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# Functional Recovery of Transected Peripheral Nerve by Means of Microwave Irradiated Collagen Nerve Guides Filled With Chick Embryonic Cerebrospinal Fluid in Rats

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## Abstract

**Objective:** The physical properties of nerve guidance channel and components of the regenerating microenvironment can significantly enhance regeneration. The aim of this research was to evaluate the effect of embryo cerebrospinal fluid (ECSF) in nerve regeneration across the microwave irradiated collagen nerve guides in comparison with autograft.

**Material and Methods:** Under general anesthesia, the left sciatic nerve was exposed and 10 mm nerve segment defect was created in 40 adult male Sprague-Dawley rats (250-300 g). Animals were randomly divided into 4 experimental groups: repair with reversed autograft, reconstruction with collagen nerve conduit filled with ECSF, reconstruction with collagen nerve conduit filled with normal saline (NS) and sham surgery. All animals were evaluated by sciatic functional index (SFI), electrophysiology, and histopathological staining at weeks 4 and 12 after surgery.

**Results:** The mean SFI value of group collagen + ECSF and autograft was significantly higher than that of group NS on days 49 and 60 post-operation ($P < 0.05$). After 90 days after the operation, the mean nerve conduction velocity (NCV) of groups collagen + ECSF and autograft were significantly faster than NS group ($P < 0.05$). The regenerated nerves of groups collagen + ECSF and autograft were more mature than that of the group NS group at day 90 ($P < 0.05$). There was no significant difference between groups collagen + ECSF and autograft.

**Conclusion:** These findings showed that chick CSF in collagen guide can enhance nerve regeneration and promote functional recovery in the injured sciatic nerve of rats.

**Keyword:** Cerebrospinal fluid, Sciatic nerve, Collagen, Nerve injury, Microwave

## Introduction

Peripheral nerve injuries are common and often cause long-lasting disability and mainly muscle weakness, sensory loss and painful neuropathy (1). Autologous nerve grafting remains the "gold standard" technique for the peripheral nerve lesions (2). However, autograft is limited with a variety of clinical complications, such as the lack of availability of donor tissue, loss of donor site function, need for a secondary surgery and mismatch in size between the donor nerve and the injured nerve (3). An alternative to autografts engineered nerve guide channels (NGCs) have been explored for the repair of nerve injury. Collagen-based biomaterials are the most common example of a guidance channel designed to create a protective environment for axonal growth across a nerve gap (4). Collagen is resorbable and semipermeable and provides an interface between the nerve and the surrounding tissue (5). Since collagen is a structural component of extracellular matrix it shows excellent advantages over other synthetic and natural polymers (6). Collagen possesses extremely good cell adhesive properties and signaling domains that promote nerve growth and proliferation (7). Recently Ahmed et al (8) studied about effective crosslinking of collagen with microwave irradiation for peripheral nerve repair. Numerous studies have shown that the local presence of growth factors plays an important role in controlling survival, migration, proliferation, and differentiation of the various cell types involved in nerve regeneration (9). It has been confirmed that nerve growth factor (NGF) can protect neurons from death and significantly improve neuronal survival and differentiation (10). Embryo cerebrospinal fluid (ECSF) contains high concentrations of NGF, transforming growth factor-α (TGFα), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and neurotrophin-3 (NT-3) (11) which are important for neural cell survival and proliferation. As a natural medium E-CSF could support viability and proliferation of cortical cells in vitro (12). Gato et al demonstrated that ECSF contributes to the regulation of the survival, proliferation and neurogenesis neuroepithelial stem cell in mesencephalic explants (13). It has been reported that chick CSF at developmental

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stages has several isoforms of FGF-2 higher concentration of total protein (14). In the present study, our purpose was to evaluate the effect of chick CSF in the collagen channel in comparison with autograft.

## Materials and Methods

### Animals

All animal procedures were conducted under a protocol approved by the ethical committee of Tabriz University of Medical Sciences. Forty adult male Sprague-Dawley rats (250 to 300 g), were randomly placed into one of 4 groups: collagen conduit + CSF (n = 10), sham surgery (n=10), collagen + NS (n = 10) and autograft (n = 10). The left sciatic nerve injury was used as experimental side and the other side to serve as the control.

### Preparation of Collagen Guide Channel

Type I collagen solution was isolated by a published method (15) from rat-tail tendons by acidic soluble techniques. A Teflon mandrel, with an external diameter of 1.6 mm, manually under sterile conditions was immersed in collagen solution and dried at room temperature. This step was repeated 20 times. Then, it was subjected to thermal dehydration at 105°C for 24 hours. After complete drying of the tube, it was removed from the Teflon mandrel and a collagen nerve guide (1.6 mm in inner diameter and 12 mm in length) remained (16). These tubes were further cross-linked by microwave irradiation (each 30 seconds with the intermittent cooling time of 2 minutes) (17).

### Collection of Chick ECSF

Fertile chicken eggs (White-Leghorn strain) were incubated at 38°C and a humidity of 50% to obtain chick embryos at developmental stage HH24 (18). After the embryos were dissected out of extraembryonic membranes in day 17 (E17) the CSF was carefully aspirated under a microscope from the right and left ventricle by using a 20 µL pulled tip glass microcapillary pipette. CSF was collected from 30 chick embryos. To minimize protein degradation, CSF samples were kept at 4°C during collection. The aliquots were centrifuged at 15 000 rpm at 4°C for 10 minutes to remove any contaminating cells and their supernatants were filtered using a 0.22 µm sterile filter (Millipore, Sigma, USA).

### Surgical Procedure

Under deep anesthesia with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg), the thigh muscles were separated and the sciatic nerve was dissected free. Using micro-scissors, 10-mm nerve segment was transected before the bifurcation of the nerve into the tibial and peroneal nerve branches. In the collagen guide groups, the transected proximal and distal stumps were being inserted into the nerve guide and two 10-0 nylon suture was placed at each end to fix the tube in place. Before inserting the distal stump, the guide was filled with NS or CSF. In the autograft group, the 10-mm nerve segment was transected and microsurgically repaired. The muscle and the skin were being subsequently closed.

### Functional Track Analysis

For SFI animal were tested one day before surgery and on the 7th, 21st, 35th, 49th, 60th and 90th days postoperation. The rat's hind feet are dipped in an Indian ink and animals were permitted to walk down the track, leaving its hind footprints on the paper. The footprints of both the operated and unoperated limbs were used to calculate the SFI as described by Bain et al (19).

### Electrophysiology

On the 30 and 90 days after nerve conduit implantation, 5 animals from each group were subjected to electrophysiological studies using Narco bio-system (USA). Animal anesthetized with the procedure described above (surgical procedure), then regenerated left sciatic nerve (operated side) was re-exposed. Stimulating electrodes were positioned in the proximal and the distal trunk of the grafted nerve and needle recording electrodes were located in the belly of the gastrocnemius muscle. The physiologic parameters (latency and amplitude of compound action potentials) were recorded, then the nerve conduction velocity (NCV) was determined.

### Histopathology

The animals were sacrificed at days 28 and 56 postoperation. To morphological analysis of regeneration, 4 mm sections of the sciatic nerves were harvested distal from the crush site. Tissue samples were fixed in buffered formalin 10% and embedded in paraffin. Tissue section were cut 5 microns across the transverse axis and stained with hematoxylin-eosin. Total myelinated fiber counts, axon diameter and myelinated fibers' diameters were calculated in each nerve cross-section with the aid of a morphometric analysis system (OLYSIA Biorefort, Olympus, Japan).

### Statistical Analysis

Statistical analysis was done by a mixed-design (within- and between-group comparisons). analysis of variance (ANOVA) was computed with 95% confidence intervals using the SPSS software (version16.0). All data are shown as mean ± SD and, $P<0.05$ was considered statistically significant.

## Results

All of the rats tolerated the surgical procedure; the animals did not exhibit clinical evidence of wound infections. At 49 and 60 days after implantation surgery, the mean SFI of collagen + ECSF and autograft groups were significantly higher than the NS group ($P<0.05$). In addition, the SFI value of collagen + ECSF group was similar to that of autograft group at 90 days' postoperation ($P>0.05$, Figure 1). At the 90th day after surgery, the NCVs were detected in all groups. The mean NCVs of group collagen + CSF were significantly faster than those of the group NS, and the difference was statistically significant ($P<0.05$). The

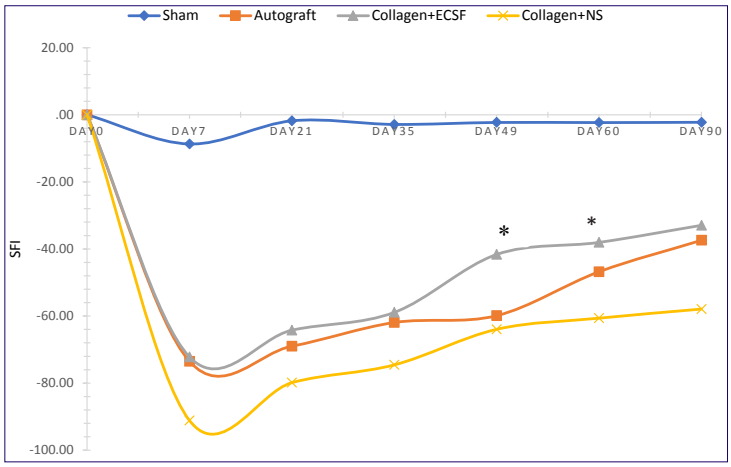


**Figure 1.** SFI Before and After Nerve Injury in the Collagen + ECSF, Collagen + NS, Autograft, and Sham Surgery Groups. *Difference the Experimental Groups ($P<0.05$, *t* test). Results presented as means ± SEM.

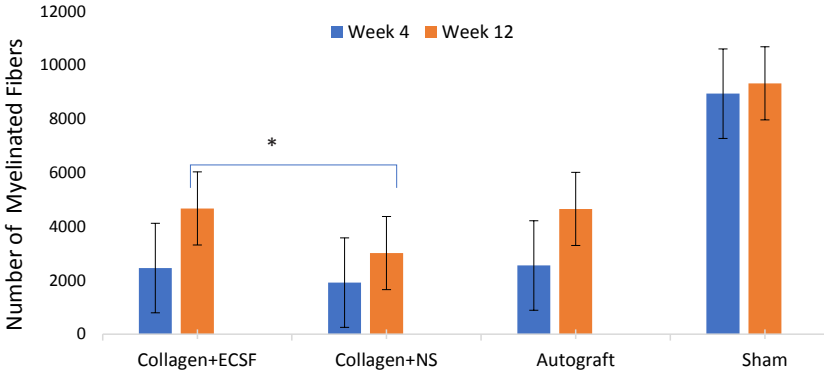


**Figure 2.** Total Number of Regenerated Myelinated Nerve Fibers After Sciatic Injury (n = 5 on week 4 and n = 5 on week 12 for each group). The difference between the collagen + ECSF and NS groups at weeks 4 and 12 after the operation (*$P<0.05$, one-way ANOVA). Results are presented as means ± SEM.

NCVs value of collagen + ECSF group was similar to that of group autograft ($P>0.05$, Figure 1). At the same time, the mean amplitude (AMP) values in the collagen + ECSF group were similar to those of group autograft respectively and the difference was not statistically significant ($P>0.05$; Table 1). In the collagen + ECSF group, the nerve cables contained fascicles of axons. At 3 months after the operation, the regenerated nerve continuously had grown cross through the guide channel and the nerve conduit was completely absorbed. A thin layer of macrophages and fibrous tissue abundant in capillaries could be seen on the outer surface of the collagen guide channel. At the 90th day, the myelinated axon numbers, the mean diameter of axon and the average thickness of myelin sheath were significantly greater for the collagen + ECSF group vs. the NS group ($P<0.05$) (Figures 2,3,4, and 5A-D).

## Discussion

In the present study, we used of ECSF in microwave irradiated (MWI) collagen nerve conduit to enhance peripheral nerve regeneration after sciatic nerve injury. Results of this study showed a better regeneration and myelination of sciatic nerve fibers. Reconstruction of severe peripheral nerve with long inter-stump distances

**Table 1.** Comparison of NCV And AMP in Each Group At Days 30 And 90 Postoperation

| Groups | NCV(m/s) | | Amp (mv) | |
|---|---|---|---|---|
| | 30th | 90th | 30th | 90th |
| NS | 11.8 ± 0.74 | 24 ± 2.1 | 1.8 ± 1.1 | 3.6 ± 1.2 |
| Collagen + ECSF | 20.8 ± 1.66 | 41.5 ± 3.1 | 2.7 ± 0.6 | 5.72 ± 1.43 |
| Autograft | 18.5 ± 2.48 | 39.07 ± 3.02 | 2.5 ± 0.02 | 4.7 ± 1.88 |
| Sham surgery | 49.4 ± 6.26 | 49.6 ± 4.5 | 9.7 ± 0.36 | 9.77 |

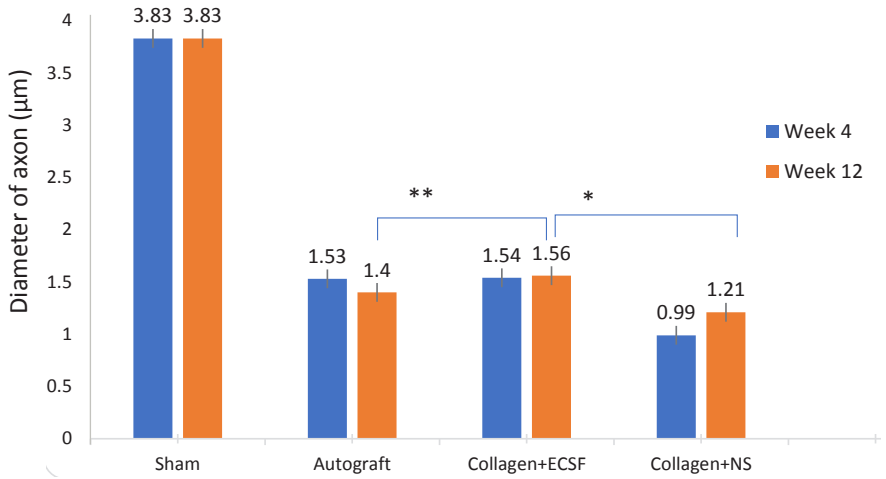


**Figure 3.** The Mean Diameter of Axon in Groups Sham, Autograft, Collagen + ECSF and NS (*$P<0.05$, **$P>0.05$)

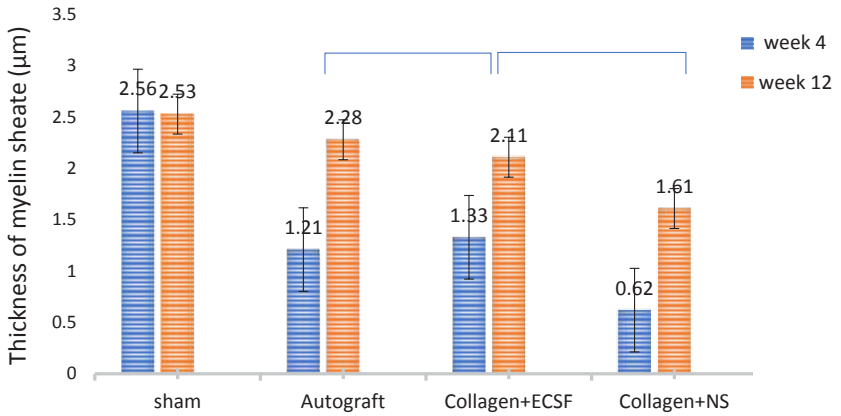


**Figure 4.** The Average Thickness of Myelin Sheath in Groups Sham, Autograft, Collagen + ECSF and NS (*$P<0.05$, **$P>0.05$)

among the cut terminals needs nerve graft techniques or nerve bridging using conduit. Various organic or synthetic materials conduit has been experimentally examined for reconstruction of peripheral nerve defects (20).

Reportedly, collagen is used for nerve conduit application due to its biocompatibility and flexibility. Collagen is bioresorbable, nontoxic, nonimmunogenic, and timely biodegradable product which is not needed to a secondary surgical site to obtain collagen guide conduit (21). Collagen nerve conduits have been shown to promote tissue repair and enhance nerve regeneration (22).

Collagen structure offers biochemical support for nerve regeneration, as it has been shown that collagen is required in the process of nerve regeneration (15). The nerve stumps slide inside the tube during the suturing process and the regenerating axons cannot escape into the surrounding tissues. Ahmed et al reported that collagen tubes cross-linked with MWI as better templates for regeneration of peripheral nerve (8).

The ECM proteins and presence of trophic factor are important for neuron survival and regeneration (23). The present study showed that ECSF-filled collagen tubes exert a significant influence on early sciatic nerve regeneration and it is better than collagen tube without ECSF that it was figured out through increased axon counts and myelin sheath thickness. Also, the results showed the mean value of SFI for ECSF groups significantly was better than the NS group. Previous reports suggested that administration of exogenous neurotrophic factors enhance nerve regeneration (23).

The chick embryo CSF is a complex fluid containing numerous neurotrophic factors (NGF, fibroblast growth factor 2, ciliary neurotrophic factor) that enhance peripheral nerve regeneration (24). The availability of neurotrophic factors appears to be essential for cell viability and migration of Schwann cells into the guide conduit, and enhance sprouting of nerve along the guide channel (9).

Previous studies showed that various nerve conduits in combination with a single administration of NGF promote sensory and motor nerve regeneration (25,26). The beneficial effects of the application of ECSF in collagen guide conduit showed a higher mean conduction velocity records along the regenerated sciatic nerves.

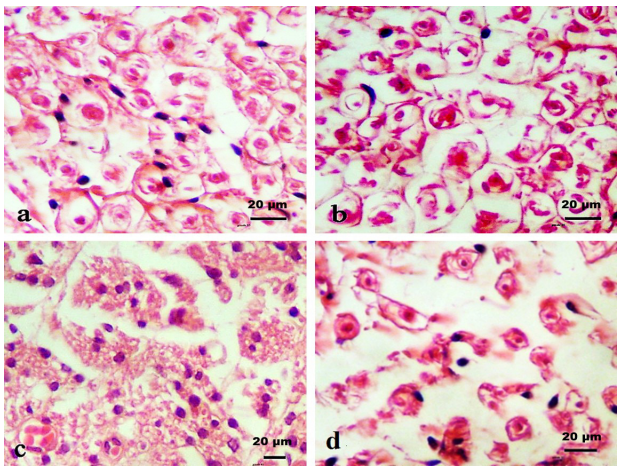


**Figure 5.** Histological Findings (H&E, ×40) of the Mid-Graft Transverse Sections of the Regenerated Nerve at 90 Days After Operation for the 4 Groups: Surgery (a), Autograft (b), collagen + ECSF (c), Collagen + NS (D). The regenerated axons were denser and organized in the autograft (group b) and in the conduit with collagen + ECSF (group c) compared with conduit with NS (groups c and d). Regenerated nerve fibers containing Schwann cells, blood vessels, and myelinated axons were present throughout the tissue (scale bar 20 µm).

It is shown that conduction velocity and fiber diameter are linearly related and conduction velocity improves significantly as the nerve is better myelinated. Following transection, the nerve conduction pathway had been completely destroyed and the NCV and SFI could not be detected at the first month after operation.

At 12 weeks after surgery, the SFI value and NCV of collagen + ECSF and autograft groups were significantly higher than the NS group. The SFI value and NCV of collagen + ECSF group were similar to that of autograft group. These findings showed that functional recovery of collagen + ECSF and autograft groups were quicker and higher than the NS group. Results indicated that the regenerated axon and Schwann cell-like cells were accompanied by the process of myelination and the functional recovery of regenerated nerve fibers. In the present study, a thin layer of the inflammatory cell and macrophage was seen on the surface of collagen tube. Macrophages secrete a number of growth factors and play an important role in the early stage after nerve injury (27).

Histological assessment showed that the count of the myelinated axon, the mean diameter of the axon and the average thickness of myelin sheath in collagen +ECSF group were similar to the autograft group but they are significantly higher than the NS group. Thus, the ECSF in collagen nerve conduit can effectively enhance axonal growth and maturity of the regenerated myelin sheath.

## Conclusion

ECSF in collagen guide conduit promotes nerve regeneration and functional recovery. The results of present study showed that for 10-mm defect in the rat sciatic nerve, nerve regeneration and functional recovery of collagen +ECSF group was similar to autograft group and also it was better than the collagen conduit without ECSF.

## Conflict of Interests

The authors have no conflict of interest in this study.

## Ethical Issues

The ethical committee of Tabriz University of Medical Sciences approved the study.

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