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Effect of Local Administration of Laminin and Fibronectin with Chitosan Conduit on Peripheral Nerve Regeneration: A Rat Sciatic Nerve Transection Model

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Abstract

Objective- Effect of local administration of laminin and fibronectin on nerve regeneration was assessed.
Design- Experimental study.
Animal- Sixty male Wistar rats.
Procedures- The animals were divided into four experimental groups (n=15), randomly: In transected group left sciatic nerve was transected and stumps were fixed in adjacent muscle. In treatment group (CHIT/LF) the defect was bridged using a chitosan conduit filled with 10 µL mixture at a concentration of 1 mg/ml laminin and 1 mg/ml fibronectin in a 1:1 volumetric addition. In CHIT group the conduit was filled with phosphate-buffered saline. In normal control group sciatic nerve was exposed and manipulated. Each group was subdivided into three subgroups of five animals each and nerve fibers were studied in a 12-week period.
Results- Behavioral, functional, gastrocnemius muscle mass findings and morphometric indices confirmed faster recovery of regenerated axons in CHIT/LF than in CHIT group ($p<0.05$). Immunohistochemical reactions to S-100 in CHIT/LF were more positive than that in CHIT group.
Conclusion and Clinical Relevance- Laminin-fibronectin improved functional recovery and morphometric indices of sciatic nerve. They could be considered as an effective treatment for peripheral nerve repair in practice.
Key Words- Nerve repair, Sciatic, Fibronectin, Laminin, Local, Rat.

Introduction

Nerve autograft remains the gold standard, however, there are several drawbacks such as sacrifice of functioning nerves, loss of sensation and mismatch between nerve and graft.[1]The ideal surgical repair technique should accomplish good wound healing with minimal scar formation and direct the nerve sprouts into their correct targets.[2] Technological advances in diagnostic imaging, neurosurgical instrumentation and the use of a surgical microscope have resulted in pronounced improvements in the diagnosis and repair of transected peripheral nerves.[3] Widely accepted method by most surgeons is bridging the defect with an autologous donor nerve. Different graft equivalents have also been applied to bridge the nerve stump and regulated through the interaction of a variety of protein and cell signals.[4]

The conduits act to guide axons sprouting from the regenerating nerve end, provide a microenvironment for diffusion of neurotrophic and neurotropic factors secreted by the injured nerve stump, as well as help protect from infiltration of fibrous tissue.[5]

Biodegradable nerve guides as a temporary scaffold are better than non-degradable biomaterials because the latter remain *in situ* as a foreign body and ultimately result in limiting recovery of nerve function.[6] Nevertheless, the resistance to biodegradation can be a cause of chronic nerve compression in the long run and a second surgery may therefore be required for its removal. Beneficial effects of chitosan as a conduit in promoting nerve regeneration have already been documented and it seems chitosan as a natural polymer has excellent properties including biocompatibility, biodegradability, non-toxicity and adsorption properties, and might be a suitable functional material for peripheral nerve regeneration.[7-9]

Both laminin and fibronectin are high molecular weight glycoproteins. Laminin is mainly produced by Schwann

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cells and widely dispersed in the peripheral nervous system while fibronectin can be found in plasma, fibrous tissue and basement membrane.[10-12] Fibroblasts also produce significant amounts of fibronectin.[13]
To the best knowledge of authors, the literature is poor regarding the functional assessments of effects of theses neurite-promoting factors on nerve regeneration. Chen et al in 2000 showed that the collagen, fibronectin and laminin mixture combined with silicone conduit could promote nerve regeneration. However, they did not approve their studies with behavioral and functional assessments.[14]
Aimed to study local effects of fibronectin and laminin on peripheral nerve regeneration, the present study was designed to determine if filling guidance tubes with these neurite-promoting factors could in fact reduce dysfunction after nerve injury in the rat sciatic nerve transection model.

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 (SHAM), transected control (TC), chitosan conduit (CHIT) and laminin-fibronectin treated group (CHIT/LF). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 weeks after surgery. 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.

Preparation of chitosan conduit

Chitosan solution was prepared by dissolving medium molecular weight, crab shell chitosan (~400kDa, 85% deacetylated) (Fluka, Sigma-Aldrich St. Louis, MO, USA) in an aqueous solution (1% v/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50^{oC}) for 3hour. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper then vacuum filtration to remove any un-dissolved particles. To overcome the fragility of chitosan, glycerol (Sigma Chemical Co., St. Louis, MO, USA) was added as 30% (w/w) of the total solid weight in solution (Ojagh et al 2010). Chitosan conduit was made according to the method described in similar studies.[24] gentle injection of the prepared solution into a home-made mold. The prepared conduit was 2 mm in external diameter, 1.8 mm in internal diameter and 10 mm in length. This internal diameter complies with optimal function in rat models.[15]

Surgical procedure

Animals were anesthetized using intraperitoneal administration of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and Xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain.[16] The University Research Council approved all experiments.
Through a muscle splitting approach, the plane between gluteus maximus and biceps femoris was developed and the right sciatic nerve was clearly visible on the underlying hamstrings muscles. Sutures were passed through the nerve epineurium (one on each side), 3 mm apart at a level of 1 cm above the trifurcation of the nerve. Sutures had the same circumferential orientation on the nerve to restore spatial longitudinal nerve continuity. Before transection, both needles were driven through chitosan conduit at each side 2 mm from the edge of the conduit. This facilitated proper and prompt insertion before endoneurial edema obscured the cut ends. Afterward, a complete transection between the sutures was undertaken and the cut ends of the nerve were driven carefully with the aid of the sutures inside the chitosan conduit and held in place. A second epineurial suture was placed, at each side and through the conduit. After placement, the chambers were filled with 10 µL a neutral pH sterile solution of mixture at a concentration of 1 mg/ml laminin (Sigmae Aldrich Chemie GmbH, Steinheim, Germany) and 1 mg/ml fibronectin (Sigma-Aldrich Chemie, Munich, Germany) in a 1:1 volumetric addition (Fig 1). In the sham operation group (SHAM), the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with Vicryl (Ethicon, Norderstedt) 4/0 sutures, and the skin was closed with 3/0 nylon (Dafilon, B/Braun, Germany). The rats were observed on a heating pad during recovery.
The animals of each group were anesthetized by intraperitoneal administration of ketamine-xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) at 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.[17] 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. 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.

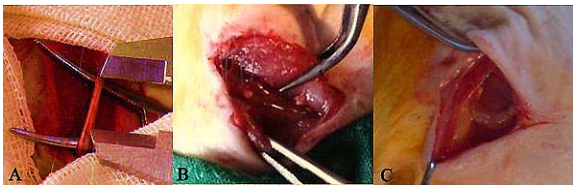

Figure 1- Intraoperative pictures showed (**A**) separated segment, (**B**) transected sciatic nerve ends leaving a 10 mm gap between the stumps, (**C**) end-to-end anastomosis of chitosan conduit to stumps of transected sciatic nerve and two 10/0 nylon sutures placed at each end of the cuff to fix the graft in place and filled with phosphate buffered saline.

Functional assessment of reinnervation

Sciatic functional index (SFI)

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method Bain et al.[18] 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 NC 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)

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

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.[19]

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

Behavioral Testing

BBB recovery

In order to assess hind limb recovery the open field locomotor was used. Fig. 2 shows BBB scores

compared to the baseline. All experimental groups, except for SHAM, showed the greatest degree of functional deficit one week after surgery. The laminin-fibronectin treated group showed significant improvement in locomotion of the operated limb compared to the CHIT group during the study period (P< 0.05).

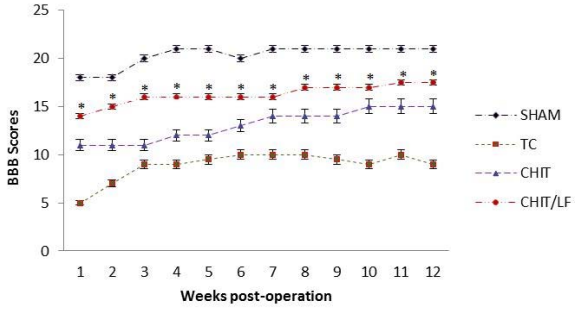

Figure 2- BBB score for all experimental groups. Topical administration of laminin-fibronectin with chitosan conduit gave better scores than in CHIT group. Standard error at each data point is shown with bars.

Recovery of sciatic nerve function

SFI outcome

Fig. 3 shows sciatic function index (SFI) values in all four experimental groups. Prior to surgery, SFI values in all groups were near zero. After the nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. The statistical analyses revealed that the recovery of nerve function was significantly (P < 0.05) different between CHIT/LF and CHIT groups and application of the lamini-fibronectin in chitosan conduit significantly accelerated functional recovery in the course of time.

SSI outcome

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

Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured at the end of the study period. There was a statistically significant difference between the muscle weight ratios of the CHIT/LF and CHIT groups (P<0.05). The results showed that in the laminin-fibronectin treated group, the muscle weight ratio was larger than in the CHIT group, and weight loss in the gastrocnemius muscle was ameliorated by laminin-fibronectin local administration (Fig. 5).

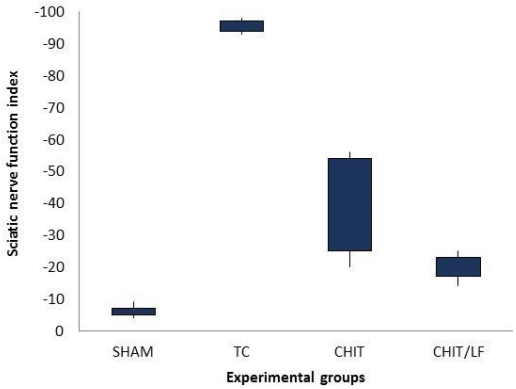

Figure 3- Box-and-whisker plots of sciatic nerve function index values in each experimental group during the study period. Topical administration of laminin-fibronectin with chitosan conduit gave better results in functional recovery of the sciatic nerve than in CHIT group.

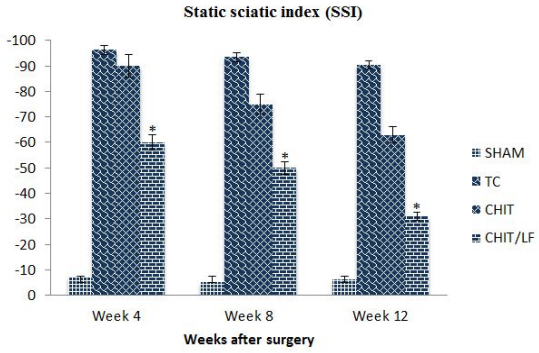

Figure 4- Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Topical administration of laminin-fibronectin with chitosan conduit gave better results in functional recovery of the sciatic nerve than in CHIT group. Data are presented as mean ± SD. * *P* < 0.05 *vs* CHIT group.

Immunohistochemistry and morphometric findings

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig.6). In both CHIT/LF and CHIT groups, the expression of S-100 and the findings resembled those of the histological evaluations. The laminin-fibronectin treated group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness during study period, compared to CHIT animals (P<0.05). Normal control group presented significantly greater

nerve fiber and axon diameter, and myelin sheath thickness compared to CHIT/LF and CHIT groups animals (Fig. 7-9). In case of myelin thickness there was no significant difference between CHIT/LF and CHIT groups, morphometrically (P>0.05).

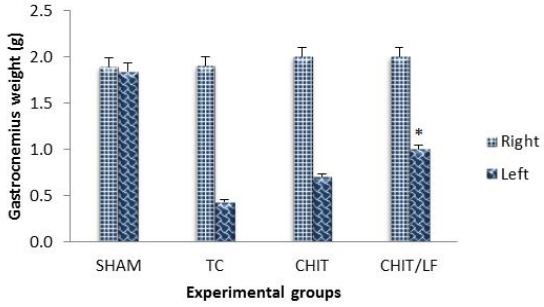

Figure 5- Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean ± SD. * *P*<0.05 *vs* CHIT group.

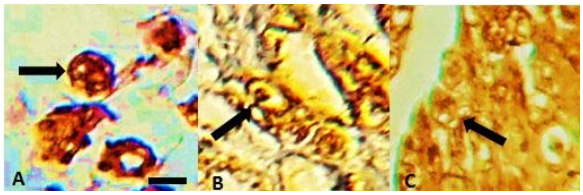

Figure 6- Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) SHAM, (B) CHIT and (C) CHIT/LF . There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in laminin-fibronectin treated nerve compared to that of the CHIT. Scale bar:10 µm

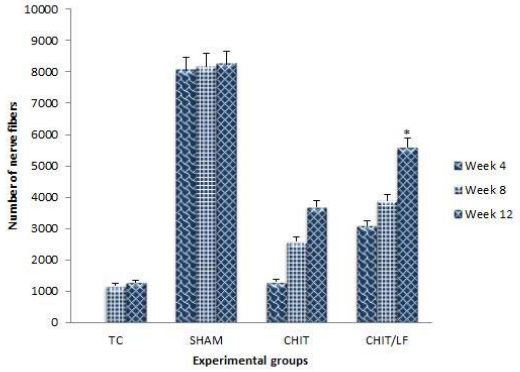

Figure7-The graph shows the quantitative results of fiber counting. Both groups of CHIT and CHIT/LF showed the ower number of fibers than the SHAM group even at the end of the study period.* P<0.05 vs CHIT group.

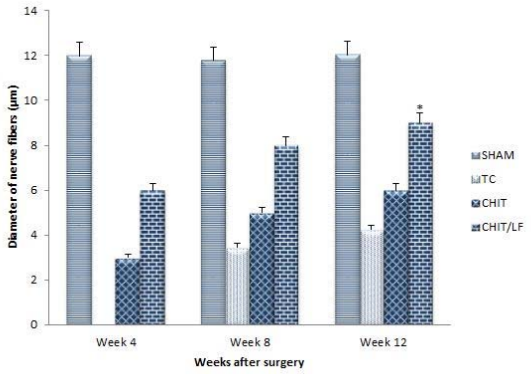

Figure 8- The graph shows the quantitative results of mean diameter of nerves fibers. Both groups of CHIT and CHIT/LF showed the lower mean diameter of nerve fibers than the SHAM group even at the end of the study. * P < 0.05 vs CHIT group.

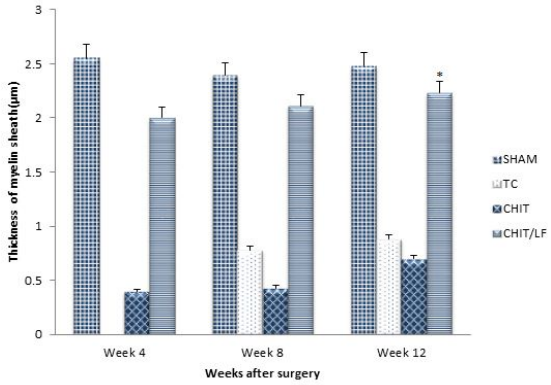

Figure 9- The graph shows the quantitative results of mean thickness of myelin sheath. Both groups of CHIT and CHIT/LF showed the lower mean diameter of axons than the SHAM group even at the end of the study period. * P<0.05 vs CHIT group.

Discussion

It is known from previous studies that regeneration process in rats would not have been completed by 12 weeks, a phenomenon which has been reported in a variety of experimental models.[20] Quantitatively, our results are consistent with these findings. However, 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.[21]

The results of the present study showed that application of laminin-fibronectin in a chitosan conduit resulted in faster functional recovery of the sciatic nerve during the study period. Left gastrocnemius muscle weight was significantly greater in the CHIT/LF group than in the

CHIT group, indicating indirect evidence of successful end organ reinnervation in the laminin-fibronectin treated animals. It has been demonstrated that morphometric indices are measures of regenerated nerve maturity and quality of regeneration.[22] Larger diameters of axons and thicker myelination give rise to improved nerve function compared to smaller and thinner myelinated fibers.[23] Loading of laminin-fibronectin into CHIT conduit at the nerve repair site increased fiber maturity.

At week 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the CHIT and CHIT/LF groups, indicating a beneficial effect of local application of laminin-fibronectin on the nerve regeneration.

Other researchers in their own results have reported that, although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor.[23-25] Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling do not necessarily predict the reestablishment of motor and sensory functions.[26-29] Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery.[24] Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment. It has been suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function.[21]

Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries.[30]

Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that the laminin-fibronectin treated animals had been improved in locomotion of the operated limb compared to the CHIT group during the study period. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models.[28,31]

SSI is a time-saving digitized static footprint analysis described by others.[31] 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.[31]

Several nerve guidance conduits and nerve protectant wraps are approved by the US Food and Drug Administration for clinical use in peripheral nerve repair. These devices cover a wide range of natural and synthetic materials, which may or may not be resorbable.[32] Surgeons are often not aware of the different (bio) materials of these conduits when performing nerve repair.[33]

Tong et al. in 1994 indicated that the presence of laminin and fibronectin in collagen grafts can dramatically increase the ability of neural components to regenerate effectively over long nerve gaps.[34] Wang et al. in 1992 found that the number of regenerated axons was significantly decreased as compared to normal controls if the laminin or the fibronectin was depleted in nerve grafts.[35] Woolley et al. in 1993 speculated a possible mechanism for this promoted regeneration: the laminin-fibronectin mixture may facilitate the influx of non-neural cells into the regenerated nerve cable in which these cells may provide additional neurotrophic factors for the growth of axons.[10,36] Since collagen, laminin and fibronectin have been shown to have neurite-promoting activity, another possible mechanism is that they can promote nerve regeneration by encouraging interactions between developing neurites and substrata.[37]

In the present study, first of all, it was important to know whether administration of laminin-fibronectin in biodegradable chitosan tubes is able to stimulate the regeneration of the transected rat sciatic nerve. With this aim, we compared the regeneration of the transected sciatic nerve within biodegradable guides. Our functional results revealed that the chitosan biodegradable guides allow laminin-fibronectin to exert the stimulation of nerve regeneration. In addition, gasterocnemius muscle mass, obtained from muscles of operated and non-operated limbs, indicated that motor functional recovery in transected sciatic nerve bridged by chitosan conduits achieved a faster rate. The functional, morphometric and immunohistochemical results indicated that the degradation of the chitosan nerve guides did not prevent the stimulating action of laminin-fibronectin.

Even though our study shows the neuroprotective action of local laminin-fibronectin in peripheral nerve injuries, data regarding the molecular mechanisms leading to the neuroprotective action remain to be investigated in depth. We have not given the histological and molecular evidence for neuroprotective action of laminin-fibronectin. 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 laminin-fibronectin on nerve regeneration. The results of the present study indicated that a single topical administration of laminin-fibronectin at the site of transected nerve could be of benefit after chitosan graft tubulization. Detailed mechanism of neuroprotective action remains to be investigated. In Conclusion results of the present study demonstrated that a single local application of laminin-fibronectin could accelerate functional recovery after transection of sciatic nerve.

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چکیده

بررسی تاثیر موضعی لامینین و فیبرونکتین توام با گرافت کیتوزانی بر روی ترمیم اعصاب محیطی: مدل قطع عصب سیاتیک رت

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هدف- هدف از این مطالعه بررسی تاثیر موضعی لامینین و فیبرونکتین توام با گرافت کیتوزانی بر روی ترمیم اعصاب محیطی در مدل قطع عصب سیاتیک رت بوده است.

طرح مطالعه- مطالعه تجربی.

حیوانات- ۶۰ سر رت ویستار نر سالم.

روش کار- 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 کیتوزان پس از ایجاد نقیصه ۱۰ میلیمتری، انتهاهای قطع شده پروکزیمال و دیستال عصب با استفاده از لوله کیتوزانی(CHIT) به هم مرتبط شدند. در گروه درمان (CHIT/LF) پس از کارگذاری لوله کیتوزانی به داخل آن ۱۰ میکرولیتر محلول لامینین و فیبرونکتین با دز ۱ میلی‌گرم 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 دو بطنی نشان داد که عملکرد عصب سیاتیک در رت‌های دریافت‌کننده گرافت وریدی بهبودی قابل ملاحظه‌ای پیدا کرده بود ($P<0.05$). شاخص های مورفومتریک و ارزیابی های ایمنوهیستوشیمیایی بیانگر ترمیم قابل ملاحظه‌ای در گروه درمان در مقایسه با گروه شاهد بود ($P< 0.05$).

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

کلید واژگان- ترمیم عصب، سیاتیک، فیبرونکتین، لامینین، موضعی، رت.