Experimental Partial Transection of Spinal Cord and Its Bioengineering Reconstruction

Bolshakov I Nikolaevich[1]*, Svetlakov A Vasilevich[2], Eremeev A Valerievich[3], Sheina Y Igorevna[2]

1. State Federal Educational Institution of Higher Education Krasnoyarsk State Medical University, Russia.
2. Krasnoyarsk Center of Reproductive Medicine, Laboratory of Cellular Technologies, Krasnoyarsk, Russia.
3. Cellthera Pharm LLC, 601125 Vladimir Region, Petushki district, pos. Volginsky Str. Vladimir 14B, Russia

Abstract

The work represents the original research devoted to cellular and issue reconstruction of a spinal cord injury in adult rats with an experimental vertebral-spinal trauma. After performing a laminectomy, partial mechanical intersection of the spinal cord at the IX thoracic vertebrae in the spinal implanted medical devices containing bovine collagen and crab chitosan, sulfated and non-sulfated glycosaminoglycans, full nutrient medium, conditioned nutrient medium with neurotrophic factors in the growth of brain cells mouse embryos and embryonic mouse stem cells, N2 neuronal additive, retinoic acid. The dynamics neurologic status in animals has shown essential reduction of deficiency on NSS scale during 1-4 weeks of the postoperative period. The indirect immune-fluorescence method of a spinal cord cells and histological sections confirms presence of active introduction of cells of a parent spinal cord into implant, high viability of the replaced cells of the mouse during all period of supervision, formation in 1 week after operation of progenitor neuronal cells with expression of neurotransmitters. This change is accompanied by a partial recovery of motor, sensory and vegetative functions of the spinal cord, reduction in the level of neuro-deficit of 5, 6 points on a scale of BBB.

Experimental article (J Int Dent Med Res 2019; 12(2): 760-779)

Keywords: Collagen-Chitosan Scaffold, Neuronal Microenvironment, Stem Cells Progenitors, Spinal Cord Injury, Neurological Deficiency, Neurotransmitters.

Received date: 27 August 2017 **Accept date:** 12 October 2017

Introduction

Primary surgical treatment of complicated spinal cord injury standard does not cover the reconstruction of the spinal cord, it includes the operation of strengthening and stabilization of the spine vertebrae relative to the spinal cord. In experimental practice in formulating the problem of reconstruction of spinal cord injury autologous, allogeneic or xenogeneic stem cells isolated and cultured from bone marrow, adipose tissue, peripheral blood, and other locations are used. Thus, implantation of cells is carried out either in the free form as an injection to the location of spinal cord injury or subarachnoid or cells are placed in a hydrogel base and in this form are introduced into the site of injury after surgical access through the bony elements of the spine and spinal cord exposure. The results of such manipulations are not satisfactory, characterized by incomplete neurological positive dynamics and the low control of morphological reconstruction.

Traditionally, the possibility of regeneration of spinal cord pathways (SC) is severely limited due to the irreversible morphological changes in the nervous tissue after injury, especially in the caudal part. Recently, however, important evidence and limited clinical data were accumulated, indicating the theoretical possibility of regeneration in the central nervous system (CNS) and its possible restoration of disturbed functions.[1-3] Received scientific facts allow us to change the current position of the canonized notion of regenerative potential and genetic mechanisms in the central nervous system, as well as to propose new strategies and concepts of treatment of injuries of the SC.[4-7] Most researchers in this field believe that the future belongs to technology of regenerative medicine. The main tool of regenerative medicine are the various cellular te-chnologies from transplantation of cells (cell therapy) up to tissue engineering. In this sense, it is the introduction of cells in traumatic spinal cord injury (SC) in its microenvironment.

Corresponding author:
Bolshakov Igor Nikolaevich,
State Federal Educational
Institution of Higher Education Krasnoyarsk
State Medical University
Krasnoyarsk, Russia
E-mail: bol.bol@mail.ru

Embryonic stem cells

The prospect of transplantation of embryonic stem cells demands a serious approach. Isolation of embryonic stem cells (ESCs) from blastocysts of the mouse[8] and transplantation of fetal tissue to the model of spinal cord injury[9] showed the possibility of its usage as a temporary intermediate matrix for the regeneration of axons in central SC. Growing axons regenerated in the embryonic graft together with the formation of interneuronal connections. The use of ESC transplantation for spinal cord injury model showed the ability of these cells to integrate into the damaged areas of the SC, differentiate according to the molecular and cellular microenvironment and to be viable for a long time.[10-12] In this form of ESC oligodendrocytes in adult rats exposed to axons myelination.[13] Axonal growth can be accompanied by germination glial scar in the surgical crossing SC.[14] Expected morphological changes accompanied by partial reduction of SC neuro-shortage.

The brain and spinal cord cells

Transplantation of embryonic cell mass of the brain or spinal cord of the rat spinal cord gap in newborn or adult rats leads to early (up to 7 days) engraft mentation and sprouting of the transplanted neurons and glia cells broadcast caudally beyond the zone of injury.[15-18] In the area of spinal cord injury (SC) in the implanted graft formed the interstitial tissue of the nervous system, filled with nerve agents, able to shape the nerve signal broadcast actively through the image between sin optical connection that points to the fact that the transplanted tissue is actively integrated with the tissue of the recipient.[19]

Neural stem cells and progenitor neuronal cells

A perspective direction in regenerative cell therapy can be considered transplantation of neural progenitor cells or neural stem cells (NSCs), which are derived from the neuroepithelium of the embryo.[20] SC directed cultivation leads to neuronal differentiation. Neural stem cells (NSCs) are most often used in experimental transplantation for the treatment of SC injuries. Regional Transplant (NSC) in rats is accompanied by SC survival and integration with the brain of the recipient, as well as differentiation into neurons and macroglia.[21,22] The reconstruction of the SC requires different types of differentiated neural cells and cellular and intermediate elements of microglia. Temporary immunological isolation of neural progenitor cells with viscous polysaccharide gel implants is one of the main conditions for the final differentiation and the start of axonal growth, genomic transformation neurons donor for the construction of the final incorporation into the brain tissue of the recipient. Reliable control of this differentiation may serve between sinoptical formation due to the presence of active forms of specialized asparaginergic, glutamatergic, serotonergic, glycinergic, cholinergic, GABA-ergic, noradrenalergic neurotransmitters.[12,13,23]

Neural progenitor cells can be obtained with the specific microenvironment in vitro conditions of the ESC and subsequently used as the actual cell transplant. Similar manipulations lead to the positive dynamics of neurological and morphological restoration of SC. The effect of translation viable transplanted cell material is significant and is accompanied by differentiation into 3 main types of neuronal cells: neurons, astrocytes and oligodendrocytes.[24] Marker analysis confirms the process of nerve conductors remyelination.[12] Injection of the suspension of neuronal stem cells (NSCs) isolated from fetal hippocampus, leads to accurate translation in the area of SC injuries, differentiation into neurons, astrocytes and oligodendrocytes and restoration of disturbed function in adult rats[11] or the primates representatives.[25]

Such differentiation of the cell mass is accompanied by activation of Schwann cells and interactions with neyrofibroblastomas. It is known that such intercellular communication provokes process of myelination of axons, new axon growth, and the distal stump sprouting SC.[26-29] In this morphological engineering SC accompanied by a reduction of neurological deficit, despite the usage of the model of complete intersection SM.[30] It is clear that the engineering process is supported by complex molecular microenvironment created by SC cells, which is based on the interaction of neurotrophic factors.[31]

In Russia, in "Neurovita" clinic about two dozen operational transplantations of autologous olfactory neuronal cells (ONC) were held based on biodegradable collagen "Sferogel". Its own cell mass is used and the combination of

autologous ONCs with autologous hematopoietic stem cells. The results indicate a 50% partial regression of neurological symptoms in the presence of postoperative complications.

Thus, prior studies of spinal cord injury, on the one hand, pose the problem of obtaining and using autologous transformed specialized neuronal cell mass, and on the other hand, the problem of a three-dimensional substrate having a full microenvironment for cell proliferation, differentiation, migration and formation of neuronal intermediate matrix, ready for direct transplantation into the spinal cord.

Preliminary studies have resulted in a 4 variants of neural matrix that can support long-term human ESC and animals (rats) in complete medium, translate them into a state of differentiation with reception on the 5th day of cells with neuronal markers exposed on its membrane (neurofilament, MBP, GFAP).[32]

Basic and applied research in the field of chitin and chitosan in the field of biology and medicine show a clear trend lasting occurrence of these biopolymers and their chemical derivatives, not only as effective food supplements, but also as independent structures that can perform specific tasks in the prevention and treatment of medical and surgical diseases. A well-known food supplement due to the global developments in the scientific research is turning into a parenteral implants, acting as the transport systems of target molecules or cells in harsh environments without losing their original activity. The approaches proposed in the work to the reconstruction of the spinal cord in experimental complicated spinal injury are based on modern biodegradable polysaccharide matrices containing the necessary microenvironment, including the products of growth and differentiation of stem cells and neuronal cells, 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, non-toxicity, 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 passage cell precursors neuronal tissue, vascular endothelial cell proliferation, tumor micro vessel recovery of intracellular substrate. Implantation of such a matrix can be done in an open manner of the operative spinal cord diastasis.

Materials and methods

Neuronal matrix getting

To create a neural matrix base polyion complex consisting of nano-micro structured ascorbate chitosan with a molecular weight of 695 kDa and degree of deacetylation of 98%, when the content of 1 g dry chitosan 1.8 g of ascorbic acid, comprising anionic salt forms hondroitin 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.

During the preparation of chitosan, the following manipulations were produced:

a) 2-3-fold purification of chitosan («Vostok-Bor-1, Dal'negorsk, Russia dissolved in 0.5% hydrochloric acid, and filtered through a glass filter number 2 and number 3, reprecipitation of the gel in 0.15 M sodium hydroxide; b) A three-time washing sediment distilled water by centrifugation in a centrifuge with a horizontal axis; c) the re-refining of chitosan: dissolution of the precipitate in a 0.5% solution of hydrochloric acid, reprecipitation in ethyl alcohol (rectified) in the ratio of 1:5, cake washing absolute acetone through a glass filter, wash the precipitate with ether (for anesthesia) through a glass filter, drying sediment in the air; d) modification (activation) of chitosan (the introduction of active functional groups - additional protonation of primary amino groups) dissolving the polymer in a solution of ascorbic acid in a ratio 1:1,2-1:1,5 (ascorbate chitosan).

The inclusion of collagen-chitosan gel heparin and serum growth factors in cattle "adgelon" (SLL «Endo-Pharm-A», Moscow region, Schcholkovo, Russia) increased the functional activity of cells. It has been suggested that culturing on collagen-chitosan matrices mouse or human ESCs in the conditioned medium from mouse embryonic neuronal cells when added to the medium of neuronal supplement N2 (Sigma) or B27 (Sigma), and retinoic acid (Sigma) can lead to neuronal differentiation.

Polyionic complexes options

A. A conditioned nutrient medium obtained after culturing embryonic neuronal cells of the brain tissue of mice added to the basic polyionic complex. B. A conditioned nutrient medium obtained after culturing embryonic allogeneic stem cells from mice (Krasnoyarsk Center for Reproductive Medicine, Krasnoyarsk, Russia) added to the basic polyionic complex.

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.[33,34]

Getting the conditioned medium of cultured embryonic neuronal cells.

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 biophisic SD RAS, Russia) after the dispersion and processing of 0.5% collagenase solution (Sigma) for 30 minutes in medium DMEM (Sigma) at 37 ºC 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 further added 4ng/ml basic fibroblast growth factor (bFGF, Sigma), 1 mM solution of essential amino acids (Sigma-Aldrich). The cell biomass was grown at 37 ºC 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 to 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 later used in as 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 ºC and then freeze-dried at 10-5Pa for 8 hours, the product is packaged and sterilized by electron beam method (neuronal dry matrix courtesy SLL «Medical Company Collachit», Krasnoyarsk Region, Russia).

The manipulation above yielded neuronal matrix of the size 50x50x2 mm, suitable for not only the culture and in vitro differentiation of embryonic stem cells into neurons and oligodendrocytes progenitor of animals and humans, but also for direct transplantation into the spinal cord gap in experimental spinal injury.

Cultivation and differentiation of mouse embryonic stem cells.

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.

Murine embryonic stem cells. Marker analysis

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

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.

Immunocytochemistry control neuronal differentiation of stem cells

Every three days, formaldehyde fixation was performed followed by immunocytochemistry of the cells with antibodies (Abcam, USA) against GFAP glial fibrillar acid protein, neurofilament and nestin. Detection of markers was carried out by the method, according to the instructions of the antibody provider. The cell nuclei were stained with DAPI (Sigma) (0.1 μg/ml) for 10 min. 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.

Experimental spinal cord injury in rats (partial rupture of the spinal cord)

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: diethyl ether. 24 white mongrel rats to female weighing 250 g with Carl Zeiss optics, individually reproduced model of spinal cord injury at the IX-X thoracic vertebrae with 50% of the intersection of the right half of the spinal cord after first performing a laminectomy.

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 spinal processes of Th9-L3. The muscles of the deep layer were separated from the spine and diluted with a microsurgical retractor, the Th9-Th12 vertebrae were bared. The Th10 arch resection was performed, the dura mater was dissected and diluted to the sides in the horizontal plane. A 50% intersection of the right half of the spinal cord was performed using a microscalpel and micro-scissors and individual optics of Carl Zeiss. A neuronal cell-matrix implant with a size of about 1 mm^3 was placed in the formed neural tissue defect. The bony wound was closed as a film with polysaccharide hydrogel mass "bolchit",[35] which does not contain animal collagen. The wound was closed layer by layer: the V-shaped seams were stitched onto the deep layer of muscles by the suture material Vicril 4-0; The muscles of the surface layer were sewn with a continuous suturing seam Vicril 4-0 thread; The skin was applied with U-shaped seams with a Polyester 3-0 thread. The stitches were treated with an alcohol solution of iodine.

The composition of transplantable matrices.

As variants of collagen-chitosan structures, 2 different implants were prepared containing the following microenvironment for neuronal cells:

1. Lyophilized collagen-chitosan matrix containing sulfated and non-sulphated glycosaminoglycans (chondroitin sulfate, sodium hyaluronate, heparin), with elements of the complete DMEM nutrient medium, conditioned by neuronal cells of the medium and neuronal N2 supplement, with retinoic acid (4 rats for each observation period);
2. Lyophilized collagen-chitosan matrix of the same composition, containing about 50,000 precursors of neuronal cells obtained by culturing and differentiating mouse embryonic stem cells (ESKM) (5 rats for each observation period).

Variants of porous matrix substrates were cut into 1 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 ºC and 6% CO2. 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 in the process of cultivation contained about 50 thousand embryonic stem cells. Before the implantation, 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.

Post-operative 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, vasodilator drugs.

Dynamic neurological control

To evaluate the neurological disturbances and recovery dynamics, a scale was used to assess the severity of the neurological deficit in partial neuronal scans (NSS scale)[36] for 1-4 weeks postoperatively.

Histology of sections of the spinal cord with direct implantation of cellular neuronal matrices in the dislocation of a spinal trauma

Spinal cord preparations obtained by careful and judicious selection of tissue from the spinal canal through the 1,2,3,4 weeks after surgery. The spinal cord was placed for 24 hours in 10% phosphate-buffered formalin. Continue to implement the classical histological tissue wiring on the equipment Leica (Germany), part of the tissue sections were stained with hematoxylin-eosin in order to review the analysis of tissue at the implant dislocation and its immediate surroundings. When the survey microscopy histological sections (longitudinal, oblique, transverse) assesses the glial reaction in the zone trauma to surrounding tissue, the state of neurons of gray matter of spinal cord. Evaluated the state of the white and gray matter of the spinal cord in fields 5 of view of each slice. The number of cells macroglia (astrocytes, oligodendrocytes), microglia (macrophages) and the number of neuronal cell number.

Immunofluorescence sections of the spinal cord with the direct implantation of neuronal cell matrices in the dislocation of spinal injury

Some of the histological sections were subjected to immunofluorescence treatment to determine the state of the implanted cell mass (search for transplanted cells expressing the green fluorescent protein GFP), 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 method included a number of sequential operations:

1. After washing histological sections of spinal cord by 5-fold washing glasses in xylol for 5 minutes, followed by 5-fold washing with 70% ethanol-st. After washing be rinsed with distilled water and buffering slices 1% phosphate buffer (Sigma).
2. Premeabilization of the slices with 0.25% Triton X100 (Sigma) in 0.1% Tween (Sigma) in 1% phosphate buffer for 10 minutes
3. Flushing sections 0.1% Tween in 1% phosphate buffer
4. Block non-specific antibody 2% non-fat dry milk, 30 minutes
5. Flushing sections 0.1% Tween in 1% phosphate buffer
6. Coloring primary antibodies. As the antibodies used rabbit polyclonal antibodies against serotonin (Abcam) at a dilution of 1:200 in 0.1% Tween in 1% PBS, and rabbit polyclonal antibodies against the acetylcholine (Abcam) at a dilution of 1:100. Coloring was performed for 60 minutes at room temperature.
7. Triple rinsing sections 0.1% Tween in 1% phosphate buffer
8. Coloring secondary goat anti-rabbit (Abcam), diluted 1:700 in 0,1% Tween in 1% phosphate buffer for 30 minutes at room temperature in the dark.
9. Two-fold washing sections 0.1% Tween in 1% phosphate buffer
10. Stained nuclei DAPI at a concentration of 0.1 mg / ml for 10 minutes in the dark.

11. Flushing sections 0,1% Tween in 1% phosphate buffer
12. Flushing cuts 95% ethyl alcohol.
    For getting and analysis using fluorescence microscope «Olympus BX-51" and software «Applied Spectral Imaging» (USA).

Immunofluorescence dispersants spinal cord. Detection of marker expression (differentiation in the assessment of the ability of transplanted cells)

Some of the spinal cord preparations were resuspended in PBS buffer, used to isolate the cell mass. The cells were washed off from the fixator and 0.1% Triton X100 solution was added to the cell suspension to permobilize the membranes. Then washed with 0.1% Tween 20 solution, followed by incubation in the same solution with the addition of 2% normal goat serum at room temperature (to block the non-specific binding of antibodies). Subsequently, they were washed repeatedly with PBS and Twin20 solutions, followed by the addition of primary antibodies to the mouse neurofilament (Abcam, USA) in a 1: 100 dilution, the mixture was incubated for 1 hour, then re-washed, then a solution of secondary rabbit antibodies with a red fluorescent label was added. The unbound label molecules were washed three times with PBS and fluorescence was evaluated on a Guava EasyCyte Mini (USA) flow cytometer. The detection by two spectra is green (assessment of the presence of cells with GFP), red is the presence of neurofilament expression.

Part of the isolated cell mass of the spinal cord was subjected to formaldehyde fixation followed by immunocytochemistry of the cells with antibodies (Abcam, USA) against GFAP glial fibrillar acid protein (marker of astrocytes), oligodendrocytes and enolase of neurons, against mouse proteins to exclude the appearance of a cross signal. Detection of markers was carried out by the method according to the instructions of the antibody manufacturer. Cell nuclei were stained with DAPI (0.1 μg/ml) for 10 min. The Olympus BX-51 fluorescence microscope and the Applied Spectral Imaging software (USA) were used to obtain images and analysis. To analyze each marker, 6 zones were identified for analysis and photo documentation. Microscopy was carried out for each zone in 30 fields of vision.

In parallel, the same preparations of GFP-labeled cells were searched. Thus, a three-color fluorescence analysis was performed - detection of green glow - GFP, red glow - marker and blue glow - of cell nuclei.

The presence of transient cells after transplantation was assessed by flow-through fluorimetry in the formulations at 1 and 2 weeks (partial transection) and 20 weeks (complete transection) after implantation.

Spinal cord samples were dispersed in 0.5% collagenase solution with PBS for 15-30 minutes at 37 °C, filtered through a nylon filter, then fixed with 1% formalin solution with PBS and stained after washing DAPI, producing estimates of the number of cells carrying the blue (cell nuclei) and green (GFP) marker.

Results and discussion

Maintenance of pluripotency of mouse embryonic stem cells (ESKM)

Previously, it was shown that the cultivation of ESC on collagen-chitosan substrates can maintain for 7 days the normal morphological state of colonies of pluripotent mouse cells, which is also characteristic of human ESC. Immunocytochemical study of pluripotency markers showed in ESK cultured on a matrix that the cells express nuclear proteins oct-4, TRA1-60, cd30 and SSEA4 antigen.[37]

The proposed modes of culturing mouse embryonic stem cells after appropriate preparation of biodegradable matrices allow to improve the quality and stability of cultivation, to exclude at the final stage of the preparation of the cell matrix, the treatment of cells by enzymes in the process of passage when changing the nutrient medium, to increase the attachment of cells to the surface of the matrix, and, therefore, to prevent them Loss upon re-entry into nutrient media due to the presence of a chitosan biopolymer in it, to provide a cell matrix suitable for direct transplantation.

Various protocols for the derivation of differentiated derivatives of neuronal cells from stem cells are known. In these protocols solutions of enzymes (trypsin, collagenase, despase, etc.) are used to transfer cells from culture flasks. These manipulations lead to an increase in the level of apoptosis, cell death, damage to surface cellular receptors.[38] The

probability of contamination increases and the procedure of transplantation becomes more complicated. In this connection, the use of matrices for cultivation and directed differentiation eliminates the above problems, however, the need for using differentiation factors remains. The use of recombinant growth factors and morphogens for this purpose when added to the matrix significantly enhances the probability of an immune response to a foreign protein. The use of cell-conditioned media as an additive to the matrix leads to the elimination of the above problems and gives the researchers a substrate for the cultivation of cells of various types, including stem cells. The addition of a conditioned medium to the basic polyionic collagen-chitosan complex from embryonic neuronal cells from mice or conditioned media from cultured mouse neuronal progenitor cells or a component of N2 and retinoic acid resulted in neuronal differentiation of both mouse and human ESCs. The possibility of such cells was differentiated in the neuronal direction (Figure 1).

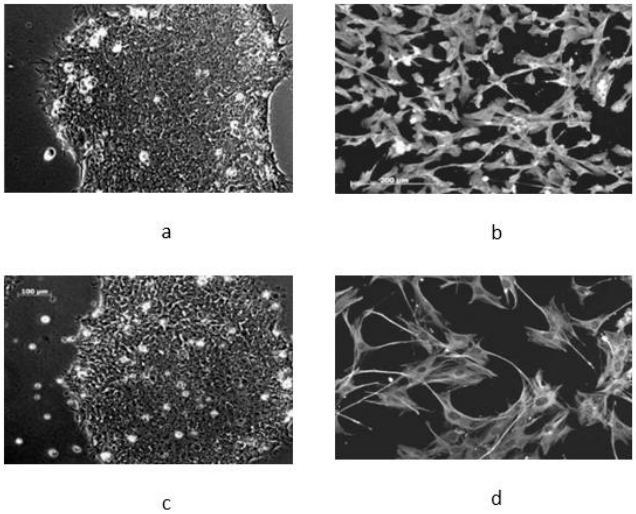


Figure 1. Phase and contrast microscopy of cultivated predecessors of neuronal mouse cells (PNCm) or the person (PNCh) browned on collagen- chitosan matrixes within 14 days in the presence of the conditioned environment (figure a - the 1st day, figure b. - the 14th day) or at addition of N2 neuronal component (figure c - the 1st day, d - the 14th day).

Analysis of the expression of one of the neuronal markers - neurofilament showed that on the first day no fluorescence signals at the specified marker (Figure 2). On the 5th day when culturedhESCs in embryonic neuronal cells conditioned medium detected neurofilament expression, and on the 7th day - and in cells cultured in medium supplemented with N2 neuronal component. A similar pattern was observed in the case of the marker GFAP and nestin (Figure 3).

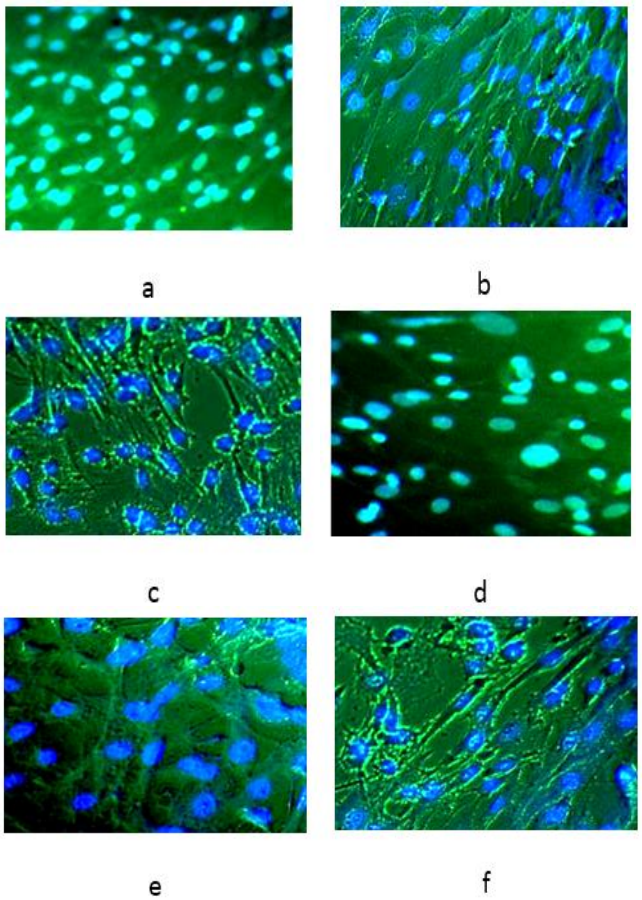


Figure 2. Dynamics of an neurofilament expression a for 1, 7 and 14 days in the cells cultivated on collagen-chitosan a complex in the presence of the conditioned environment (a,b,c) or at addition of N2 neuronal component (d,e,f).

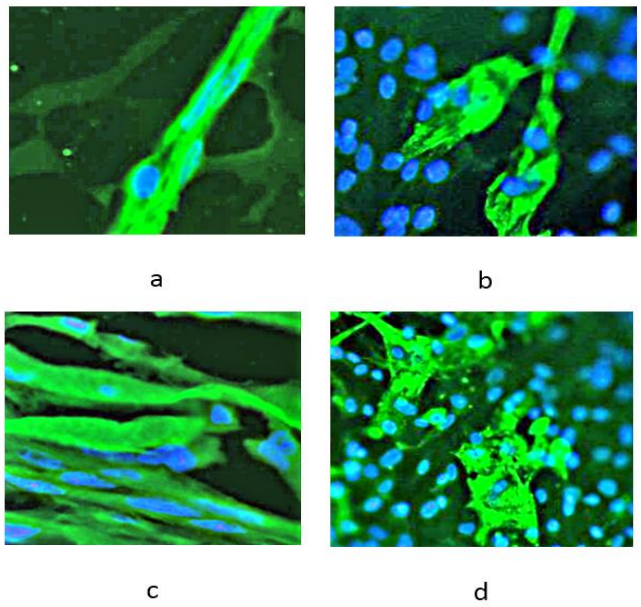


Figure. 3. Immunocytochemistry of predecessors of neuronal mouse cells (PNCm) cultivated in environment conditioned by embryonic neuronal cells (a, b) or in the environment with addition of N2 neuronal component (c, d). Cells are fixed by 1% formaldehyde in the phosphatic buffer. Cells processed antibodies against nestin, glial fibril sour protein, with the subsequent secondary antibodies and fluorescence detection.

Thus, the results showed the presence of a matrix conditioned medium obtained after culturing embryonic neuronal cells or medium supplemented with N2 neuronal component, on the 14th day stimulates the expression of ESC markers characteristic of neuronal cells, and shapes their morphology.

The results of neurological analysis with partial spinal cord injury.

Modeling spinal cord injury at the level of IX-X thoracic vertebrae with partial (50% by volume) of the intersection of the trunk side of the brain and spinal implantation diastasis between central and peripheral segments of the spinal cord of collagen-chitosan matrices with embryonic neuronal cell mass showed the adequacy of the model used. The animals in the postoperative period without treatment within 4 weeks there was a steady loss of certain sensory and motor areas of the innervation of the lower limbs, pelvis, the lower half of the body. The model used allows the technician to carry out the implantation of collagen-chitosan matrix in the lateral spinal cord rupture, creating an opportunity to nurse the animals safely in the postoperative period with zero mortality.

Analysis of neurological disorders after spinal cord injury model and implanted in the spinal cord diastasis control and test matrices showed a positive trend recovery of sensory and motor functions of the spinal cord (Table 1 and Table 2).

Test of "narrowing road" is going through the rat on the road length of 165 cm and a width at the beginning - 9 cm, at the end of the road - 3 cm track located at a height of 120-130 cm above the floor, creating a rat motivation to overcome it. Rats with spinal cord injury in advancing began to stumble (advance pelvic limbs by track). Depending on the severity of spinal cord injury in general, and the degree of violation of proprioception in particular, rats could accurately pass on a path different distances. Intact rats crossed completely the track.

The results showed that in the presence of only a full matrix microenvironment, and the matrix with the embryonic cell mass - neuronal precursor cells is a significant improvement in neurological indicators, such as motor and sensory activity of the hind limbs, the absence of abnormal motor activity, restoration of balance, improving run tests on a horizontal surface and narrowing a movement on the track. The positive effect of the implantation of the matrix without cells in the absence of inflammatory processes associated with most likely a sufficient set of factors included in its structure, needed to stimulate the migration of its own cells involved in regeneration (Table 2). In other words, the matrix itself regulates bioengineered processes in the wound.

The results of fluorescence detection in samples of spinal cord with partial rupture of flow cytometry in the terms of 1-2 weeks.

Cytometry analysis showed that direct transplantation of neural progenitor cells obtained by cultivation and the creation of neuronal microenvironment remains viable neuronal precursor that proliferate within 2 weeks. The relative number of cells expressing factor GFP, does not change during the two weeks, and is about 34% (Figure 4).

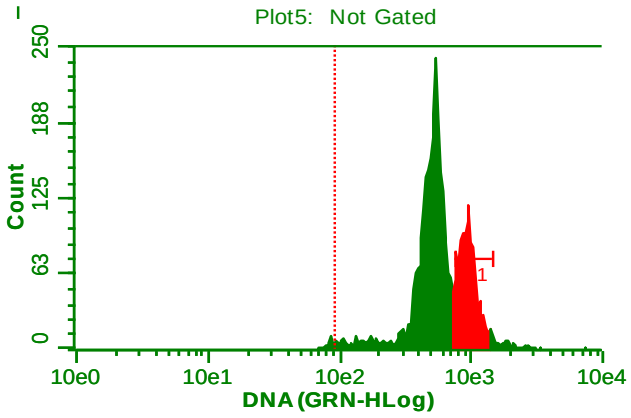
Histological analysis of the partial rupture of the spinal cord.

In the implanted matrix with ESC with the extension of the period of implantation was observed more severe resorbtion of its fibers. By 28th days most of the neuronal progenitor cells migrated to the periphery of the matrix close to the nerve tissue. Analysis of histological sections show that cells in the matrix not only survive, but to fill its entire structure, migrate toward the nervous tissue of the recipient. Moreover, the reduction of biopolymer fibers indicates a high metabolic activity in the area of repair (Figure 5).

Around the area of injury in both cases (with and without a precursor of neuronal cells in the matrix) revealed a large number of macrophages, the cytoplasm which is filled with phagocytosed detritus. Number of microglial cells was increased by 7-day 14 and decreased to 21 - 28 days (Figure 6).

When implanted matrix precursor neuronal cells showed an increase in all cells macroglia (astrocytes, oligodendrocytes). When using the matrix without cells in the spinal cord in the area of transplantation, an increase oligodendroglial cells and decrease of astrocytic glial cells.

The number of neurons in the gray matter of the spinal cord after implantation of matrix precursor neurons remained at the same level, with a slight peak in 2-3 weeks. In addition, 28 days around the area of implant matrix precursor neurons observed emergence of a large number of poorly differentiated cells (Figure 7). In the absence of a matrix precursor cells of neurons decreases with time.



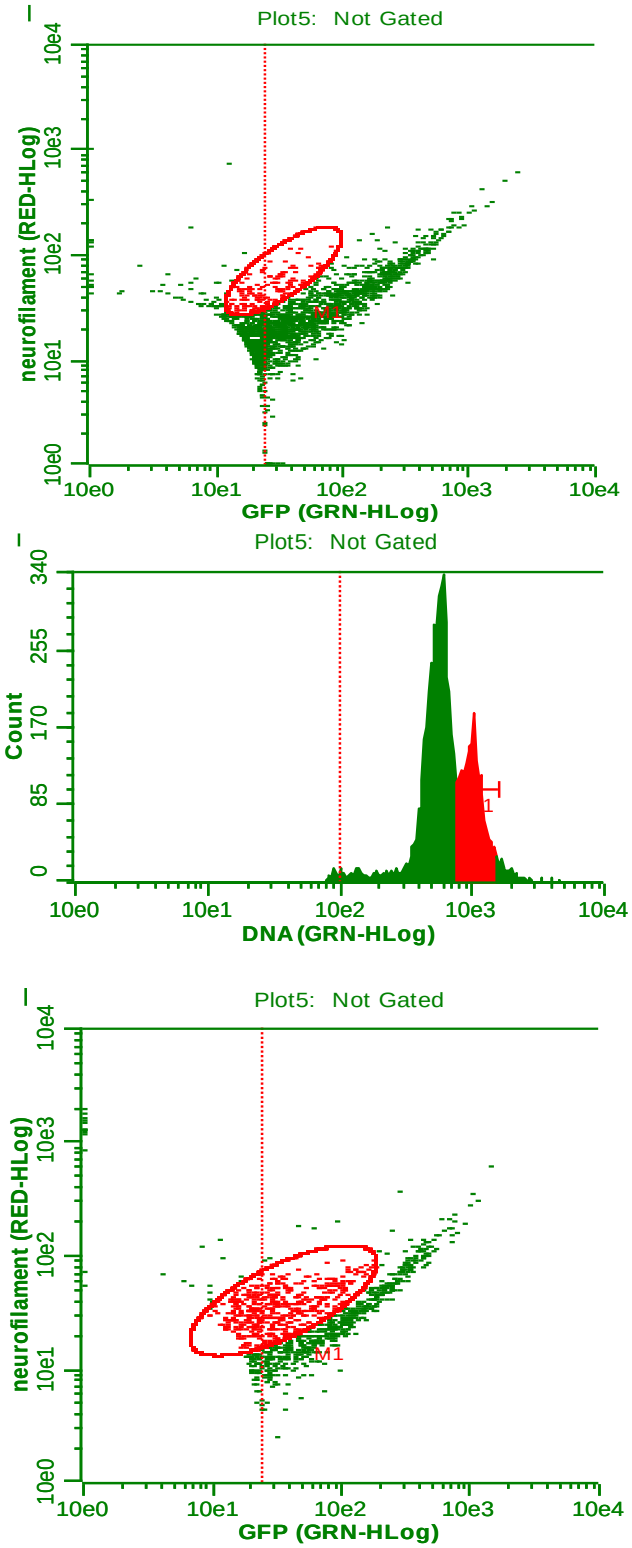
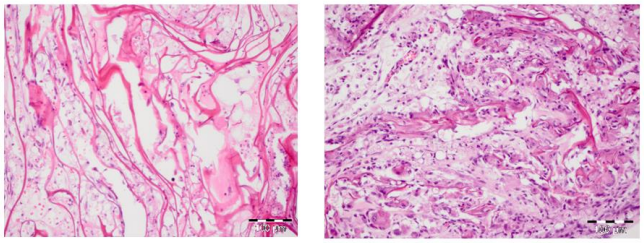


Figure. 4. Neurofilament expression (red color) in the transplanted cages with GFP fluorescence (green color) dispersants a spinal cord in 1 week (the top number of drawings) and in 2 weeks after transplantation (the bottom number of drawings).



a b

Figure. 5. The implanted matrix to a spinal trauma zone (the 14th days): a) absence of mouse neuronal cages predecessors (PNCm); b) PNCm existence.

Thus, studies have shown signs of a possible full recovery structure of the damaged spinal cord. Transplantation of cells to the matrix leads to a full and rapid recovery of histological tissue integrity after injury.

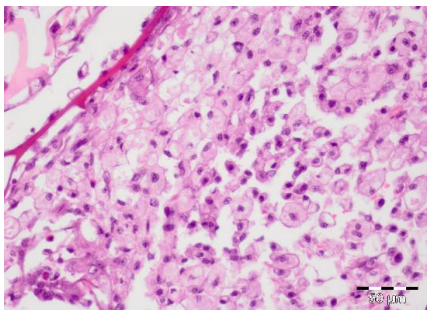


Figure. 6. The expressed macrophage reaction in a spinal trauma zone by the 7th days after matrix implantation.

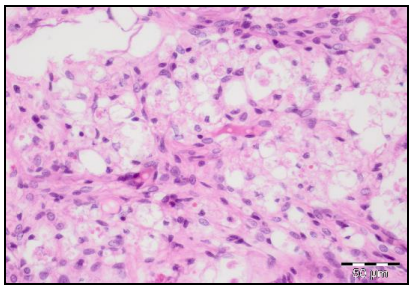
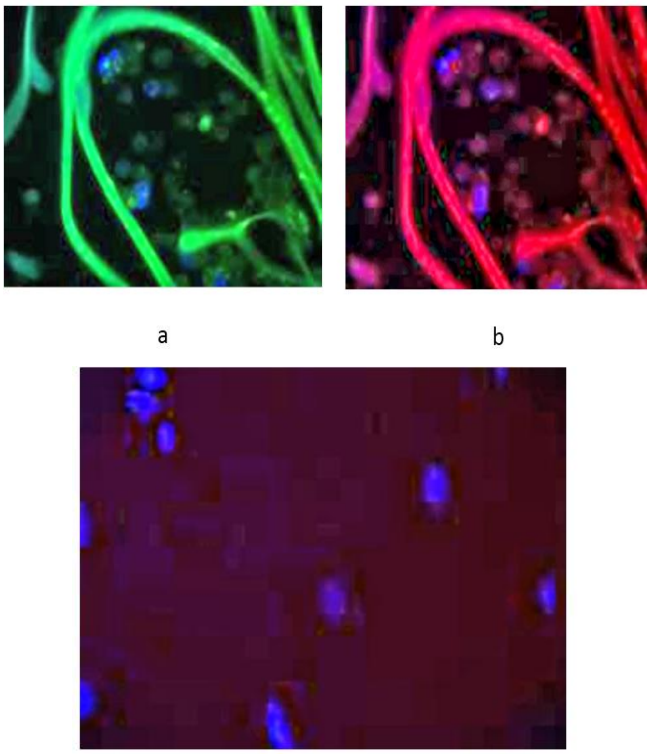


Figure. 7. The low-differentiated cells in a implantation zone of a matrix with neuronal cages predecessors by the 28th days.

Immunofluorescent analysis of spinal cord slices.

When immunofluorescence fixed precursor cells in the spinal cord sections were pre-treated cells with antibodies against neurofilament, followed by secondary antibody labeling and detection of fluorescence. The analysis of paraffin sections of rat spinal cord showed that the addition of a matrix implant neural progenitor cells results in engraftment and migration into the wound area, followed by differentiation into neuronal direction regardless of the composition of the matrix within 1-4 weeks. Probably the key influence is not only the three-dimensional structure of the support, but also the cytokine milieu of cells in the wound.

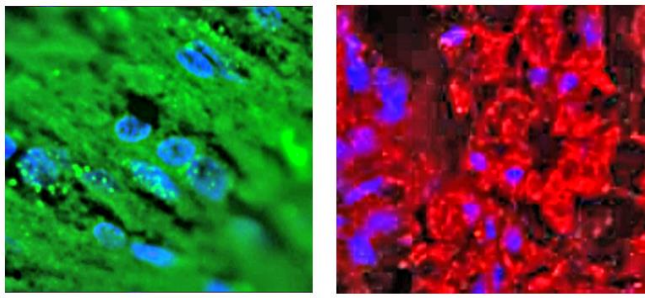
In rats, as in the early stages, and in the later period there was the presence of transplanted cells differentiated into tissue-specific types. When cells in neuronal differentiation in the longitudinal direction of the cut is the presence of spinal cord neurofilaments in cell strands (Figure 8), enolase (Figure 9), the formation of synapses (Figure 10) and to glial GFP protein (Figure 11).



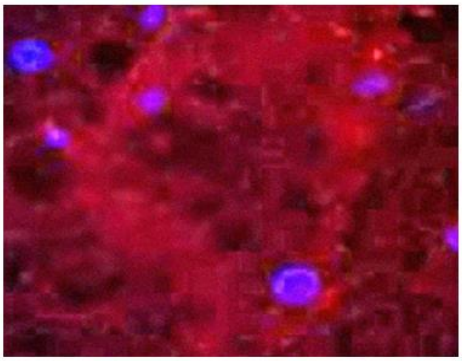
a b

c

Figure. 8. a) an expression neurofilament-GFP, 2 weeks, experience; b) a neurofilament expression, 2 weeks, experience; c) lack of an expression of a neurofilament, control.



a b



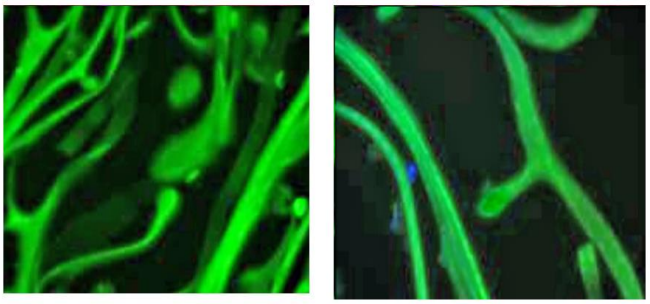
c

Figure. 9. a - enolase expression - GFP, 2 weeks, experience; b - an enolase expression, 2 weeks, experience; c - lack of an expression, enolase control.

For analyzing the presence of enolase in preparations spinal cord after treatment of the cells with antibodies against enolase and subsequent labeling of secondary antibodies with detection of fluorescence detected specific protein neuron. When processing an antibody against glial acidic protein it is also found in the cell mass of the transplanted cells.

When used in the implants spinal cord neural progenitor cells treated with antibodies against oligodendrocytes, followed by secondary antibody labeling and detection of fluorescence detected in the last line-transplantation.

Analysis of the morphology of the spinal cord in rats indicates that the technology matrix implanted in the spinal cord and provided stability to the composition of the spongy structure within 4 weeks, the viability of transplanted neural progenitor cells and oligodendrocytes, absence of a pronounced inflammatory reaction at the implant site, the formation inter-synaptical compounds in cell-cell contacts. This picture confirms substrate reconstruction of spinal cord at the break as the newly formed nerve tissue.



a b

Figure. 10. a, b - axonal terminations synapses existence on the replaced cells. GFP expression, experience.

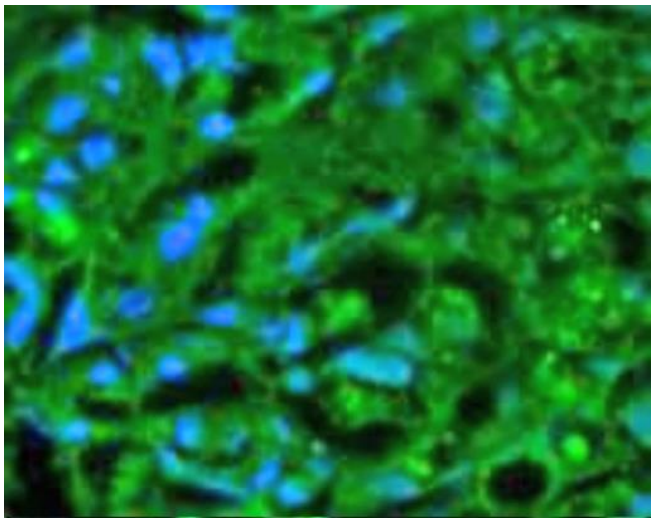


Figure. 11. GFP existence to glial protein, experience.

Analysis of dispersants spinal cord

Study dispersant spinal cord showed that the samples are present GFP-labeled cells (Figure 12). The results show stable expression of GFP at 3 and 4 weeks after transplantation in the area of spinal cord injury, which indicates the ability of transplanted cells homing to the site of injury.

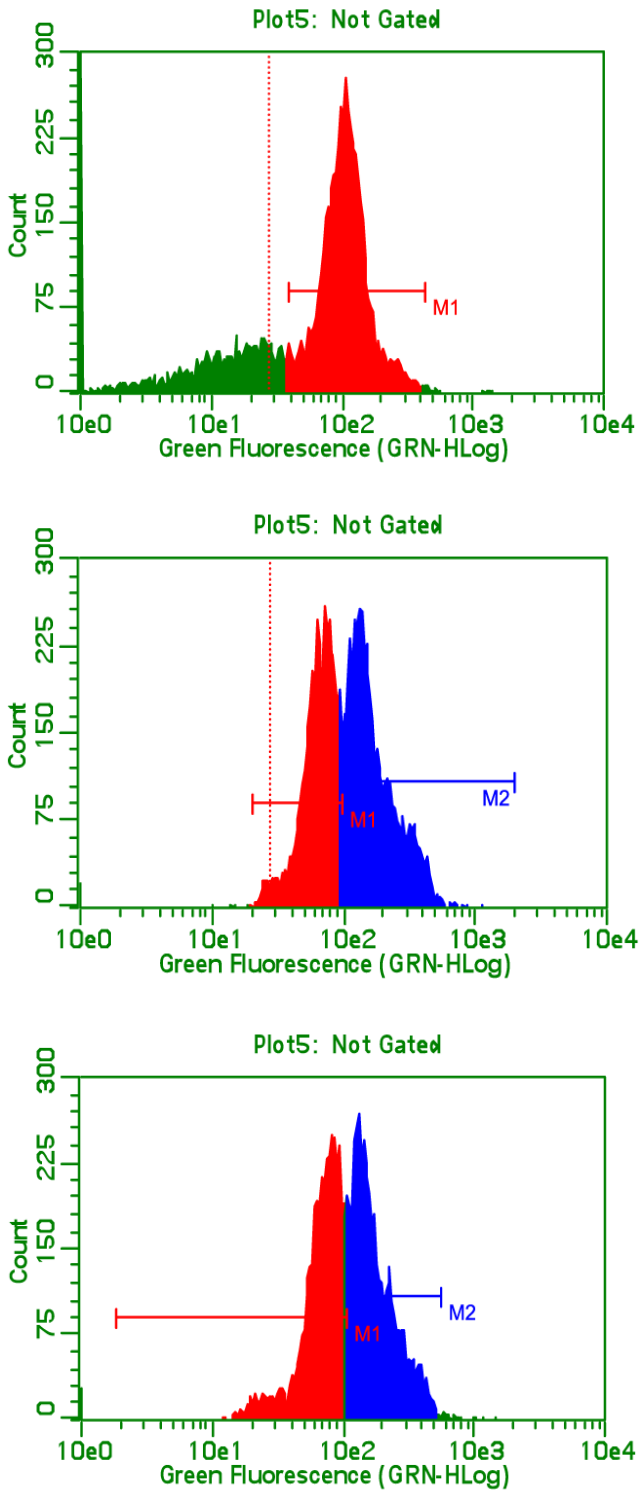


Figure 12. Flowing cytofluorometry spinal cord dispersants. Detection of green fluorescence (in red-green area): a - control (without cells); b – the 3rd week with cells; c – the 4th week with cells. Fig. 12b and fig. 12c – existence of the second peak of fluorescence irrespective of matrixes type.

Marker analysis of spinal cord sections in the area of transplantation

Further samples were analyzed by histological sections for markers of neuronal differentiation and restore normal synaptic cell-cell contacts. The data presented in Figure 19 show that the transplanted cells expressed markers of oligodendrocytes and neurofilaments forming between sinoptic communication.

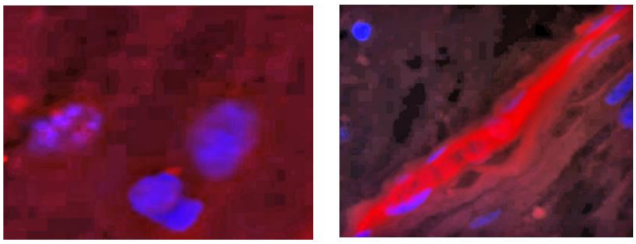
In preparations with transplanted cells were labeled with anti neurofilament cells, indicating that the differentiation in the direction of the transferred predecessors. The study showed markers for oligodendrocytes, the cells that form the characteristic morphological structures (Figure 14).

A green light indicates that the transplanted cells function, and differentiate into oligodendrocytes. Transplanted cell three-dimensional structure can create the conditions for the differentiation of transplanted cells in the matrix precursor in neuronal and glial directions.

Results of the analysis of neurotransmitters in the area of transplantation of neural matrix with partial rupture of spinal cord

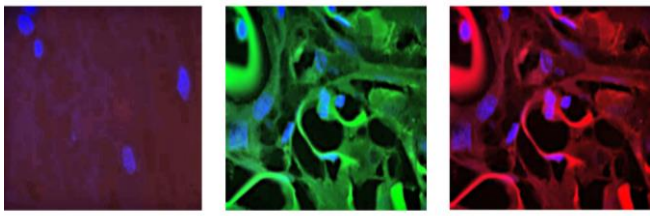
Markers analysis of transmission of nerve signals with partial spinal cord injury in is showed that in the segments of the central nervous system, which are higher than the control a cellular graft detected cells expressing GABA, acetylcholine and serotonin in the period 1-4 weeks after surgery (Figure 15). In the control area of the matrix is filled with interstitial implantation of tissue with cells of the spinal cord of the parent expressing the above mentioned neurotransmitters (Figure 16). In the tail of the spinal cord revealed viable tissue with cells producing mediators of transmission of nerve signals (Figure 17).

A green light indicates that the transplanted cells function, and differentiate into oligodendrocytes. Transplanted cell three-dimensional structure can create the conditions for the differentiation of transplanted cells in the matrix precursor in neuronal and glial directions.



a b

Figure 13. Immunofluorescent analysis of samples of a spinal cord of implantation zone. Primary antibodies - against a neurofilament, secondary - red. The blue kernels painted by DAPI. Increase 12x60, longitudinal cut. a – control (transplantation without cells, kernels are visible only); b– transplantation with cells.



a b c

Figure 14. Spinal cord samples immunofluorescent analysis of implantation zone. Primary antibodies - against oligodendrocytes, secondary antibodies - red. The blue kernels painted by DAPI. Increase 12x60, longitudinal cut: a – control (transplantation without cells) – kernels are visible only; b -cells, expression GFP; c – transplantation with cells, an expression oligodendrocytes.

Results of the analysis of neurotransmitters in the area of transplantation of neural matrix with partial rupture of spinal cord

Markers analysis of transmission of nerve signals with partial spinal cord injury in is showed that in the segments of the central nervous system, which are higher than the control a cellular graft detected cells expressing GABA, acetylcholine and serotonin in the period 1-4 weeks after surgery (Figure 15). In the control area of the matrix is filled with interstitial implantation of tissue with cells of the spinal cord of the parent expressing the above mentioned neurotransmitters (Figure 16). In the tail of the spinal cord revealed viable tissue with cells producing mediators of transmission of nerve signals (Figure 17).

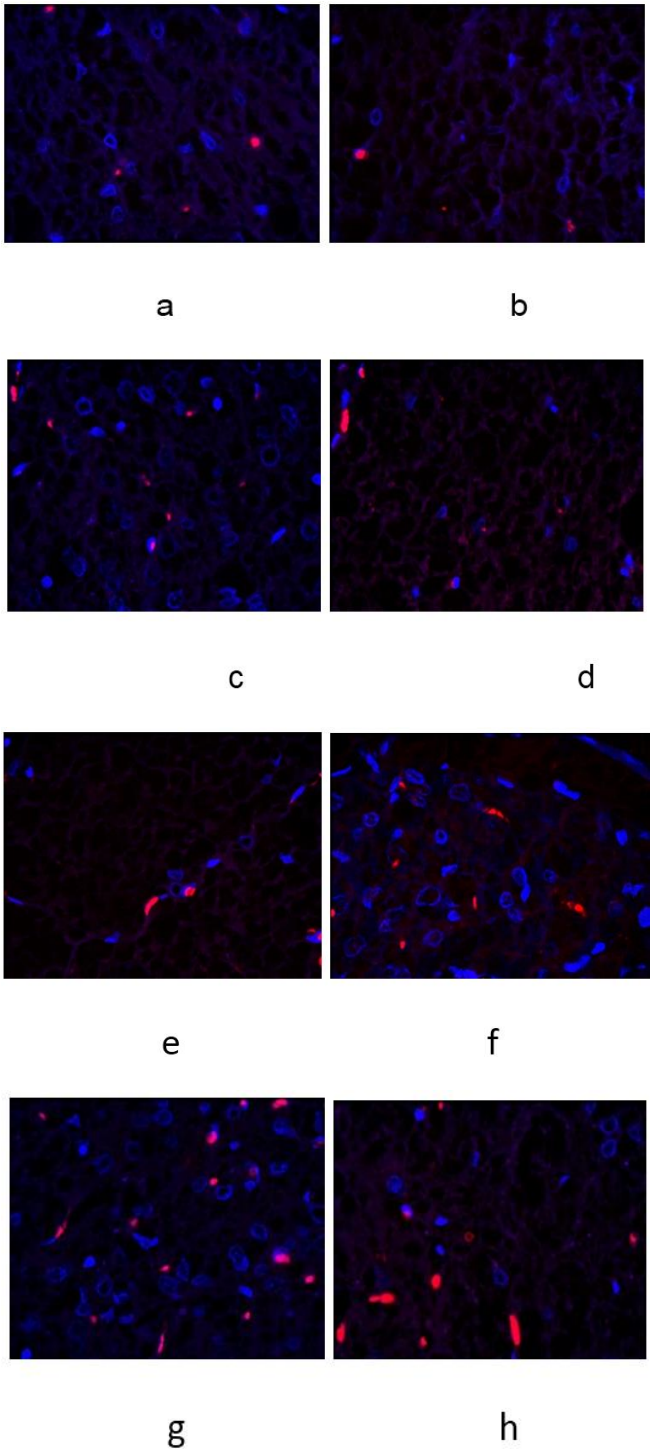


Figure 15. The immunofluorescent analysis of a series spinal cord cuts (from top to down, control) over a transplant at a partial trauma: GABA (a, b), acetylcholine (c, d, e), serotonin (f, g, h).

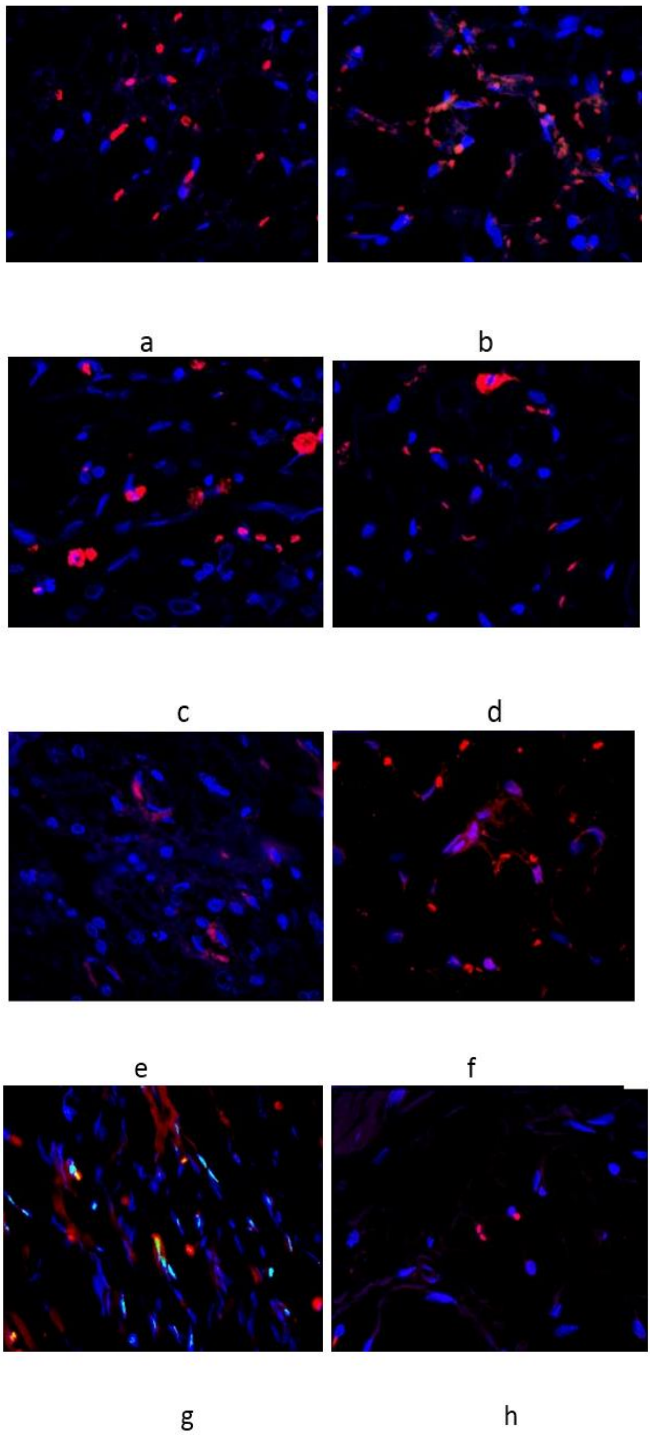


Figure 16. A series spinal cord cuts in a zone of a partial trauma and matrix transplantation (from top to down, control): GABA (a, b), acetylcholine (c,d, e), serotonin (f, g, h).

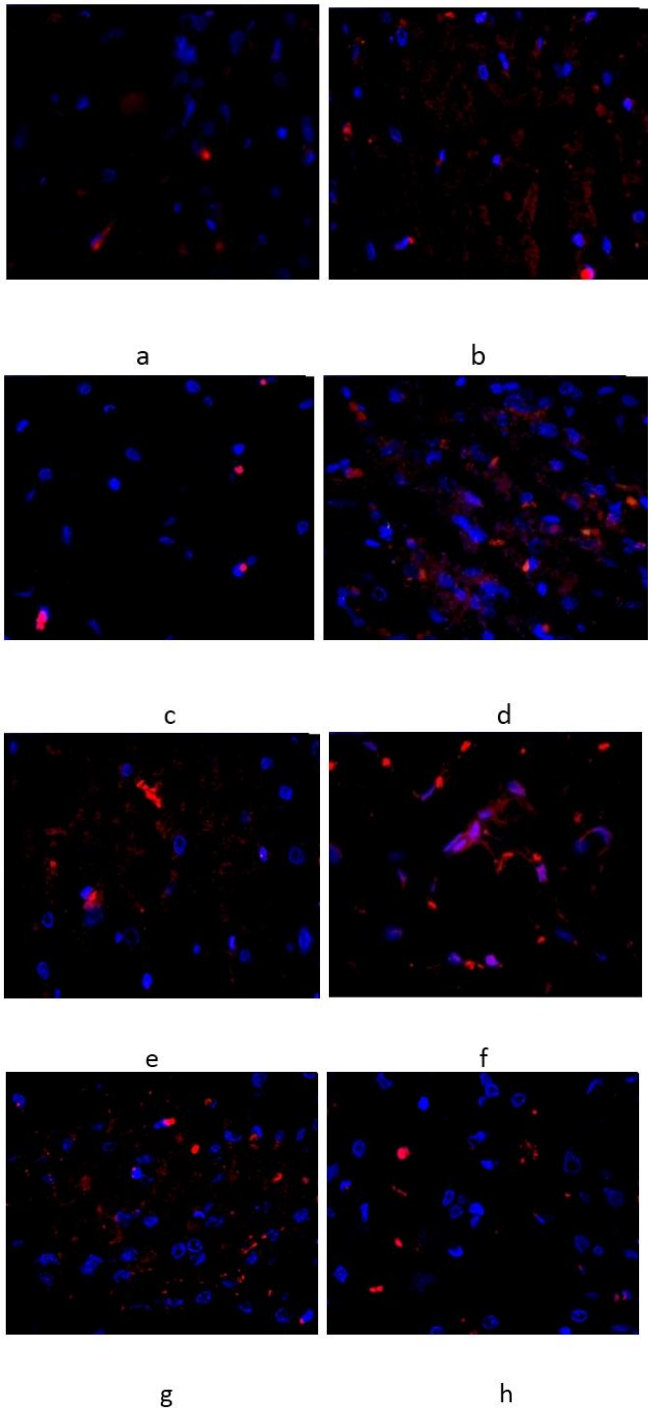


Figure 17. A series of cuts of a spinal cord is lower than a zone of a partial trauma (from top to down, control): GABA (a, b), acetylcholine (c, d, e), serotonin (f, g, h).

In animals, experimental series with partial spinal cord injury in the areas of the head and tail of the central nervous system revealed identical expression pattern control neurotransmitters. However, the phenomenon is registered sprouting a transplanted neural progenitor cells

from the area of transplantation in proximal maternal spinal cord. In the cytoplasm of cells besides detecting green fluorescent protein revealed expression of mediators (Figure 18). Throughout the area of transplantation into the spinal cord of collagen-chitosan matrix precursor cells of neuronal cells in addition to maternal tissue detected a high number of cells containing cytoplasmic GFP. In addition, these cells express GABA, acetylcholine and serotonin (Figure 19). In the tail of the spinal cord below the transplanted cell matrix observed sprouting neuronal progenitor cells (cells expressing the acetylcholine-GFP, 2 weeks after transplant period) (Figure 20).

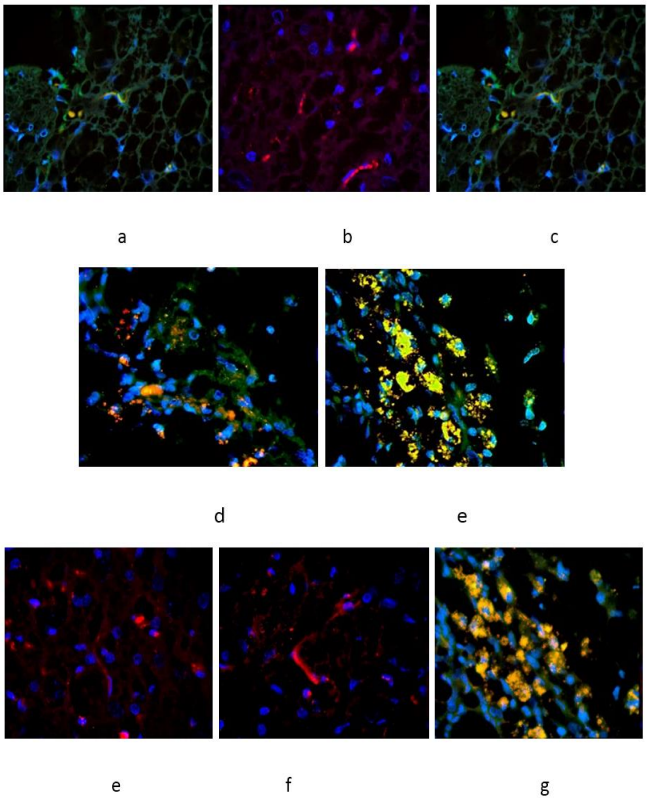


Figure 18. The immunofluorescent analysis of a series spinal cord cuts (from top to down, experience) above a cellular transplant at a partial trauma: GABA (a, b), acetylcholine (c) and acetylcholine-GFP (e), serotonin (f, g) and serotonin -GFP (h).

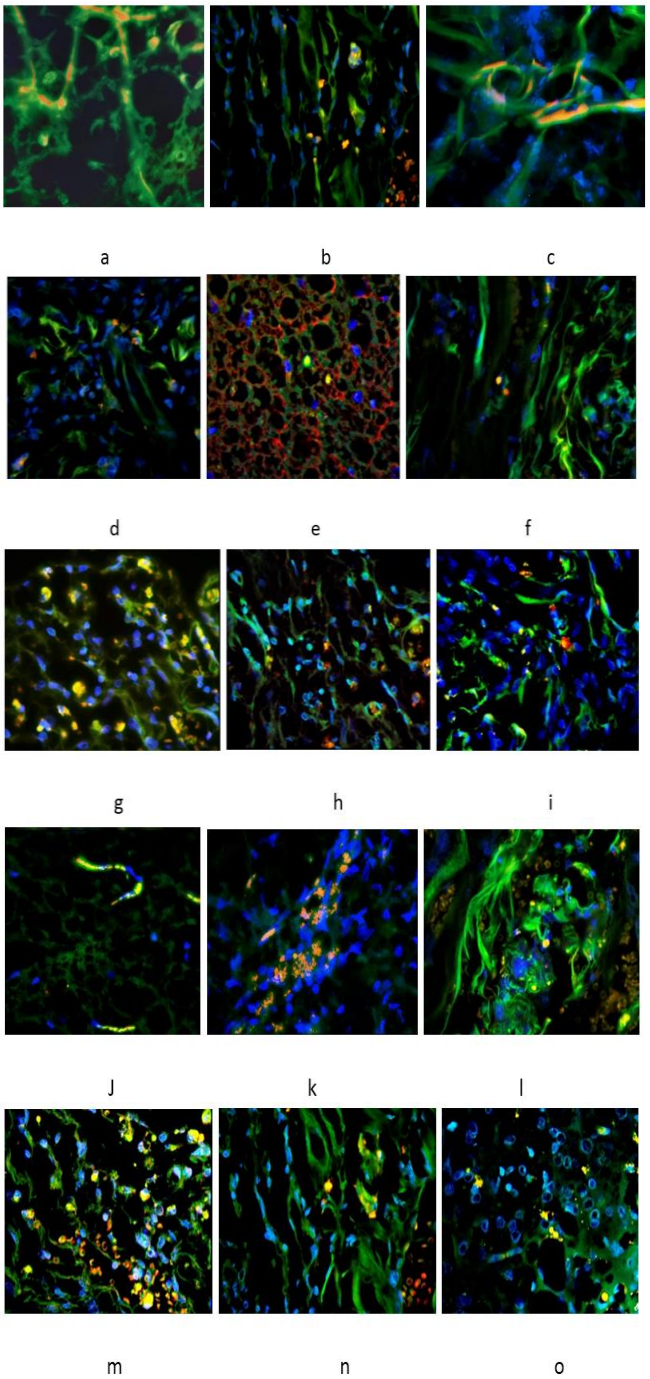


Figure 19. A series of spinal cord cuts in a zone of a partial trauma and transplantation of a cellular matrix with predecessors of neuronal cells, GFP (from top to down, experience): GABA (a-e), acetylcholine (f-j), serotonin (k-o).

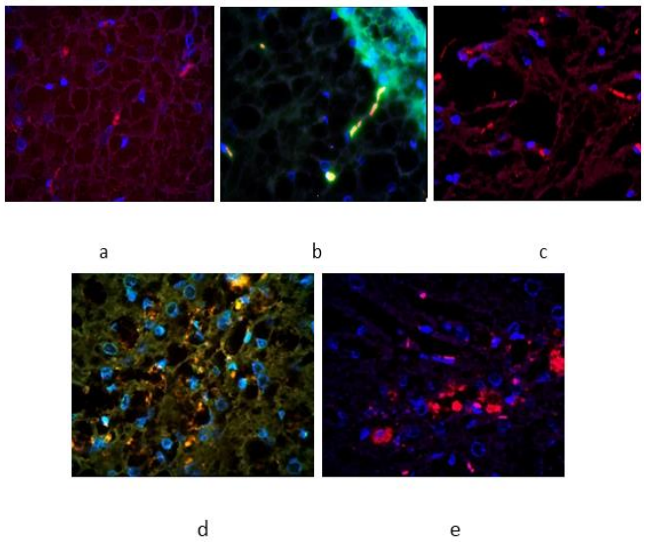


Figure 20. The immunofluorescent analysis of a series of spinal cord cuts (from top to down, experience) below a cellular transplant at a partial trauma: GABA (a), acetylcholine-GFP(b), acetylcholine (c), serotonin -GFP (d) and serotonin (e).

Conclusions

Thus, the results, obtained in the researches, allow to assume that the collagen-chitosan matrix containing in its composition factors of neurogenic differentiation of neural progenitor cells, is suitable for implantation with the aim of restoring the functions of the damaged spinal cord without risk of teratoma during the cultivation of cell mass of neural cells in artificially created three-dimensional environment. Transplantation of acellular collagen-chitosanase substrate with incomplete experimental transection of the spinal cord is accompanied by active sprouting maternal cell mass of neural origin, actively expressing markers of neural differentiation and neurotransmitters. Such transplantation is accompanied by the partial restoration of motor, sensory and autonomic functions of the spinal cord, reaching a level of reduction neural deficit equal to 5,6 points in NSS scale. Transplantation of collagen-chitosan matrix, containing 50 thousands of neural progenitor cells, is accompanied during 4 weeks with a preservation of their viability, the formation of numerous neurons, forming inter-synaptical connection, in addition to expressing markers of neural mediators in the transmission of nerve signals. The transplanted cell mass encounters a stream of their axons towards the central segment of the maternal spinal cord beyond the graft.

Acknowledgements

This work was supported by grant from State Educational Institution Krasnoyarsk State Medical University. prof. V.F.VoynoYasenetsky MH-SD RF (2009), grants the State Fund for Assistance to Small Innovative Enterprises in Science and technology (contract number 6746r/9167 from 10.04.2009, contract № 8775 dated 11.01.2011 r/13993 city, contract number 10494 r/16892 from 06.08.2012).

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

Motorfunctionevaluation	Maximumpoints
rat suspension by the tail: 1 - forelimb flexion bending 1 - the back leg bending 1 - move your head more than 10 degrees from the vertical axis for 30 sec	3
Putting the rat on the floor: 0 - normal movement of the floor 1 - failure to maintain directional movement 2 - circling towards the paretic limb 3 - falling on the paretic side	3
Evaluation of sensory function: 1 - laying (visual and tactile tests) 1 - proprioceptive test (crushing feet to the edge of the table)	2
Assessment of balance: 0 - to maintain balance and stable body position 1 - gripping rocker 2 - grasp of the balance and a sagging paretic limbs along the rocker 3 - grasp balancer and two sagging paretic limbs along the rocker, or spinning on a rocker (60 sec) 4 - an attempt to keep his balance on a rocker (40 sec), but drop it 5 - an attempt to keep his balance on a rocker (40 sec), but drop it 6 - fall without trying to balance or grab the lever (less than 20 sec)	6
The absence of pathological reflexes or motor activity 1 - reflex ear (when touched to the hearing tubercle - shaking his head) 1 - corneal reflex (when touched to the cornea with a piece of cotton wool - blinking) 1 - startle reflex (motor response to a short noise flipping the paper clip) 1 - seizures, myoclonus, miodystonia.	4
Maximum points	18

Table 1. Scale of assessment of neurological deficit (Neurological Severity Scores - NSS). Note: One point corresponds to the inability to perform a task or absence of the test reflex, 13 - 18 points - marked damage, 7 - 12 points - moderate heavy damage, 1 - 6 points - moderate damage.

Test'sName	1 Week		2 Week		3 Week		4 Week	
	Monitoring	Experience	Monitoring	Experience	Monitoring	Experience	Monitoring	Experience
Motor function Evaluation	1,0	0,94	0,8	0,29	0,20	0,28	0	0,12
Surfacetest	1,4	0,95	0,4	0,64	0,20	0,42	0	0,49
Evaluation of sensory function	1,6	1,37	1,4	1,11	0,60	1,2	0	0,62
Balance assessment	4,8	5,36	3,6	4,93	4,20	3,99	2,0	3,75
Absence of reflexes, abnormal motor activity	0	0.31	0	0,46	0,20	0,21	0	0,12
Running "narrows" road (cm)	-	13,8	-	28,5	-	36,0	-	47,5
Integrated sum of points	8,8	8,94	6,2	7,46	5,4	6,12	2,0	5,6
Observations numbers	5	19	5	17	5	14	2	8

Table 2. Rats with partial spinal cord injury and implantation of collagen-chitosan matrices with neuronal precursor cells of mice. Note: Control - implantation of collagen-chitosan matrix with neuronal microenvironment without cell mass, experience - implantation of collagen-chitosan matrix microenvironment and neuronal precursor cells of the mouse neuronal

References

1. Kakulas BA. Neuropathology: the foundation for new treatments in spinal cord injury. Spinal Cord. 2004;42(10):549-63.
2. Young W. Bases for Hope in Spinal Cord Injury. / / Http://sci.rutgers.edu.
3. Tsai EC, Tator CH. Neuroprotection and regeneration strategies for spinal cord repair. Curr Pharm Des. 2005;11(10):1211-22.
4. Bryukhovetskiy AS. Transplantation of neural cells and tissue engineering brain nerve disease. Moscow: ZAO clinic of restorative neurology and interventional therapy. NeuroVita. 2003;398.
5. Bersenev AV. Cellular Transplantation - history, present status and prospects. Cell Transplantation and Tissue Engineering. 2005;1:49-56.
6. Borschenko IA. Modern possibilities of active treatment of traumatic spinal cord injury. Proceedings of the 4th Annual All-Russian scientific-practical conference "Society spinal cord." Moscow. 2005;4-10.
7. Tator C.H. Strategies for recovery and regeneration after brain and spinal cord injury. Inj. Prev. 2002;8:33-6.
8. Guest JD, Rao A, Olson L, Bunge MB, Bunge RP. The ability of human Schwann cell grafts to promote regeneration in the transected nude rat spinal cord. Exp. Neurol. 1997;148:502-22.
9. Evans MJ, Kaufman MH. Establishment in culture of pluri-potential cells from mouse embryos. Nature. 1981;292:154-6.
10. Bjorklund A, Stenevi U. Intracerebral neural transplants neuronal replacement and reconstruction of damages circuitries. Ann. Rev. Neurosci. 1984;7:279-308.
11. Liu S, Qu Y, Stewart TJ, Chakrabortty S, Holekamp TF, McDoonald JM. Embrionic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc. Nat. Acad. Sci. 2000;97:6126-31.
12. McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK. TuretskyD, Gottlieb DI, Choi DW. Transplanted embryonic stem cells survive, differentiate, and promote recovery in injured rat spinal cord. Nat. Med. 1999;5(12):1410-2.
13. Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differention of embrionic stem cells into motor neurons. Cell. 2002;110(3):385-97.
14. Lanza R, Gearhart J, Hogan B, Melton D, Pedersen R, Thomas ED, Thomson J, West M. Essentials of Stem Cell Biology. Elsevier; 2009.
15. Diener PS, Bregman BS. Fetal spinal cord transplants support the development of target reaching and coordinated postural adjustments after neonatal cervical spinal cord injury. J. Neurosci. 1998;18:763-76.
16. Shibayama M, Matsui N, Himes BT, Murray M, Tessler A. Critical interval for rescue of axotomized neurons by transplants. Neuroreport. 1998;9:11-4.
17. Das GD. Neural transplantation in the spinal cord of adult rats. Conditions, survival, cytology and connectivity of the transplants. J. Neurol. Sci. 1983;62:191-210.
18. Goldberg WJ, Bernstein JJ. Transplant-derived astrocytes migrate into host lumbar and cervical spinal cord after implantation of E14 fetal cerebral cortex into adult thoracic spinal cord. J. Neurosci. Res. 1987;17:391-403.

19. Bernstein JJ, Underberger D, Hoovler DW. Fetal CNS transplants into adult spinal cord: techniques, initial effects and caveats. Cent. Nerv. Syst. Trauma. 1984;1:39-46.
20. Mendez I, Sadi D, Hong M. Reconstruction of the nigrostriatal pathway by simultaneous intrastriatal and intranigral dopaminergic transplants. J. Neurosci. 1996;16:7216-27.
21. Tsymbalyuk VI, Medvedev VV. Neurogenic stem cells. Kiev;2005.
22. Liu Y, Himes T, Solowska-Baird J, Moul J, Chow S, Tessler A, Snyder E, Fischer I. Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. Exp. Neurol. 1999;158:9-26.
23. Onifer SM, Cannon AB, Whittemore SR. Altered differentiation of CNS neural progenitor cells after transplantation into the injured adult rat spinal cord. Cell Transplant. 1997;6:327-38.
24. Whittemore SR. Neuronal replacement strategies for spinal cord injury. J. Neurotrauma. 1999;16:667-73.
25. Fujiwara Y, Tanaka N, Ishida O, Fujimoto Y, Murakami T, Kajihara H, Yasunaga Y, Ochi M. Intravenously injected neural progenitor cells of transgenic rats can migrate to the injured spinal cord and differentiate into neurons, astrocytes and oligodendrocytes. Neurosci. Lett. 2004; 366(3):287-91.
26. Raisman G. A promising therapeutic approach to spinal cord repair (ed). J. R. Soc. Med. 2003;96:259-61.
27. Tiansheng S, Jixin R, Wu J. et al. Transplantation of olfactory ensheathing cells for the treatment of spinal cord injury. First International Spinal Cord Injury Treatment and Trials Symposium. Abstracts and free papers. 2005.
28. Huiyong S, Tang Y, Wu YF, et al. Experimental and clinical observation olfactory ensheathing cells: Migratory property after being transplanted in spinal cord. First International Spinal Cord Injury Treatment and Trials Symposium. Abstracts and free papers. Hong-Kong, 2005.
29. Shen HY, Tang Y, Wu YF, et al. The influences of transplanted olfactory ensheathing cells of axonal regeneration in adult rat spinal cord. First International Spinal Cord Injury Treatment and Trials Symposium. Abstracts and free papers. Hong-Kong, Ab060, 2005.
30. Ramer LM, Au E, Richter MW. Peripheral olfactory ensheathing cells reduce scar and cavity formation and promote regeneration after spinal cord injury. J. Comp. Neurol. 2004;473(1):1-15.
31. Perry C, Bianco JI, Harkin DG, Mackay-Sim A, Feron F.. Neurotrophin 3 promotes purification and proliferation of olfactory ensheathing cells from human nose. Glia, 2004;45(2):111-23.
32. Eremeev AV, Svetlakov AA, Bolshakov IN, Sheina YI, Polstyanoy AM. Method for producing a neural matrix. PCT/RU000213, No WO/2011/142691; 2011.
33. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282(5391):1145-7.
34. Kima HS, Oha SK, Park YB, Ahn HJ, Sung KC, Kang MJ, Lee LA, Suh CS, Kim SH, Kim DW, Moon SY. Methods for Derivation of Human Embryonic Stem Cells. Stem Cells. 2005;23(9):1228-33.
35. Patent RU 2301675, 2007.
36. Combs DJ, D'Alecy LG. Motor Performance in Rats Exposed to Severe Forebrain Ischemia: Effect of Fasting and 1,3-Butanediol. Stroke. 1987;18(2):503-11.
37. Eremeev AV, Svetlakov AV, Bolshakov IN, Vlasov AA, Arapova VA. Function of cultured embryonic cells on collagen-chitosan matrix. J. Cell Transplantation and Tissue Engineering. 2009;IV(2):55-62.
38. Eremeev AV, Svetlakov AV, Bolshakov IN, Vlasov AA, Arapova VA. Viability and function of pluripotent cells and fibroblasts dermal-epidermal layer of animals in their culture on collagen-chitosan coatings. Siberian Med Rev. 2008;6(54):24-7.