Original Article

The use of biodegradable scaffold based on bacterial cellulose in the treatment of open brain injury

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ABSTRACT

Traumatic brain injury (TBI) occurs as a result of direct mechanical action on the brain and causes degeneration and cell death in the central nervous system. Cell transplantation has proven itself not only as an experimental method for studying structural and functional relationships, development, neuroplasticity, and regeneration in the adult central nervous system but has also recently shown optimistic results in restoring functions after traumatic brain injury. Unfortunately, TBI leads to the death of a large number of brain parenchyma, therefore, one of the promising approaches used in regenerative medicine is the use of three-dimensional biocompatible scaffolds capable of supporting the growth and integration of nerve cells at the site of injury. The biocompatibility of a biodegradable scaffold, which acts as a carrier of transplanted cells and replaces the matrix of nervous tissue during neuro transplantation, was studied on the model of experimental open brain injury in mice. The 3D biodegradable scaffold was created using micro stereolithographic techniques by combining bacterial cellulose and hyaluronic acid. In an experiment on the parameters of cognitive behavior of mice (a study of long-term conditioned reflexes and short-term memory during recognition of a new object), visualization of the integrity of brain tissue using high-field MRI (9.4 T), it was shown that transplantation of a 3D scaffold based on bacterial cellulose during reconstructive therapy of a brain tissue defect reduces cognitive deficit after injury, restores the integrity of brain tissue 5 months after injury.

Keywords: Neurotrauma, Cell therapy, Biodegradable scaffold, Biocompatibility, Cognitive disorders

Introduction

Traumatic brain injury (TBI) occurs as a result of direct mechanical action on the brain, causes degeneration and cell death in the central nervous system (CNS), which leads to a disorder in the transmission of nerve impulses, and, in turn, causes memory loss [1, 2]. The lack of significant progress in the effectiveness of drug therapy, and the severely limited internal reserve capabilities of the brain about the restoration of nerve cells arouse high scientific interest in the development of fundamentally new methods of treating severe TBI [3, 4]. Neurotransplantation of stem or progenitor cells may be one of the most promising methods [5]. Restoration of functions is possible due to the integration of transplanted cells in the areas of damage and stimulation of compensatory repair processes in pathologically altered recipient cells [6, 7]. Currently, there is little experience in the world of using stem cells for the treatment of brain injuries [8, 9]. Several transplants of poorly differentiated neural and hematopoietic cells were performed in patients with severe traumatic brain injuries [10]. It has been shown that cell therapy reduces the number of adverse outcomes

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by almost 4 times and ensures good recovery in almost half of the patients who participated in clinical trials [11].

An urgent area of work is the development of adequate carriers for transplantable cells. The clinical and experimental effects of cell transplantation as part of three-dimensional carriers based on synthetic biodegradable materials have not been sufficiently studied [12]. In the literature, the problem of adequate carriers supporting survival, proliferative activity, and the ability to migrate transplanted cells comes to the fore [13-15]. At the same time, the artificial carrier must remain in the recipient's brain tissue during transplantation for a long time so that stem cells, maintaining their phenotype, can be evicted into adjacent tissues, stimulating the post-traumatic survival of neurons and the growth of nerve fibers in them [16].

According to the literature, there is an extremely high interest in cellulose produced by evolutionarily different species of organisms in the world [16, 17]. For example, the biosynthesis and properties of bacterial cellulose (BC) are widely studied in various fields of human activity, especially in medicine [18-20]. During surface cultivation of Gluconacetobacter xylinus, the assembly of BC macromolecules ends at the nanoscale, which leads to the formation of a hydrocolloid film unique in the hierarchy of structure. This makes it possible to introduce a variety of systems into it while maintaining high mechanical tensile strength [21-23]. The study of the properties of bacterial cellulose in medicine is conducted in many directions: wound coatings, burn treatment, implantation, cartilage defects, etc. [24-26]. Previously, the physicochemical properties of BC were studied and it was shown that the local application of coatings based on it optimizes the wound process [19, 20]. However, no comparison has been made with existing analogs and traditional wound treatments, and it has not been established in which phases of the wound process their use is preferable. Thus, the work aimed to study the biocompatibility of a biodegradable three-dimensional scaffold based on bacterial cellulose modified with hyaluronic acid in experiments with open brain injury in mice.

Materials and Methods

The work was performed on 8–10-week-old outbred male mice weighing 20-22 g (n=40). The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes [27].

The 3D biodegradable scaffold was created using a micro stereolithographic technique by combining BC and high molecular weight hyaluronic acid [28, 29].

Modeling of open traumatic brain injury (TBI) was carried out by the "weight-drop" method [30] according to the original methodology. The mechanical injury was inflicted by falling a load with a blunt surface, which provides acceleration of the head with minimal local impact at the point of application of the traumatic force. The use of this model most fully reproduces the clinical picture of focal damage, including brain injury. This allows us to study local changes and traumatic changes accompanied by secondary death of nerve cells in remote parts of the brain that are sensitive to injury, such as the hippocampus, dentate gyrus, and visual tubercle, as well as to assess gross motor disorders, changes in fine coordination of movements, and cognitive deficits.

Before the injury was inflicted, it was fixed under isoflurane anesthesia in a stereotactic installation for mice "Narishige" (Japan). The animal's head was pressed against a steel plate to avoid a jaw fracture and achieve a horizontal position of the skull arch to the end section of the load. Then, an incision was made on the scalp free of wool, and trepanation was performed with a cutter of the skull bones (d 4 mm, Bregma 0.82 mm). The dura mater remained intact. The load, which is a steel cylinder, was lifted to a predetermined height, then dropped and struck the area of the trepanation window (the diameter of the impact part corresponding to the trepanation window is 3 mm; the weight of the load is 4 grams; the height of lifting the load is 0.80 meters). After injury, damage occurred in the underlying medulla in the form of rupture of the dura mater, the formation of foci of hemorrhagic bruising or crushing of the medulla, characteristic of severe traumatic brain injury. After the operation, the skin of the animals was sutured, and the suture was treated with an antiseptic solution.

During the experiment, the animals were randomly divided into groups: the first group – intact animals (n=10), the second – control (falsely operated) animals (n=10), the third group – control animals (n=6), and the fourth – experimental (n=5).

On the seventh day after the TBI simulation, either buffered saline solution (PBS, 30 µl) or a biodegradable 3D scaffold (size 2x1 mm) was implanted into the lesion in the control and experimental mice, respectively. The same procedures (anesthesia, trepanation of the skull bones) were performed in the group of falsely operated animals, except for TBI modeling and transplantation. The mice of the intact group were not subjected to anesthesia, TBI modeling, or transplantation.

Morpho-functional parameters for evaluating the biocompatibility of scaffold in vivo were visualization of the integrity of brain tissue using MRI and testing of the cognitive behavior of mice.

To morphologically assess the biocompatibility of the developed scaffolds with mouse brain tissues [31], high-field MRI methods were used on an Agilent Technologies DD2-400 9.4 T (400 MHz) tomograph with an M2M (H1) volumetric coil. During mouse brain tomography, the physiological parameters of animals such as temperature, respiration, and ECG were monitored using SA Instruments equipment using the PC-SAM program at an ambient temperature of 37 °C. The MGEMS (multi gradient echo multi-slice) pulse sequence was used to obtain T2-weighted images. MGEMS is a standard sequence (it was preinstalled in the VnmrJ program working with the tomograph) for creating anatomical images, which consists of using two bipolar pulses of a gradient magnetic field instead of a focusing 180-degree RF pulse. The repetition time (TR) is 1000 ms, the echo time (TE, TE2) is from 1.5 to 2 ms, the number of echoes is from 6 to 8, the angle of rotation (flip angle) is 90, the

matrix size is 128×128, the number of slices is 15, the thickness of the slices was 1 mm, the field of view is from 20×20 mm2. The total duration of the sequence was 8 min 32 sec [32].

For the functional assessment of learning disorders in animals, a conditioned passive avoidance reflex (URPI) was developed on the 10th day of the post-traumatic period. The installation (Shuttle Box LE895, PanLab / Harvard Apparatus Spain; Stoelting, USA) consisted of a chamber (46×27×25) with an electrified lattice floor divided into two compartments by a sliding door. A fixed resistance source was used for electrical stimulation (LE100/26 Harvard Apparatus Spain; Stoelting, USA). The supply of electrocutaneous irritation, the position of the door between compartments, and the latent transition time to the dark compartment, measured from the moment the animal was placed in the light compartment of the camera, were controlled by the Shutavoid v1.8.03 program (PanLab / Harvard Apparatus Spain; Stoelting, USA). The latent transition time (LTT) into the dark compartment of the camera was recorded with the door between the compartments open. The test ended when the animal entered a dark compartment or if the animal did not do so for 3 minutes. The functions of long-term memory were studied by evaluating the preservation of a conditioned passive avoidance reaction in mice on day 30.

A new object recognition test was used to study disorders of the non-spatial hippocampus-mediated short-term memory. The test consisted of three stages: habituation, training, and testing. On days 23 and 24 after the TBI simulation, the animals were placed in the center of a square arena (45×45×40 cm) (LE802S PanLab / Harvard Apparatus Spain; Stoelting) for 10 minutes to get used to and reduce stress, during which the animals explored an empty arena. On day 25, two identical objects were placed in the arena, located at an equal distance. The mice were placed in the center of the field for 10 minutes to study objects (training). After 24 hours, the animals were again placed in the center of the arena with one familiar object and one new one to register research behavior for 10 minutes (testing). During the experiment, the cumulative time spent exploring familiar and new objects was recorded at the training and testing stages. The research behavior included sniffing reactions (pointing the nose at an object at a distance of no more than 2 cm, touching the object). During training and memory testing, animal behavior was considered exploratory if reactions to objects were at least 10 seconds.

Video recording of individual behavioral acts of animal behavior was carried out using a SONY SSC-G118 video camera (Japan). Data registration and analysis were controlled by the Smart v.3.0.03 program (Panlab Harvard Apparatus Spain; Stoelting, USA). The state of memory functions was determined as the discriminatory time of the study of a new object (DIR) according to the formula:

$$\text{Dir} = [(\text{the proportion of time spent exploring a new object by an animal is the proportion of time spent exploring a familiar object during a testing session}) / \text{total time spent exploring during a testing session}] \times 100 \quad (1)$$

The obtained results were processed using Statistica 5.5 application software packages. The data were checked for the normality of the distribution using the Shapiro-Wilk W-test. For small sample sizes ($n \leq 10$), nonparametric criteria were applied: for two dependent samples, the Wilcoxon criterion, and for two independent samples, the Mann–Whitney criterion. The differences between the groups were considered statistically significant at a significance level of $p<0.05$.

Results and Discussion

The obtained results of behavioral tests indicated a violation of the ability to learn in animals of the control group due to TBI of the brain. During the training session in the control group of mice, the latent transition time (LTT) significantly exceeded similar indicators of the intact and control groups of falsely operated animals ($p<0.05$), due to impaired motor functions of the central nervous system as a result of TBI. 24 hours after training, the latent time of transition to the dark compartment of the camera in this group did not significantly differ from LTT during training ($p>0.05$) **(Figure 1)**.

24 hours after the training session in the group of animals implanted in the lesion of the biodegradable BC scaffold, as well as in the intact and control groups of falsely operated animals, LTT in the dark compartment of the chamber increased statistically significantly relative to the time during training ($p<0.05$). In addition, in the experimental group with implantation of an LTT scaffold into the lesion, the transition time of animals in the control group with the introduction of a PBS solution into the lesion was 1.82 times ($p<0.05$).

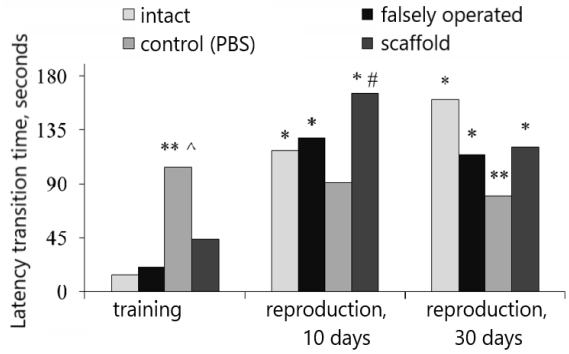


Figure 1. Study of the ability to learn and reproduce the conditioned reaction of passive avoidance in C57BL/6 mice in the post-traumatic period (* - $p<0.05$ concerning the latent transition time to the dark compartment during training, Wilcoxon criterion; # - $p<0.05$ with respect to the control group of animals, Mann-Whitney criterion; ** - <0.05 with respect to the intact group of animals, Mann-Whitney criterion; ^ - <0.05 with respect to the control group of falsely operated animals, Mann-Whitney criterion)

Long-term memory functions were assessed by the ability of animals to reproduce a conditioned passive avoidance reaction on day 30 of the post-traumatic period. In the group of mice implanted a week after the TBI simulation into the lesion of the

biodegradable BC scaffold, LTT into the dark compartment of the chamber significantly exceeded the training time ($p<0.05$), as well as in the intact and control groups of falsely operated animals. It did not differ from the time when checking the safety of the conditioned reflex skill on the 10th day of the post-traumatic period ($p>0.05$).

The short-term memory functions of mice were evaluated in the "new object recognition test" (NOR, Novel object recognition test) during the 23-25 days of the post-traumatic period.

Analysis of the results showed that modeling of TBI in animals of the control group led to significant violations of hippocampal-dependent short-term memory. At the testing stage, the duration of the research behavior of a new object did not exceed the time of the study of a familiar object. The discriminatory ratio of the study in the control group with the introduction of PBS solution into the lesion was significantly lower than that of the intact and control groups of falsely operated animals ($p<0.05$) **(Figure 2)**. In mice of the group with implantation on the 7th day after TBI modeling in the lesion of the biodegradable BC scaffold, the time to study a new object at the testing stage exceeded the time to study a familiar object. The discriminatory ratio of the study duration did not differ from the ratio of the time of the intact and control groups of falsely operated animals ($p>0.05$), but at the same time statistically significantly exceeded the indicator of the control group with the introduction of PBS solution into the lesion ($p<0.05$), which indicates the restoration of the functions of CA1-CA3 fields of the hippocampus-dependent short-term recognition memory **(Figure 2)**.

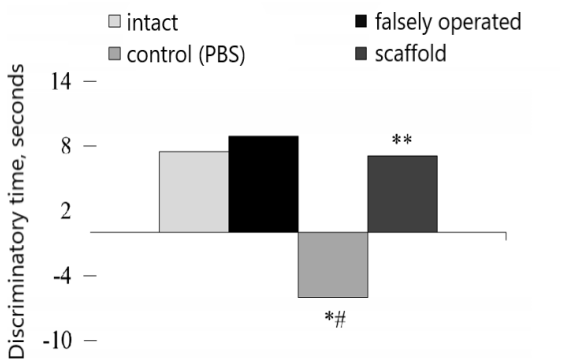
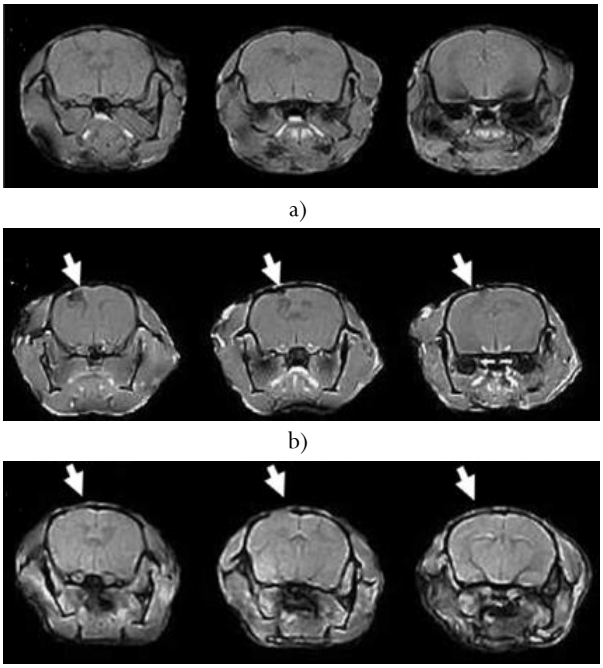


Figure 2. Investigation of the short-term memory functions of C57BL/6 mice in the post-traumatic period
* - $p<0.05$ in relation to the intact group; Mann-Whitney criterion; # - $p<0.05$ in relation to the group of falsely operated animals; Mann-Whitney criterion; ** - $p<0.05$ in relation to the control group of animals (PBS); Mann-Whitney criterion

MRI data obtained 5 months after the injury showed that the tissue formed at the site of the scaffold injection is comparable to intact brain tissue in terms of the intensity of the tomogram. There was no accumulation of fluid in the area of the lesion, which would be expressed in a hyperintensive signal and an overexposed area on the MRI image. There was also no tissue compaction in the injury area, which could be indicated by a hypointensive signal and a decrease in intensity (darkening). **Figures 3a-3c** shows MRI slices of the brain of intact mice (a), mice with an injection of PBS solution (b), and mice with biodegradable BC scaffold embedded in the lesion (c). As can be seen from **Figure 3**, the brain of a mouse with a scaffold is structurally unchanged and comparable to an MRI of an intact brain.



a)

b)

c)

Figure 3. MRI images of brain slices of intact mice a), mice with an injection of PBS solution into the lesion b), and mice with biodegradable BC scaffold implanted into the lesion c). T2-weighted images were obtained using the MGEMS pulse sequence. The arrows indicate the area of injury.

Thus, 5 months after neurotransplantation of a biodegradable BC-based scaffold in combination with hyaluronic acid, MRI data revealed the formation of a homogeneous tissue in the area of the lesion, which is comparable in intensity to intact tissue, has no pronounced boundary with intact tissue, does not accumulate fluid and has no seals, which corresponds to the literature data [33]. TBI of the brain leads to a violation of learning processes and long-term memory in the formation of a conditioned reaction of passive avoidance, as well as short-term memory functions in the post-traumatic period. Implantation a week after TBI modeling into the lesion of the biodegradable BC scaffold had a positive effect on the ability of animals to learn conditioned reflexes on day 10 and actualize traces of short-term and long-term memory in the long term, as shown in [34-36].

Conclusion

When modeling TBI in animals, due to impaired neurological functions, and motor and emotional behavior, the ability to learn was impaired, which is consistent with the literature data. Behavioral tests revealed significant improvements in the restoration of cognitive and motor functions of the central nervous system in animals against the background of neurotransplantation. Implantation a week after the TBI

simulation into the lesion of the biodegradable scaffold had a protective effect, probably restoring the synaptic plasticity of the neurons of the brain (cortex, hippocampus) underlying the processes of learning and memory. An optimizing effect was observed on the ability of animals to learn the conditioned reaction of passive avoidance on day 10 and the actualization of traces of short-term and long-term memory in the long term.

The revealed morphological and functional parameters of the vital activity of mice testified to the biocompatibility of the biodegradable 3D scaffold material based on BC in combination with hyaluronic acid. An interesting fact was that an attempt was made to partially restore the tissue lost as a result of injury, which in the usual case remains forever unfilled since a cyst surrounded by a glial scar and filled with cerebrospinal fluid remains in place of the damage zone. Transplantation of a three-dimensional designed biodegradable structure contributed to the formation of brain tissue in place of the damaged area during injury.

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Ethics statement: The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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