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Effect of Local Administration of Brain Derived Neurotrophic Factor with Silicone Conduit on Peripheral Nerve Regeneration: a Rat Sciatic Nerve Transection Model

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Abstract

Objective- The objective was to assess local effect of brain derived neurotrophic factor (BDNF) on functional recovery of peripheral nerve in rat sciatic nerve transection model.
Design- Experimental study.
Animals- Sixty male healthy white Wistar rats
Procedures- The animals were randomized into four experimental groups of 15 animals each: In sham-operated group (SHAM), sciatic nerve was exposed and manipulated. In transected group (TC), left sciatic nerve was transected and nerve cut ends were fixed in the adjacent muscle. In silicone graft group (SIL) a 10-mm defect was made and bridged using a silicone tube. The graft was filled with phosphated-buffer saline alone. In treatment group the silicone tube (SIL/BDNF) was filled with 10 microliter BDNF (100ng/kg BW). Each group was subdivided into three subgroups of five animals each and regenerated nerve fibers were studied at 4, 8 and 12 weeks post operation.
Results- Behavioral testing, sciatic nerve functional study, gastrocnemius muscle mass and morphometric indices showed earlier regeneration of axons in SIL/BDNF than in SIL group ($p < 0.05$). Immunohistochemical study showed clearly more positive location of reactions to S-100 in SIL/BDNF than in SIL group.
Conclusion and Clinical Relevance- When loaded in a silicone graft, BDNF improved functional recovery and morphometric indices of sciatic nerve. This finding supports the role of BDNF after peripheral nerve repair and may have clinical implications for the surgical management of patients after nerve transection.
Key Words- Nerve repair, Sciatic, BDNF, Local.

Introduction

Regeneration of the damaged peripheral nerve depends on the microsurgical procedure performed. Currently, there are several operating techniques used to repair injured nerves such as direct epineural repair, grouped fascicular repair, fascicular repair, and nerve grafting. The results following nerve repairs are influenced by many parameters, such as the nature, location, and extent of the injury, the level and timing of the repair, the fascicular anatomy, and appropriateness of re-alignment of the injured nerve, and the surgical technique, as well as patient factors.[1,2]

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In addition to these factors in the regeneration of nerve repair, some pharmaceutical agents which are used locally at the site of nerve repair also have an effect. Several studies have shown that the most frequently applied topical substances are tacrolimus (FK506), hyaluronic acid and its derivatives, melatonin, and methylprednisolone. These substances contribute to fibroblast proliferation suppression at the site of the peripheral nerve repair thus reducing scar formation in the injured peripheral nerve.[3]
Experimental studies and clinical reports indicate that insertion of a conduit could be an interesting alternative to direct end-to-end suturing of nerve stumps or interposition of an autograft.[4-6] The conduits act to guide axons growing from the regenerating nerve stump, provide a microenvironment for dissemination of neurotrophic and neurotropic factors secreted by the injured nerve end, and prevent infiltration of fibrous

tissue. [7] It has been reported that using silicone tubes in bridging of nerve defects could be promising because it is inert and does not induce extensive scarring or degeneration after implantation.[8] The advantages like no donor morbidity, availability, affordability and no foreign reactions make silicone rubber chamber an attractive alternative to other standard grafts.[9,10]

Despite a wealth of research to date, few treatments are available to accelerate or improve recovery after facial nerve injury and none prevents the adverse effects of aberrant regeneration and its clinical correlate, synkinesis.[11] Nerve regeneration can be enhanced by various growth factors that help to sustain the growing nerve.[12]

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and has an important role in the maintenance and formation of neuronal synapses in the brain.[13,14] Glial cells express and respond to BDNF stimulation, favoring the notion of their pivotal role in neuroprotection.[15] BDNF helps to maintain cortical neuron size and dendrite structure rather than the initial development of these features.[16] Physiological roles for BDNF in peripheral nerves have also been suggested. BDNF enhances axonal regeneration in vitro and promotes axonal sprouting from the proximal end of cut nerves into denervated nerve stumps.[17,18] Neurotrophic factors also affect Schwann cells in the distal nerve stump, including the promotion of Schwann cell migration.[19] Schwann cells modulate axonal sprouting by releasing BDNF, thereby promoting nerve regeneration.[20] BDNF is a crucial signaling molecule between microglia andneurons in neuropathic pain .[21]

Takemura et al (2012) suggested that the attenuated recovery of the BDNF+/2 mice was rescued by the transplantation of BDNF and that BDNF from had an essential role in nerve repair in crush model injury.[22]

To the best knowledge of authors, the literature is poor regarding the functional assessments of effects of these neurite-promoting factors on transected nerve regeneration.

In the present study we aimed to assess the effect of a silicone conduit loaded BDNF on peripheral nerve regeneration in a rat model of sciatic nerve transection. The assessment of nerve regeneration was based on behavioral, functional, histomorphometric, and immunohistochemical (Schwann cell detection by S-100 expression) criteria 4, 8, and 12 weeks after surgery.

Materials and Methods

Study design and animals

Sixty male Wistar rats weighing approximately 290g were divided into four experimental groups (n = 15), randomly: sham-operation group as normal control (SHAM), transected control (TC), silicone graft (SIL) and BDNF treated group (SIL/BDNF). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 weeks after surgery. BBB and SFI were taken for 12 weeks on a weekly basis in the group that was kept for 12 weeks. In that group after 12 weeks the nerve segments were harvested for immnunohistochemical and morphometric analyses and the gastrocnemius muscles were also harvested for muscle mass measurement. In the groups of 4 and 8 weeks only the nerve segments were harvested for immnunohistochemical and morphometric analyses. The SSI was taken after each time point. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of (23±3) ° C, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups.

Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.[23] The University Research Council approved all experiments.

Following surgical preparation in the sham-operated group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin was closed with 3/0 nylon. In TC group, the left sciatic nerve was transected proximal to the tibio-peroneal bifurcation where a 7mm segment was excised, leaving a 10mm gap due to retraction of nerve ends. Proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture. No graft was interposed between the stumps. In the SIL group, a 7 mm nerve segment was resected to produce a 10 mm nerve gap after retraction of the nerve transected ends. The gap was bridged using a silicone tube, entubulating 2 mm of the nerve stump at each end. Two 10/0 nylon sutures were used to anchor the graft to the epineurium at each end. In BDNF treated group (SIL/BDNF) the graft was filled with 10 µl BDNF (SigmaeAldrich Chemie GmbH, Steinheim, Germany) (100 ng/mL). The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2% paraformaldehyde and 1%glutaraldehyde buffer (pH 7.4) 4, 8 and 12 weeks after surgery.

Behavioral Testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function.[24] Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated

that it could be very useful in assessment of never repair processes in peripheral nerve injuries.[25] Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination.[24,25] BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-minute exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 12 weeks.

Functional assessment of reinnervation
Sciatic functional index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others.[26] The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:
SFI=-38.3× (EPL-NPL)/NPL+109.5× (ETS-NTS)/NTS +13.3× (EIT-NIT)/NIT-8.8
In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the SHAM group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

Static sciatic index (SSI)

SSI is a time-saving digitized static footprint analysis described by others.[27] A good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others.[27] The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:
SSI = [(108.44 × TSF) + (31.85 × ITSF)] − 5.49
Where:
TSF = (ETS-NTS)/NTS and ITSF = (EIT-NIT)/NIT
Like SFI, an index score of 0 was considered normal and an index of −100 indicated total impairment. When no footprints were measurable, the index score of −100 was given.

Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance.

Immunohistochemical analysis and morphometric studies

In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% paraformaldehyde for 2h and embedded in paraffin. Prior to immunohistochemistry nerve sections were dewaxed and rehydrated in PBS (pH 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 minutes. To block non-specific immunoreactions the sections were incubated with normal swine serum (1:50, DAKO, USA). Sections were then incubated in S-100 protein antibody solution for 1h at room temperature. They were washed three times with PBS and incubated in biotynilated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labelled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases.[25]

Statistical Analysis

The results were expressed as means ± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were set at $P< 0.05$.

Results

BBB recovery

In order to assess hind limb improvement the open field locomotor was used. Figure 1 shows BBB scores compared to the baseline. All experimental groups, except for sham, demonestrated the maximum degree of functional insufficiency one week after surgery. The BDNF treated group showed significant improvement in locomotion of the operated limb compared to the SIL group during the study ($P< 0.05$).

Sciatic nerve function index
SFI outcome

Figure 2 shows sciatic function index (SFI) values in all four studied groups. Before operation, SFI values in all animals were nearly zero. Following the nerve transection, the mean SFI diminished to -100 as a result of the complete deficit of sciatic nerve function in all groups. The statistical analyses showed that the improvement of nerve function was significantly ($P < 0.05$) different between SIL/BDNF and SIL groups and application of the BDNF in silicon graft significantly accelerated functional recovery in the course of time.

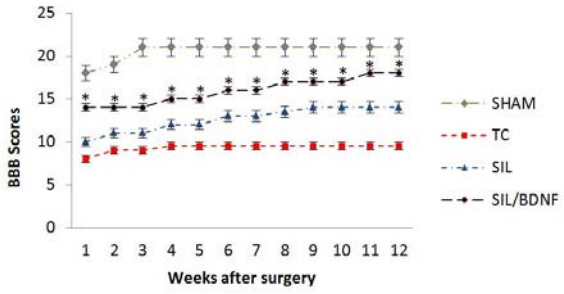

Figure 1- BBB score for all experimental groups. Topical administration of BDNF with silicone grafting gave better scores than in SIL group. * $P < 0.05$ vs SIL group.

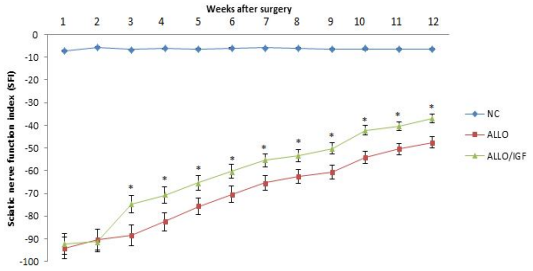

Figure 2- Line graph showing sciatic nerve function index values in each experimental group during the study period. Local administration of BDNF with silicone grafting gave better results in functional improvement of the sciatic nerve than in SIL animals.

SSI outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Fig. 3). Changes in SSI were significant at weeks 4, 8 and 12 of recovery ($P < 0.05$). The contrasts indicate SSI values in group SIL/BDNF at week 12 to differ significantly from those obtained from SIL, a trend also noticed for SFI ($P < 0.05$).

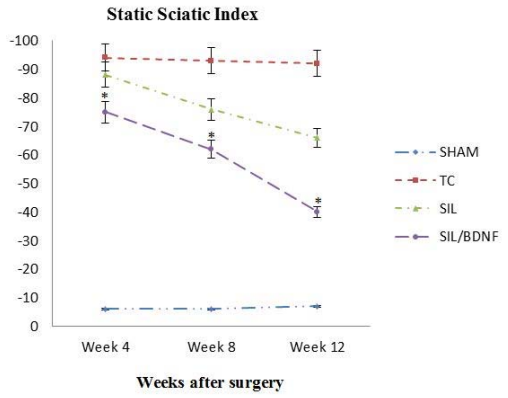

Figure 3- Line graph indicating static sciatic index (SSI) values in each experimental group during the study period. Topical administration of BDNF with silicon conduit gave better results in functional recovery of the sciatic nerve than in SIL group. Data are presented as mean ± SD. * $P < 0.05$ vs SIL group.

Muscle mass assessment

The mean ratios of gastrocnemius muscle mass were assessed at the end of the experiment. There was a significant difference between the muscle mass ratios of the SIL/BDNF and SIL groups ($P < 0.05$). We found that in the BDNF treated group, the muscle mass ratio was greater than in the SIL group, and mass deficient in the gastrocnemius muscle was improved by local application of BDNF (Fig. 4).

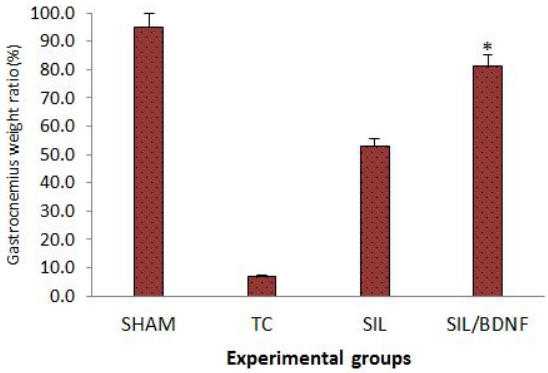

Figure 4- Bar graph showing percentage of gastrocnemius muscle weight ratio. The gastrocnemius muscles of both sides were excised and weighed in the experimental groups at 12 weeks after surgery. Data are shown as mean ± SD. * $P < 0.05$ vs SIL group.

Immunohistochemistry and morphometric findings

In immunohistochemistry S-100 protein was broadly detected in the semi thin sections of regenerated nerves. The expression of S-100 protein signal was located mostly in the myelin sheath. The axon also presented a feeble expression signifying that Schwann cell-like phenotype was present around the myelinated axons (Fig. 5). In both SIL/BDNF and SIL groups, the expression of S-100 and the outcomes were similar to those of the morphometrical assessments.

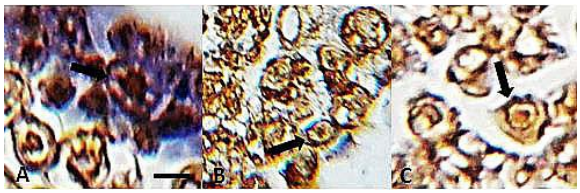

Figure 5- Immunohistochemical assessment of the nerves 12 weeks post operation from (A) midpoint of SHAM, (B) SIL and (C) SIL/BDNF. Positive staining of the myelin sheath-associated protein S- 100 (arrows) around the nerve fiber is shown. This indicates well organized structural nerve reconstruction in BDNF treated nerve compared to that of the SIL. Scale bar:10µm

Figures 5–8 show the morphometrical analyses of regenerated nerves of the study groups. The significantly greater nerve fiber, axon diameter, and myelin sheath thickness were found in BDNF treated group 4 weeks post operation, compared to SIL group ($P < 0.05$). Sham-operation animals showed significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to SIL/BDNF and SIL groups animals. In case of myelin thickness there was no significant difference between SIL/BDNF and SIL groups, morphometrically ($P>0.05$).

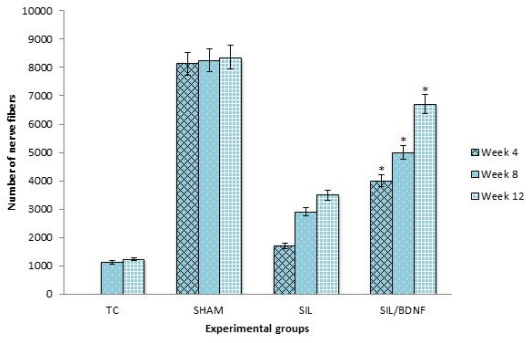

Figure 6- Bar graph shows the results of number of nerve fibers. SIL/BDNF group showed the greater number of fibers than SIL at the end of the study period. Data are presented as mean ± SD. * $P < 0.05$ vs SIL group.

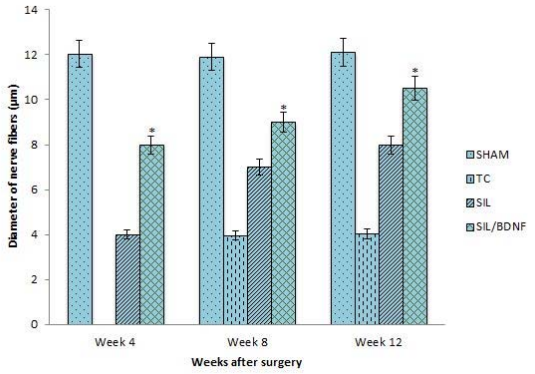

Figure 7- Bar graph shows the quantitative results of mean diameter of nerves fibers. SIL/BDNF group showed the greater mean diameter of nerve fibers than SIL at the end of the study period. Data are presented as mean ± SD. * $P < 0.05$ vs SIL group.

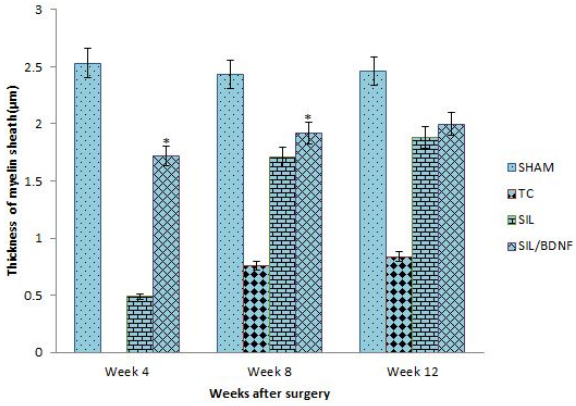

Figure 8- The graph shows the quantitative results of mean thickness of myelin sheath. SIL/BDNF group showed the greater mean thickness of myelin sheath than SIL at the end of the study period. * $P < 0.05$ vs SIL group.

Discussion

A 12-week experimental period is sufficient for evaluation of regeneration process because in rats functional recovery after repair of a transected peripheral nerve occurs during this timeline.[28]

There are several tests for functional evaluation of the nerve repair. One of the best known tests is the sciatic function index (SFI) in rats.[29] Many experiments on nerve regeneration are performed in recent years using SFI as a measure of functional loss. According de Medinaceli et al. (1982), there are a wide variety of techniques that can be used to study the recovery of the peripheral nerve injury.[30] Nerve regeneration in animal

experiments can be assessed with histomorphometry and measurements; however, these methods do not always correlate with recovery motor and sensory functions.[31] It is recommended to use various methods for overall assessment of nerve function.[32] One of these methods is a walking track which was designed to visualize and record gait of rats. Rats were allowed to walk freely in a walkway in order to analyze their visible footprints by stepping in developer on X-ray film or paint on paper.[30] Bervar introduced a new method that can perform credible and fast footprint analysis.[27] For evaluation of the footprints, three different parameters are used: print length (PL), toe spread (TS), and intermediary toe spread (ITS).[30]

The findings of this study demonstrated that use of BDNF in a silicon graft resulted in faster functional improvement of the sciatic nerve during the study period. Left gastrocnemius muscle mass was considerably greater in the SIL/BDNF than in the SIL animals, representing indirect indication of successful end organ reinnervation in the BDNF-treated animals.

Although morphologic and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two assessment types is usually poor.[28,33,34]

At week 12 morphometric values of regenerated nerve fibers indicated significant differences between the SIL and SIL/BDNF animals, demonstrating a positive effect of local use of BDNF on the nerve repair.

We did not perform nerve conduction tests because electrophysiological studies have poor correlation with functional indices.[35] Nerve conduction velocity and peak action potential amplitude do not evaluate total nerve function but a fraction of nerve fibers population. Compound action potential is derived from extrinsic direct nerve excitation and does not correlate with proper central or peripheral connections.[36]

Castaneda et al.,[28] suggested that entrance of sprouts from the proximal nerve stump at the distal nerve stump does not essentially indicate improvement in nerve function. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that the BDNF treated animals were improved in locomotion of the operated limb compared to the SIL group during the study period. Walking track analysis has repeatedly been used to reliably conclude functional improvement after nerve regeneration in rat model.[26]

Various materials have been employed to be used as nerve guides, including non-biodegradable and biodegradable materials. Due to its inert and flexible characteristics, the silicon tube was one of the first and most widely used conduits to repair the transected nerves. [9]

Others have demonstrated that BDNF protein secreted from neurons in the dorsal root ganglia and the anterior horn of the spinal cord was transported to the regeneration site via axons.[37-39] It has also been demonstrated that BDNF protein secreted at the neuromuscular junction was transported via axons to the regeneration site.[40] It is thus possible that the local administration of the BDNF protein in the present study might have been transported there in an antegrade and/or retrograde manner.

Although our initial study indicated the neuroprotective effect of topical BDNF in peripheral nerve damages, findings regarding the molecular mechanisms resulting in the neuroprotective effect are still lacking. We have not given the histological and molecular evidence for neuroprotective action of BDNF. This may be considered as a limitation to our study.

Therefore, the authors stress that the aim of the current investigation was to evaluate a single local dose and clinical treatment potential of BDNF on nerve repair. The results of the present study indicated that a single topical administration of BDNF at the site of transected nerve could be of benefit after silicon graft tubulization. Mechanism of neuroprotective action remains to be investigated.

The present study demonstrated that local application of BDNF could accelerate functional recovery after transection of sciatic nerve. It is available and easily performed without limitations and complications of adverse effects compared to its chronic systemic administration. Thus, dose–response investigations should be performed for BDNF to conclude the combination of the graft and the compound that achieve the highest effectiveness in nerve transection models.

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چکیده

بررسی تاثیر تزریق موضعی(Brain Derived Neurotrophic Factor (BDNF توام با گرافت سیلیکونی بر روی ترمیم اعصاب محیطی: مدل قطع عصب سیاتیک رت

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هدف- هدف از این مطالعه بررسی تاثیر تزریق موضعی فاکتور نوروتروفیک مغزی (BDNF) توام با گرافت سیلیکونی بر روی ترمیم اعصاب محیطی در مدل قطع عصب سیاتیک رت بوده است.

طرح- مطالعه تجربی

حیوانات– ۶۰ قطعه رت ویستار نر سالم.

روش‌کار- رت‌ها بطور تصادفی به چهار گروه ۱۵ تایی تقسیم شدند. در گروه کنترل نرمال (SHAM) عصب سیاتیک سمت چپ پس از برش پوست و عضله سرینی دستکاری شده و پس از خونبندی موضع بخیه گردید.درگروه کنترل منفی (TC) پس از دستیابی به عصب سیاتیک با قطع عصب، نقیصه‌ای به طول ۱۰ میلیمتر ایجاد گردید و انتهاهای قطع شده به عظلات مجاور بخیه گردید. در گروه سیلیکون پس از ایجاد نقیصه ۱۰ میلیمتری، انتهاهای قطع شده پروکزیمال و دیستال عصب با استفاده از لوله سیلیکونی(SIL) به هم مرتبط شدند. در گروه درمان (SIL/BDNF) پس از کارگذاری لوله سیلیکونی به داخل آن ۱۰ میکرولیتر محلول BDNF با دز ۱ میلی گرم بر میلی‌لیتر تزریق گردید. هر گروه متعاقبا به سه زیر گروه ۵ تایی تقسیم گردیده و در مقاطع زمانی ۴، ۸ و ۱۲ هفته بعد از جراحی مورد مطالعه قرار گرفتند.

نتایج- تست‌های رفتاری و عملکردی عصب سیاتیک و وزن عضله دوبطنی نشان داد که عملکرد عصب سیاتیک در رت‌های دریافت‌کننده گرافت سیلیکونی بهبودی قابل ملاحظه‌ای پیدا کرده بود ($P < 0.05$). شاخص‌های مورفومتریک و ارزیابی‌های ایمنوهیستوشیمیایی بیانگر ترمیم قابل ملاحظه‌ای در گروه درمان در مقایسه با گروه شاهد بود ($P < 0.05$).

نتیجه‌گیری و کاربرد بالینی- کاربرد موضعی BDNF عملکرد عصب سیاتیک و شاخص‌های مورفومتریک عصبی را بهبود بخشید. می‌توان از آنها به عنوان درمان موثر در ترمیم جراحات اعصاب محیطی استفاده کرد.

کلمات کلیدی- ترمیم عصب، سیاتیک، فاکتور نوروتروفیک مغزی، موضعی، رت.