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The Effects of Chicken Embryo Brain Extract on Sciatic Nerve Regeneration of Male Rat; An Experimental Study

***Fatemeh Rasouli- Sangani*[1], *Gholam Hossein Farjah*[2*], *Sima Nasry*[1]**

[1]*Department of Biology, Payame Noor University of Tehran Shargh, Tehran, Iran*
[2]*Neurophysiology Research Center, Department of Anatomy, Urmia University of Medical Sciences, Urmia, Iran*

***Corresponding author:** *Gholam Hossein Farjah*
Address: Neurophysiology Research Center, Department of Anatomy, Urmia University of Medical Sciences, Urmia, Iran.
Tel: +98-441-2770698; **Fax:** +98-441-2780800,
e-mail: hfarjah@hotmail.com

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ABSTRACT

Objective: To determine the effects of chicken embryo brain extract (BE) on transects sciatic nerve in male rats.
Methods: Thirty adult male Sprague-Dawley rats weighing 200 to 250 g, were randomized into three groups treated with (1) sham surgery, (2) normal saline (NS), and (3) brain extract (BE). The BE was taken from incubating chick embryos at day 8. The sciatic nerve was exposed and sharply transected at the mid thigh level. Immediate epineurial repair was then performed. The BE treated animals were given 400 µl/kg of the chick embryo BE intraperitoneal, once daily, for 2 weeks. All animals were evaluated by sciatic functional index (SFI), electrophysiology, histology, and immunohistochemistry at days 28, 90 after surgery.
Results: The mean SFI difference between BE and NS groups at days 28, 60 and 90 after surgery was statistically significant (p=0.086). The mean number of myelinated fibers in the BE group was significantly greater than that of the NS group on days 28 and 90 after surgery (p=0.034). At days 28 and 90 after surgery, the mean nerve conduction velocity (NCV) in the BE group was significantly faster than that of the NS group (p=0.041).
Conclusion: These results indicate for the first time that chick embryo brain extract can enhance peripheral nerve regeneration in rat.

Keywords: Brain extract; Chicken embryo; Regeneration; Sciatic nerve; Rat.

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Introduction

The sciatic nerve can be often damaged by injections, division, crush, and lacerations cause [1]. Injuries to sciatic nerves cause partial or total loss of motor, and sensory functions due to the axon degeneration, and final results in substantial functional loss and decreased quality of life [2, 3].

Previous studies have demonstrated that platelet rich plasma [4], heparin binding neurotrophic factor combined with Chitosan entubulization [5], 17-beta-estradiol [6], and methylprednisolone laden hydrogel enhances regeneration of the sciatic nerve in rat [7].

It has been shown that the extract from the differentiated cells change that fate of the other cell types [8]. In addition, it can promote cell reprogramming in somatic cells such as fibroblasts [9], lymphocytes [10], and granulosa cells [11]. Talaei-Khozani *et al*., [12] showed that fibroblasts were also able to express cardiomyocyte markers by extract treatment. Zhou *et al*., [13] showed that spinal cord extract could promote the proliferation of rat embryonic neural stem cells. Brain possesses neurotrophic agents such as nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). NGF is an important growth factor in cerebral cortical development due to fact that it stimulates neuronal precursor cell proliferation [14]. BDNF is widely distributed in the CNS, and serves an essential role in maintaining and promoting the growth, survival of a variety cells through binding to its receptors [15].

Previous studies showed that the extract from embryonic chicken tissue can to reduce the severity of radiation injury through the decrease of serum C-reactive protein level [16], and may promote the reversion of metastatic phenotypes phenotypes of osteosarcoma cells [17]. It has been previously reported that the administration of chick embryo cerebro-spinal fluid [18], and chick amniotic fluid enhance peripheral nerve regeneration injuries [19]. Despite the easy availability of chick embryo and its use in traumatic lesions of various tissues, there are no studies assessing the effect of brain extract (BE) of chick embryo on peripheral nerve injury. For this reason, we planned this study to evaluate of chick embryo brain extract on sciatic nerve injury in a rat model. In the present study, our purpose was to evaluate the effect of chick embryo brain extract on repair of sciatic nerve injury in rats by functionally, electrophysiology, histology, and immunohistochemistry evaluation.

Materials and Methods

Experimental Design

Thirty adult male Sprague-Dawley rats (220 to 250 g), were randomized into three groups: brain extract (BE), normal saline (NS), and sham surgery. The experimental procedures were approved by Biology Department, Payame Noor University. The left sciatic nerve was used as the experimental side end the right sciatic nerve as the control.

Cell-Free Chick Embryo Brain Extract Preparation

Thirty Fertilized chick eggs from Lohmen selected white Leghorn hens were incubated at 38±1˚ C and a humidity of 50% for 8 days. Then, the eggs were carefully broken into a Petri dish. The whole brain of each fetus was removed from the skull on the ice and washed in cold sterile PBS. To prepare the brain extract, the brain was put on ice and washed twice in cold sterile PBS. The specimen was transferred into 2 ml grinder and added to 1 ml 4ºC sterile PBS. They were homogenized and centrifuged at 3000 rpm at 4ºC for 10 min. The supernatant was filtered using 0.22 µm syringe filters, aliquoted in 100 µl portions, and stored at -80˚C until use [13].

Surgical Procedure

Following general anesthesia with intraperitoneal (IP) (ketamine: 90 mg/kg; xylazine: 10 mg/kg). In the left hind limb, the sciatic nerve was exposed via a gluteal muscle splitting incision and sharply transected at the mid thigh level. Immediate epineurial repair was performed under a surgery microscope, using two 10-0 nylon sutures. In a sham surgery group, the left sciatic nerves were briefly exposed. Then, muscle and skin were sutured (4-0 Dexon). After nerve injury, the BE treated animals were given 400 µl/kg of the chick embryo BE (IP injection) once a day for up to 2 weeks. The NS group, rats received the sterile NS injection in the same manner as BE treated animals. After the surgery, animals were housed in individual cages with ad libitum food and water and a cycle of 12h light/12h dark.

Sciatic Functional Index (SFI)

The SFI was evaluated at the day before surgery (Day 0) and the 7, 21, 28, 60, and 90 days after the surgery. Black Ink was applied to the plantar surface of the hind feet and the rats were allowed to walk on a white paper track and leave footprints. The animals were evaluated to obtain three footprint parameters including: the distance between the first and the fifth toes: toe spread (TS), the distance between the second and the fourth toes: intermediary toe spread (IT), and the distance between the tip of the third toe and the most posterior part of the foot in contact with the ground: print length (PL). Measurements of footprint parameters were taken from the normal (N) and the experimental (E) limbs. The footprints of both (operated and unoperated) limbs were used to calculate SFI using the Bain formula [20].

SFI= -38.3 [(EPL-NPL)/NPL] + 109.5 [(ETS –NTS)/NTS] + 13.3 [(EIT – NIT)/NIT] – 8.8.

An SFI value of 0 was considered normal, whereas -100 meant total impairment, that could be obtained by a complete transaction of the sciatic nerve.

Electrophysiological Study

The animals in each group were subjected to electrophysiological study using Narco bio-system (USA) at days 28 and 90 after the surgery. Under IP urethane anesthesia (1 g/kg; IP), the left sciatic nerve (operated side) was re-exposed. Stimulating electrodes were placed on each side of the epineurial

sutures, and a recording electrode was inserted into the gastrocnemius muscle. The length of nerve between stimulating and recording electrodes was measured. Then, the nerve conduction velocity (NCV/ms) was determined by stimulus latency and the distance between the two electrodes. During the test, the animal's body temperature was kept constant between 36.5-37˚C using a temperature control unit (Narco, USA) [21].

Histology Examination

After electrophysiological study (28 and 90 days after the surgery), the animals were sacrificed and 4 mm sections distal from the epineurial suture set of the sciatic nerves were removed and immediately fixed at 10% buffered formalin. Then, the samples were embedded in paraffin, cut in 5 µm, and stained with H-E. The total myelinated fiber was counted in each nerve cross-section.

Immunohistochemistry

At days 28 and 90 after the surgery, anti S-100 (Dako, 1:200 dilution) was used as a myelin sheath marker for all groups. Briefly, the specimens was fixed at 10% buffered formalin, post-fixed in 4% paraformaldehyde for 2 h, embedded in paraffin and cut into 5 µm thick sections. Prior to immunohistochemistry evaluation, the dewaxing paraffin sections were rehydrated in PBS (pH.7.4). According to the instructions of staining kits, non-specific immunoreactions were blocked. Then, sections were incubated in S-100 protein antibody solutions for 1 h (at room temperature), washed three times with PBS (pH 7.4), and incubated with biotinylated anti-mouse, rabbit IgG solution for 1 h. Secondary antibody (horseradish peroxidase labelled) solution was added to the sections using the diaminobenzidine method. The sections were incubated for 10-20 min at room temperature and washed with PBS, pH 7.4) 3 times. Immunohistochemical results were analyzed qualitatively using positive, more positive and clearly more positive terms [21].

Statistical Analysis

Data were expressed as means±standard error. Statistical analysis was done by a mixed-design (within and between group comparisons) ANOVA followed by Tukey´s post hoc test for multiple comparison using the statistical package for social sciences (SPSS Inc., Chicago, Illinois, USA) version 16.0. A 2-sided p-value of less than 0.05 was considered statistically significant.

Results

Sciatic Functional Index

The all animals tolerated the surgical procedure. The Sciatic functional index (SFI) prior to surgery in all groups was near zero. The SFI greatly decreased for BE and normal saline groups at day 7 after the operation. The sciatic function index (SFI) prior to surgery in all groups was near The SFI improved from the first week to the last week evaluation in the experimental groups, and the difference between BE and NS groups was statistically significant at days 28, 60, and 90 after surgery (p=0.086), So that, the recovery of nerve function was faster in BE group than in NS group. No statistically significant differences were found, between the BE and NS groups at day 21 after the surgery (p=0.821) (Figure 1).

Electrophysiological Study

At days 28 and 90 after the surgery, the mean NCV of the BE group was statistically significant faster than the NS group (p=0.041). There were no statistically significant differences between sham group and BE and NS group 90 days after surgery

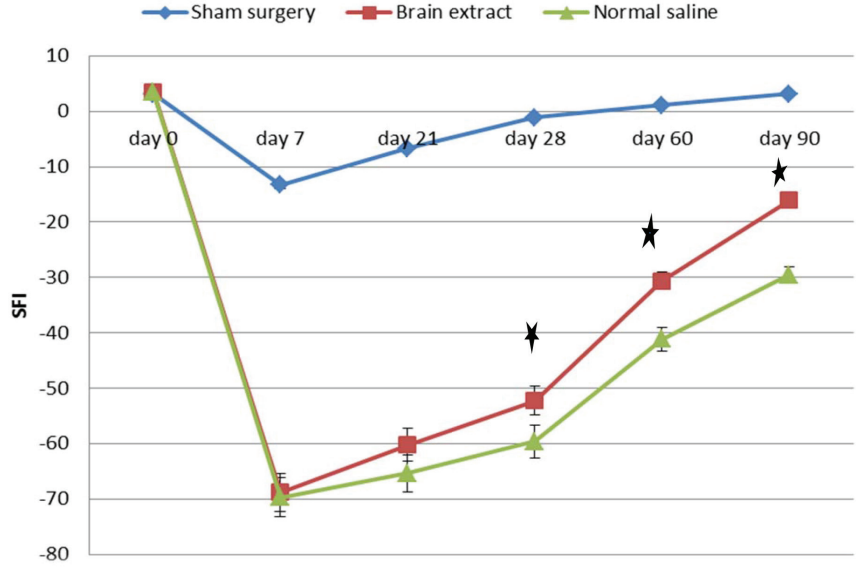

Fig. 1. Sciatic nerve index (SFI) before and after nerve injury in chicken embryo brain extract (BE), normal saline (NS), and sham surgery groups. *Difference between BE treated and NS group (p=0.086). Result presented as means±SEM

(p=0.567). The results of the electrophysiological study are presented in Table 1.

Table 1. Comparison of Nerve conduction velocity (NCV) in each group at days 28 and 90 after the surgery

Groups	Day 28	Day 90
Brain extract (BE)	23.34±11[a]	37.21±4.51[a]
Normal saline (NS)	15.14±3.44	30.28±3.97
Sham surgery	44±7.2	45.1±7.1

[a]p=0.041, the difference between BE and NS groups were significant. Results are presented as means±SEM

Histology and Immunohistochemistry Evaluation

The mean myelinated axon numbers were significantly greater for the BE group (7410±259, 9918±226 nerve fibers) *vs.* NS group (5073±63, 7286±103 nerve fibers) at days 28 and 90 after surgery, respectively (p=0.034) (Figure 2). At day 90 after the operation, Immunoreactivity to S-100 was extensively observed in the cross sections of the sciatic nerve in the BE group. In the experimental groups, the expression of the S-100 protein was located mainly in the myelin sheath, and the Schawnn cell was present around the myelinated axons. In the BE group, the myelinated fiber nerves were more similar to those of a normal nerve compared to the NS group (Figure 3).

Discussion

In the current study, we evaluated chick BE

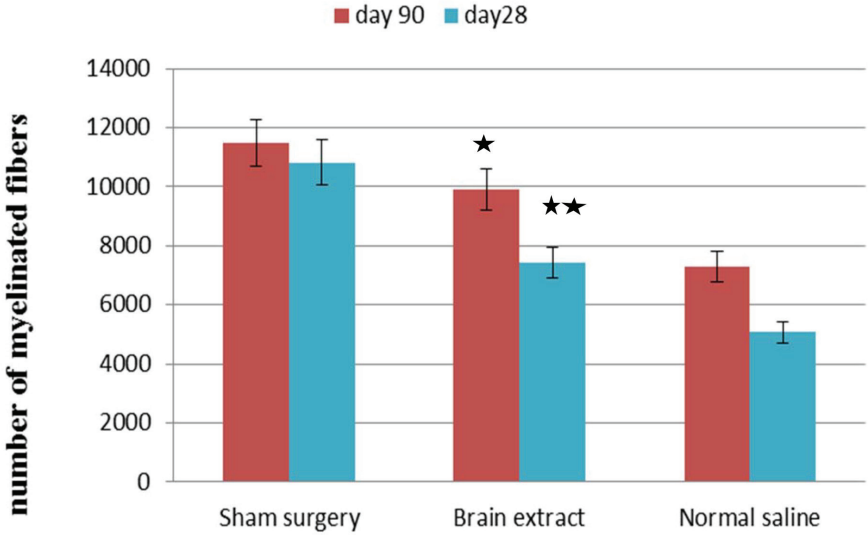

Fig. 2. Total number of regenerated myelinated nerve fibers after sciatic nerve injury at days 28 and 90 after surgery. The significant difference between chicken embryo brain extract (BE) and normal saline (NS) groups at days 28* and 90** after the surgery, (p=0.034). Results are presented as means±SEM

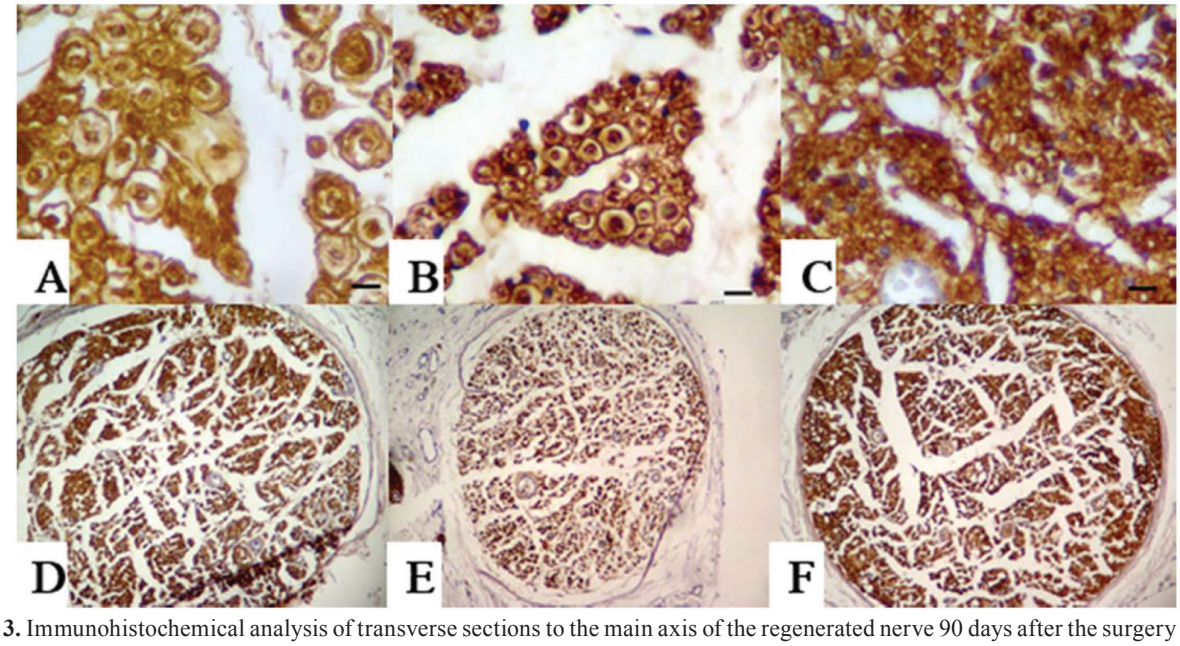

Fig. 3. Immunohistochemical analysis of transverse sections to the main axis of the regenerated nerve 90 days after the surgery distal from the transected site of the chicken embryo brain extract (BE) (A, D), Normal saline (NS) (B, E), and sham surgery group (C, F). Positive staining of the myelin sheath-associated protein S-100 was observed. The Schwann cells, blood vessels, and myelinated axons were present throughout the tissue were present (Scale bar 20 µm). A, B, C (×400), and D, E, F (×100).

administration for sciatic nerve regeneration in adult male rats. The results demonstrated that chick embryo BE significantly enhanced peripheral nerve regeneration in vivo. With our knowledge, this is the first study to examine the effect of chick BE on sciatic nerve regeneration. This is in agreement with the other study that showed the rat BE improved peripheral nerve regeneration in rats [22]. In the present study, the results revealed that functional sciatic index (SFI), nerve conduction velocity (NCV), and the mean myelinated axon numbers in the BE treatment group than the NS group difference were statistically significant. There is a hypothesis that the functional evaluation is more comprehensive and reliable than histomorphometric methods in peripheral nerve repair studies [23]. This study supports the idea that the combination of histomorphometric and electrophysiological assessment with functional analysis is more comprehensive than electrophysiological methods alone.

The remarkable finding of this study was the accelerating effect of chick embryo BE administration on peripheral nerve regeneration in male rats. Several growth factors have been previously discovered in the BE including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) [14].

The studies showed that chick embryo extracts contain growth factors that are active in differentiation and proliferation of chick hematopoietic cells [24], murine neural crest stem cells [25], and diminish the metastatic potential of osteosarcoma cells [17]. Neurotrophic factors have been extensively investigated in animal models of nerve injury to further enhance and accelerate the process of nerve regeneration and functional recovery, and support the survival of axotomized neurons and enhance the intrinsic regenerative capacity after retrograde uptake and induction of specific signaling cascades [26].

There are mechanisms by which chick embryo brain extract affects peripheral nerve regeneration. The several studies showed that human, rat and chicken NGFs possess very similar biological activities [27], and chicken IGF-IR is 85% identical to that of humans [28], and IGF-II is 60% identical to humans and bovine [29]. The biological function of NGF is the maintenance and survival of the nervous system [27]. In addion, NGF inhibits Schwann cell apoptosis by activating the P13k/Akt/GSK3ß and ERK1/2 pathways [30]. Presence of Schwann cells is necessary for advancement of sxons [31].

In addition, Talaei-Khozani *et al*., [12] showed that factors in the cell-free extract can also induce stem cells isolated from different species to differentiate into the other cells.

According to our data, there were no statistically significant difference between chick embryo BE and sham surgery number of myelinated fibers at 90 days after surgery. Administration of chick embryo extract causes a considerable increase in the content of serum IL-1ß and IL-6, plays an important role in stimulation of hematopoiesis and provides a basis to consider this substance as an immunomodulator in pathologic immunosuppresive states [16]. Previous study illustrated that IL-6 cytokine induces angiogenesis [32]. The recently, more attentions have been given to the close relationship between tissue regeneration and angiogenesis. So that nerve regeneration and angiogenesis were activated at 1 day immediately after sciatic nerve transaction [33].

The limitations of our study were the inability to determine the level of nerve growth factors in the BE, and BE was prepared from chick embryos at day 8.

In conclusion, the present study shows that chick embryo brain extract effectively enhances peripheral nerve regeneration. Although the mechanism of rat peripheral nerve regeneration by chick embryo brain extract is unclear.

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Conflicts of Interest: None declared.

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