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ORIGINAL PAPER

Effect of Silk Fibroin on Neuroregeneration After Traumatic Brain Injury

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Abstract

Traumatic brain injury is one of the leading causes of disability among the working-age population worldwide. Despite attempts to develop neuroprotective therapeutic approaches, including pharmacological or cellular technologies, significant advances in brain regeneration have not yet been achieved. Development of silk fibroin-based biomaterials represents a new frontier in neuroregenerative therapies after brain injury. In this study, we estimated the short and long-term effects of silk fibroin scaffold transplantation on traumatic brain injury and biocompatibility of this biomaterial within rat neurovascular cells. Silk fibroin microparticles were injected into a brain damage area 1 day after the injury. Silk fibroin affords neuroprotection as judged by diminished brain damage and recovery of long-term neurological functions. We did not detect considerable toxicity to neuro-vascular cells cultured on fibroin/fibroin-gelatin microparticles in vitro. Cultivation of primary cell cultures of neurons and astrocytes on silk fibroin matrices demonstrated their higher viability under oxygen-glucose deprivation compared to 2D conditions on plastic plates. Thus, we conclude that scaffolds based on silk fibroin can become the basis for the creation of constructs aimed to treat brain regeneration after injury.

Keywords Scaffold · Neurons · Astrocytes · Neuroprotection · Fibroin · Ischemia

Introduction

The lack of effective therapeutic strategies to assist healing to maintain the integrity of neural tissue after acute cerebral injuries, such as stroke or trauma, remains a serious clinical problem and requires the search for new approaches, in addition to conventional therapies [1]. One such approach is tissue repair using bioscaffold-based grafts. In the field of regenerative engineering, the use of different acellular biomaterials has introduced a new approach to treat different human disorders in which cell death and/or degeneration constitute a common factor [2]. Brain damage by mechanical trauma and hemorrhagic or ischemic stroke and several degenerative disorders, such as Alzheimer's, Huntington's or Parkinson's disease, represent the main causes of neurological dysfunction in humans. Effective therapies to promote substantial recovery after brain injury are currently not available. Brain ischemia, as well as traumatic brain injury, impair both the mechanical integrity of the nervous tissue and the functional links between neural cells and the extracellular matrix, which plays a key role in the positioning of cells, cell-to-cell crosstalk and extracellular signaling. This is particularly important for the extracellular matrix surrounding endothelial cells of vessels, which changes can lead to dysfunction of the blood–brain barrier [3]. As a result of stroke or traumatic brain damage, the formation of a lesion occurs which further forms a glio-mesodermal scar. Normal extracellular matrix (ECM) is absent in this

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area, and there is no mechanical support for the formation of new neurites, angiogenesis and other growth processes [4]. Consequently, cells adjacent to a lesion suffer from ischemia, yielding activation of astroglia [5], which may be considered as a starting point of massive local inflammation and infiltration of immune cells [6], which can also corrupt the processes of restoration of the structural and functional architecture of brain tissue. Over time, inflammation and proliferation of astrocytes lead to remodeling of brain tissue in the injury zone with the formation of a fibrotic scar instead of nervous tissue [7]. The cavity formed in the injury locus is a convenient place for transplantation of cellular allografts or biomaterials, because it allows introduction of significant amounts of injected substance without affecting the areas of the intact part of brain unaffected by damage [8]. Moreover, both in the case of traumatic injury, and especially in focal ischemia, the zone of destruction of the nervous tissue, in which a scaffold could be introduced, is surrounded by the penumbra zone, the place of the greatest activity of neovascularization and neurogenesis, the impact of which can be therapeutically studied [9].

The most successful strategy for recovery of the brain after injury involves stimulation of angiogenesis, neural regeneration, reduction of inflammation and ensuring the directed growth of axons and neurites by delivery of scaffolds to the damage area, providing a physical basis for formation of a new neuro-vascular structures. As we have already said, with pathological interventions such as trauma, not only cells are damaged, but also extracellular structures, primarily the ECM and potentially, the scaffold as a 3-D tissue mimic, would substitute for ECM of the brain. However, there is still some controversy regarding the role of ECM. Previous studies have shown that ECM removal improves post-traumatic regeneration in the brain [10] and to some extent restores plasticity, memory and enhances cognitive flexibility [11–13]. However, subsequent research proved that the ECM plays an important role in the retention of existing connectivity in mature neurons (possibly through optimization of ambient glutamate concentration preventing its spillover and protecting extrasynaptic glutamate receptors from activation and desensitization), while ECM removal provides better connectivity in a newly-formed neuronal network [14]. Taken together, the data demonstrates the important role of extracellular architecture in organizing and maintaining neuronal connectivity, supporting the necessity of restoration of this architecture when it is damaged even by using acellular materials, namely biopolymers. Recent advances in technology used to create biopolymers, hydrogels and other scaffolds have made it possible to obtain different ECM mimetics that directly support the survival of neural cells and the invasion of blood vessels and nerve sprouts from the surrounding tissue [15]. Biomaterials used for this purpose, such as matrigel [16], PLGA [17], alginate [18], collagen [19] or hyaluronic acid [20] have been demonstrated to improve functional recovery in rodents experienced brain injury. Using adequate materials that can produce minor adverse effects compared with the dysfunction caused by the disease itself may compensate for the aggressive character of this procedure and justify this type of therapy, especially in patients with more severe neurological deficits. Silk fibroin meets most of the standards of a biomaterial suitable for the above-mentioned applications. Although this biomaterial has never been used in preclinical models of brain repair, silk fibroin composites have been employed as anti-epileptic drug carriers [21] and in the regeneration of peripheral nerves in rats as nanoparticles or in fibrillar structures [22, 23]. Due to the emerging potential of silk fibroin for brain therapeutic opportunities and to the possibility for getting silk fibroin in the form of microparticles [24], we examined the short and long-term effects of silk fibroin scaffolds transplantation on traumatic brain injury and the biocompatibility of this biomaterial.

Materials and Methods

Scaffolds and Microparticles Assembly

Fibroin scaffolds and composite fibroin matrices supplemented with 30% gelatin were prepared as previously described [25]. Briefly, sericin-free silk fibers (LLC «Optikum») were dissolved in a mixture of $CaCl_2$:C_2H_5OH:H_2O (Sigma Aldrich) in 1:2:8 molar proportion in water bath for 5 h at 80 °C temperature. The resultant solution was centrifuged and dialyzed against distilled water for 24 h. Next, the solution was centrifuged and its concentration was brought to 20 mg/ml by adding distilled water. This solution was used to prepare fibroin scaffolds.

To prepare composite scaffolds, type A gelatin from Sigma Aldrich was dissolved in distilled water at 37 °C, fibroin and gelatin solutions (20 mg/ml) were mixed in 7: 3 volume ratio. In order to make experimental fibroin-based scaffold and composite fibroin scaffold specimens with gelatin by the freezing–thawing method, 1% DMSO was added to polymers solutions. Scaffolds were formed in the well of 24-well plates; 700 µl solution was added to each well. The plates kept for 7 days at − 35 °C. Frozen scaffolds were processed with 96% ethanol for 1 h, and then transferred to 70% ethanol for storage.

Fibroin-gelatin microparticles were generated by cryodestruction of fibroin sponge substituted with 30% gelatin. Made microparticles were passed through the filter with pore size of 500 µm, 250 µm and 100 µm. The target fraction was represented by microparticles that passed through the pore size of 500 µm and 250 µm, but did not pass through the pore size of 100 µm.

To describe scaffold structure in aqueous medium, confocal laser scanning microscopy was used. The scaffold material was conjugated with tetramethylrhodamine (TRITC, Invitrogene). The samples were incubated for 1 h in 90 mM TRITC solution in PBS (pH 7.5) in dark conditions at room temperature. The reaction was stopped by transferring a sample to 0.1 M Tris for 30 min. Unbound dye was washed out with phosphate saline buffer.

Traumatic Brain Injury Model (TBI)

The animal protocols used in this work were evaluated and approved by the institutional animal ethics committee in accordance with FELASA guidelines. The experiments were performed on outbred white male rats (350–400 g). The animals had unlimited access to food and water and were kept in cages with a temperature-controlled environment (20 ± 2 °C) with light on from 9 a.m. to 9 p.m. For all surgical procedures, rats were anesthetized with i/p injections of 300 mg/kg (12%) chloral hydrate. A feedback-controlled heating pad maintained the core temperature (37.0 ± 0.5 °C) supplemented with an infrared lamp until awake.

In the present work, we employed our own modification of the earlier used model of focal open severe brain trauma in rats [26, 27]. To generate the trauma, the rats were positioned in a stereotaxic frame (NeuroStar Robot Stereotaxic, Germany), the left frontal part of the skull was trepanized above the sensorimotor cortex zone and a movable Teflon piston 4 mm in diameter with depth of insertion of 2.5 mm was placed into it; this piston was struck from the height of 10 cm with a 50 g load sliding along a directing rail. For localization of the sensorimotor cortex zone, we used the following stereotaxic coordinates; +4 to −3 mm anterior and posterior from bregma and +1 to +4.5 mm lateral from the midline. In sham-operated rats, the experiments were done using the same protocol except that trauma was excluded. Rats were randomly divided by three groups as follows: (1) Sham + Saline (n = 8), (2) TBI + Saline (n = 8), (3) TBI + fibrioin (n = 8). The volume of damage was quantified by analyzing brain magnetic resonance (MR)-images obtained 14 days after the TBI as described previously [28].

One day after TBI induction and confirmation of neurological defecit, the rats were placed in a stereotaxic frame and, under aseptic conditions, the sutures were removed and the skull was exposed. In the TBI + fibroin group of animals, suspension of fibroin particles in a saline was transplanted into a single locus in the center of lesion area of the sensorimotor cortex using a Hamilton syringe with a needle (Microliter 702 LT). The coordinates of the administration point were taken using the stereotaxic's software. The syringe needle was inserted up to a depth of 2.9 ± 0.2 mm through the hole in the skull. The solution was then infused in a total volume of 20 µl at a rate of 1 µl/min using a micro-screw in order to reduce the pressure on the surrounding tissue. When the solution was infused, the needle was pulled out with a rate of 0.5 mm/min. In the control and sham groups, rats received the same volume of saline.

Limb-Placing Test

A modified version of the limb-placing test, consisting of seven tasks, was used to assess forelimb and hindlimb responses to tactile and proprioceptive stimulation [29]. The rats were habituated for handling and tested before operation and at 1st, 4th, 7th and 14th post-injury days. For each task, the following scores were used: 2 points, normal response; 1 point, delayed and/or incomplete response; 0 points, no response. The total score over seven tasks was evaluated.

Cylinder Test

Asymmetry of forelimbs use was evaluated in the cylinder test during spontaneous exploration of the cylinder walls [30]. The tests were performed on day 14 after TBI modeling. The rat was placed into a transparent cylinder (30 cm height and 20 cm in diameter) and its movements were recorded over 5–8 min with a camcorder positioned above the cylinder. The independent use of the contra- and ipsilateral forelimbs during cylinder wall exploration in a rear posture and their simultaneous (combined) use were counted. The frequency of forelimb use was calculated by the formula $(contr + 1/2 \times simult)/(ipsi + simult + contr) \times 100$, where *contr* and *ipsi* corresponded to the use of contralateral (damaged) and ipsilateral limbs and *simult* corresponded to simultaneous use of both limbs.

Cell Culture Experiments

The effects of fibroin-gelatine microparticles and scaffolds on the neurons in vitro were examined on 7-day cultures of cerebral cortical neurons isolated from rat embryos of 16–18 days gestation by enzymatic and mechanical dissociation as described by Brewer [31]. The cerebral cortex was transferred to a Petri dish with PBS and purified from meninges; the tissue was incubated for 15 min at 37 °C in PBS containing 0.05% trypsin and 0.02% EDTA, the culture was washed twice with PBS and once with Neurobasal medium (NBM) with 2% B27 supplement (Invitrogen) and then mechanically dissociated with a Pasteur pipette in NBM/2% B27. The cell suspension was centrifuged for 2 min at 210*g* and 21 °C and the pellet was resuspended in growth medium (NBM with 2% B27 and 0.5 mM L-glutamine). The cells were cultured on scaffolds, fibroin microparticles or in 96-well plastic plates coated with poly-L-lysine. Cultures

were grown in a CO_2 incubator at 37 °C with 5% CO_2. The medium was changed on day 4 of culturing.

Cultures of astroglial cells were prepared from the brain of 2–3-day-old rats according to a modified method [32]. The cerebral cortex was isolated, purified from the meninges, and incubated in PBS containing 0.05% trypsin and 0.02% EDTA at 37 °C for 30 min. After the enzymatic dissociation, the tissue was washed twice in PBS and then mechanically dissociated using a Pasteur pipette in enriched DMEM/F-12 (1:1) culture medium supplemented with 10% fetal calf serum (PAA), glutamine, vitamins (PanEco), and amino acids (Gibco). The cell suspension was seeded in 75-cm^2 ventilated flasks (Costar) and grown in enriched medium in a CO_2 incubator at 37 °C and 5% CO_2. In 8–10 days, the cultures were shaken on an orbital shaker at 250 rpm for 16 h to detach and remove microglia. Passage 3 cultures were transferred on scaffolds placed in 6-well plastic plates and used in the experiments.

Endothelial cells EA.hy926 provided by Prof. C. J. Edgel (Carolina University, USA) reproduce the main morphological, phenotypic and functional features of microvascular endothelial cells. These cells were cultured in an enriched medium consisting of DMEM/F-12 (1:1), 10% fetal calf serum (PAA), penicillin and streptomycin (100 U/ml), 2 mM L-glutamine (PanEco) and HAT (PanEco), supplements containing hypoxanthine (5 mM), aminopterin (20 mM), and thymidine (0.8 mM). The cultures were seeded on scaffolds placed in 6-well plastic plates and used in the experiment.

Oxygen-Glucose Deprivation (OGD) Model

Ischemia in vitro was modelled as described previously [33]. Cultured cells were washed twice with balanced salt solution containing (in mM) 116 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 NaH_2PO_4 (pH 7.3) and then incubated in this solution in a humidified chamber filled with nitrogen at 37 °C for 5 h. For control cultures, culturing conditions remained unchanged throughout the experiment. Immediately after OGD, saline was replaced with culture medium.

Cell Viability Estimation

Cells were collected 24 h after OGD and incubated with Muse Count & Viability Kit (Merck Millipore, USA) for 5 min. The number of viable cells was determined by using the MUSE Cell Analizer (Merck Millipore, USA). For estimation of neurons viability 1, 4 and 7 days of in vitro culture on fibroin/gelatin microparticles Live/Dead staining was performed with LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (ThermoFisher) according to the manufacturer's instructions. Stained cells were observed using an Eclipse Ti-E microscope with an A1 (Nikon Corporation, Japan) confocal module and a CFI Plan Apo VC 10×/0.75 objective. Samples were maintained at 37 °C in an ambient atmosphere containing 5% CO_2 during the experiment.

MTT-Test

Cell cultures were established seeded on fibroin/gelatin microparticles as described above. After 1, 4 and 7 days, 200 µl of MTT solution (5 mg/ml in culture medium) were added and incubated at 37 °C for 4 h. The medium with the microparticles was collected and centrifuged at 14,500*g*. DMSO was added to the precipitate and then the solution was centrifuged at 14,500*g*. Colorimetric measurements were performed at 540 nm.

Immunofluorescence Imaging

Cells were fixed after 24 h post-seeding in 4% paraformaldehyde solution in PBS for 30 min at room temperature and rinsed three times with PBS. Samples were permeabilized in 0.1% Triton X-100 in PBS for 15 min at 4 °C and rinsed twice with PBS before 1 h incubation at room temperature in 1% FBS and 0.1% Tween in PBS. Mouse primary antibody directed against NCAM (Thermo Fisher Scientific) was diluted (1:100) in PBS containing FBS 0.1% and Tween 0.1% (w/v) and incubated at room temperature for 1 h. The cells were washed five times with 0.1% BSA/ 0.1% Tween/ PBS solution. Goat anti-Mouse IgG (H + L) secondary antibody, conjugated with Alexa Fluor® Plus 647 (Thermo Fisher Scientific) was diluted (1:200) in PBS containing FBS 0.1% and Tween 0.1% (w/v) and incubated at room temperature in the dark for 1 h. Alexa Fluor® 488 anti-βIII Tubulin antibody (Biolegend), and Hoechst 33342 (Thermo Fisher Scientific) were added and incubated at room temperature in the dark for 1 h. The samples were then rinsed with PBS, and observed using a Eclipse Ti-E microscope with an A1 (Nikon Corporation, Japan) confocal module and a CFI Plan Apo VC 20×/0.75 objective.

Results

The Structural Organization of Scaffolds

To study the effectiveness of fibroin and fibroin-gelatin scaffolds for brain tissue regeneration, three-dimensional spongy scaffolds (Fig. 1a) were developed as well as microparticles based on these scaffolds (Fig. 1c). The surface structure of the obtained fibroin-gelatin scaffolds and microparticles was characterized by confocal laser scanning microscopy. Spongy scaffolds had a three-dimensional porous structure with complex topography (Fig. 1b), providing a substrate for cell adhesion. Microparticles from fibroin-gelatin were

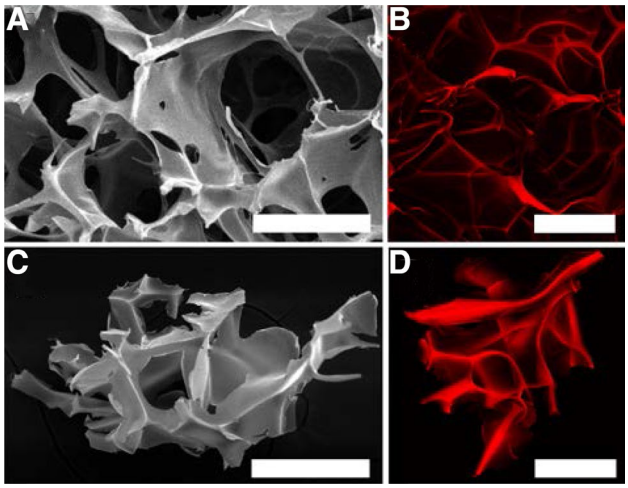

Fig. 1 Scaffolds and microparticles based on fibroin supplemented with 30% gelatin. **a, c**—Scanning electron microscopy. Scale bar, 100 µm, **b, d**—Confocal images of TRITC bounding material. **a, b**—Porous scaffold, generated by freezing–thawing method; **c, d**—Microparticles generated by cryodestruction of porous scaffold. Scale bar, 100 µm

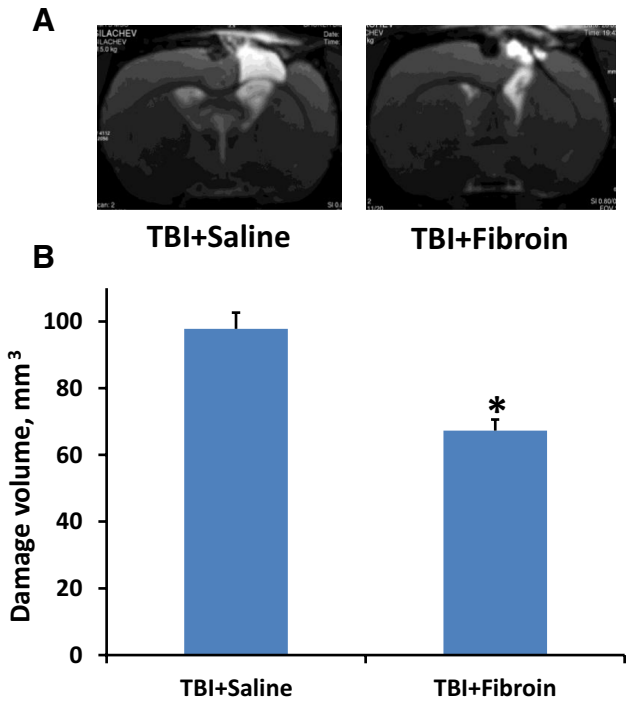

Fig. 2 Protection against traumatic brain injury by the fibroin-gelatin microparticles transplantation starting 24 h after trauma. **a**—Representative T2-weighted MR-images from coronal brain sections [0.8 mm thick, from rostral (top) towards caudal (bottom)] obtained on the 14th day after TBI induction. Light regions refer to damage areas. **b**—Damage volume was evaluated by using MRI with analysis of T2-weighted images. Data is expressed as mean ± SEM. *$p < 0.05$, T-test

formed by the method of cryo-grinding and consisted of the fragments of scaffolds that preserve spongy structure and characterized by a size of 100–250 µm (Fig. 1a).

The Effect of Fibroin-Gelatin Microparticles Transplantation on Traumatic Brain Injury

In our experiments, TBI caused extensive damage of sensorimotor cortex (Fig. 2a). When compared with the control group, the transplantation of fibroin-gelatin microparticles in the damage zone reduced the damage volume by 30% ($p < 0.05$, Fig. 2b). The results of the limb-placing test revealed the development of a post-traumatic functional deficit in the right limbs (contralateral side of damage), whereas it was absent in the left limbs. Before the induction of trauma, the intact rats scored 14.0 ± 0 in limb-placing test, while on the first day after TBI rats scored only 1.7 ± 0.2. The transplantation of fibroin-gelatin microparticles restored the neurological status by 25% starting from 4th days after TBI induction compared with saline treated group (Fig. 3a). Using the cylinder test, we found that TBI results in asymmetry in the use of the forelimbs. Normally, rats use both the left and right limbs in the same proportion when exploring the walls of the closed space in the glass cylinder test (Fig. 3b). Quantitative analysis showed that before TBI modeling, the animals used each forelimb with a frequency of 50% (Fig. 3c). On the other hand, 14 days after the simulation of TBI, the frequency of the contralateral paw usage was reduced from 22 to 3% (Fig. 3b). Injection of fibroin-gelatin microparticles into damaged area partially restored the contralateral paw use to 8% (Fig. 3b) or by 30% when counting the percentage of simultaneous use of both limbs (Fig. 3c).

The Effect of Fibroin Scaffolds and Microparticles on the Survival of Neurons, Astrocytes and Endothelial Cells In Vitro

Ischemic injury of primary cultures of neurons, astroglia, and endothelium was simulated in vitro by OGD for 5 h. To analyze the effect of three-dimensional culturing on the cells tolerance to ischemia, cells were seeded on scaffolds formed of fibroin or fibroin containing 30% gelatin. We found that OGD caused significant cell death; the average number of viable cells after OGD in the case of cells cultured in 2D conditions on the plastic dish was 38 ± 2.9% for neurons, 78 ± 2.7% for astrocytes and 73 + 12% for endothelial cells (Figs. 4a, 5a, 6a). Cultivation in a three-dimensional fibroin scaffold increased the survival of neurons: viability was 51 ± 3.9% for the fibroin-based scaffold (Fig. 4b, d) and 48 ± 3.5% for fibroin-gelatin (Fig. 4c, d). The viability of

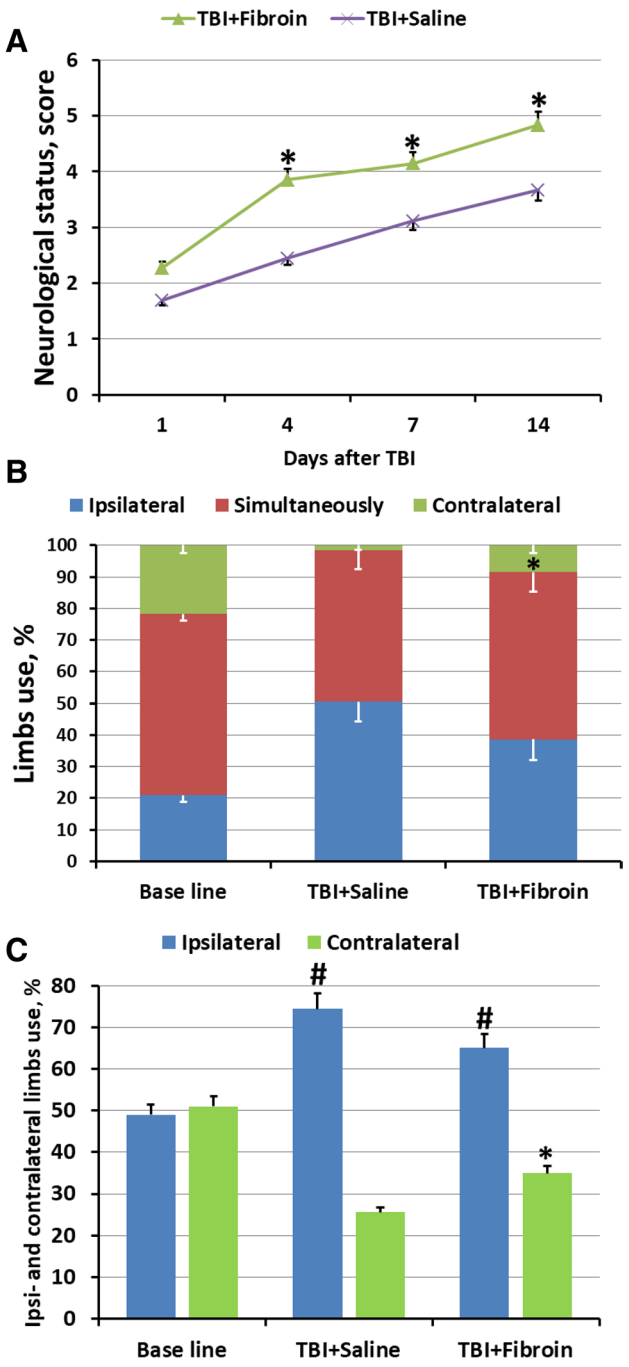

Fig. 3 Effect of fibroin-gelatin microparticles transplantation on the neurological status of animals. **a**—Neurological deficit scores estimated in the limb-placing test. **b, c**—TBI induced limb asymmetry measured in the cylinder test on 14 day after the surgery. Data is expressed as mean ± SEM. $^{\#}p < 0.05$ denotes significant difference from base line; $^{*}p < 0.05$ denotes significant difference from TBI + Saline group (Kruskal–Wallis test with the Mann–Whitney u-test)

astrocytes after OGD was also increased when culturing on matrices, rising to 84 ± 3.1% in the case of fibroin scaffold (Fig. 5b, d) and 87 ± 4.8% in the case of fibroin-gelatin (Fig. 5c, d). The survival of endothelial cells after OGD on scaffolds did not significantly change. The viability was 70 ± 4.2% and 61 ± 3.3% for cultivation on fibroin and fibroin-gelatin, respectively (Fig. 6b–d).

The protective effect of scaffolds on neurons exposed to OGD was confirmed for the injectable form—microparticles. MTT assay and LIVE/DEAD staining were used to study the increased tolerance to OGD of neurons immobilized on microparticles.

Metabolic activity of primary neurons cultured on fibroin-gelatin microparticles was determined at 1, 4 and 8 days of cultivation in standard conditions and 24 h after the OGD. By the 8th day of cultivation on fibroin-gelatin on microparticles in standard conditions, MTT signals significantly increased compared to 1 day (Fig. 7k). In addition, 24 h after OGD, the metabolic activity of cells on microparticles was higher compared to cells on a plastic dish (Fig. 7l).

LIVE/DEAD Staining

To examine neuron viability during cultivation on microparticles, CLSM was used. Cells were stained with Calcein-AM and ethidium homodimer-1 at 1, 4 and 8 days of cultivation. Viable cells exhibited green fluorescence that was generated by the esterase activity hydrolyzing the membrane-permeant dye, Calcein-AM. Dead cells acquired red fluorescence of ethidium homodimer-1. Neurons cultured on polylysine-coated glass slides were used as a control (Fig. 7a–j1). About 500–550 cells were counted for each group. The percentage of viable cells immobilized on microcarriers was higher than 75% at all studied time intervals (Fig. 7m). Results of the viability-test were not different for cells either on microcarriers or on PL glass, which can be acknowledged as a microcarrier to be a suitable substrate for neuronal cells (Fig. 7m). To evaluate the potential protective effect of microparticles after exposure to OGD, cells were stained with Calcein-AM and ethidium homodimer-1 24 h after the challenge. The percentage of viable cells was 49.35%, which is close to the results obtained for the fibroin-gelatin scaffolds (Fig. 7n).

Since the neurites were also stained with the Calcein dye of the LIVE/DEAD stain, captured confocal images could be used to evaluate neurite morphology. On the 8th day of culturing neurons on the microparticles, the neurites of the cells formed extensive branched networks that connected the cell body with different microparticles. Single microcarriers with dimensions of 100–250 microns were organized into assembles up to 700 microns in size, consisting of several microparticles that were joined together by cultivated cells (Fig. 7f1, j1). The size of the assemble was affected by the OGD challenge (not shown).

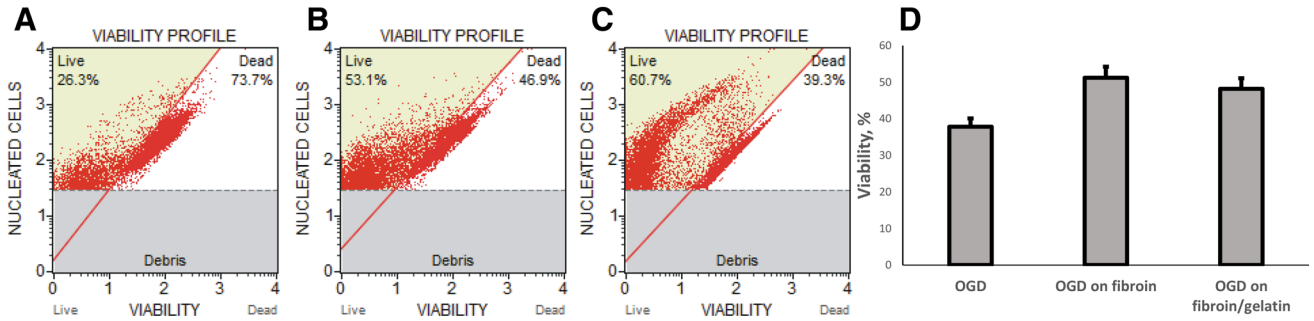

Fig. 4 Neuronal death in the simulation of OGD in a two-dimensional culture (**a**), on a scaffold made of silk fibroin (**b**) and of fibroin copolymerized with gelatin (**c**). Representative images of MUSE count/viability dot-plots. Distributions of live and dead cells after analysis on MUSE Cell Analyzer and the average viability obtained by three independent experiments (**d**)

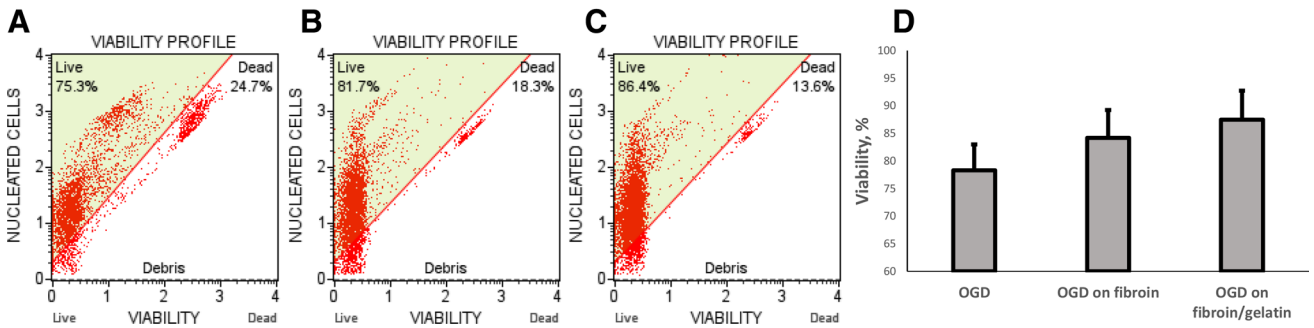

Fig. 5 Astrocytes cell death in the simulation of OGD in a two-dimensional culture (**a**), on a scaffold made of silk fibroin (**b**) and of fibroin copolymerized with gelatin (**c**). Representative images of MUSE count/viability dot-plots. Distributions of live and dead cells after analysis on MUSE Cell Analyzer and the average viability obtained by 3 independent experiments (**d**)

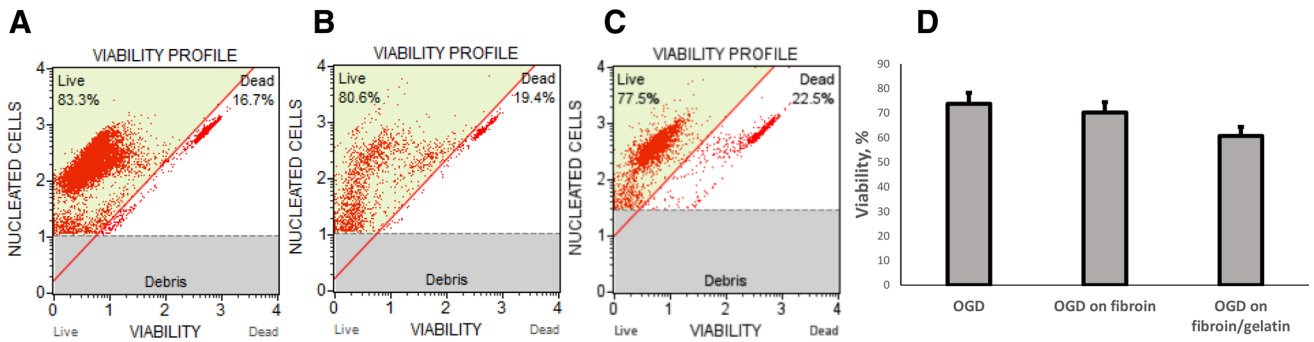

Fig. 6 Cell death of endothelium in the simulation of OGD in a two-dimensional culture (**a**), on a scaffold of silk fibroin (**b**) and fibroin copolymerized with gelatin (**c**). Representative images of MUSE count/viability dot-plots. Distributions of live and dead cells after analysis on MUSE Cell Analyzer and the average viability obtained by three independent experiments (**d**)

Immunofluorescent Staining

To study the adhesion of primary neurons to the surface of microparticles, as well as cell–cell junctions, the neurons were probed with antibodies to the neuronal cell adhesion molecule (NCAM) and β-III tubulin (Fig. 8a–h). On the first day of cultivation, it is seen that the cells are attached to the substrate and begin to form lamellipodia, both on

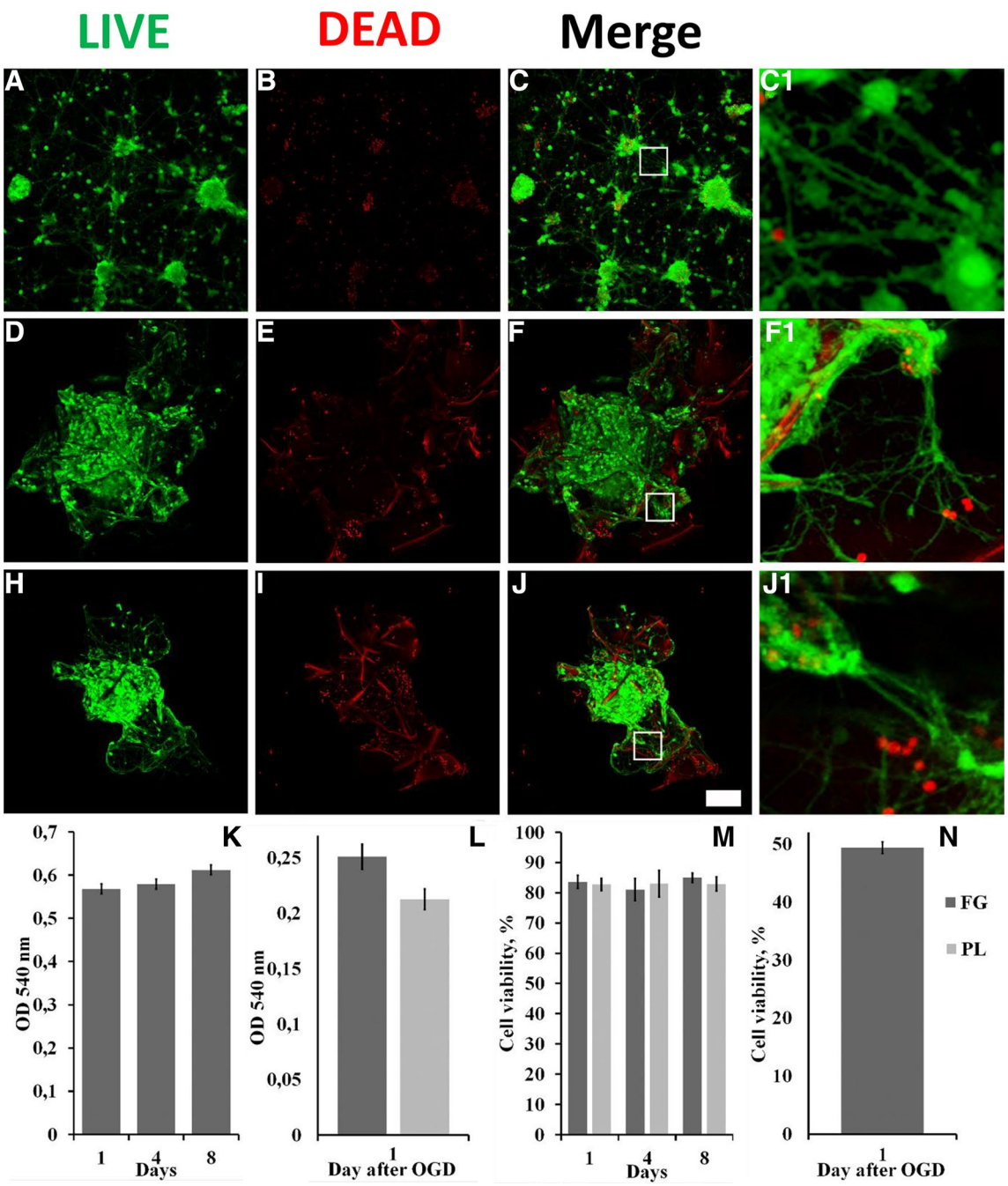

Fig. 7 Evaluation of viability of neurons on fibroin-gelatin microparticles. **a**–**j1**—LIVE/DEAD staining of primary neurons, viable cells exhibit green fluorescence of Calcein, dead cells acquire red fluorescence due to penetration of ethidium homodimer-1. Scale bar—100 µm. **a**–**c** and **c1**—Cells cultured on polylysine coated glass slides (PL) for 8 days; **d**–**f** and **f1**—cells cultured on fibroin-gelatin (FG) microparticles for 8 days; **h**–**j** and **j1**—cells cultured on fibroin-gelatin microparticles on the first day after OGD; **c1, f1** and **j1** show magnification of selected areas, **k, l**—the results of MTT test, for neurons cultured in standard conditions and for neurons on the first day after OGD, respectively; **m, n**—percentage of the viable cells for neurons cultured in standard conditions and for neurons on the first day after OGD, respectively

fibroin-gelatin microparticles and on polylysine coated glass (Fig. 8b, f). In addition, the cells express neuronal markers NCAM and β-III tubulin on both kinds of surfaces (Fig. 8c, g).

Discussion

It is believed that a three-dimensional architecture for organizing cell culture systems, mimicking the cytoarchitecture of

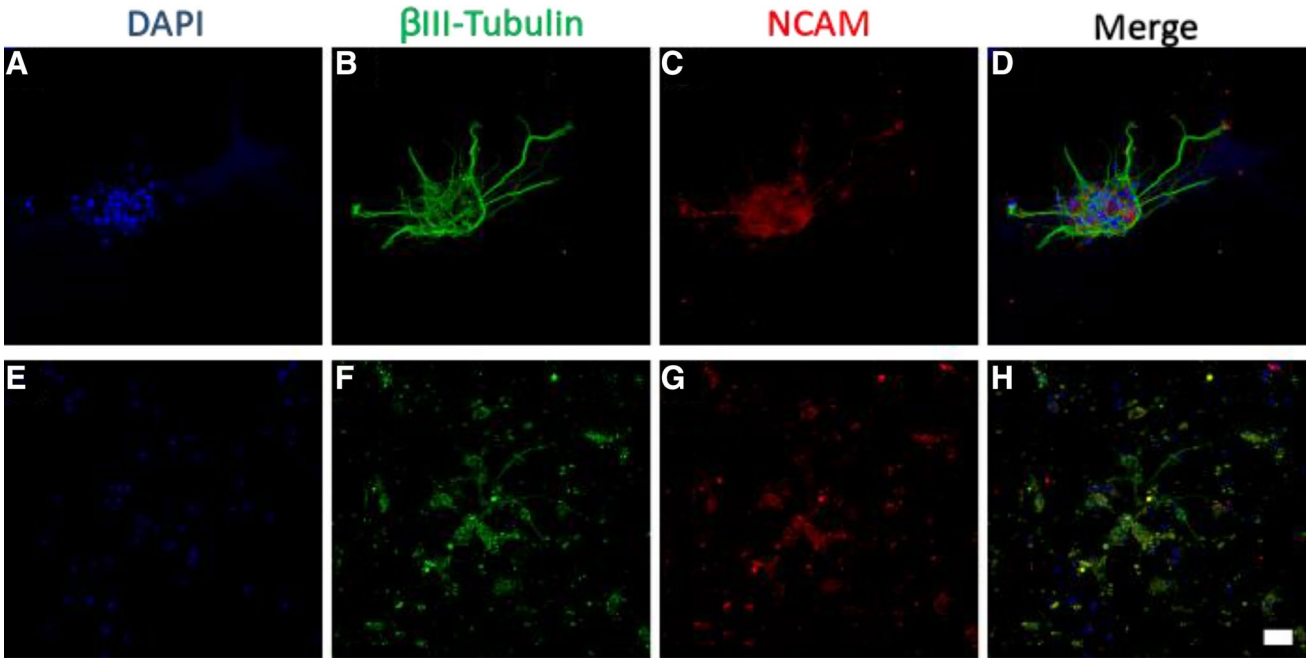

Fig. 8 Adhesion of neurons on fibroin-gelatin microparticles. **a**–**d**—Neurons cultured on fibroin-gelatin microparticles for 24 h; **e**–**f**—neurons cultured on polylysine coated glass for 24 h. **a, e**—DAPI stained cell nuclei, **b, f**—β-III tubulin reactivity, **c, g**—anti-NCAM reactivity, **d, k**—merged images. Scale bar—20 µm

tissue, provides more physiologically reliable conditions for cell cultivation than conventional systems of cell growth in a monolayer. Due to their complex surface, three-dimensional structures provide a larger area for cell adhesion, growth, migration and interaction, as well as other factors affecting cell differentiation and maturation. Three-dimensional cultivation avoids physiological and morphological disorders in cells, such as excessive spreading (tension) of cells and changes in the shape and size of the nucleus. Moreover, scaffolds can have a protective effect, preventing adverse effects of microenvironments, such as changes in pH, pressure, etc. Scaffolds can increase cell survival through positive intercellular cooperation. Recently, it was shown that dopaminergic neurons isolated from the embryonic brain retain their viability for a longer time if cultured under 3D conditions [34]. Hippocampal neurons also formed longer neurites and remained viable when exposed to neurotoxic substances in the 3D cultural system, in contrast to 2D culture [35].

Classical tissue engineering strategies are not well suited for brain repair because they require the invasive implantation of the tissue construct, and it is unclear whether if functional brain tissue can be engineered/made ex vivo. In situ tissue regeneration aims to completely circumvent the ex vivo generation of the engineered organ by implanting a scaffold directly at the site of injury in order to stimulate the development of an endogenous tissue repair skeleton through the use of local or transplanted progenitors. One of the preferred materials for creating scaffolds is silk fibroin because of its numerous properties that are superior to other natural biopolymers [36]. This fibrillar protein is used to regenerate both hard tissues such as bone and connective tissues, as well as nervous tissue. Fibroin, being not immunogenic, has high biocompatibility and an optimal biodegradation rate. Additionally, by changing the concentration of silk or adding plastificators, it is possible to obtain soft substrates, which makes it an attractive material for use in regeneration of nervous tissue. Recently, comparison of fibroin gels with different degrees of stiffness revealed that elongation of dorsal root neurites ganglia occurred on substrates with a stiffness modulus less than 5–80 kPa [37]. Hopkins et al. studied the possibility of culturing primary cortical neurons on hydrogels with 1, 2, 4, and 8% w/v fibroin modified by neurotrophin-3 (NT-3) and having values of Young's modulus of 4.8, 7.4, 22.4 and 33.1 kPa, respectively [38]. The maximum length of neurites was achieved at 2 and 4% hydrogel with the value of the Young's modulus of 7.4 and 22.4 kPa, which is explained both by the optimal stiffness of the substrate and the immobilization of the growth factor in the gel. The stiffness of fibroin scaffolds used in this study was 5.9 kPa for the pure fibroin and 9.0 kPa for the fibroin-based scaffolds containing 30% gelatin, which corresponds to the rigidity of the substrate that is required for neurogenesis [39].

After TBI in adults, injured axons cannot regenerate past the lesion due to the formation of a astroglial scar, which consists predominately of reactive astrocytes and proteoglycans. To overcome the inhibitory environment of the scar,

treatments should ideally provide a growth supportive root across the lesion cavity, intrinsically enhancing the ability of neurons to elongate and minimize the effect of extrinsic inhibitors that block growth in the immediate environment of the scar [40]. Probably, the restoration of local volumes of the brain tissues appeared to be possible by reducing the formation of the glial scar and the production of artificial intercellular matrix as a basis for the sprouting of axons through the area of injury. The use of biodegradable scaffolds for this purpose should meet the following requirements: biocompatibility, a three-dimensional structure, the ability to maintain adhesion, proliferation and migration of cells. In addition, scaffolds should have an optimal biodegradation rate for gradual replacement by regenerating tissue, providing cellular invasion and physical support for the growing of axons [41]. We suggested that the transplantation of fibroin-gelatin microparticles into the lesion area of brain should contribute to the creation of the extracellular matrix, as a basis for the attachment of endothelial cells and astrocytes, and guide the direction for the growing of neuronal sprouts. Indeed, in vitro cultivation of neural cells on fibrin scaffolds contributed to their survival under ischemic conditions. It is known that silk fibroin can support the growth of several cell populations that are native to nervous tissue, such as neurons and astrocytes [42–44]. Supplementation with gelatin in the composition of the fibroin-based scaffold permits an improvement in its properties through the introduction of integrin-binding amino acid sequences, as well as to increase the hydrophilicity of the substrate [45]. Gelatin is a natural biopolymer obtained by hydrolysis of collagen, containing conservative Arg-Gly-Asp (RGD) motifs. Such sequences are able to bind with integrin receptors, which is involved in adhesion, migration, and proliferation [46]. In addition, some studies indicate that including specific neuronal adhesion molecules NCAM and L1-CAM can also interact with RGD adhesion and cell–cell binding through homophilic interaction, and interacts with other cellular proteins, such as receptors and membrane components of the cytoskeleton. It has been shown that NCAM plays a critical role in increasing the length of neurites [47]. L1-CAM is a neural cell adhesion molecule, affecting adhesion, migration, and sprouting of neurons, myelination and neuronal differentiation [48]. Thus, the presence of RGD sequences in the scaffold composition not only improves cell adhesion, but also contributes to more accurate simulation of the extracellular matrix in vivo.

Our analysis of cell survival showed that cultivation of primary neurons on fibroin-gelatin microparticles retains high cell viability throughout the entire cultivation, and neurons in these conditions are able to form assemblies up to 700 microns in size due to migration of neurites to neighboring microcarriers. This observation confirms our assumption that scaffolds based on fibroin are promising objects for the regeneration of nervous tissue, as they are able to maintain the elongation of neurites. In addition, the ability of microparticles to assemble into larger structures can be used for a so-called "bottom-up" tissue engineering approach or modular tissue engineering [49]. This approach has several advantages over traditional tissue engineered "from top to bottom", when cells are passed into a macroscopic scaffold.

Engineered micro-carriers can be used for one of the varieties of modular tissue engineering [50]. Cell cultivation on microcarriers contributes to their homogenous distribution and maintenance of the uniform conditions of cultivation, and a small size of microscaffolds reduces the probability of necrotic area occurrence, which is common for static cultures at high cell density [50–52].

Modular tissue engineering techniques are in demand for the development of organotypic three-dimensional cell cultures, which can be used as model systems for the study of the pathogenesis of neurodegenerative diseases [53]. It is also possible to use them to obtain structures for replacement of damage in the central nervous system. For example, by combining artificially obtained surrogates of white and gray matter of the spinal cord, a prototype transplant was obtained to replace the damaged area. However, our attempt to restore the damaged brain by transplantation of fibroin-gelatin microparticles was made for the first time.

We showed that transplantation of fibroin microparticles into the injury locus of the brain resulted in a decrease of damage volume, as well as the restoration of sensorimotor functions. We assumed that the silk fibroin had a pleiotropic protective effect. Firstly, it contributed to the regeneration of nervous tissue, which led to functional recovery. For example, after 14 days, the frequency of limb usage increased, which is contralateral to the damaged hemisphere. On the other hand, in a limb-placing test, statistically significant functional changes were observed on the 4th day, which can be explained by the anti-inflammatory effects of fibroin. Note, it has been previously shown in various models that silk fibroin exhibits anti-inflammatory properties [54–56]. For example, oral administration of silk fibroin contributed to the regeneration and reduction of the inflammatory response in the lungs after burn injury in rats [56]. Previously, Fernández-García and colleagues have shown that transplantation into the brain of a hydrogel based on silk fibroin is a safe procedure. The authors did not detect considerable cognitive or sensorimotor deficits, examined either by different behavioral tests or by an electrophysiological analysis [57]. Ultimately, we may predict that scaffolds based on silk fibroin can become the basis for the creation of constructs usable in brain regeneration.

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