Features of Primary Hippocampal Cultures Formation on Scaffolds Based on Hyaluronic Acid Glycidyl Methacrylate

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The aim of the study was to investigate the morphological and metabolic features of primary hippocampal cultures formation on hydrogel films and scaffolds based on hyaluronic acid glycidyl methacrylate.

Materials and Methods. Hydrogel films and scaffolds with certain architectonics were developed by micromolding technique on the basis of hyaluronic acid glycidyl methacrylate. Primary hippocampal cells obtained from C57BL/6 mouse embryos (E18) were cultured on the created constructs more than 14 days. Testing cell viability, morphometric assessment, and analysis of spontaneous calcium activity of primary hippocampal cultures were performed on day 14 of cultures development *in vitro*.

Results. This study revealed that the material for the development of scaffolds with given architectonics is non-toxic for the nervous system cells. Dissociated hippocampal cells were actively attached to the scaffold surface and were assembled into cell conglomerates, which exhibited spontaneous calcium activity.

Conclusion. Scaffolds designed on the basis of hyaluronic acid glycidyl methacrylate have a high biocompatibility with the nervous system cells. Architectonics and adhesive properties of scaffold contribute to the formation of functionally active cell conglomerates.

Key words: scaffold; hyaluronic acid; biocompatibility; calcium imaging; primary hippocampal cultures.

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Introduction

In recent decades, there has been a growing interest in research and development of high-efficiency methods for morphological and functional brain tissue reconstruction. Loss of structural and functional elements of the central nervous system due to the development of pathological processes induced by trauma or diseases with various etiology (ischemia, neoplasm, neurodegenerative diseases, etc.), entails growth of an acute neurological deficit, violations in mnestic and cognitive functions that leads to severe patient disability. Engineering reconstruction of nervous tissue is necessary not only for negation the induced pathological changes but also for stimulation endogenous neuroreparative processes in the damaged area [1–5]. The effectiveness of using three-dimensional constructs (scaffolds) for replacement therapy after a spinal cord injury, in bone and cartilage tissue reparation was already shown [6–10].

Application of three-dimensional constructs with a given architectonics for brain injury correction also seems promising. Strict requirements imposed on materials of scaffolds for neurotransplantation are the absence of cytotoxicity, provision of cell adhesion, proliferation or differentiation. In addition, the material should be efficiently degraded without forming toxic products. These conditions are necessary to minimize risk of neurotransplant rejection and to maintain certain anatomical structure in the lesion focus for providing free transport of biological fluids and its gradual substitution with a natural tissue [3, 5, 11, 12].

Hyaluronic acid is regarded as a potent material for the development of biocompatible three-dimensional constructs [13–16]. Recent studies have shown that hyaluronic acid is an active component of a brain extracellular matrix, which not only structures the nervous tissue but also actively participates in synaptic plasticity, neuronal outgrowth, and cell migration [17–18].

In this regard, **the aim of the study** was to investigate the morphological and metabolic features of primary hippocampal cultures formation on hydrogel films and scaffolds based on hyaluronic acid glycidyl methacrylate.

Materials and Methods

Formation of hydrogel films. Spacers (thickness 150 μm) were imposed on coverslip. Photocurable composition consisting of 22% hyaluronic acid glycidyl methacrylate, 2% poly(ethylene glycol) diacrylate (Mn=575) (Sigma Aldrich, USA), 0.005% riboflavin mononucleotide dissolved in 2% collagen solution (Belcozin, Russia) was placed between spacers and then top covered with the second coverslip. The exposure was conducted at 450 nm wavelength (intensity 70 mW/cm^2) for 20 min. Then the coverslips were carefully separated. The sample was treated with ultraviolet (intensity 10 mW/cm^2). The obtained sample was stored in phosphate buffered saline before the cell culturing.

Fabrication of scaffolds. For the preparation of photocurable composition, 20 wt% hyaluronic acid glycidyl methacrylate (>4 MDa), 2.5 wt% poly(ethylene glycol) diacrylate (Sigma Aldrich, USA), 0.0004 wt% riboflavin mononucleotide were dissolved in 2% collagen solution (Belcozin, Russia) under sonication during 30 min. Thereafter, the composition was incubated at room temperature for 12 h and then placed in ultrasonic bath until formation of uniform consistency.

Scaffolds were produced using micromolding technique [19]. Photocurable composition was placed in a specially made silicone master mold and covered with a coverslip. The exposure was carried out for 20 min using the light emitting diode at 365 nm wavelength (intensity 20 mW/cm^2). Then the coverslip was carefully removed and the crosslinked hydrogel scaffold was pulled out from the master mold and placed in a phosphate buffered saline before the cell culturing (Figure 1).

Primary hippocampal cultures were obtained according to the previously developed technique from C57BL/6 mouse embryos (E18) [20]. The main rules of keeping and caring for experimental animals corresponded to the norms provided in the order of the Ministry of Health of the Russian Federation No.708n dated 23.08.2010 “Concerning Approval of the Rules for Laboratory Practice in Russian Federation” and the ethical principles established by European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 2006) and approved by the Ethics Committee of Lobachevsky State University of Nizhni Novgorod.

After enzymatic digestion of hippocampal tissue by 0.25% trypsin solution (Life Technologies, USA) cells were resuspended in Neurobasal™ (Thermo Fisher Scientific, USA) containing B27 supplement (Thermo Fisher Scientific, USA), L-glutamine (Thermo Fisher Scientific, USA), and fetal bovine serum (PanEco, Russia) and then were centrifuged at 1000 rpm within 3 min. The cells were

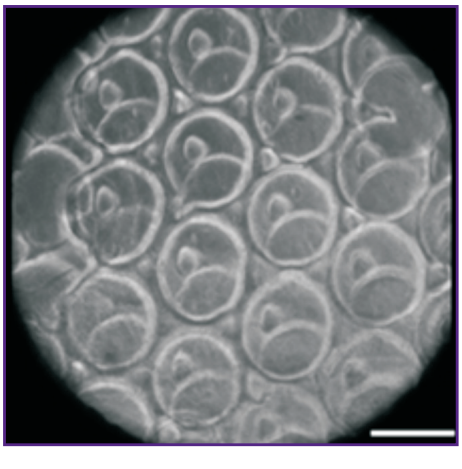

Figure 1. Representative microscopy imaging of scaffold based on hyaluronic acid glycidyl methacrylate. Scale bar is 250 μm

subsequently seeded on hydrogel films and scaffolds in approximate initial density of the cells — 9000 cells/mm^2. In control cultures, the same concentration of cells was placed on culture dish and coverslips (18×18 mm) pre-treated by polyethyleneimine (PEI) for increasing cell adhesion. The viability of the primary hippocampal cultures was maintained under the constant conditions of 35.5°C, 5% CO_2 and a humidified atmosphere in a cell culture incubator. All experimental cultures were divided into the following groups: "Control 1" — primary hippocampal cultures, obtained by the standard protocol with using specific adhesive substance PEI; "Control 2" — primary hippocampal cultures, cultivated on culture dish without PEI; primary hippocampal cultures cultured on hydrogel films; primary hippocampal cultures cultured on scaffolds.

The viability of primary hippocampal cells were estimated on day 14 of culture development *in vitro* (DIV) by counting in a inverted fluorescence microscope Leica DMIL HC (Leica, Germany) the number of cell nuclei stained by propidium iodide (Sigma, USA) (dead cells) and the number of cell nuclei stained by bis-benzimide (Sigma, USA) (all cells in a culture). The proportion of dead cells was calculated as the percentage ratio between bis-benzimide-positive and propidium iodide-positive cells [20].

To investigate the features of metabolic activity of primary hippocampal cell cultures a method of *functional calcium imaging* was used. Detection of calcium events was performed by using a specific calcium-sensitive dye Oregon Green 488 BAPTA-1 AM (Invitrogen, USA) and a confocal laser-scanning microscope Zeiss LSM 510 (Zeiss, Germany). The time series of the fluorescent images were registered. Determination and analysis of calcium events were conducted in original software Astroscanner (registration certificate for computer program No.20114662670). It was analyzed the records of the *F(t*) function of the average fluorescence intensity of calcium-sensitive dye in selected region (coinciding with the cell body) from time. To determine the start time (*Tstart*) and the end time (*Tend*) of the oscillations, the deviation threshold of the fluorescence level was taken from the mean value at the size of the standard quadratic error *F*(*t*). The following parameters were analyzed: the duration of the calcium oscillations (s), the frequency of the calcium oscillations (the number of oscillations per minute), and the percentage of cells exhibited calcium activity [21].

Scanning electron microscopy. This part of the study was carried out on the equipment of the Collective Usage Center "New Materials and Resource-saving Technologies" (Lobachevsky State University of Nizhni Novgorod). Experimental samples were placed in 2.5% glutaraldehyde solution for 12 h at 4°C. After fixation, the samples were washed in 0.01 M phosphate buffered saline (pH 7.4) (Sigma, USA) and then consistently dehydrated at 4°C in a battery of aqueous ethanol solution at increasing concentration: 30, 50, 70, 90, 100%. To remove ethanol, the samples were placed in hexamethyldisilazane (Sigma Aldrich, USA) for 30 min and then were dried in air. The samples were examined in an electronic scanning microscope JEOL JSM-IT300 (Jeol, Japan). Two types of signal were detected: the backscattered electrons and the secondary electron. In freeware ImageJ, the surface area of individual cells and their conglomerates (μm^2) and the Feret diameter (μm) (maximum distance between the edges of cell body) were calculated.

Statistical analysis. All quantified data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using ANOVA implemented in the Sigma Plot 11.0 software (Systat Software, Inc.). Differences between groups were considered significant if the corresponding p-value was less than 0.05.

Results

One of the key requirements offered for developing scaffold for neurotransplantation is the absence of cytotoxicity of material for the nervous system cells. Thus, the first stage of the research dedicated to the toxicity assessment of hydrogel films based on hyaluronic acid glycidyl methacrylate for primary hippocampal cultures. It was shown the intensive attachment of the dissociated hippocampal cells to the culture dish near the hydrogel film during the first day of culture development *in vitro*. Further observations revealed the formation of neuronal and glial outgrowth, that during the cultivation transformed into complex intertwined structures, evidencing the creation of a network-like structure (Figure 2). Neurons and glial cells had a typical structure in the field of view there were no cells with morphopathology (rounded, swollen or cells without outgrowth). Directly on the experimental material, a fewer number of cells that formed cellular conglomerates were visualized. Similar conglomerates, but in greater number, were observed in the PEI-untreated group of cultures ("Control 2"). It is assumed that low concentration of cells on the material is mediated by the lack of architectonics facilitated the effective cell attachment to the hydrogel film.

Cell viability assessment on 14 DIV did not reveal significant differences in the number of dead cells relative to control groups of cultures. The percentage of living cells in the "Control" 1 was 97.0±2.3%, in "Control 2" — 94.0±3.5%, in the experimental group — 95.0±1.9%. Thus it was shown that the tested material is non-toxic for the nervous system cells.

Based on hyaluronic acid glycidyl methacrylate we produced scaffolds with given architectonics (the main parameters and method of preparation are described in "Materials and Methods") on which further cultivation of primary hippocampal cultures was conducted.

We found that scaffolds have more adhesive properties compared with hydrogel films. During the first 24 h of cultivation, the dissociated hippocampal cells were actively attached to the scaffold surface. Neuronal and

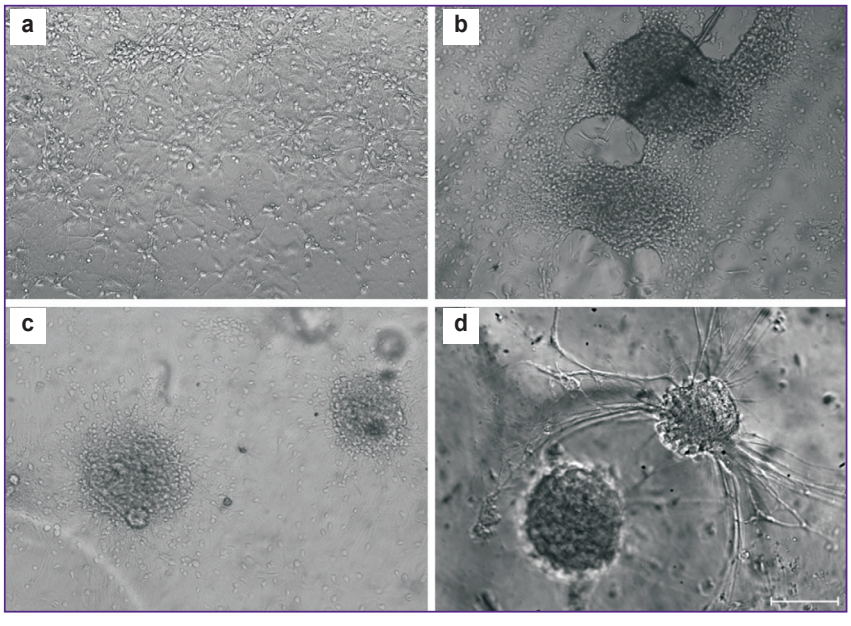

Figure 2. Representative microscopy images of primary hippocampal cultures cultivated on different substrates, 14 DIV:
(a) “Control 1”; (b) “Control 2”; (c) culture on hydrogel film; (d) culture on scaffold. Scale bar is 50 μm

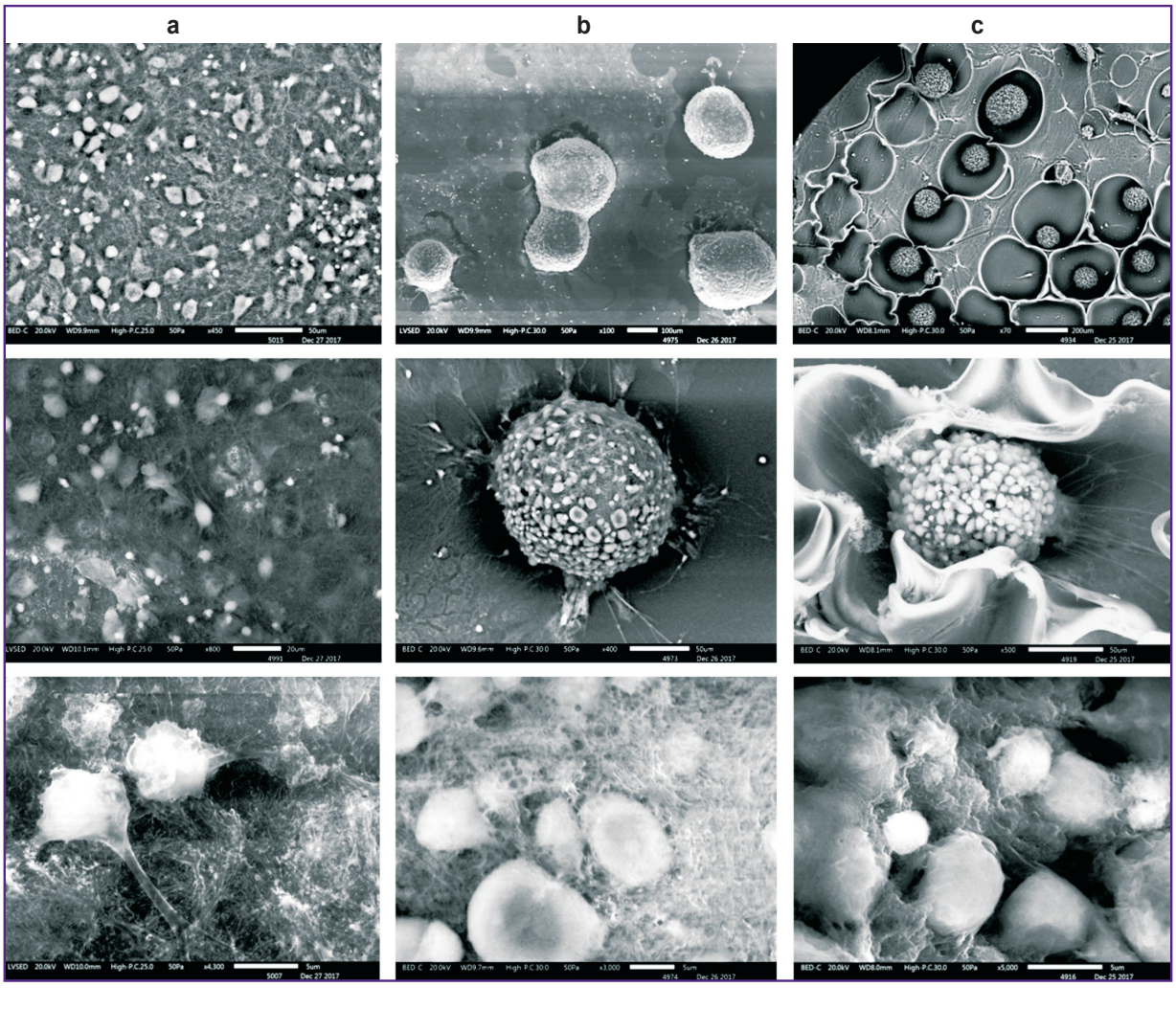

Figure 3. Representative scanning electron microscopy images of primary hippocampal cultures cultivated on different substrates, 14 DIV:
Vertical columns: (a) “Control 1”; (b) “Control 2”; (c) culture cultivated on scaffolds

glial cells were assembled into clusters, which subsequently formed the cell conglomerates (see Figure 2). These conglomerates were connected to each other by complex plexuses of neuronal and glial outgrowth but in a fewer number than conglomerates in the "Control 2" group.

Electron scanning microscopy data revealed that morphometric parameters of single cell and cell conglomerates formed on scaffold had some differences from the parameters of the "Control 2" group (Figure 3). On the background of indistinguishable Feret diameter ("Control 2" — 11.77±0.44 μm, "Scaffold" — 10.84±0.39 μm), the surface area of single cells and the surface area of cell conglomerates were statistically lower than in the control group of cultures (See the Table) ($p<0.05$, ANOVA).

The main morphometric parameters of primary hippocampal cultures cultivated on different substrates

Groups	Feret diameter (μm)	The surface area of single cells (μm^2)	Surface area of cell conglomerates (μm^2)
"Control 1"	13.67±0.36	127.26±3.41	No conglomerates
"Control 2"	11.77±0.44*	74.96±1.89*	33,138.98±4535.89
"Scaffold"	10.84±0.39*	61.53±0.99*#	13,216.04±1253.20#

N o t e: * versus "Control 1"; # versus "Control 2"; $p<0.05$, ANOVA, N=4 in each group.

Using the method of calcium imaging, functional characteristics of the primary hippocampal cultures cultured on the scaffold were evaluated on 14 DIV. On this day of culture development, we observed the spontaneous calcium activity in the control cultures ("Control 1") (Figure 4). The percentage of cells exhibiting calcium activity was equal to 74.4±15.8%; duration of calcium oscillations was 8.8±0.1 s and the frequency of calcium oscillations per minute was 1.0±0.07. Such level of calcium activity is typical for this period of primary cultures development of medium density [22]. Dissociated hippocampal cultures cultivated on scaffolds also showed functional calcium activity. However, the number of cells exhibited calcium activity (25.2±11.6%) was significantly lower than in the "Control 1" group. Changes in the oscillatory profile manifested in fivefold decrease in the frequency of calcium events (0.2±0.01 osc/min) and in the increase of the duration of calcium oscillations (12.6±1.2 s) are

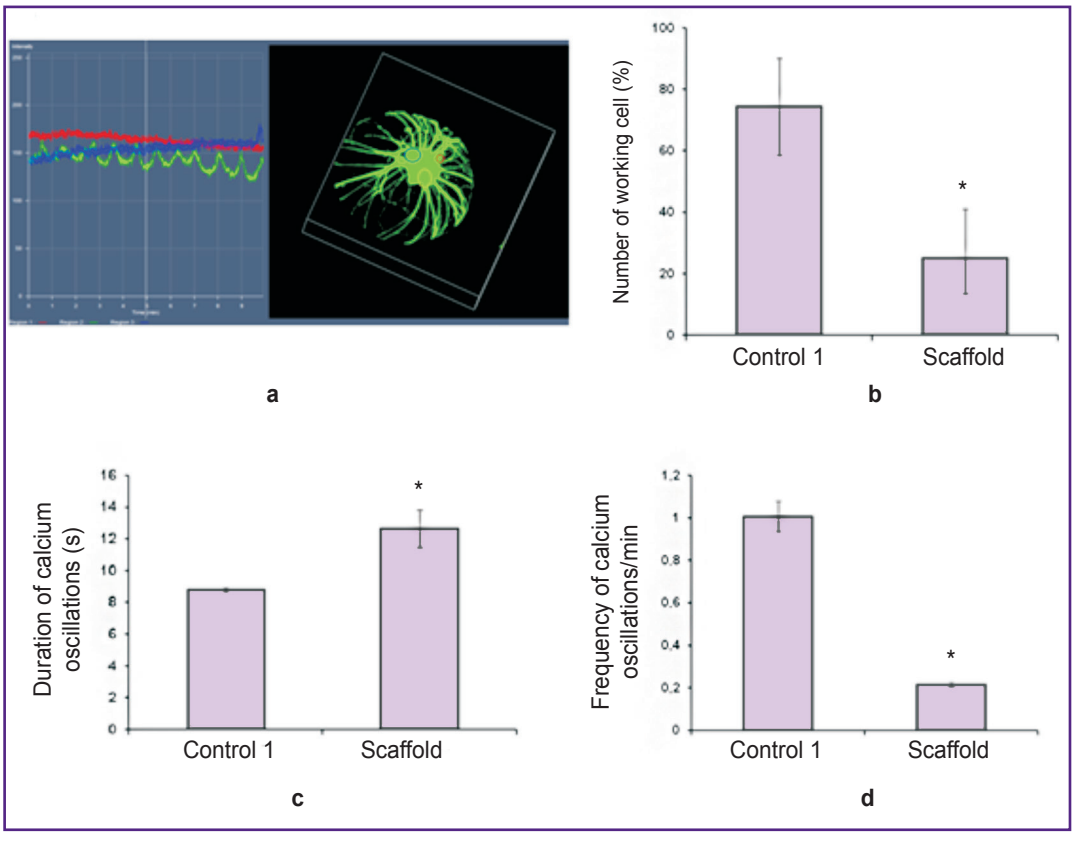

Figure 4. Functional characteristics of primary hippocampal cultures cultured on scaffold: (a) representative recording of spontaneous calcium activity; (b) proportion of cells exhibiting calcium activity; (c) duration of calcium oscillations; (d) frequency of calcium oscillations per minute; * versus "Control 1", $p<0.05$, ANOVA, N=4 in each group

also noted. The decrease in the frequency of calcium events against the background of the increase of calcium oscillations duration suggests the predominant role of glial elements (astrocytes) in the realization of the functional metabolic activity of the culture.

Discussion

Development of three-dimensional constructs with a given architectonics opens promising prospects in regenerative medicine, in particular for brain tissue reconstruction. Extensive use of tissue engineering in substitution therapy offers a solution to the problem of donor tissues accessibility, donor selection, and reduces risk of transplant rejection. Recovery of functionally active elements of neural network supposed to reduce a level of disability and improve a quality of patients life, and also will decrease economic costs for therapy and course of rehabilitation procedures.

The complexity of task for developing scaffolds for neurotransplantation is a satisfaction of constructs to numerous physicochemical and biological requirements. The first mandatory requirements for scaffold materials are the absence of cytotoxicity, the provision of cell adhesion, proliferation, and differentiation.

We hypothesized that compounds actively synthesizing in the brain and include in its structural components can be used as a material for developing scaffold. Application of endogenous substances will negate the development of side effects (neuroinflammation, autoimmune processes, etc.) and thereby minimize risk of neurotransplant rejection.

The basis for the development of scaffold in our research was the derivative of the main component of the brain extracellular matrix — hyaluronic acid. The brain extracellular matrix synthesizes and secretes by neurons and glial cells, which forms stable aggregates in the intercellular space [23]. In the postnatal period, the brain extracellular matrix supports a variety of physiological processes, including synaptic plasticity and homeostatic regulation [17, 18].

Our studies revealed that the material based on hyaluronic acid glycidyl methacrylate (hydrogel film) non-toxic for the nervous system cells. The morphological parameters and the level of cell viability of primary hippocampal cultures cultivated on hydrogel films did not differ from the control group.

To improve the adhesive properties of the material for nervous cells, it was structured into a three-dimensional construction with a given architectonics (scaffold). We showed that the developed construction promotes active attachment of neural and glial cells and contribute to the development of neuronal outgrowth and the formation of network-like structure exhibiting functional metabolic activity.

Thus, scaffolds based on hyaluronic acid glycidyl methacrylate could be considered as a promising material for neurotransplantation.

Conclusion

Scaffolds designed on the basis of hyaluronic acid glycidyl methacrylate have a high biocompatibility with the nervous system cells. Architectonics and adhesive properties of scaffold contribute to the formation of functionally active cell conglomerates.

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Conflict of interests. The authors declare no conflict of the interests mandatory to be reported.

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