stimulation of breeding passaged cell precursor neuronal tissue, vascular endothelial cell proliferation, and tumor micro-vessel recovery of the intracellular substrate. Implantation of such a matrix can be done in an open manner of the operative spinal cord diastasis.

## 2. Materials and methods

### 2.1 Neuronal matrix getting

To create a neural matrix base poly-ion complex consisting of nano-micro-structured ascorbate chitosan with a molecular weight of 695 kDa and a degree of deacetylation of 98%, when the content of 1 g dry chitosan 1.8 g of ascorbic acid, comprising anionic salt forms chondroitin acid (Sigma) (20 mg\d), hyaluronic acid (Sigma) (10 mg\d) and heparin (5 mg\d) (Russia), serum growth factors in cattle “adgelon” (110 mcg\d) were used.

To obtain the basic collagen-chitosan complex, we used a triply purified chitosan obtained in Vostok-Bor-1, Dal'negorsk, Russia with a molecular weight of 695 kDa and a deacetylation degree above 95%, protonated with an ascorbic acid solution (dissolution of the polymer in ascorbic solution acid in a ratio of 1: 1.5). Introduction of a heparin solution, serum low-molecular growth factor "adgelon" (SLL "Endo-Pharm-A," Moscow region, Shchyolkovo, Russia), neural supplement N2 (Sigma), and retinoic acid (Sigma) stimulated embryonic stem cells (ESCs) in the chitosan gel to neuronal differentiation. A conditioned nutrient medium obtained after culturing embryonic neuronal cells of brain tissue of mice or conditioned nutrient medium obtained after culturing embryonic allogeneic stem cells of mice was added to the base poly-ionic complex (Krasnoyarsk Center for Reproductive Medicine, Russia). Embryonic stem cells (ESCs) were obtained from mouse blastocysts by eluting the uterus with DMEM medium of the anesthetized animal on the 4th–5th day after copulation. Getting the inner cell mass (ICM) and the further expansion of ESC colonies was performed according to the protocol [50, 51]. Overage human blastocysts were taken from patients undergoing IVF. Full consultation was done and informed consent was received before embryo processing. This research followed the directives of the Russian Association of Human Reproduction. Embryos were obtained for research use only, without other restrictions and compensation.

Cultivation of cells was performed in DMEM supplemented with 10% fetal calf serum (FCS F0926, Sigma), 100 mg/ml kanamycin sulfate (Sigma), 1 mM L-glutamine (G7513, Sigma) in bottles Corning, gelatin-coated (Sigma).

In the experiments to obtain the conditioned medium from neural stem embryonic mouse cells (cells from the brains of 17–20 day fetus outbred mice (Institute of biophyisic SD RAS, Russia) after the dispersion and processing of 0.5% collagenase solution (Sigma) for 30 minutes in medium DMEM (Sigma) at 37°C for increasing cell biomass used DMEM under light microscopy with 10% fetal calf serum (FCS), 100 mg/ml canamycin sulfate, 1 mM L-glutamine, which is added 4 ng\ml basic fibroblast growth factor (bFGF, Sigma), 1 mM solution of essential amino acids (Sigma-Aldrich). The cell biomass was grown at 37°C in vials coated with 0.1% gelatin solution. The environment was collected daily. The condition of cells was estimated by light microscope. After subculturing with 0.5% collagenase solution, the matrix cells were cultured in medium supplemented with neuronal differentiation agent—N2 component, according to the manufacturer's instructions. The medium was collected, filtered through a 0.22 mcm cellulose acetate filter, and used hereinafter as a conditioned medium.

Next performed covalent compounds derived polysaccharide gel structure (The Developer is SBEU HPT Krasnoyarsk State Medical University, Russia) with bovine collagen gel (SLL Belkosin, Russia) in a ratio of 1: 3, a freeze-drying deep frozen samples to install FC500 (Germany). This mixture was poured on pallets of duralumin, a layer thickness of 2 mm, frozen at −20°C, and then freeze-dried at $10^{-5}$ Pa for 8 hours; the product is packaged and sterilized by the electron beam method (neuronal dry matrix courtesy SLL "Medical Company Collachit," Krasnoyarsk Region, Russia).

The manipulation above yielded a neuronal matrix of the size 50 × 50 × 2 mm, suitable for not only the culture and in vitro differentiation of embryonic stem cells into neurons and oligodendrocyte progenitor of mice, but also for direct transplantation into the spinal cord gap in experimental injury in adult rats.

When using a sponge matrix, they need to be previously soaked in sterile bicarbonate buffer (Sigma) to reduce their acid properties. After neutralization phase of collagen-chitosan substrate was washed three times with sterile phosphate-buffered Dulbecco's modified eagle's medium (Biolot), placed in a vial, and carefully layered on top of them the cell suspension in a medium with all the components, depending on the cell type.

The cultivation of pluripotent cell experiments on collagen-chitosan substrates initially used for biomass growth basal medium DMEM (Sigma) supplemented with 10% SR (serum substitute), 100 μg/ml kanamycin sulfate, 1 mM L-glutamine, 4 ng/ml primary engine of growth fibroblast (bFGF), 1 mM solution of amino acids, and the inhibitor Rock 5 ng/ml kinase (Sigma). Capacity cell biomass was performed in flasks coated with 0.1% gelatin. The environment was changed daily. The colonial state was assessed visually using a microscope AxioVert-200. To assess the state of maintenance of pluripotency, limmune-cytochemical analysis of the expression of markers—oct4, TRA-1-60, SSEA4, and cd30 (Sigma)—was performed.

To differentiate embryonic cells in the neuronal direction, they were seeded into vials in a medium with all additives except bFGF, with the addition of retinoic acid and N2 component.

Every three days, formaldehyde fixation was performed followed by immune-cytochemistry of the cells with antibodies (Abcam, USA) against GFAP glial fibrillar acid protein, neuro-filament, and nestin. Detection of markers was carried out by the method according to the instructions of the antibody manufacturer. The cell nuclei were stained with DAPI (Sigma) (0.1 μg/ml) for 10 minutes. The Olympus BX-51 fluorescence microscope (Japan) and the software products "Applied Spectral Imaging" (USA) were used to obtain images and analysis. For the analysis of each marker, the experiment was repeated three times, increasing three vials, each of which was randomly assigned into 6 zones for carrying out immunocytochemistry. Microscopy was carried out for each zone in 30 fields of vision.

### 2.2 The experimental complete transection of the spinal cord in rats

The conditions of biological test systems in the CDI CI correspond to Guide for the Care and Use of Laboratory Animals, 8th edition, 2011, NRC, USA (Manual on the content and use of laboratory animals, 8th edition, 2011, national research Committee, USA). The content of animals in individually ventilated cells from polysulfone seal-safe, 461 × 274 × 228 mm (production TECHNIPLAST.P. A.)

The rooms contain biological test systems, controlled temperature (18–24°C), humidity (30–70%), illumination (12/12 hours), and the multiplicity of air (X11 without recirculation). Control of climatic parameters is carried out in accordance with the SOP "control of climatic parameters in the premises of the vivarium."

Distribution of feed and water is carried out at a fixed time; the change of litter is made once a week in accordance with the SOP "preparation of cells for biological test systems. Marking. Change of bedding, feed, water."

Biological test systems receive food " CHARA" for the maintenance of laboratory animals of the SPF-category, production of LLC "Assortment agro" and "VAKA," and production of LLC "BIOSPHERE," auto-clavable, with confirmation of the absence of microbial contamination. The feed is balanced in amino acid composition, minerals, and vitamins, made of high quality components. Laboratory tests for toxicity have been carried out. The food is not toxic, but a environmentally friendly product. Certificates of feed quality are present. Sterilization of feed is carried out in the steam sterilizer TOUCHCLAVE LAB K14 369 manufactured by LTE Scientific LTD, with confirmation of sterilization (multi-parameter chemical indicators of sterilization 3 M Comply Steri Gage manufactured by 3 M). After sterilization, the feed is stored in closed containers for no more than 7 days (SOP "operation of steam sterilizer with automatic control Touchclave-LAB model TC/R300E/PL/PASS").

For watering of biological test systems, we used water that had been trained in the reverse osmosis production unit JSC "Scientific-production company

Mediana-Filter," confirming the absence of microbial contamination, and bottled in pro-anti-cianidinas bottle. Access to food and water is unrestricted throughout the study.

The cells consist of a mixture of litter "Golden cat," produced by "the zone" "the Gold Ear," Russia and "Lignosol," produced by J. Rettenmaier and Sohne Gmbh, Germany, in the ratio of 1:4 subjected to autoclaving, control of microbiological purity in accordance with SOP "Operation of a steam sterilizer with automatic control Touchclave-LAB model TC/R300E/PL/PASS."

Veterinary protocol was considered and approved at the meeting of the Commission on Bioethics, minutes of the meeting No 57 dated 01.10.2016.

The research methods used in the planned experiment, capable of causing pain and stress to animals, cannot be replaced by more humane ones. The possible negative effects on animals are minimized and the principles of their humane use are observed.

Premedication: 30 minutes prior to surgery—Sol. Tramadoli 2.5 mg/m; Sol. Atropini sulfatis 0.1%–0.1 ml/m; Sol. Dimedroli 0.1%–0.1 ml/m Anesthesia: anesthesia (diethyl ether). About 12 white female rats, Wistar line, weighing 250 g with Carl Zeiss optics, individually reproduced model of spinal cord injury at the IX-X thoracic vertebrae with a complete transverse intersection of the spinal cord after first performing a laminectomy.

### 2.3 Operation course

After preliminary treatment of the operating field with a 70% solution of ethyl alcohol under anesthesia, an incision was made along the midline of the animal's back at a level of Th7 to L4 4–5 cm in length. Hemostasis was performed during the operation. After dissection of the skin, subcutaneous fat layer, the wound edges were mobilized and diluted with Edson's retractors. The acristoid-trapezius and the broadest muscle were cut off from the places of their attachment to the spinous processes of Th9-L3. The muscles of the deep layer were separated from the spine and diluted with a microsurgical retractor, and the Th9-Th12 vertebrae were bared. The Th10 arch resection was performed, and the dura mater was dissected and diluted to the sides in the horizontal plane.

A complete transverse intersection of the spinal cord was performed using a microscalpel and micro-scissors and individual optics of Carl Zeiss. A neuronal cell matrix with a size of about 2 $mm^3$ containing about 100 thousand precursors of neuronal cell implant was placed immediately in the formed neural tissue defect. The bony wound was closed as a film with polysaccharide hydrogel mass "bolchit" [52], which does not contain animal collagen. The wound was closed layer by layer using suture material Vicril 4–0 (muscle) and Polyester 3–0 (skin). The stitches were treated with an alcohol solution of iodine (**Figures 1–6**).

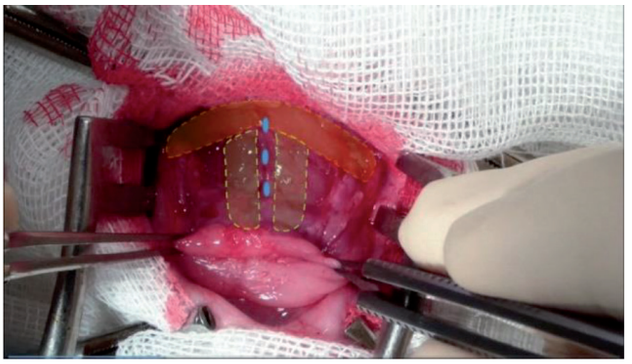

**Figure 1.**
*Quick access to back extensor and awned appendages of vertebras Th VIII-X.*

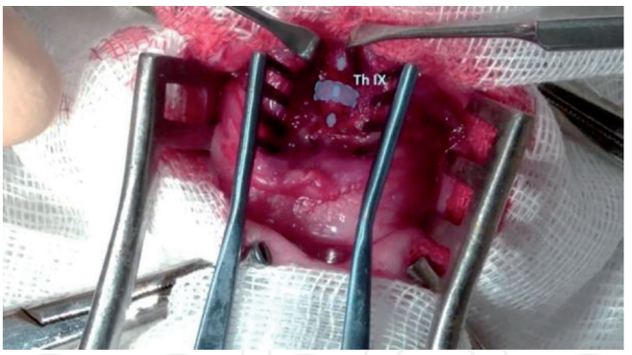


**Figure 2.**
*Skeletization of the ninth chest vertebra handle.*

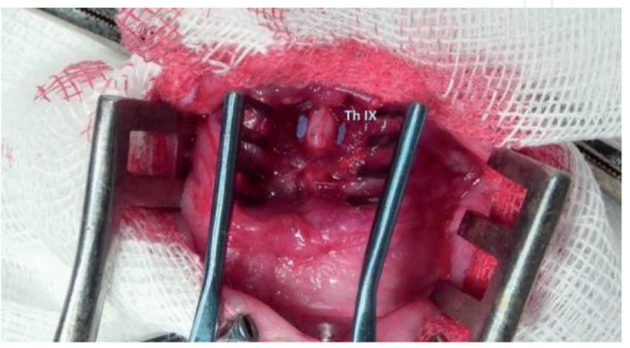


**Figure 3.**
*Resection of the ninth chest vertebra handle with removal of a firm brain cover, and spinal cord exposure.*

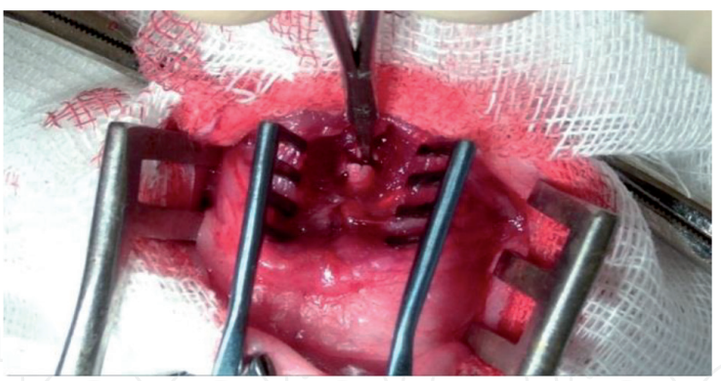

**Figure 4.**
*Full spinal cord transection.*

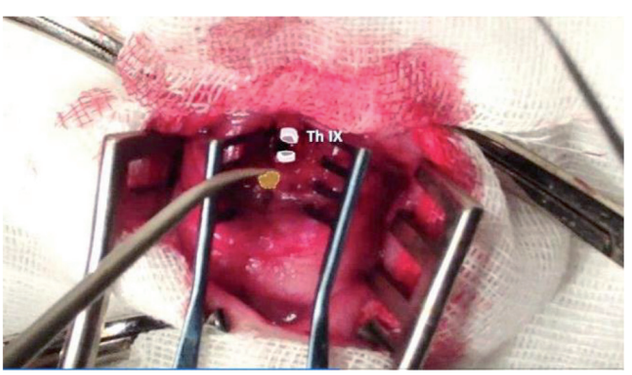


**Figure 5.**
*Cellular neuromatrix implantation of spinal cord diastases.*

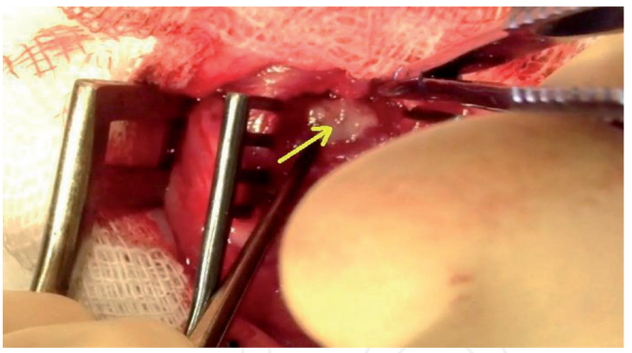

**Figure 6.**
*Restoration of vertebral channel integrity by means of a chitosan film (it is pointed by an arrow).*

### *2.3.1 The composition of transplantable matrices*

On the basis of collagen-chitosan structures, two different implants were prepared containing the following components:

1. A lyophilic collagen-chitosan matrix obtained as described above, which does not contain cells, including in its composition the complete DMEM nutrient medium, conditioned medium from neuronal cells and neural supplement N2, and retinoic acid (6 rats of the control group);

2. A lyophilic collagen-chitosan matrix, obtained as indicated above in the control group, containing about 100,000 neuronal precursor cells obtained by culturing and differentiating mouse embryonic stem cells (ESCm) (6 rats in the test group).

Variants of porous matrix substrates were cut into 1 or 2 $mm^3$ before embryonic stem cells were placed on them and, in sterile conditions, were unfolded in the wells of a 96-well plate without coating with 0.1% gelatin solution. The cells from the culture flasks were removed with a 0.5% collagenase solution, then washed three times with DMEM medium from the enzyme and transferred to wells with a cut matrix in the ESC medium. Cells were cultured for at least 3 days prior to the appearance of neuronal markers under humid conditions at 37°C and 6% $CO_2$. The number of attached cells was evaluated by dispersing 5–6 pieces of the matrix in the enzyme solution, followed by counting the cells in the Goryaev chamber. Each future neuron implant during cultivation contained about 100 thousand of neuronal precursor cells. Before the implantation in the spinal cord, the pieces with a micro pincer were carefully transferred to microtubes with DMEM/F12 culture medium (Nutrient mixture F-12 HAM, Sigma) and transported to the operating room.

## 2.4 Postoperative care

Within 3–5 days after the operation, the animals received Sol as an analgesic; Tramadoli 2.5 mg 3 times daily IM. Sutures were removed after 10 days. During the first 24 hours, animals were admitted to the water. Feeding was performed 48 hours after the operation solely with a mixture of "Polyproten-nephro" (SLL "Protenpharma," Russia) for 1–4 weeks. The rats were kept in separate boxes with a double bottom. Drug support was provided with a broad-spectrum antibiotic, antispasmodics, and vessel-dilator drugs.

### 2.5 Dynamic neurological control

To assess the neurological disturbances and recovery dynamics, a scale was used to assess the severity of the neurological deficit for complete transection (BBB scale) [53] within 20 weeks after implantation.

### 2.6 The immunofluorescence analysis of sections of the spinal cord after direct implantation of cellular neuronal matrices in the dislocation of a spinal trauma

Histological sections were subjected to immunofluorescence treatment for the search for transplanted cells expressing the green fluorescent protein GFP and the presence of neurotransmitters in the upper and lower areas of the spinal cord adjacent to the graft, as well as in the collagen chitosan transplant itself: acetylcholine, serotonin, and GABA (Abcam, USA). The Olympus BX-51 fluorescence microscope and the software products "Applied Spectral Imaging" (USA) were used to obtain images and analysis.

Spinal cord preparations obtained by careful and judicious selection of tissue from the spinal canal through the 20 weeks after surgery.

## 3. Results and discussion

Analysis of neurological deficits in rats after complete spinal cord transection indicates that transplantation of the cell-free collagen-chitosan matrix into spinal cord diastase at the level of thoracic vertebra IX leads to a marked reduction in the volume of violations by restoring the function of the pelvic organs in full, and provides 5–6 level recovery of motor and sensory functions of the spinal cord within 20 weeks of follow-up (**Table 1**). Implantation into the spinal cord diastasis collagen-chitosan substrate of about 100,000 mouse neuronal precursor cells leads to virtually eliminate neuronal lack, reaching over 20 weeks 19.5 replacement level (**Table 1**, **Figure 7**). The serial photo shows the animals in the control and experiment at different times of the postoperative period with different levels of reduction of neurological deficit (**Figures 8–14**).

### 3.1 Immunofluorescent analysis of neurotransmitters in the spinal cord sections at its complete transection

Immunofluorescence neurotransmitter control of serial sections of the cerebral spinal cord rats 20 weeks after its full transection (the zone above the transplant area) shows that the interstitial tissue filled with nuclear cell mass actively express GABA, acetylcholine, and serotonin. The number of these cells is the uniformity of the distribution center to the area of the graft (**Figure 15**).

Serial immunofluorescence zone collagen-chitosan graft shows that 20 weeks after the operation area is filled with interstitial brain tissue with a large number of viable neuronal cells, maternal spinal cord actively expresses markers GABA, acetylcholine, and serotonin. Furthermore, zone graft contains many newly formed micro-capillaries containing the body of erythrocytes with auto-fluorescence effect. The number of nucleated cells in maternal spinal cord transplant decreases toward the tail region (**Figure 16**). In the rear area of the spinal cord (below the graft), the number of nucleated cells is substantially less than in the head area and in the control graft. However, viable cells express neurotransmitters GABA, acetylcholine, and serotonin (**Figure 17**).

| **Lower limb\week** | | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Neuronal lack's level | Experimental group (with predecessors NC) | left paw | 0 | 1 | 2.5 | 5 | 7 | 7 | 7.5 | 9 | 10 | 11.5 | 12.5 | 13 | 14.5 | 16 | 19 | 19.5 | 19.5 | 19.5 | 19.5 | 19.5 |
| | | right paw | 0 | 1 | 1 | 4 | 4.5 | 5.5 | 7 | 8.5 | 10.5 | 11.5 | 12.5 | 13 | 14.5 | 16 | 19 | 19.5 | 19.5 | 19.5 | 19.5 | 19.5 |
| Neuronal lack's level | The control group (without predecessor NC | left paw | 0 | 0 | 0.5 | 1.2 | 1.8 | 3.4 | 4.8 | 5 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 | 5.4 |
| | | right paw | 0 | 0.4 | 0.8 | 0.8 | 2.2 | 4.4 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |

**Table 1.**
*Analysis results with complete neurological spinal cord transection and transplantation of collagen-chitosan matrix predecessor mouse neuronal cells (PNCm).*

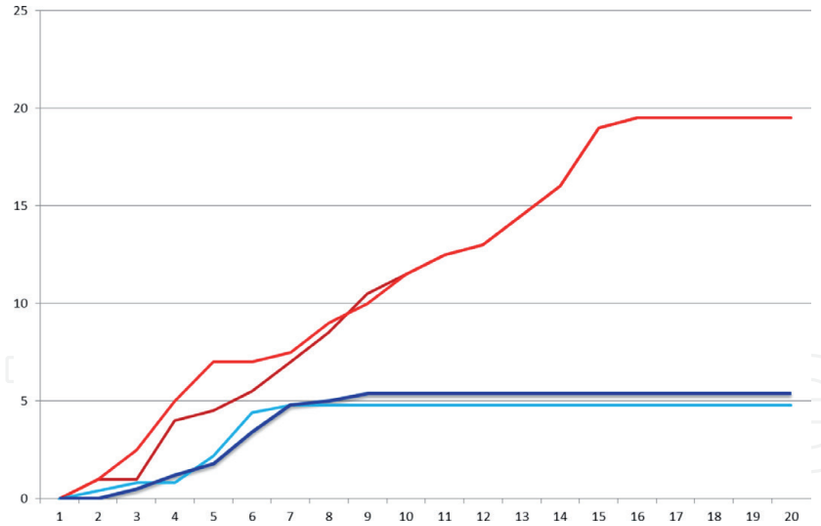


**Figure 7.**
*The neurological analysis results of a full transection of a spinal cord. Red (left) and burgundy (right) color (experience) back extremities, blue (right) and navy blue (left) color (control) back extremities. Down – levels of reduction of neuronal deficiency, across – weeks of supervision.*

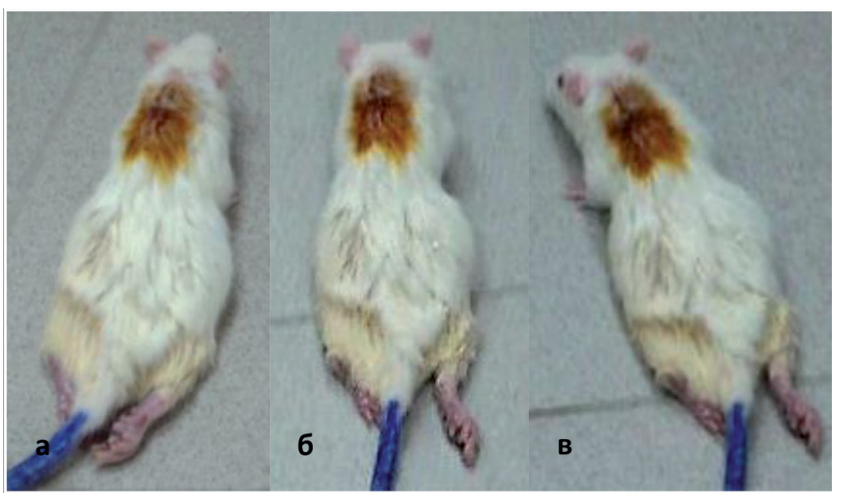


**Figure 8.**
*The first week after transplantation, level of reduction of neurological deficiency 0 (control).*

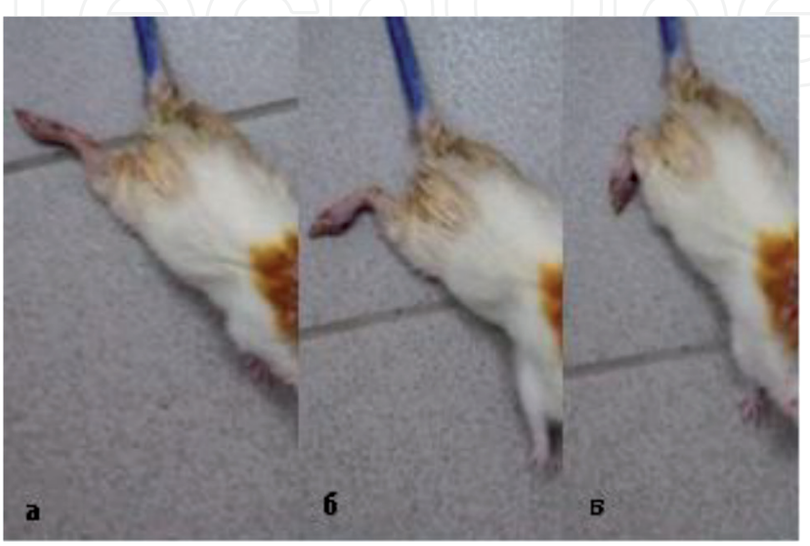


**Figure 9.**
*The fourth week after transplantation, level of reduction of neurological deficiency 1 (control).*

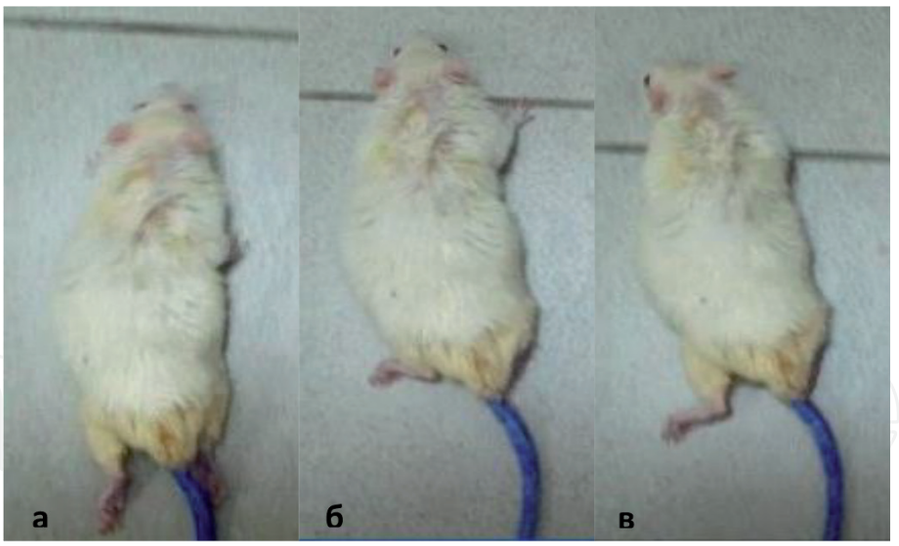


**Figure 10.**
*The sixth week after transplantation, level of reduction of neurological deficiency 3.9 (control).*

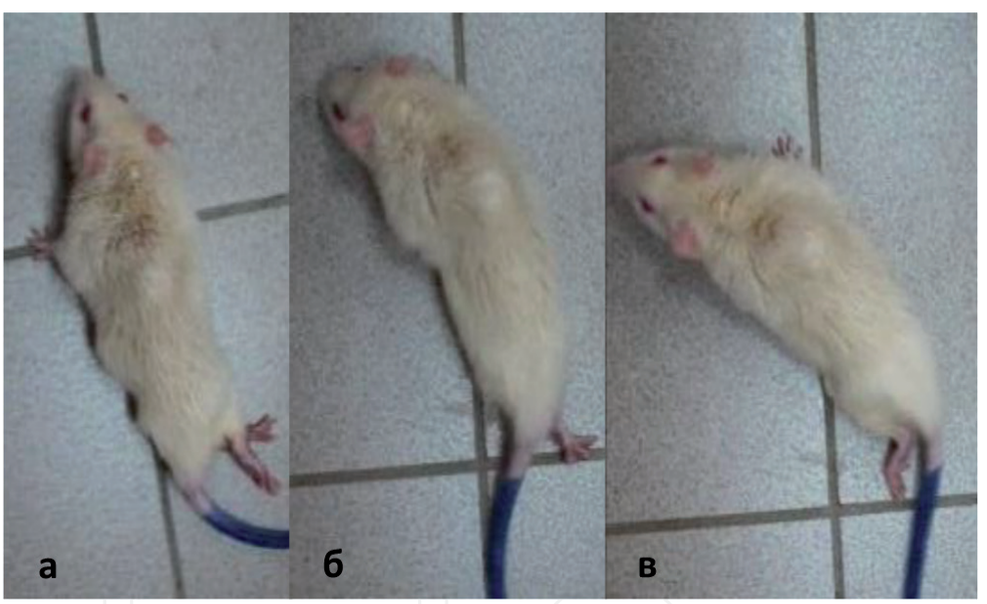


**Figure 11.**
*The eighth week after transplantation, level of reduction of neurological deficiency 4.9 (control).*

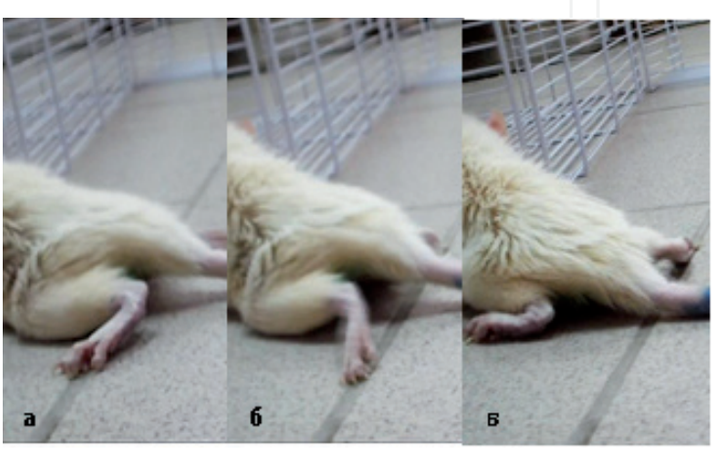


**Figure 12.**
*The twentieth week after transplantation, level of reduction of neurological deficiency 5.1 (control).*

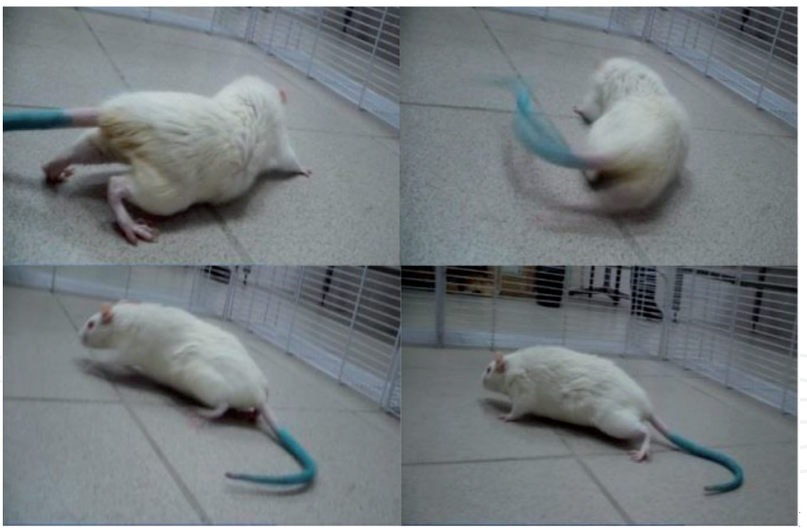

**Figure 13.**
*The eighteenth week after transplantation, level of reduction of neurological deficiency 19.5 (experience).*

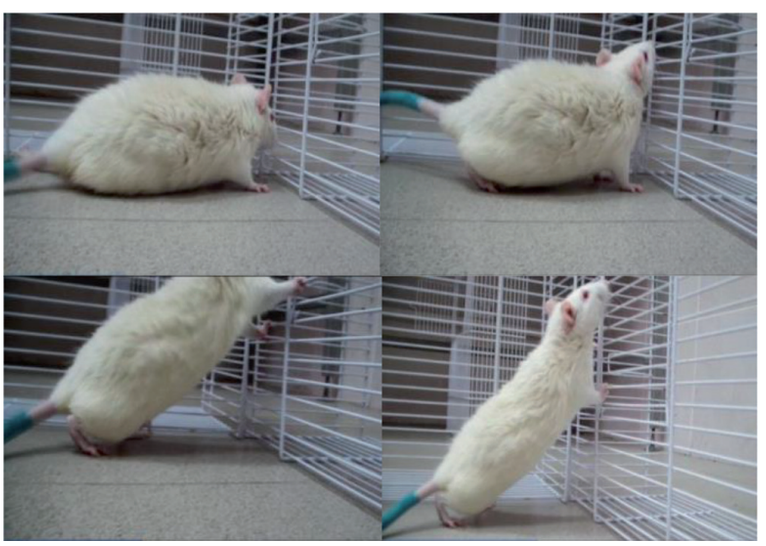

**Figure 14.**
*The eighteenth week after transplantation, level of reduction of neurological deficiency 19.5 (experience).*

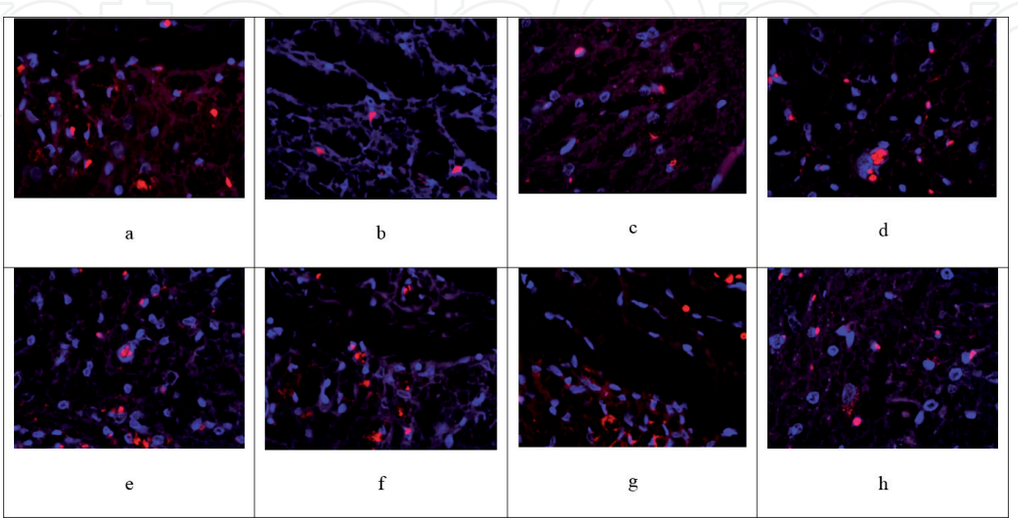


**Figure 15.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, control): head department of a spinal cord in 20 weeks after its full transection (above-transplant zone): GABA (a and b), acetylcholine (c–e), and serotonin (f–h).*

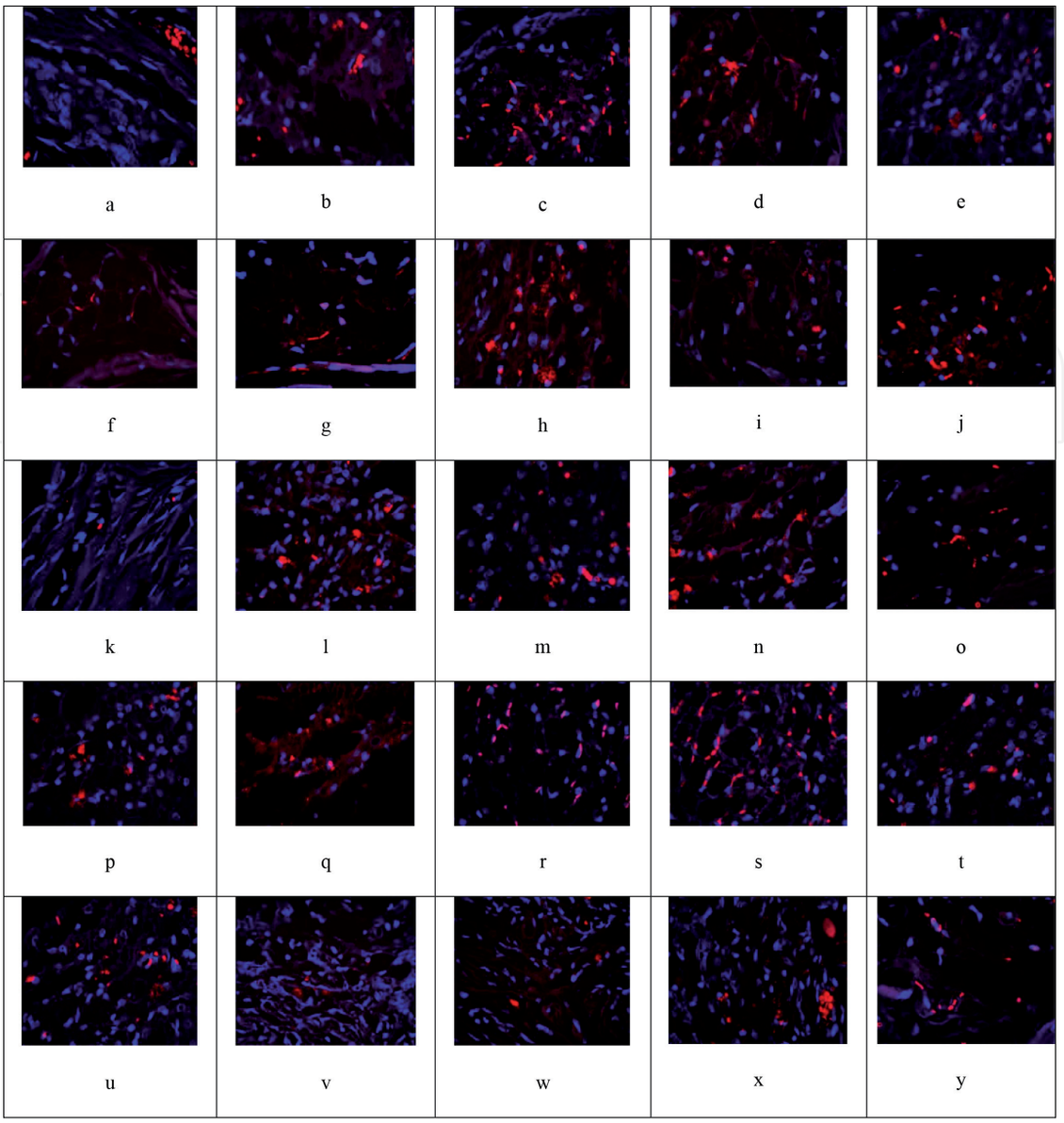


**Figure 16.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, control): zones of a spinal cord transplant in 20 weeks after its full transection: GABA (a–g), acetylcholine (h–o), and serotonin (p–y).*

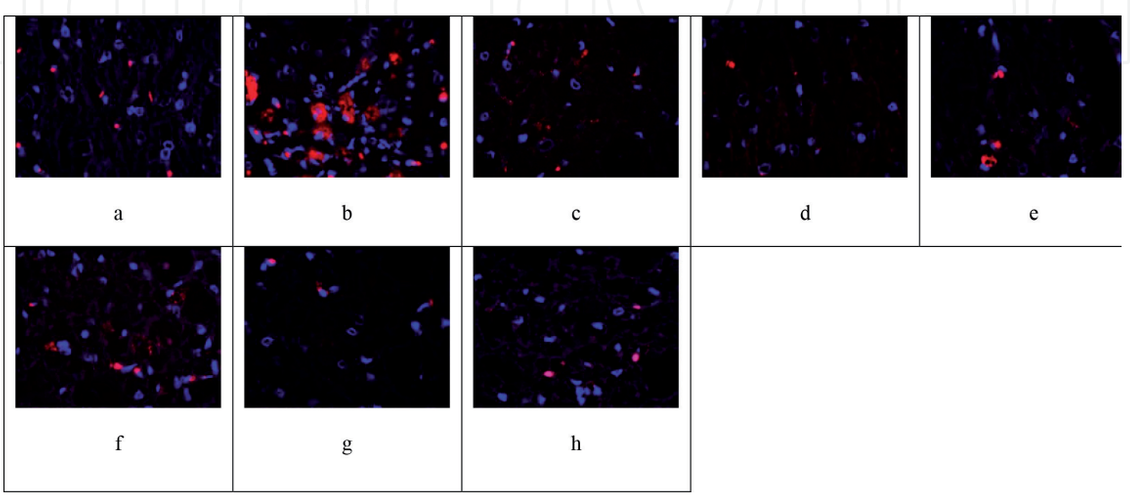


**Figure 17.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, control): a tail zone of a spinal cord in 20 weeks after its full transection: GABA (a and b), acetylcholine (c–e), and serotonin (f–h).*

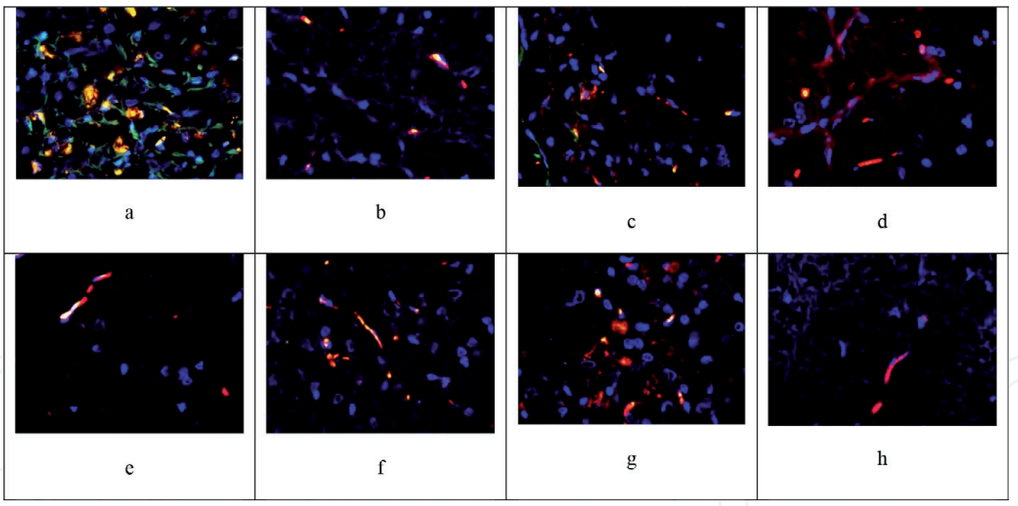


**Figure 18.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, experience): head department of a spinal cord in 20 weeks after its full transection (above-transplant zone): GABA (a and b), acetylcholine (c–e), and serotonin (f–h).*

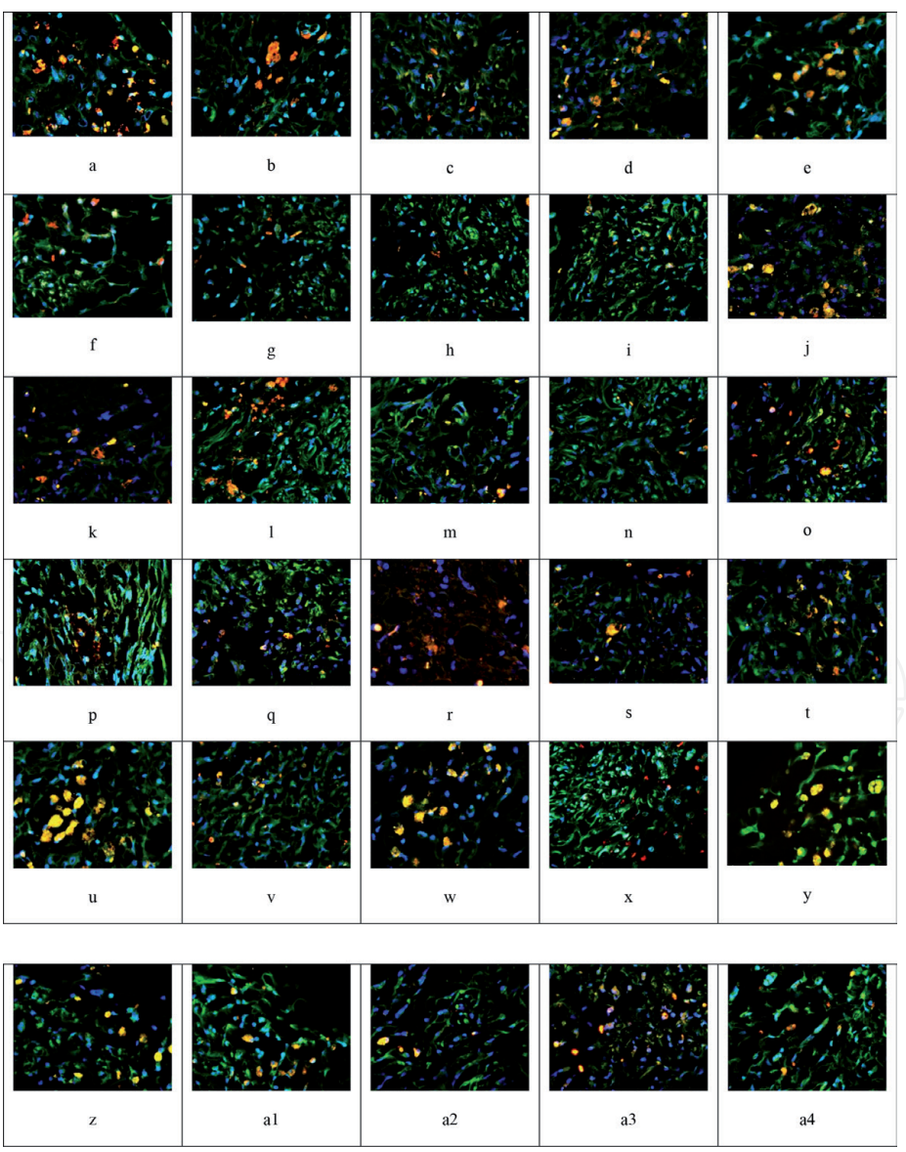


**Figure 19.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, experience) zones collagen-chitosan of a transplant of a spinal cord in 20 weeks after its full transection: GABA (a–i), acetylcholine (j–r), and serotonin (s-a4).*

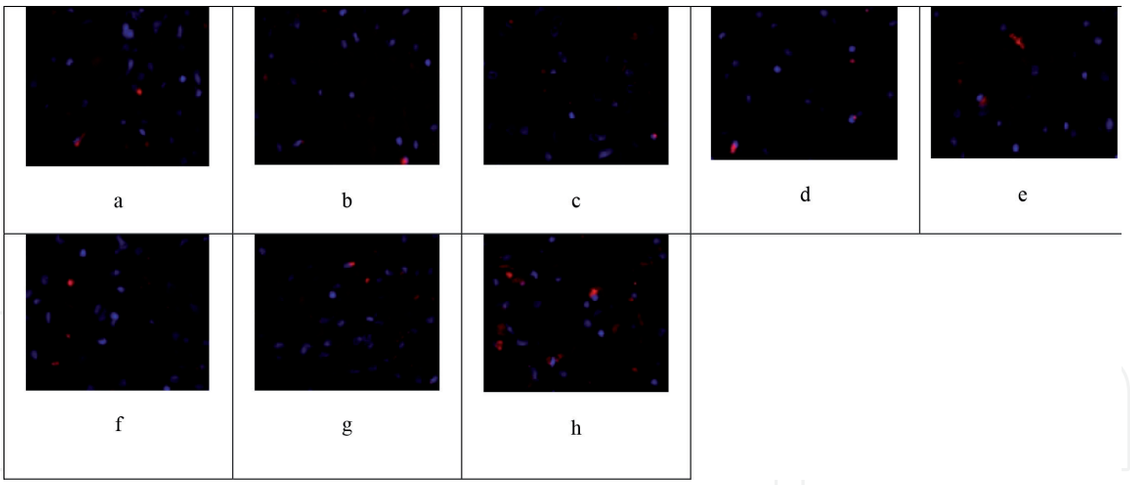


**Figure 20.**
*The immune fluorescent analysis of serial cut neurotransmitters (from top to down, experience): a tail zone of a spinal cord in 20 weeks after its full transection: GABA (a and b), acetylcholine (c–e), and serotonin (f–h).*

Research prototypes spinal cord, containing besides the neuronal microenvironment of growth factors 100,000 neuronal progenitor cells of mice, showed that by the transplant register sprouting cells producing GFP, in the area of the central end of the parent spinal cord. Broadcast nucleated cells are accompanied by the expression of neurotransmitter GABA, acetylcholine, and serotonin (**Figure 18**). A detailed analysis of the serial sections of the actual donor cells in the spinal cord indicates a rich content of viable neurons, producing GFP while expressing neurotransmitters. Transplanted cell mass in addition to the parent who came to neuronal cell occupies the entire volume of collagen-chitosan scaffold (**Figure 19**). In the tail of the spinal cord of the experimental group, the number of registered nucleated cells with expression and without expression of neurotransmitters reduced. The study of serial sections below collagen-chitosan cell transplant (tail of the spinal cord) did not reveal the GFP-cell sprouting phenomenon (**Figure 20**).

## 4. Conclusion

Thus, the results obtained in the course of the studies suggest that the collagen-chitosan matrix containing the neural progenitor cells is suitable for the repair of the damaged cells in the artificial three-dimensional environment. Transplantation of acellular collagen-chitosan substrate at full experimental spinal cord transection is accompanied by active sprouting maternal cell mass neuronal origin, actively expressing markers of neurotransmitters. This change is accompanied by a partial recovery of motor, sensory, and autonomic functions of the spinal cord, reaching the level of reduction, which is 5.6 point neuronal lack in BBB scale. Transplantation of collagen-chitosan matrix containing 100,000 progenitor neuronal cells followed for 20 weeks of the maintenance of their viability, in addition to expressing neuronal marker mediators of transmission of nerve signals. Transplanted cell mass broadcast their axons in the maternal side of the central segment of spinal cord beyond the graft. The tail part of the spinal cord after its complete intersection demonstrates a reduced number of viable neurons. However, the transplantation of a matrix containing precursors of neurons, resulting in significant recovery of lost motor and sensory functions of the spinal cord, reaching the level of reduction neuronal lack is equal to 19.5 on a BBB scale. Recent studies show that the inclusion of

implantable structures and aminated polysaccharide polymer in a state of compaction to nanoscale alert direct contact of immune cells with allogeneic or xenogeneic cells of nervous tissue that is included in the implant, which substantially reduces the immunological conflict in this area of privilege [54–56].

It is known that primary objectives of SCI therapy are processes of neuron re-myelination, reduction in apoptosis, damaged neuron and oligodendrocyte regeneration, neural cell sprouting, formation of new synaptic connections, reduction in astrogliosis intensity, neurotrophic support, reduction in leucocytes, and macrophage reactions. [57]. Combined therapy of SCI is dictated by complicated mechanisms of pathophysiological changes in critical and chronic periods after trauma [36, 58–61]. Based on the multifactorial mechanism of spinal trauma the main directions of the problem solution of spinal cord bioengineering after its damage are formed. The authors of the paper confirm that the cell therapy is an attractive therapeutic way of spinal cord function regeneration. The main idea formulates the necessary regeneration of deficiency in specialized cells, remaining after the injury, which leads to auto-allo-transplantation of exogenic neural stem cells (NSCs) and neural precursor cells (NPCs), and stimulation of endogenic potential of neural cells. Using optimal neurotrophic support for spinal cord cells such as exogenic injections including cocktails from conditioned environments and endogenic stimulation is an indispensable component of an integral strategy for repairing spinal cord injury. The use of cells as part of biodegrading and highly compatible 3D matrix, constituting carcass and benevolent conditions for cultivation, proliferation, and differentiation of cell material and the possibility of direct translation into problematic areas in spinal cord constitute an important part of the algorithm in the treatment of spinal cord injury. Biopolymer substrates should play the role of protection against permeabilization and progressing destruction of neural tissue, and also should regenerate barrier function of damaged membranes.

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## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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