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Regenerative Effects and Development Patterns of Solid Neural Tissue Grafts Located in Gelatin Hydrogel Conduit for Treatment of Peripheral Nerve Injury

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Background: The regeneration of the peripheral nerves after injuries is still a challenging fundamental and clinical problem. The cell therapy and nerve guide conduit construction are promising modern approaches. Nowadays, different sources of cells for transplantation are available. But it is little known about the interaction between fetal central nervous system cells and peripheral nerve tissue. In this study, we analyzed the development of the fetal neocortex and spinal cord solid grafts injected into the gelatin hydrogel conduits and their effects on sciatic nerve regeneration after cut injury.

Methods: Frontal neocortex tissue was obtained from E19.5 and spinal cord tissue was obtained from E14.5 fetuses harvested from transgenic EGFP mice. The grafts were injected into the hydrogel conduits which were connected to the nerve stumps after cut injury. The recovery of motor function was estimated with walking track analysis at 2, 5, and 8 weeks after surgery. Then immunohistochemical study was performed.

Results: The histological examination showed that only fetal neocortex solid graft cells had survived after implantation. Immunostaining revealed that some of the transplanted cells expressed neural markers such as neurofilament protein and NeuN. But the cells mostly differentiated in glial lineage, which was confirmed with immunostaining for GFAP and S100β. The walking-track analysis has shown that 8 weeks after surgery bioengineered conduit differed significantly from the control.

Conclusions: We revealed that the hydrogel conduit is suitable for nerve regrowth and that the fetal neocortex grafted cells can survive and differentiate. Bioengineered conduit can stimulate functional recovery after the nerve injury. (*Plast Reconstr Surg Glob Open 2020;8:e2610; doi: 10.1097/GOX.0000000000002610; Published online 11 February 2020.*)

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INTRODUCTION

Damage of the peripheral nerves of the upper and lower limbs is one of the most widespread and severe types of injury. This type of injury can lead to long-term loss of the working capacity with a high frequency of chronic disability of the victim. There are currently several approaches to treating peripheral nerve injury. For minor injuries, microsurgery is performed to stitch the nerves. The principles of fine microsurgery of the peripheral nerve were independently described by Kurze and Smith in 1964 and became the "gold standard."[1] These principles are still relevant and are most often applied in medical practice. However, these approaches cannot be used in the case of significant damage. Autologous nerve transplantation is the preferred method to treat such injuries. However, this

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approach has a number of drawbacks. It requires double surgical intervention and leads to a loss of sensitivity of the donor tissues. Moreover, complications often develop due to scar formation and degeneration processes of the transplanted nerve. Therefore, it is necessary to seek other methods of treating such injuries, as well as methods for the long-term maintenance of nerve regeneration after transplantation. Potential approaches include tissue engineering strategies and cell therapy. At the moment there is a significant variety of conduits for implantation. Fundamentally, these conduits can be divided into 2 groups: conduits of biological origin and those created on the basis of synthetic materials.[2] The first group includes decellularized nerves and muscles, veins, fibrin, and collagen. The second group includes silicone, polyglycolic acid, polyvinyl alcohol hydrogel, etc.[2] Also, conduits can be divided into those that are biodegradable and those that are not. However, the use of hollow conduits also has a limitation. In clinical practice, empty conduits are used only to repair small-diameter nerves with nerve defects of <3 cm.[3] Moreover, there are data that shows that hollow conduits have lower regenerative potential and functional recovery than autograft.[2] But other research demonstrated that chitosan hollow conduits used for peripheral nerve repair had similar results as autologous nerve graft.[4] Another approach is to use conduits as a carrier for different types of cells. Thus, it is possible to use cultures of one's own Schwann cells; however, this approach requires cutting a healthy nerve from a patient and spending a long time in cell culture. The alternative is to use different stem cells, such as mesenchymal stem cells from different sources, neural stem and progenitor cells, neural crest stem cells, etc. It was shown that using these cells can lead to significant benefits, such as functional recovery.[5] However, despite the results, the problem of treating peripheral nerve injuries still persists and it is necessary to study the development of implanted cells and their effect on regeneration. It is well known that fetal neural stem cells are the "gold standard" for neurotransplantation studies, when we talk about the central nervous system.[6] Nowadays, solid grafts of the fetal neocortex and spinal cord are rarely used to repair injured peripheral nerves and little is known about their interaction with host tissue and their fate after the implantation. It should be noticed that in experiments with fetal nervous tissue, the solid grafts or the cell suspension are directly injected into the nerves.[7–9] We suggest that cell implantation for nerve regeneration is more promising tool if used as a part of 3d bioengineered construct (conduit).

Here, we investigate the regenerative effects and development patterns of the solid fetal neocortex and spinal cord grafts located in the gelatin hydrogel conduit, which connects parts of the cut nerve. We demonstrated that the hydrogel conduit is suitable for recipient nerve regrowth. We revealed that the fetal neocortex grafted cells can survive after implantation, differentiate into neuronal and mostly glial lineage. Moreover, bioengineered conduit can stimulate functional recovery after the nerve injury. Transplantation of the fetal neural tissue (which is native) can serve as a specific standard for comparison with modified cells (cultured, dedifferentiate, or reprogrammed cells).

MATERIALS AND METHODS

Animals and Fetal Nerve Tissue Harvesting

All the experimental protocols were approved by the Ethics Committee for Animal Research of the Koltzov Institute of Developmental Biology RAS in accordance with the Recommendations for Laboratory Practice in Russian Federation. The study was performed on C57Bl/6 and Transgenic heterozygous C57BL/6-Tg(ACTB-EGFP)1Osb/J mice (Jackson Laboratories, Bar Harbor, ME). To obtain dated pregnancy, C57Bl/6 females were caged with a male C57Bl/6-Tg(ACTB-EGFP)1Osb/J mouse. The morning on which the vaginal plug was found was designated as E0.5. Frontal neocortex tissue was obtained from E19.5 and spinal cord tissue was obtained from E14.5 fetuses harvested from transgenic EGFP mice.

Gelatin Hydrogel Conduit

The gelatin hydrogel conduits were made based on the modified protocol described by Sowa et al and Yamamoto et al.[10,11] Briefly, gelatin hydrogel was made by mixing the glutaraldehyde solution and gelatin from porcine skin (Sigma) and dispensed in a mold around a 21-gauge needle and dehydrated into tube shape overnight. After that, the conduits were rinsed in a large amount of deionized water and put in PBS with gentamicin (25 µg/ml). The lumen diameter of each conduit was 0.8 mm and the wall thickness was 0.4 mm. The conduits were cut into fragments with a length of 5 mm.

Surgical Procedures

Adult C57Bl/6 mice (n = 24) were anesthetized with an intraperitoneal injection of chloral hydrate (Fluka Chemie GmbH, Buchs, Switzerland, 300 mg/kg). Immunosuppression was not performed. The operating surface was shaved and the right sciatic nerve was exposed. The animals were randomly distributed to 1 of the following 5 experimental groups: (1) "Cortex" group (n = 5). The nerves were cut and the ends were inserted 1 mm into the conduits, leaving a 3-mm gap of conduit into which the fetal neocortex was injected with a glass needle. It is important to note that the conduit was not sutured to the nerve. (2) "Spin" group (n = 5). The procedure was the same, except for the fact that the fetal spinal cord was injected. (3) "Tube" group (n = 5). The procedure was the same but the conduit was hollow. (4) "Control" group (n = 5). Nerves were simply cut, and the ends were left in front of each other. (5) "Sham" group (n = 4). Nerves were exposed but not cut (Fig. 1). Analgesic ketoprofen (5 mg per kg) was administered after surgery. Eight weeks after surgery, the mice were sacrificed by intraperitoneal injection of a lethal dose of Chloral hydrate (Sigma-Aldrich, USA).

Immunohistochemistry

Nerves were removed and post-fixed in the 4% paraformaldehyde in 0.1M PBS for 24 hours, washed 3 times with PBS and transferred to 30% sucrose in PBS. Longitudinal serial sections were sliced with a cryostat (Leica CM1900)
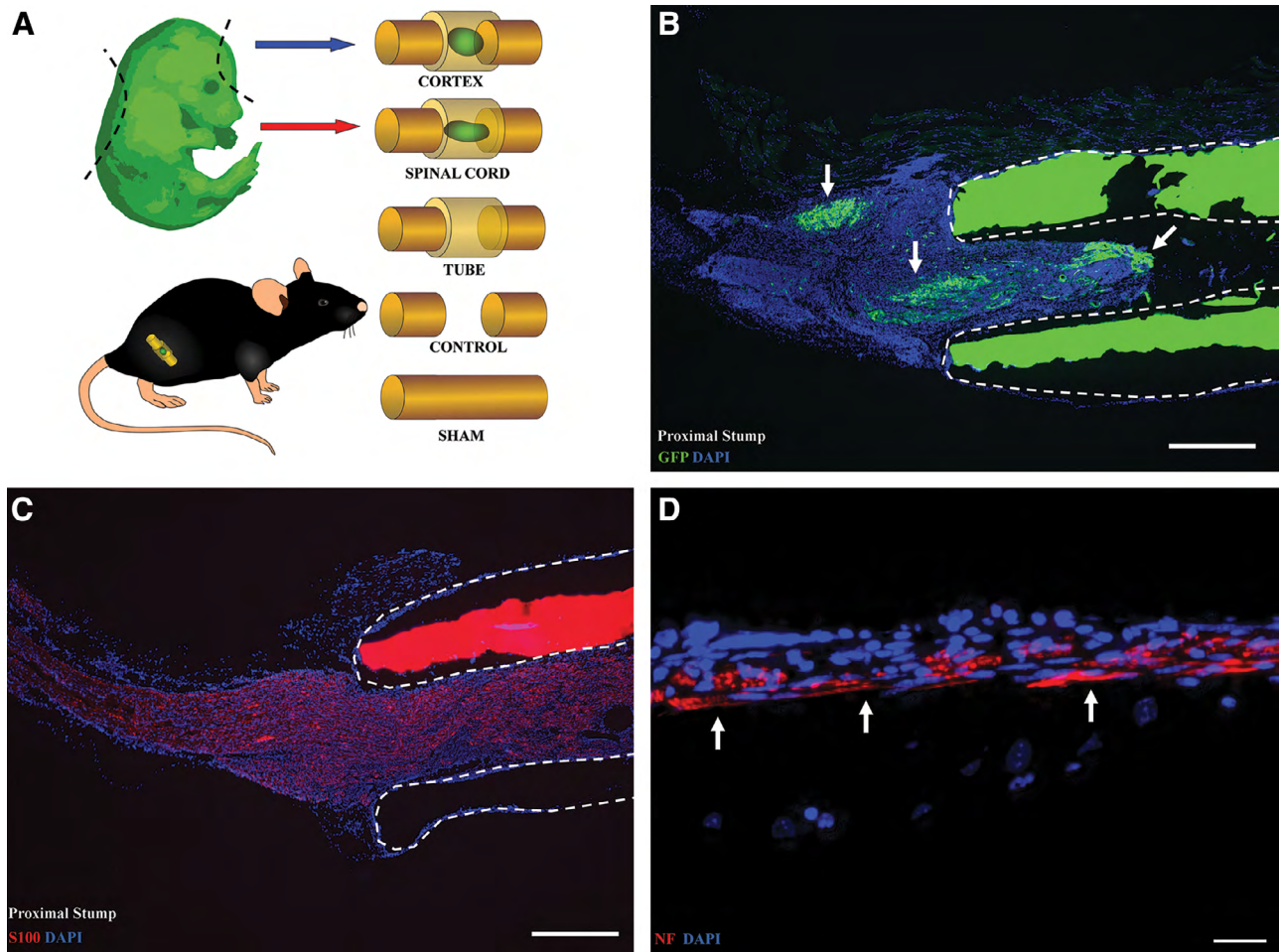
Fig. 1. A, Schematic illustration of the experiment design. The mice nerves were cut and the ends were rejoined with the conduits containing the fetal neocortex or spinal cord tissue. B, The GFP⁺ fetal neocortex cells detected 8 weeks after surgery. Scale bar: 500 µm. C, Schwann cells (stained with anti-S100β antibody) inside repaired nerve. Scale bar: 500 µm. D, Nerve fibers (stained with anti-neurofilament antibody) located in connection tissue capsule around conduit. Scale bar: 20 µm.

to a thickness of 14 µm in 6 sets of sections spaced 98 µm apart. The sections were incubated for an hour at room temperature in a block solution: a mixture of 5% normal goat serum (Sigma-Aldrich), 0.3% Triton x-100 (Triton), and 0.01 M PBS (pH 7.4). Then the sections were incubated overnight at +4°C in a mixture of a block solution and primary antibodies. The following antibodies were used: anti-neurofilament heavy polypeptide (rabbit polyclonal, 1: 200, Abcam), anti-GFP antibodies (chicken polyclonal, 1:500, Molecular Probes), Anti-S100 (rabbit polyclonal, 1: 500, Abcam), anti-doublecortin (rabbit polyclonal, 1: 500, Abcam), anti-NeuN (rabbit polyclonal, 1: 500, Abcam), anti-Myelin basic protein (rabbit polyclonal, 1: 800, Abcam), and Anti-αSMA (rabbit polyclonal, 1:500, Abcam) (Table 1). The sections were then washed and incubated for 2 hours in a mixture of 0.3% Triton x-100 (Triton), 0.01 M PBS (pH 7.4), and the following various secondary antibodies: Goat Anti-Chicken IgY H & L (Alexa Fluor 488, 1: 600, Abcam), Goat Anti-Rabbit IgG H & L (Alexa Fluor 594, 1: 600, Abcam). The sections were rinsed with PBS and the nuclei were counterstained for 20 minutes by incubating for 10 minutes at RT with DAPI solution

(2 µg/ml, Sigma). The histological images were acquired with the BZ-9000E fluorescence microscope (Keyence, Japan). The research was done using equipment of the Core Centrum of Institute of Developmental Biology RAS.

Nerve Fiber Density Measurement

To estimate the fiber density in longitudinal sections, the following method was proposed. Images of

Table 1. Antibody Description

Antibody Name	Description
Anti-GFP antibodies	Green fluorescent protein which expressed in transplanted cells
Anti-Neurofilament heavy polypeptide	Class of intermediate filaments that are found in neurons
Anti-S100	Schwann cells and astrocyte marker
Anti-Doublecortin	Marker of migrating neuroblasts
Anti-NeuN	Marker of postmitotic neurons
Anti-Myelin basic protein	Localized in the myelin sheath surrounding myelinated axons
Anti-alpha smooth muscle Actin antibody	Marker of smooth muscle cells in vessel walls

anti-neurofilament-stained longitudinal sections were divided into zones: proximal part of the nerve (prox), proximal part of tube (tp), middle part of tube (t_mid), distal part of tube (td), and distal part of the nerve (dist). Using ImageJ, the gray value was measured in 10 areas of each zone and analyzed.

Muscle Mass Estimation

Immediately after the mice were sacrificed, gastrocnemius muscles were excised and weighed. The ratio of the experimental side muscle mass to the control side muscle mass was estimated.

Evaluation of Functional Recovery

The recovery of motor function was estimated with walking track analysis at 2, 5, and 8 weeks after surgery, based on the protocol described by Inserra et al.[12] Briefly, mice hind paws were painted with ink, then the mice were allowed to walk along a track on white paper. Functional recovery was assessed by calculating the sciatic functional index value with the following formula. Sciatic functional index = 118.9 × [(ETS − NTS)/NTS] − 51.2 × [(EPL − NPL)/NPL] − 7.5. In this formula, ETS, NTS, EPL, and NPL represent experimental toe spread, normal toe spread, experimental print length, and normal print length, respectively.

Statistical Analysis

The data are represented as a mean ± SE. The multiple group comparisons were analyzed with the one-way ANOVA and nested ANOVA followed by Tukey's post hoc test. The statistical analysis was performed using R studio software. A P-value of <0.05 was considered significant.

RESULTS

Fetal Neocortex Tissue Cells Can Survive up to 8 Weeks and Differentiate after Implantation

To analyze the development patterns of the fetal nervous tissue grafts and its regenerative effects after nerve injury, the following experiment was conducted. The solid pieces of GFP mouse fetal neocortex or spinal cord were injected into the gelatin hydrogel conduits, which were joined the stumps of the cut sciatic nerves (see Methods). The histological and microscopic examination of harvested nerves was performed 8 weeks after surgery. The nerve defect was repaired in all groups. The conduits were enclosed in a connective tissue capsule. The GFP+ cells were found only in the "Cortex" group, predominantly in part of the nerve before the tube. We can assume that donor cells have migrated from the solid tissue graft (Fig. 1). The cells were arranged in groups and some of them have processes. We did not find any signs of spinal cord graft. Immunostaining revealed that some of the neocortex transplanted cells expressed neural markers such as neurofilament protein and NeuN, which is normal for mouse brain tissue at stage E19,5. Most cells differentiated into glial lineage, which was confirmed with immunostaining for GFAP and S100β. Additionally, we did not find any DCX and MBP positive cells. Interestingly, we not only found blood vessels in the nerves but some of the transplanted cells also expressed αSMA (see figure, Supplemental Digital Content 1, which demonstrates the differentiation of the transplanted cells, http://links.lww.com/PRSGO/B283).

Gelatin Hydrogel Conduit Acts as a Bridge for Nerve Repair

We revealed that the recipient's nerves can easily grow through the conduits, which confirmed by anti-neurofilament immunostaining. An interesting fact is that nerve fibers were also found in connection tissue capsule around conduits. High expression of S100β confirmed that there were a lot of Schwann cells inside the repaired nerve (Fig. 1). Also, we revealed that MBP was weakly expressed in a part of the nerve located in the conduit.

Nerve Fibers Density Is Increased While Re-growing through Gelatin Hydrogel Conduit

To evaluate the regeneration process, the nerve fibers re-growth density distribution was analyzed. The gray value was measured in 5 zones of the longitudinal section: proximal part of the nerve, proximal part of the tube, the middle part of the tube, distal part of the tube, and distal part of the nerve. The "Cortex" group, "Spin" group, and "Tube" group were studied. It was shown that in "Tube" group there was a significant difference between the proximal and distal part of the nerve and tube zones ($P < 0.0005$). In "Spin" group, there was the same tendency, except the distal part of the tube, which was significantly different from other zones. The "Cortex" group was more complex. Two middle zones did not significantly differ as in previous groups ($P > 0.05$) but there was a great difference between proximal and distal parts of the nerve ($P < 0.001$) (Fig. 2).

Fetal Nerve Tissue Did Not Restore Muscle Atrophy

Eight weeks after surgery gastrocnemius muscles were excised and weighed to estimate muscle atrophy. Unfortunately, just "Sham" group was significantly different from others ($P > 0.0005$). There was no restoration of muscle atrophy regardless of conduit and fetal nerve tissue implantation during experiment time (Fig. 3).

Bioengineered Conduit Can Stimulate Functional Recovery

To estimate the motor function recovery the walking-track analysis was performed. The sciatic functional index was analyzed. The "Sham" group was significantly different relatively than others during all experiment ($P < 0.0001$). But there was no difference between other groups until 8 weeks after surgery. At that point "Cortex" group significantly differed from the control group ($P < 0.05$) but the difference between other groups was not significant (Fig. 3).

DISCUSSION

The regeneration of the peripheral nerves after injuries is still a challenging fundamental and clinical problem. A promising solution is stem cell therapy. This approach presumes the placement of the cells into a new microenvironment. The fetal stem cells are the "gold
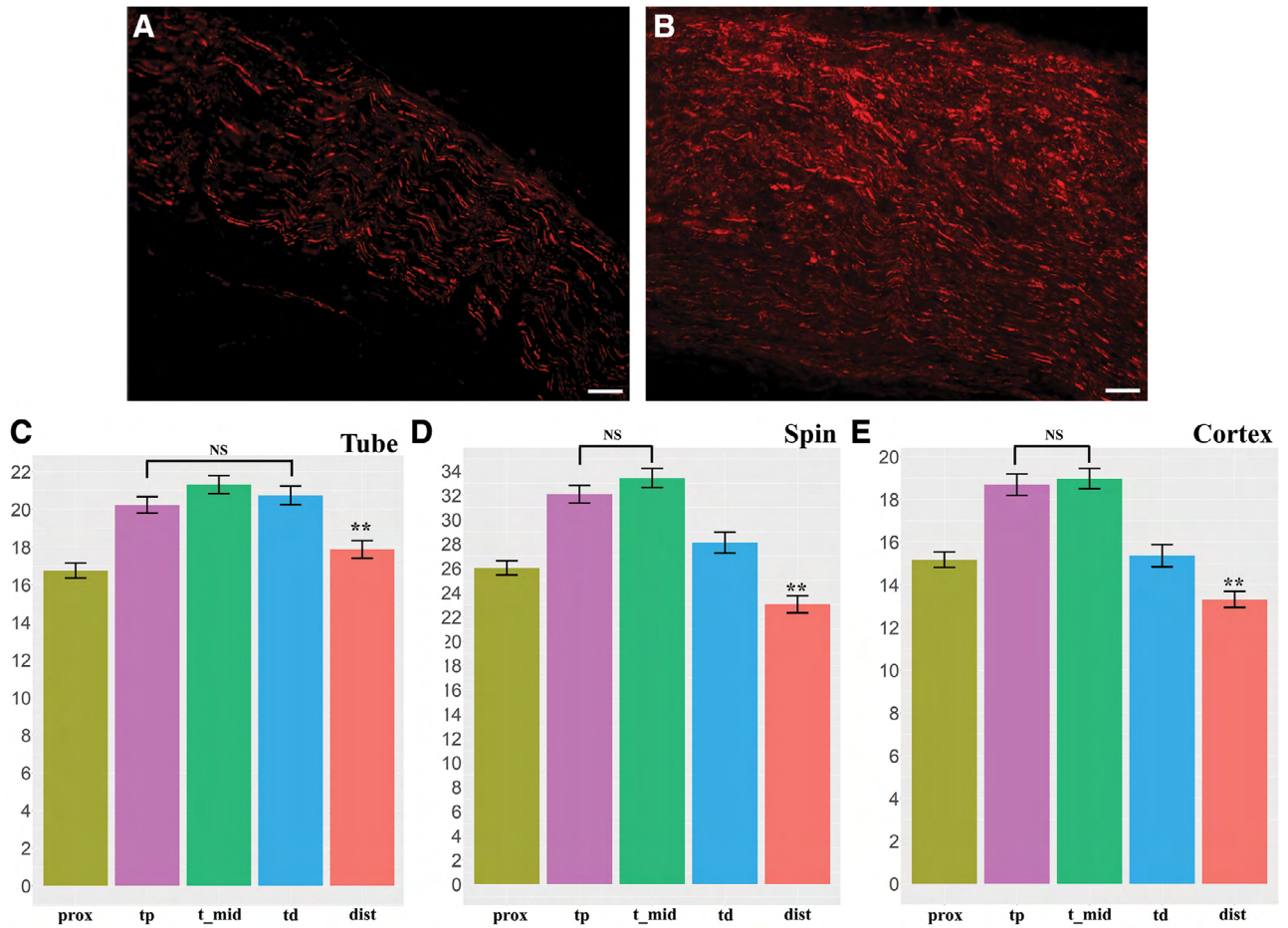
Fig. 2. Nerve fibers density inside the gelatin hydrogel conduit and nerves stumps. A and B, Low and height density of nerves fibers stained with anti-neurofilament antibody inside nerve stump and tube conduit respectively. Scale bar: 50 μm. C–E, Bar plots show gray value of different parts of the regenerating nerve. Error bars show SE. The P values are indicated as follows: ns = not significant, $**P \leq 0.0005$.
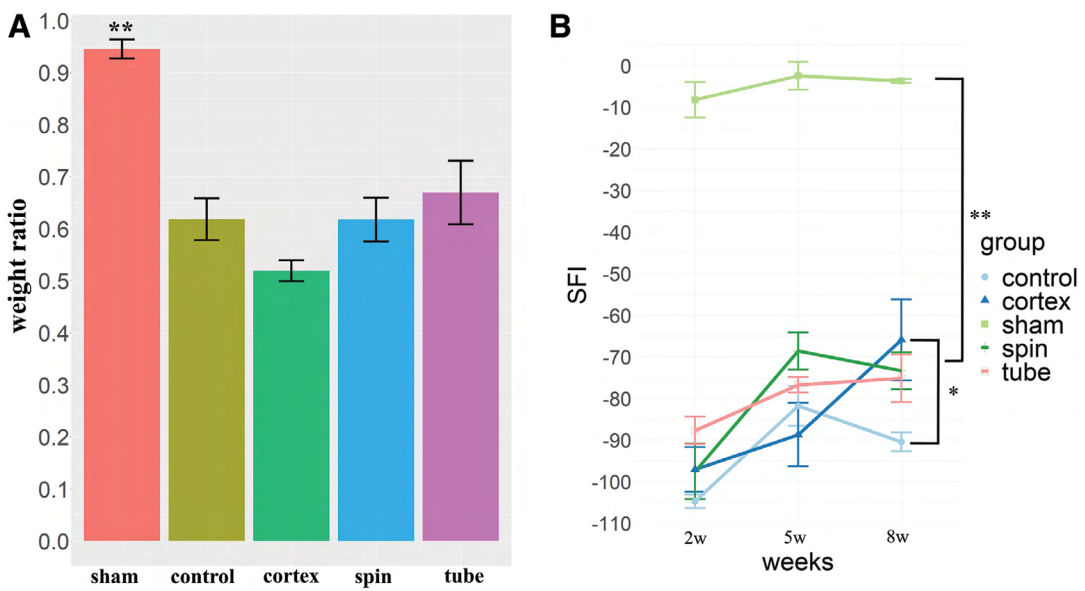

Fig. 3. Muscle and functional restoration. A, Gastrocnemius muscles atrophy was not restored. B, Restoration of motor function in "Cortex" group. The sciatic functional index (SFI) was measured. Error bars show SE. The P values are indicated as follows: ns = not significant, $*P \leq 0.05$, $**P \leq 0.0005$.

standard" source for neurotransplantation. In this study, we analyzed the development of the fetal nervous tissue solid grafts located in the gelatin hydrogel conduits and their effects on nerve regeneration after cut injury. We revealed that the hydrogel conduit is suitable for nerve re-growth and that the fetal neocortex transplants can survive, differentiate, and stimulate functional recovery after the nerve injury.

Eight weeks after surgery, the histological examination showed that only fetal neocortex solid graft cells but not spinal cord solid graft cells had survived after implantation. Other works, on the other hand, revealed that spinal cord cells can survive as well.[7,13,14] However, in that experiment, the solid grafts or the cell suspension were directly injected into the nerves, while in our research the gelatin hydrogel conduits were used as graft carrier. Notably, the grafted neocortex cells were mostly located inside the recipient nerve proximal stump. We can assume that the cells migrated to this part of the nerve and survived because of blood supply and a more preferable microenvironment compared with the one inside the conduit. An interesting fact (previously noticed by Ruven[13]) is that the GFP signal had weakened after the transplantation, and we had to use the anti-GFP antibody to detect the cells. Some investigators recommend delaying the cell transplantation for 1 week because of neurotoxic effects during the acute phase of inflammation which occur after nerve transection.[15]

The immunohistochemical study demonstrated the differentiation potential of the neocortex fetal cells after transplantation into the microenvironment of the peripheral nerve. We have shown that most of the cells differentiated to astrocytes (GFAP+) and only a few neurons (NeuN+) were found. This may be due to the fact that we used the fetal neocortex at stage E19.5 which is the time point of the end of the neurogenesis. As a result, mature neurons mostly did not survive after transplantation. Petrova has shown that GABAergic, NO-ergic, cholinergic, and catecholaminergic neurons persist in the solid graft of the fetal neocortex in the sciatic nerve after implantation.[8] In contrast with our work, Petrova used E14 rat embryos, at a stage of development when the neurogenesis is active. In another work, E14.5 rat embryonic spinal cord fetal cell suspension was used. That research revealed that, after transplantation, cells were mostly positive for neurofilament marker NF200 and NeuN, while some of the neurons were positive for anti-choline acetyltransferase. But if the cells were previously cultured for 12 days, they mostly differentiated into astrocytes revealed by their positive GFAP staining.[13] We also found GFP+/S100+ cells. It is well known that S100B expressed in particular by astrocytes and Schwann cells. Schwann cell-like differentiation was noted after implantation of cultured E17 rat hippocampus cells into the silicone tube bridge with collagen medium.[16] Neural crest cells which were differentiated from hESCs and seeded into the lumen of biodegradable conduits in fibrin matrix also differentiated into S100+ cells.[17] We further revealed that some of the transplanted cells also expressed αSMA and they were usually located around blood vessels.

We have shown that the gelatin hydrogel conduit is suitable for direct fiber re-growth and Schwann cells migration. In other work, the Schwann cells migration in hydrogel conduit was not so pronounced, perhaps due to the fact that the gap was bigger.[10] We also found nerve fibers in the connective tissue capsule around conduits, so we can conclude that connective substrate is suitable for nerve growth. We measured nerve fibers re-growth density distribution through longitudinal sections to evaluate the regeneration process. We revealed differences between the middle part and distal/proximal part in the "Tube" group, but in other groups, the difference was not so striking. We can assume that solid grafts can obstruct the fiber growth. Petrova and Isaeva has shown that solid grafts can grow in volume and they can be an obstacle to nerve growth. Moreover, suspension, but not solid grafts, increase myelination after transplantation.[9]

Muscle atrophy was not restored in our experiment. But in another work, the muscle wet weight analysis showed that fetal spinal cord grafts retained muscle weight, and muscles were bigger than the control, with the differences visually noticeable.[13] In an experiment where the adipose-derived stem cells and Schwann cells in gelatin hydrogel tube were used, the muscle weight was also significantly increased compared with the "Control" group.[10] However, stem cells have greater neurotrophic activity than E19.5 neocortex neurons used in our work. Induced pluripotent stem cell-derived motor neurons also increase muscle weight after transplantation.[18] Walking-track analysis in our experiment has shown that the bioengineered conduit can stimulate functional recovery after 8 weeks in comparison to the "Control" group. This trend will require further investigation. The positive effect may be due to the fact that transplanted cells mostly differentiated into astrocytes. And it is known that astrocytes express BDNF and S100B which exerts trophic effects on neurons, reduces microglia reactivity, and improves regeneration after injury.[19,20] On the other hand, transplanted astrocytes may impede regeneration of injured sciatic nerve.[21] Xiong et al[7] conclude that transplanted embryonic E12 spinal cord solid graft can promote both host motor nerve regeneration and target muscle reinnervation. They showed that electromyogram patterns of the transplantation group returned to nearly normal on the eighth week. Other cell types, such as adipose-derived stromal cells also promoted functional restoration after transplantation.[10,22]

CONCLUSIONS

This study describes the regenerative effects and development patterns of solid neural tissue grafts located in gelatin hydrogel conduit after sciatic nerve injury. We revealed that the gelatin hydrogel conduit is suitable for nerve regeneration. We demonstrate that fetal neocortex cells can survive up to 8 weeks. The grafted cells differentiate mostly in glial lineage. Our bioengineered conduit can stimulate functional recovery after the nerve injury. We can assume that the positive effect was the result of the paracrine impact of the astrocytes. But, this assumption requires further study.

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