치의학박사 학위논문

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신경성장인자 공급 임플란트를 이용한 하치조신경 손상 치료에서 신경 재생 및 골유착에 관한 연구

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- Abstract -

Therapeutic effect of nerve growth factor supplying implant on the inferior alveolar nerve regeneration and osseointegration

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Purpose Among the various complications of dental implant procedure, sensory disturbance caused by the damage of inferior alveolar nerve (IAN) is one of the most difficult and challenging issues with continually increasing incidence. The purpose of this study was to evaluate the functional regeneration of IAN by supplying nerve growth factor (NGF) using specially designed dental implant and its effect on the

osseointegration of NGF-supplying implant.

Materials and Methods Under general anesthesia with intravenous injection of the mixture of tiletamine and zolazepam, bilateral IAN of beagle dogs (n=9, 18 week-old, 10 ~ 12kg) was exposed, transected and directly repaired. NGF-supplying implants were installed just above the nerve damage site after the third premolar extraction and connected with osmotic pump by microcatheter.. For experimental group (right IAN), total 300μg of NGF mixed with 2mL PBS solution was loaded in the pump and same amount of PBS for control group (left IAN). The amplitude and latency were measured with needle-type electrodes before surgery and at postoperative 3, 6 week. The conduction velocity (CV) and peak voltage (PV) were measured with custom-made hook type electrodes before transection, after nerve repair and at postoperative 6 week. At 6 week, nerve specimens were harvested and embedded to calculate axon count and density. After that, TEM analysis was done to measure the myelin thickness and G-ratio. Dental implants were observed clinically with checking the implant stability quotient (ISQ). After 6 weeks, each dental implant was prepared for the decalcified and non-decalcified sections for the measurement of bone-implant contact ratio (BIC) and the

new bone area inside the thread area (BA).

Results In the amplitude and latency, there were no significant differences between two groups. The median CV of NGF group (2.675 m/s) was significantly higher than that of PBS group (1.892 m/s) at 6 week ($p < 0.01$) and same tendency was also observed in PV (1.940 μV in NGF group and 1.300 μV in PBS group). The axon count of NGF group (4576.107±270.413) was higher than that of PBS group (3606.972±242.876) with significance ($p < 0.001$) and same as axon density (NGF group : 10707.458±638.835/mm2, PBS group : 7899.781±1063.625/mm2,$p < 0.001$). In myelin thickness, mean of NGF group (1.670±0.555 μm) was higher than that of PBS groups (1.173±0.388 μm) with significance ($p < 0.01$), but no significant difference was found in G-ratio between two groups (NGF vs. PBS, 0.594±0.110 vs. 0.635±0.092). The BIC of NGF group (46.609±6.521 %) was higher than that of PBS group (42.884±6.489 %), but no significant difference was found. The same tendency was found in the BA (36.993±7.0434 % in NGF group and 33.327±6.551 % in PBS group) and ISQ (42.375±3.017 in NGF group and 38.714±2.533 in PBS group). Overall survival rate of NGF-implant was 88.89% in NGF group (88.89%), and 77.78% in PBS group.

Conclusions NGF group showed higher conduction velocity and peak voltage across the nerve repair with significance. Same tendencies were also observed in amplitude and latency of CNAP, but there were no significant differences between two groups. In histomorphometric analysis, NGF group exhibited significantly higher axon count, axon density and myelin thickness than those of PBS group, but there were no significant differecence in G-ratio, BIC and BA. Except three exfoliated implants, no significant difference was found in ISQ between NGF group and PBS group at 6 week. These results showed that NGF supplying via specially designed dental implant could promote the functional regeneration of IAN transection-repair injury. Furthermore, supplied NGF and the design of implant did not interrupt the osseointetegration of implant. This approach could be a novel technique in the treatment of IAN injury, in which nerve regeneration and prosthetic rehabilitation could be achieved simultaneously.

Key Words : Nerve growth factor, Specially designed dental implant, Inferior alveolar nerve damage, Peripheral nerve regeneration

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I. Introduction

Over the last 40 years, dental implant procedures have become the preferred option to rehabilitate partial or complete edentulism, with a high success rate of greater than 90%. However, many complications have been reported, and sensory disturbances due to injury of the inferior alveolar nerve (IAN) remains one of the most challenging issues and is occurring increasingly more frequently.[1] Kiyak et al.[2] reported that sensory disturbance was found in 43% of patients at 2 weeks after implant surgery and van Steenberghe et al.[3] reported that sensory disturbance remained in 6.5% of patients 1 year after surgery. According to a retrospective, multicenter study by Ellis and Hawker[4], permanent IAN damage was found in 13% of the patients who underwent mandibular implant surgery.

When IAN damage is identified during or after implant surgery, it should be managed as soon as possible with appropriate care, which includes medication, physical therapy, implant removal, neuroma excision, direct repair or nerve grafting. However, many cases of IAN damage result from direct contact

between the nerve and the implant fixture or drill, and involve severe damage including complete neurotmesis, neuroma formation or segmental loss of nerve stump, in which case, surgical intervention should be considered.[5] Ever since Heuter[6] introduced the epineural repair technique, it has become the standard method of nerve repair. Although there have been overall advancements in microsurgical techniques and instruments, complete regeneration takes months to years and function rarely returns to pre-injury levels.[7] Therefore, many clinical and experimental approaches have been attempted to promote functional regeneration of the injured nerve.

Neurotrophic factors are primarily involved in the survival, maintenance and regeneration of nervous system.[8-10] Nerve growth factor (NGF) is a neurotrophic factor that was discovered by Levi-Montalcini and Hamburger almost 60 years ago and is the first identified member of the neurotrophin family.[11] NGF is considered to be a very powerful growth factor for sympathetic and sensory neurons and for the neuronal crest cells.[12, 13] NGF mediates its signals by activating two NGF-specific receptors: p75 neurotrophin receptor ($p75^{NTR}$) and

the tropomyosin kinase receptor A (TrkA) receptors.[14] NGF is correlated with innervation density, cell body size, axonal terminal sprouting, dendrite arborization and control of neuropeptides and neurotransmitters or transmitter-producing enzymes.[15-18] NGF can also promote regeneration of the peripheral nerve.[19-21]

However, despite extensive previous research, the clinical application of NGF for IAN damage has been impeded, primarily due to its very short *in vivo* half-life[22]. Hence, NGF should be applied directly to the damaged site in order to maintain the effective dose for as long as needed. Although various types of controlled NGF-supplying systems have been introduced, none of them can effectively supply NGF to the intraosseously-located IAN and *in vivo* studies of their application are still insufficient. Moreover, completion of total treatment would be delayed due to the time required for functional regeneration of IAN and subsequent osseointegration of the reinstalled implant.

For these reasons, the authors designed an NGF-supplying implant that can supply a constant amount of NGF solution. With this system, nerve regeneration

and osseointegration can be achieved simultaneously and prosthetic rehabilitation can then be started immediately without additional implant placement procedures. Consequently, the total treatment period could be shortened with improved regeneration of the repaired IAN injury.

The purpose of this study was to evaluate the functional regeneration of IAN by supplying NGF using specially designed dental implant and its effect on the osseointegration of the IAN.

II. Materials and Methods

1. Experimental design

1.1. Fabrication of NGF-supplying implant

The design of the NGF-supplying implant was based on the commercially available SimpleLine® II implant (Dentium Inc., Republic of Korea) and manufactured as an internal fixture with an internal lumen of 1.3 mm in diameter (Dentium Inc., Korean patent No.10-2011-0103682). Body diameter and platform diameter were 3.4 mm and 4.8mm, respectively. The fixture was 8 mm long and the gingival height of the collar was 2.2 mm. The collar has a morse taper internal design with an 8-degree angle and octagonal shape. The titanium surface was modified with sandblasting with large grit and acid etching (SLA) treatment (Fig. 1).

1.2. Dosage and supplying system of NGF

Immediately before surgery, 3.0 mg of human β-NGF (Peprotech Inc., Rocky Hill, NJ, USA) was mixed in 20 mL of phosphate buffered saline (PBS) and stored on ice. Just prior to osmotic pump insertion, the NGF-loaded pump was prepared by injecting an aliquot (300 μg of NGF in 2.0 mL PBS) of this solution into an Alzet® osmotic pump (model #. 2ML2, Alzet Inc., Cupertino, CA, USA). The PBS-loaded pump was prepared by loading an Alzet® osmotic pump with the same amount (2.0 mL) of PBS.

1.3. Surgical procedures

The 18-week-old male beagle dogs (n=9, 10-12kg) were placed under general anesthesia using an intravenous injection (cephalic vein) of a 1:1 mixture of tiletamine and zolazepam (0.2ml/kg; Zoletil®50, Virbac Inc., France) and xylazine hydrochloride (0.15ml/kg; Rumpun®, Bayer Korea, Republic of Korea). The IAN was exposed according to the modified protocol of Kahnberg and

Ridell.[23] Briefly, after intraoral disinfection with betadine scrubbing, a mucosal incision and periosteal dissection were performed and extended to the retromandibular area. After identifying the mental foramen, a bony window was created with a round bur. The bone fragment was carefully removed with a periosteal elevator and chisel, and the nerve bundle was identified. Extraction forceps were then used to surgically extract the third premolar (P3). The NGF-supplying implant (Fig. 1B) was installed to a depth where the implant apex was directly over the IAN. Using a microscope, the nerve was transected at the implant apex-nerve contact site and 9-0 nylon was used for epineural sutures (3 stitches). A micro-catheter (rat jugular, 15cm in length) was inserted through the internal lumen of the implant, and the catheter tip was sutured onto the transection-repaired site with 9-0 nylon (Figs. 1C, D). The osmotic pump and its reservoir were placed into the retromandibular area. Gaps between the micro-catheter and the implant collar were filled with Caviton® (GC Inc., Japan). After the periosteal releasing incision was made, wound closure was done with 4-0 Nylon. All procedures were conducted in strict accordance with the animal care

guidelines of the Institute of Laboratory Animal Resources at Seoul National University (Approval No. SNU-120416-4).

2. Evaluation

2.1. Gross clinical observation

At three and six weeks postoperation, a clinical inspection was performed to assess wound healing, inflammation, implant exposure and loss of fixture.

2.2. Evaluation of nerve regeneration

2.2.1. Electrophysiological assessment

2.2.1.1. Indirect assessment: compound nerve action potential (CNAP)

Compound nerve action potential (CNAP) was assessed before nerve transection and at three and six weeks postoperation (prior to IAN exposure) with Neuro-EMG-Micro system® (Neurosoft Co., Russia). Dogs were anesthetized as previously mentioned and stimulating electrode needle was placed above the mental foramen and for reference at the mandibular symphysis. A recording electrode needle was placed over the mandibular foramen and for reference in the ipsilateral zygomatic area. The stimuli were repeated 20 times with an intensity of 4mA, a duration of 0.1 ms each and a frequency of 4Hz. Digitally converted signals were recorded and analyzed with Neruo-MEPω® software (version 3.1.14.0; Neurosoft Co., Russia).

2.2.1.2. Direct assessment: conduction velocity (CV) and peak voltage (PV)

Conduction velocity (CV) and peak voltage (PV) were assessed with Neuro-EMG-Micro system® (Neurosoft Co., Russia) just before nerve transection, after nerve stump suture and at six weeks postoperation. After general anesthesia,

custom-made hook electrodes were placed 5mm proximally (recording electrode) and 5 mm distally (stimulating electrode) from the transection site. The 1mA stimuli was repeated 20 times; each stimuli had a duration of 0.1ms and a frequency of 4Hz. After stimulation, latency (ms) and peak voltage (μV) were recorded with Neruo-MEPω® software version 3.1.14.0; Neurosoft Co., Russia) and the distance between recording and stimulating electrodes (10 mm) was divided by this latency to calculate CV.

2.2.2. Histomorphometric analysis of axon count, axon density, myelin thickness and G-ratio

After electrophysiological assessment at six weeks postoperation, a 12-mm segment of IAN was harvested from the center of the transection site. All specimens were fixed with 2.5% glutaraldehyde in PBS at 4℃ for 24 hours and post-fixed in 2% osmium tetroxide for 1.5 hours. After dehydration in a graded concentration of ethanol, the specimens were embedded in epon resin, and 1-μm-

thick sections were prepared and stained with toluidine blue. Light microscopic images were visualized with an Olympus BX51® microscope (Olympus Co., Japan) and digitally converted with DP2-BSW® software (version 2.2; Olympus Co., Japan). Average axonal density was calculated from three randomly selected areas near the center with Image J® software (version 1.46r; National Institute of Health, Bethesda, MD, USA). Total axon count was calculated using a proportional ratio of the total area. Specimens were then trimmed, sectioned (80 nm) and double stained with uranyl acetate and lead acetate. Myelin thickness and G-ratio (ratio of axon diameter to fiber diameter) were measured using a transmission electron microscope (JSM 1200 II EX, JEOL, Japan).

2.3. Evaluation of osseointegration

2.3.1. Implant stability quotient (ISQ) and survival rate of NGF implant

Implant stability quotient (ISQ) was measured with Osstell Mentor® (Osstell

AB, Sweden) at six weeks postoperation. Overall implant survival rates for each group were also determined.

2.3.2. Histomorphometric analysis of bone-implant contact ratio (BIC) and the ratio of bone area inside the thread area (BA)

After all parameters were measured, dogs were sacrificed with an intravenous potassium chloride injection six weeks postoperatively. Mandibular segments around the implant site were harvested with a disk bur and fixed with 4% paraformaldehyde solution. Each segment was divided into two blocks that contained longitudinal sections of the implant: one non-decalcified and one decalcified specimen.

For non-decalcified specimens, harvested segments were fixed with 10% phosphate buffered formalin. After gradual dehydration with ethanol, they were embedded in light-curing resin (Technovit 7200 VLC, Heraeus Kulzer, Germany) and incubated at 60℃ for 3 days. An EXAKT system (Exakt Apparatebau,

Germany) was used for trimming and grinding to make 30-μm-thick sections. These sections were stained with hematoxylin and eosin (H&E).

For decalcified specimens, segments were fixed with 10% phosphate buffered formalin for 2 weeks. After two weeks, the segments were decalcified with 1% EDTA (ethylenediaminetetraacetic acid) solution. These samples were embedded in paraffin wax and 10-μm-thick sections were prepared. Masson's trichrome (MT) was used for staining. Under a light microscope, (Olympus BX51® microscope, Olympus Co., Japan) images were digitally converted with DP2-BSW® software (version 2.2; Olympus Co., Japan) and assessed.

Digital images of decalcified and non-decalcified specimens were organized sequentially and merged with Photoshop® software (CS6 extended version 13.0.1; Adobe systems, San Jose, CA, USA). Based on these images, the bone-implant contact ratio (BIC) and the ratio of bone area inside the thread area (BA) within apical 5 threads were measured using Image J® software (version 1.46r; National Institute of Health, Bethesda, MD, USA).

3. Statistics

Data normality was verified with Lilliefors' modified Kolmogorov-Smirnov test and all data except conduction velocity (CV) and peak voltage (PV) were verified ($p > 0.05$). Student's *t*-test was used to compare axon count, axon density, myelin thickness, G-ratio, ISQ, BIC and BA between groups. Consecutive comparisons of CNAP amplitude and latency were performed with repeated measured analysis of variance, while relative comparisons were made with a paired t-test.

Since Lilliefors' modified Kolmogorov-Smirnov test did not verify the normality of CV and PV ($p < 0.05$), consecutive comparisons were performed with a non-parametric Friedman test followed by a post-hoc analysis using Bonferroni's correction method. Relative comparisons were performed with the Wilcoxon signed rank test. *P*-values less than 0.05 were considered statistically significant. SPSS ver. 20.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

III. Results

1. Gross clinical observations

The beagles used in this study remained healthy for the duration of the experiment. There were four cases of gingival bleeding (two cases in each group) and one case of pus discharge in PBS group. The implant was exposed in 7 cases (three from the NGF group and four from the PBS group). Three fixtures were lost (one in NGF group and two in PBS group).

2. Evaluation of nerve regeneration

2.1. Electrophysiological assessment

2.1.1. Indirect assessment: CNAP

The mean amplitude of CNAP in the NGF groups before surgery was 0.928±0.598 μV; this value decreased after 3 weeks (0.733±0.404 μV) and then increased after 6 weeks (1.331±0.663 μV). In contrast, the mean amplitude of CNAP in the PBS group decreased consistently over time (1.123±0.551 μV, 0.900±0.703 μV and 0.653±0.347 μV before surgery, after 3 weeks and after 6 weeks, respectively). However, there were no significant differences between the two groups (Fig. 2A). At 3 and 6 weeks post-surgery, average CNAP amplitudes had gradually increased compared to day of surgery in the NGF group, but not in the PBS group (Fig. 2B).

Mean latency of NGF group had increased slightly at 3 weeks, but decreased at 6 weeks (0.944±0.482 ms, 1.043±0.417 ms and 0.992±0.676 ms before surgery, after 3 weeks and after 6 weeks, respectively) and this same pattern was also observed in PBS group (0.903±0.231 ms, 1.104±0.395 ms and 1.032±0.292 ms before surgery, after 3 weeks and after 6 weeks, respectively). There were no significant differences between the two groups (Fig. 3A). A comparison of the relative ratio of average latency was performed as mentioned above. The average

amplitudes in both groups gradually decreased, although this change was not significant (Fig. 3B).

2.1.2. Direct assessment: CV and PV

The median CV for both groups decreased just after surgery and then increased at 6 weeks. There were no significant differences in median values between the two groups before or after surgery (NGF vs. PBS; 2.671 m/s vs. 2.531 m/s before surgery and 1.698 m/s vs. 1.694 m/s after surgery). However, the median CV of NGF group (2.675 m/s) was significantly higher than that of PBS group (1.892 m/s) at 6 weeks postoperation ($p < 0.01$; Fig. 4A). The relative ratio of average CV for both groups gradually increased and that of NGF group at 6 weeks was almost close to the preoperative level. (Fig. 4B).

Similar tendencies were observed for PV. The NGF group (1.940 μV) had a significantly higher PV than the PBS group (1.300 μV) at 6 weeks ($p < 0.01$; Fig. 5A). The relative ratio of average latency for the NGF group increased

considerably by 6 weeks, but there were no notable changes in PBS group (Fig. 5B).

2.1.3. Histomorphometric analysis of axon count, axon density, myelin thickness and G-ratio

Mean axon count in NGF group (4576.107±270.413) was significantly higher than the PBS group (3606.972±242.876) ($p < 0.001$; Fig. 6C). The same pattern was observed for axon density; mean axon density of NGF group and the PBS group were 10707.458±638.835/mm^2 and 7899.781±1063.625/mm^2, respectively ($p < 0.001$; Fig. 6D).

Mean myelin thickness in NGF group (1.670±0.555 μm) was significantly higher than in PBS group (1.173±0.388 μm) ($p < 0.01$; Fig. 7C). There were no significant differences in G-ratio between the two groups (NGF vs. PBS, 0.594±0.110 vs. 0.635±0.092), although the mean G-ratio in the NGF group was lower than that of the PBS group (Fig. 7D).

3. Evaluation of osseointegration

3.1. Implant stability quotient (ISQ) and survival rate of NGF implant

There were no significant differences in ISQ between the two groups (42.375±8.535 in NGF group and 38.714±6.701 in PBS group; Fig. 8). The overall success rate was 83.33% (15 out of 18), with a survival rate of 88.89% (8 out of 9) for the NGF group and 77.78% (7 out of 9) for the PBS group.

3.2. Histomorphometric analysis of bone-implant contact ratio (BIC) and the ratio of bone area inside the thread area (BA)

In the non-decalcified specimens, new bone formation around the implant was slightly more prominent in the NGF group. There were no severe pathologic findings in either group (Fig. 9). Similar findings were also observed in the decalcified specimens (Fig. 10). The BIC of NGF group (46.609±18.445 %) was

higher than that of PBS group (42.884±17.168 %) although this difference was not significant (Fig. 11A). There was no significant difference in BA between the NGF and PBS groups (36.993±19.923 % and 33.327±17.332 %, respectively) (Fig. 11B).

IV. Discussion

Local administration of NGF enhances the functional recovery of IAN,[24-26] but its clinical application in implant-related IAN injuries can be problematic. First, NGF has a short half-life (5.4 minutes of distribution and 2.3 hours of elimination), and it is very difficult to maintain the predetermined dose for a given period.[22] Second, the IAN is located in the mandibular canal, which acts as a protective barrier. Third, NGF is a multifunctional biomolecule, possibly due to the wide distribution of its receptors in non-neural tissues.[27] For example, NGF is known to be involved in the release of 5-HT (5-hydroxytryptamine) from mast cells and the following sensitization of nociceptors,[28-31] healing of wounds and fractures[32, 33] and survival of memory B lymphocytes.[34] To overcome these problems, NGF must be supplied directly to the damaged IAN in a controlled manner. Although many types of supplying systems have been introduced, such as collagen matrix[35] and fibrin gel,[36] none of them were successfully used alone in this clinical situation. Thus, the authors designed the NGF-supplying implant,

which supplies NGF through an internal lumen by mini osmotic pump (5.0 μL/hr).

A literature review was conducted to determine the appropriate NGF dose for IAN regeneration. Eppley et al.[24] created a 7-mm mandibular nerve defect model in New Zealand white rabbits (body weight: 3 kg) that was repaired with a 10-mm silastic tube conduit filled with 25 μL of NGF solution (3 mg/mL) using a total NGF dose of 75 μg. Wang et al.[25] created a 3kg New Zealand white rabbit mandibular distraction osteogenesis model, injected two doses of 40 μg NGF (total 80 μg) into the IAN and concluded that morphological recovery of the IAN was accelerated. Another study using the same animal model with NGF gel (total dose was 20 μg) also showed recovery of the IAN.[37] A previous study of crush injuries to the mental nerve in Sprague-Dawley rats found that 1.0 μg/mL was the optimal dose of NGF for nerve regeneration (total 84 ng).[38] The present study adopted the dose used in Eppley et al. [24] (75 μg) because their experimental design was most similar to the present study. Since beagle dogs were used in this study and their average body weight was ranged from 10 to 12 kg, the NGF dose

was calculated from proportional conversion (fourfold) and a total of 300 μg was used as the experimental dose in this study.

The electrophysiological assessment in this study showed that functional recovery in NGF group was more prominent than in the PBS group. This finding was consistent with a previous study by Savignat et al.,[39, 40] in which they used an NGF-coated polymeric membrane in crush injuries of rat mental nerve. The activation potential at 1 month postoperation increased significantly in the NGF group, whereas it decreased significantly in the control group. Histomorphometric studies also showed that IAN regeneration with an NGF-supplying implant could enhance functional recovery with significantly higher total numbers of axons and axon density, as shown by other studies.[25, 26, 37] In addition, although there was no significant difference between the two groups, the mean value of the G-ratio in NGF group was close to 0.6, which is considered the optimal value for the peripheral nerve.[41,42] This result corroborated the higher CV of NGF group in this study.

It is also important to consider the effect of NGF on the osseointegration of the

NGF-supplying implant. Previous studies demonstrated that NGF could promote the bone formation around the regenerated axons, fracture healing and consolidation.[24,33,43,44] NGF is known to stimulate a calcitonin gene-related peptide (CGRP)[18] which is involved in the production of interleukin-6 and insulin-like growth factor-I by osteoblasts[45,46] and the inhibition of osteoclastic bone resorption.[47] Moreover, NGF and its receptors are found in osteoblastic cells and seem to regulate bone formation through autocrine and paracrine mechanisms.[48] In this study, there were no significant differences between the NGF and PBS groups in terms of ISQ, BIC and BA. Average ISQ values were somewhat lower than reported in other animal studies, which might be due to the immediate implantation after premolar extraction; implantation was performed approximately 2 to 3 months after extraction in other studies.[49,50] Although the effects of NGF on the ISQ, BIC and BA were not clear in this study, it did not seem to have a deleterious effect on osseointegration of the NGF-supplying implant.

To the best of the author's knowledge, this is the first *in vivo* study on the development and experimental verification of a controlled NGF-supplying device for IAN regeneration. However, this study has some limitations. First, although the NGF dose in this study was effective in nerve regeneration, further studies are necessary to determine the optimal therapeutic dose. Another limitation was the hollow structure of the NGF-supplying implant, which may not be as strong as conventional fixtures, warranting biomechanical evaluation under occlusal load. In addition, due to its internal lumen, microleakage around the abutment could result in unwanted complications in the alveolar bone around the apical hole of implant. Although NGF can be supplied using the methods described in this study, or by repeated injections through the implant lumen in clinical situations, it is worth considering a more sophisticated and controlled NGF-supplying system using a drug-supplying vehicle filled in the lumen of the NGF-implant. In addition, the clinical safety of NGF should be verified, as the expression of NGF and its receptors are correlated with tumorigenesis, although the underlying mechanism is not clearly understood.[51-53] Despite these limitations, this study

showed that IAN regeneration with NGF-supplying implant in this study may enhance functional recovery of IAN injury and did not hinder osseointegration of the NGF-supplying implant.

V. Conclusions

A controlled supply of NGF with a specially designed dental implant could enhance functional regeneration of the IAN. Osseointegration of the NGF-supplying implant was not hindered by the NGF or implant design. This approach could be a novel technique in the treatment of IAN injuries, making it possible to achieve nerve regeneration and prosthetic rehabilitation simultaneously.

References

1. Delcanho RE. Neuropathic implications of prosthodontic treatment. *J Prosthet Dent*. 1995;73:146-152.

2. Kiyak HA, Beach BH, Worthington P, Taylor T, Bolender C, Evans J. Psychological impact of osseointegrated dental implants. *Int J Oral Maxillofac Implants*. 1990;5:61-69.

3. van Steenberghe D, Lekholm U, Bolender C, Folmer T, Henry P, Herrmann I, et al. Applicability of osseointegrated oral implants in the rehabilitation of partial edentulism: a prospective multicenter study on 558 fixtures. *Int J Oral Maxillofac Implants*. 1990;5:272-281.

4. Ellies LG, Hawker PB. The prevalence of altered sensation associated with implant surgery. *Int J Oral Maxillofac Implants*. 1993;8:674-679.

5. Hegedus F, Diecidue RJ. Trigeminal nerve injuries after mandibular implant placement - Practical knowledge for clinicians. *Int J Oral Maxillofac Implants*. 2006;21:111-116.

6. Heuter K. [The General Surgery]. Leipzig: Vogel Verlag: 1873. (in German)

7. Hessling KH, Reich RH, Hausamen JE, Feistner H. [Long-term results of microsurgical nerve reconstruction in the area of the head-neck]. *Fortschr Kiefer Gesichtschir*. 1990;35:134-138. (in German)

8. Gordon T. The physiology of neural injury and regeneration: The role of neurotrophic factors. *J Commun Disord*. 2010;43:265-273.

9. Magill CK, Moore AM, Yan Y, Tong AY, MacEwan MR, Yee A, et al. The differential effects of pathway- versus target-derived glial cell line-derived neurotrophic factor on peripheral nerve regeneration. *J Neurosurg*. 2010;113:102-109.

10. Henderson CE. Role of neurotrophic factors in neuronal development. *Curr Opin Neurobiol*. 1996;6:64-70.

11. Levi-Montalcini R, Hamburger V. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J Exp Zool*. 1951;116:321-361.

12. Cowan WM. Viktor Hamburger and Rita Levi-Montalcini: the path to the discovery of nerve growth factor. *Annu Rev Neurosci*. 2001;24:551-600.

13. Rush RA, Chie E, Liu D, Tafreshi A, Zettler C, Zhou XF. Neurotrophic factors are required by mature sympathetic neurons for survival, transmission and connectivity. *Clin Exp Pharmacol Physiol*. 1997;24:549-555.

14. Wiesmann C, de Vos AM. Nerve growth factor: structure and function. *Cell Mol Life Sci*. 2001;58:748-759.

15. Otten U, Schwab M, Gagnon C, Thoenen H. Selective induction of tyrosine hydroxylase and dopamine beta-hydroxylase by nerve growth factor: comparison between adrenal medulla and sympathetic ganglia of adult and newborn rats. *Brain Res*. 1977;133:291-303.

16. Mearow KM, Kril Y. Anti-NGF treatment blocks the upregulation of NGF receptor mRNA expression associated with collateral sprouting of rat dorsal root ganglion neurons. *Neurosci Lett*. 1995;184:55-58.

17. Verge VM, Richardson PM, Wiesenfeld-Hallin Z, Hokfelt T. Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J Neurosci*.

1995;15:2081-2096.

18. Lindsay RM, Harmar AJ. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature*. 1989;337:362-364.

19. Lee AC, Yu VM, Lowe JB, 3rd, Brenner MJ, Hunter DA, Mackinnon SE, et al. Controlled release of nerve growth factor enhances sciatic nerve regeneration. *Exp Neurol*. 2003;184:295-303.

20. Sun W, Sun C, Lin H, Zhao H, Wang J, Ma H, et al. The effect of collagen-binding NGF-beta on the promotion of sciatic nerve regeneration in a rat sciatic nerve crush injury model. *Biomaterials*. 2009;30:4649-4656.

21. Chen J, Chu YF, Chen JM, Li BC. Synergistic effects of NGF, CNTF and GDNF on functional recovery following sciatic nerve injury in rats. *Adv Med Sci*. 2010;55:32-42.

22. Tria MA, Fusco M, Vantini G, Mariot R. Pharmacokinetics of nerve growth factor (NGF) following different routes of administration to adult rats. *Experimental neurology*. 1994;127:178-183.

23. Kahnberg KE, Ridell A. Transposition of the mental nerve in orthognathic

surgery. *J Oral Maxillofac Surg*. 1987;45:315-318.

24. Eppley BL, Snyders RV, Winkelmann TM, Roufa DG. Efficacy of nerve growth factor in regeneration of the mandibular nerve: a preliminary report. *J Oral Maxillofac Surg*. 1991;49:61-68.

25. Wang L, Zhao Y, Cheng X, Yang Y, Liu G, Ma Q, et al. Effects of locally applied nerve growth factor to the inferior alveolar nerve histology in a rabbit model of mandibular distraction osteogenesis. *Int J Oral Maxillofac Surg*. 2009;38:64-69.

26. Du ZJ, Wang L, Lei DL, Liu BL, Cao J, Zhang P, et al. Nerve growth factor injected systemically improves the recovery of the inferior alveolar nerve in a rabbit model of mandibular distraction osteogenesis. *Br J Oral Maxillofac Surg*. 2011;49:557-561.

27. Shibayama E, Koizumi H. Cellular localization of the Trk neurotrophin receptor family in human non-neuronal tissues. *Am J Pathol*. 1996;148:1807-1818.

28. McMahon SB, Bennett DLH, Priestley JV, Shelton DL. The biological

effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. *Nat Med*. 1995;1:774-780.

29. Andreev NY, Dimitrieva N, Koltzenburg M, McMahon SB. Peripheral administration of nerve growth factor in the adult rat produces a thermal hyperalgesia that requires the presence of sympathetic post-ganglionic neurones. *Pain*. 1995;63:109-115.
30. Svensson P, Cairns BE, Wang K, Arendt-Nielsen L. Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain*. 2003;104:241-247.
31. Theodosiou M, Rush RA, Zhou XF, Hu D, Walker JS, Tracey DJ. Hyperalgesia due to nerve damage: Role of nerve growth factor. *Pain*. 1999;81:245-255.
32. Kawamoto K, Matsuda H. Nerve growth factor and wound healing. *Prog Brain Res*. 2004;146:369-384.
33. Grills BL, Schuijers JA, Ward AR. Topical application of nerve growth factor improves fracture healing in rats. *J Orthop Res*. 1997;15:235-242.

34. Torcia M, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, et al. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell*. 1996;85:345-356.

35. Madduri S, Feldman K, Tervoort T, Papaloïzos M, Gander B. Collagen nerve conduits releasing the neurotrophic factors GDNF and NGF. *J Control Release*. 2010;143:168-174.

36. Bhang SH, Jeon O, Choi CY, Kwon YHK, Kim BS. Controlled release of nerve growth factor from fibrin gel. *J Biomed Mater Res A*. 2007;80:998-1002.

37. Wang L, Cao J, Lei DL, Cheng XB, Yang YW, Hou R, et al. Effects of nerve growth factor delivery via a gel to inferior alveolar nerve in mandibular distraction osteogenesis. *J Craniofac Surg*. 2009;20:2188-2192.

38. Jin WP. Exogenous nerve growth factor promoted the nerve regeneration at a crush injured mental nerve of rat (MS Thesis). Seoul National University; 2012.

39. Savignat M, De-Doncker L, Vodouhe C, Garza JM, Lavalle P, Libersa P. Rat

nerve regeneration with the use of a polymeric membrane loaded with NGF. *J Dent Res*. 2007;86:1051-1056.

40. Savignat M, Vodouhe C, Ackermann A, Haikel Y, Lavalle P, Libersa P. Evaluation of early nerve regeneration using a polymeric membrane functionalized with nerve growth factor (NGF) after a crush lesion of the rat mental nerve. *J Oral Maxillofac Surg*. 2008;66:711-717.
41. Rushton WA. A theory of the effects of fibre size in medullated nerve. *J Physiol*. 1951;115:101-122.
42. Chomiak T, Hu B. What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. *PLoS One*. 2009;4:e7754.
43. Wang L, Zhou S, Liu B, Lei D, Zhao Y, Lu C, et al. Locally applied nerve growth factor enhances bone consolidation in a rabbit model of mandibular distraction osteogenesis. *J Orthop Res*. 2006;24:2238-2245.
44. Cao J, Wang L, Lei DL, Liu YP, Du ZJ, Cui FZ. Local injection of nerve growth factor via a hydrogel enhances bone formation during mandibular distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol*.

2012;113:48-53.

45. Millet I, Vignery A. The neuropeptide calcitonin gene-related peptide inhibits TNF-alpha but poorly induces IL-6 production by fetal rat osteoblasts. *Cytokine*. 1997;9:999-1007.

46. Vignery A, McCarthy TL. The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts. *Bone*. 1996;18:331-335.

47. Zaidi M, Fuller K, Bevis PJ, GainesDas RE, Chambers TJ, MacIntyre I. Calcitonin gene-related peptide inhibits osteoclastic bone resorption: a comparative study. *Calcif Tissue Int*. 1987;40:149-154.

48. Asaumi K, Nakanishi T, Asahara H, Inoue H, Takigawa M. Expression of neurotrophins and their receptors (TRK) during fracture healing. *Bone*. 2000;26:625-633.

49. Huh JB, Kim SE, Kim HE, Kang SS, Choi KH, Jeong CM, et al. Effects of anodized implants coated with Escherichia coli-derived rhBMP-2 in beagle dogs. *Int J Oral Maxillofac Surg*. 2012;41:1577-1584.

50. Calvo-Guirado JL, Satorres M, Negri B, Ramirez-Fernandez P, Maté-Sánchez JE, Delgado-Ruiz R, et al. Biomechanical and histological evaluation of four different titanium implant surface modifications: an experimental study in the rabbit tibia. *Clin Oral Investig*. 2013 (article in press)

51. Sasahira T, Ueda N, Yamamoto K, Bhawal UK, Kurihara M, Kirita T, et al. Trks are novel oncogenes involved in the induction of neovascularization, tumor progression, and nodal metastasis in oral squamous cell carcinoma. *Clin Exp Metastasis*. 2013;30:165-176.

52. Kolokythas A, Cox DP, Dekker N, Schmidt BL. Nerve growth factor and tyrosine kinase A receptor in oral squamous cell carcinoma: is there an association with perineural invasion? *J Oral Maxillofac Surg*. 2010;68:1290-1295.

53. Ye Y, Dang D, Zhang J, Viet CT, Lam DK, Dolan JC, et al. Nerve growth factor links oral cancer progression, pain, and cachexia. *Mol Cancer Ther*. 2011;10:1667-1676.

Figures and Legends

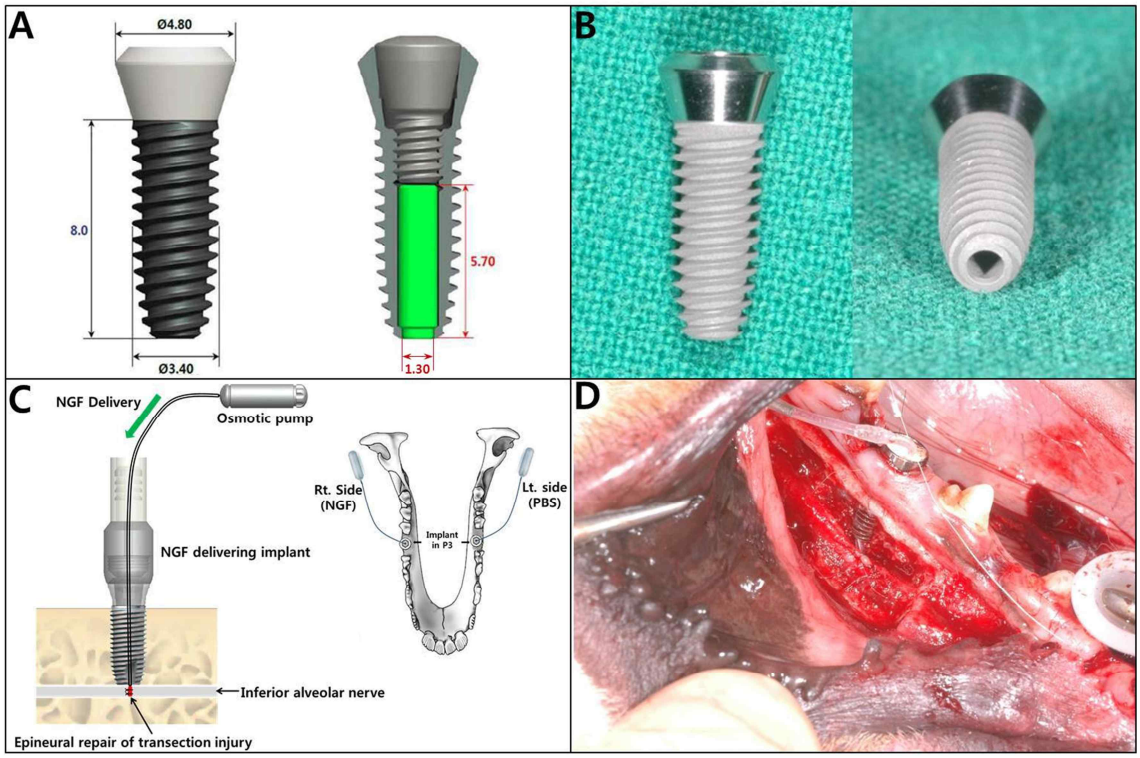

Fig 1. Design of the NGF-supplying dental implant and diagrammatic and intraoperative views of animal surgery.

(A) Design and cross-sectional diagram of NGF-supplying dental implant. Body diameter and platform diameter were 3.4 mm and 4.8mm, respectively. The fixture was 8-mm-long and the gingival height of the collar was 2.2 mm. The collar has a morse taper internal design with an 8-degree angle and octagonal shape. **(B)** Photograph of NGF-supplying dental implant with an internal lumen, **(C)** scheme of animal surgery and **(D)** completed repair of transection injury and micro-catheter and mini-osmotic pump connected to the IAN transection site.

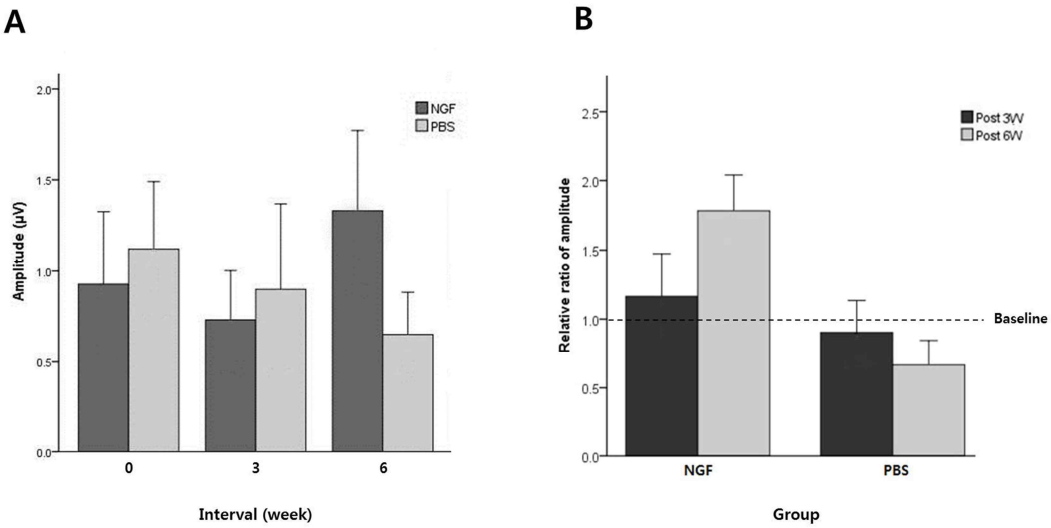

Fig 2. Comparison of compound nerve action potential (CNAP) amplitude by interval and group.

(A) Consecutive comparison of average amplitude for the NGF and PBS group with repeated measured analysis of variance. The NGF group showed improved recovery, although this finding was not statistically significant. **(B)** Comparison of the relative ratio of average amplitude using a paired *t*-test. Baseline was set as the average value measured on day of surgery. At 3 and 6 weeks postoperation, the average amplitude of NGF group had increased slightly, but there were no changes to the average amplitude in the PBS group.

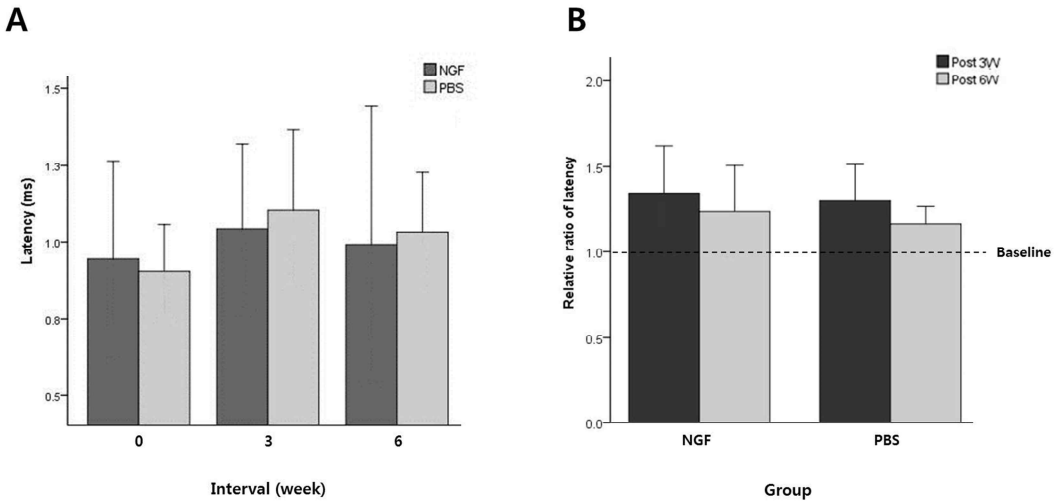

Fig 3. Comparison of compound nerve action potential (CNAP) latency by interval and group.

(A) Consecutive comparison of the average latency of the NGF and PBS groups with repeated measured analysis of variance. In both groups, average latencies had increased slightly after 3 weeks and decreased at 6 weeks postoperation, but these changes were not statistically significant. **(B)** Comparison of relative ratio of average latency with a paired *t*-test. Baseline was set as the average value measured on day of surgery. The average amplitude gradually decreased in both groups although this change was not statistically significant.

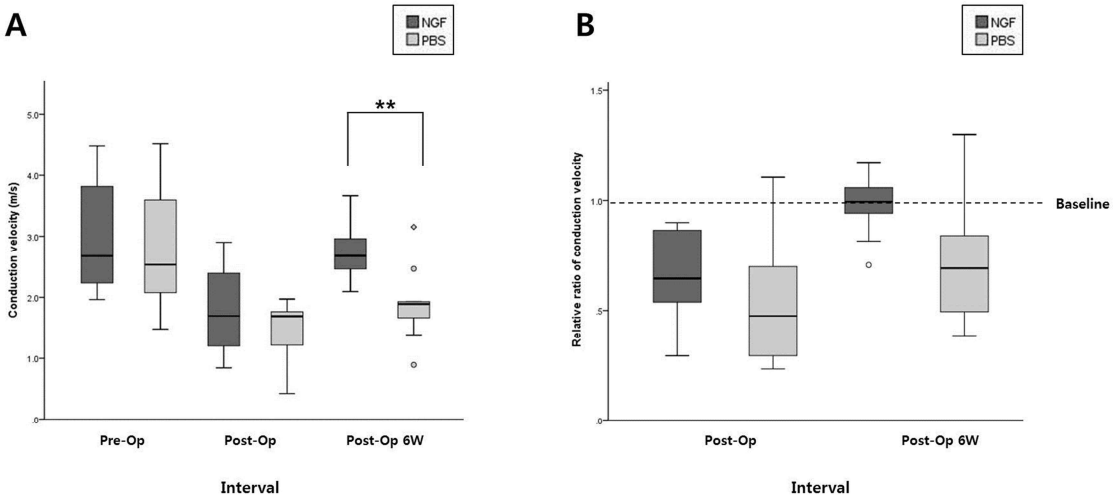

Fig 4. Comparison of conduction velocity (CV) between groups.

(A) Consecutive comparison of average CV of the NGF and PBS groups using the Friedman test. After transection, both groups exhibited a decreased CV, which then increased by 6 weeks postoperation. At 6 weeks, the average CV of NGF group was significantly higher than that of PBS group (**$p < 0.01$). **(B)** Comparison of relative ratio of average CV with Wilcoxon signed rank test. Baseline was set as the average value measured on day of surgery. In both groups, average CV gradually increased. The average CV of the NGF group was close to preoperative values.

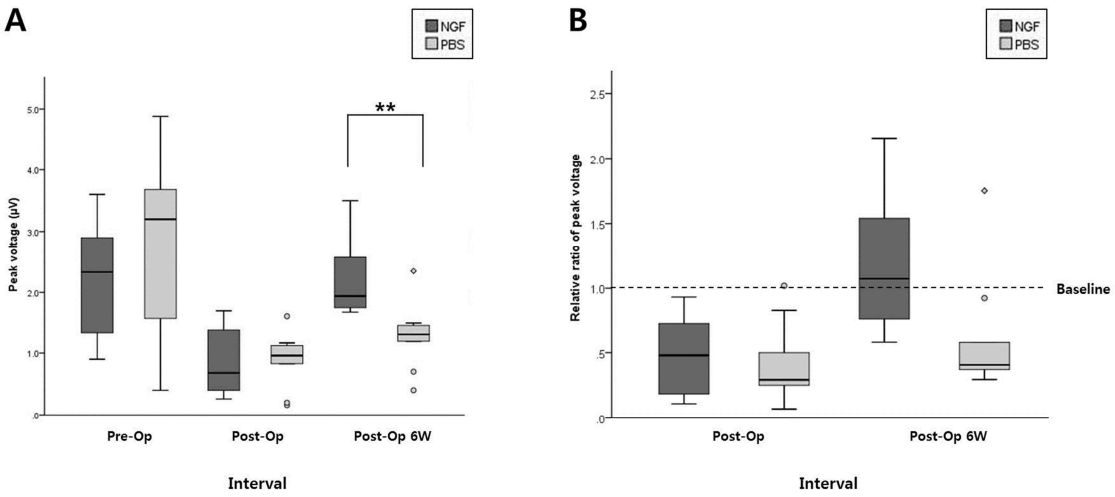

Fig 5. Comparison of peak voltage (PV) between groups.

(A) Consecutive comparison of average PV for the NGF and PBS groups using the Friedman test. Decreased PV in the NGF group had increased by six weeks and was significantly higher than the PBS group (**$p < 0.01$). There were no noticeable changes in the PBS group at six weeks. **(B)** Comparison of relative ratio of average latency with the Wilcoxon signed rank test. Baseline was set as the average value measured on day of surgery. Average PV increased considerably in the NGF group but not in PBS group.

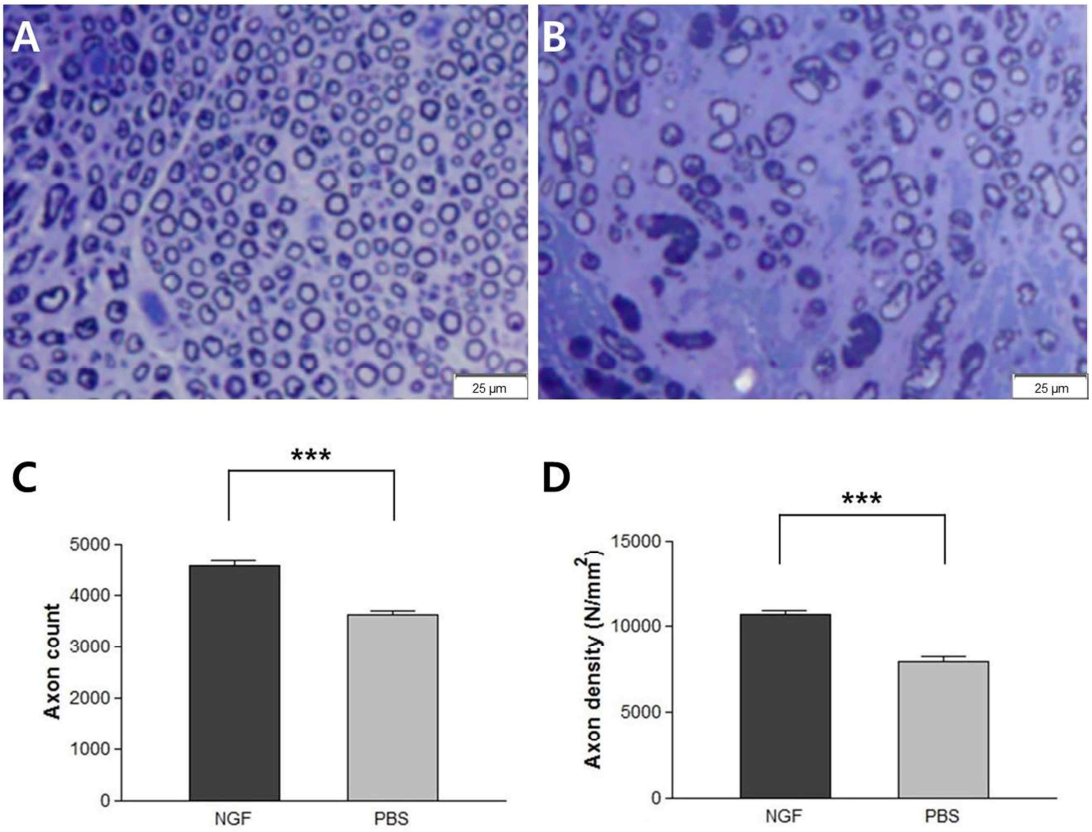

Fig 6. Histomorphometric analysis of axons (toluidine blue staining, 100× magnification).

Microscopic images of a 1-μm-thick specimen from the NGF **(A)** and PBS groups **(B)**. These images were digitally converted and analyzed to determine axon count and density. Comparison of axon count **(C)** and density **(D)** were done with Student's *t*-test. Both axon count and density in the NGF group were significantly higher than those of PBS group (***$p < 0.001$).

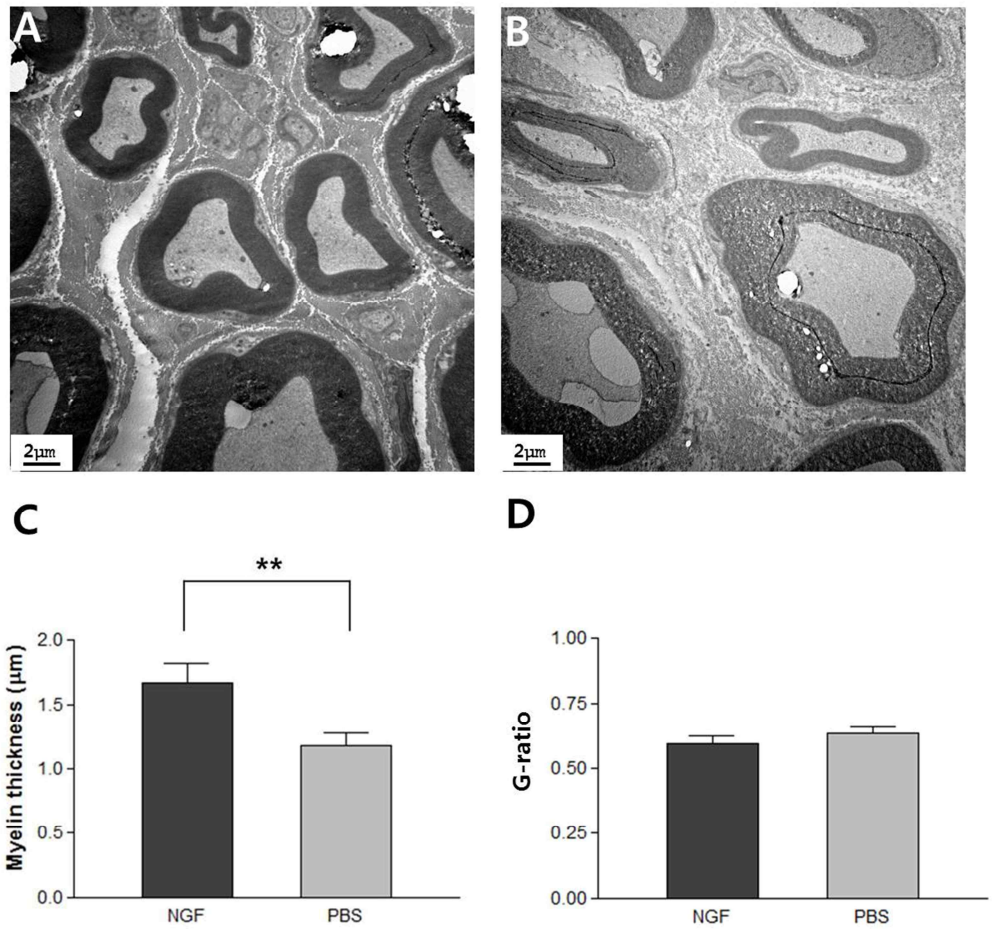

Fig 7. Histomorphometric analysis of myelin (5000× magnification).

Transmission microscope image of an 80-nm-thick specimen from the NGF **(A)** and PBS groups **(B)**. Comparison of myelin thickness **(C)** and G-ratio **(D)** were done using Student's *t*-test. Myelin from the NGF group was significantly thicker than myelin from the PBS group (**$p < 0.01$). The average G-ratio was slightly lower in the NGF group compared to the PBS group, although this difference was not significant.

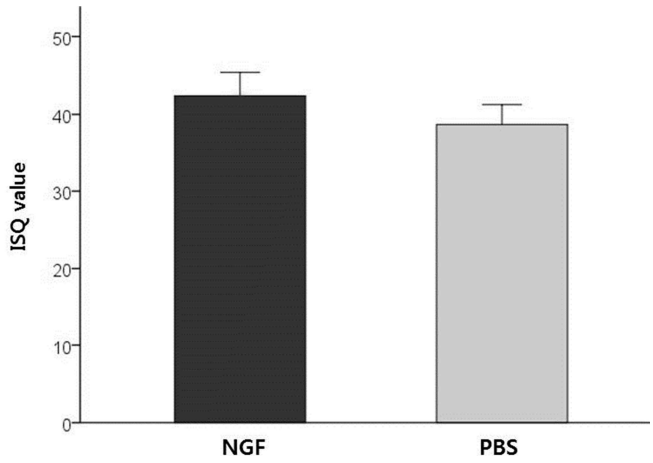

Fig 8. Comparison of ISQ at 6 weeks in the NGF and PBS groups. The average ISQ of NGF group (42.375±8.535) was slightly higher than the PBS group (38.714±6.701), but there was no significant difference between the two groups.

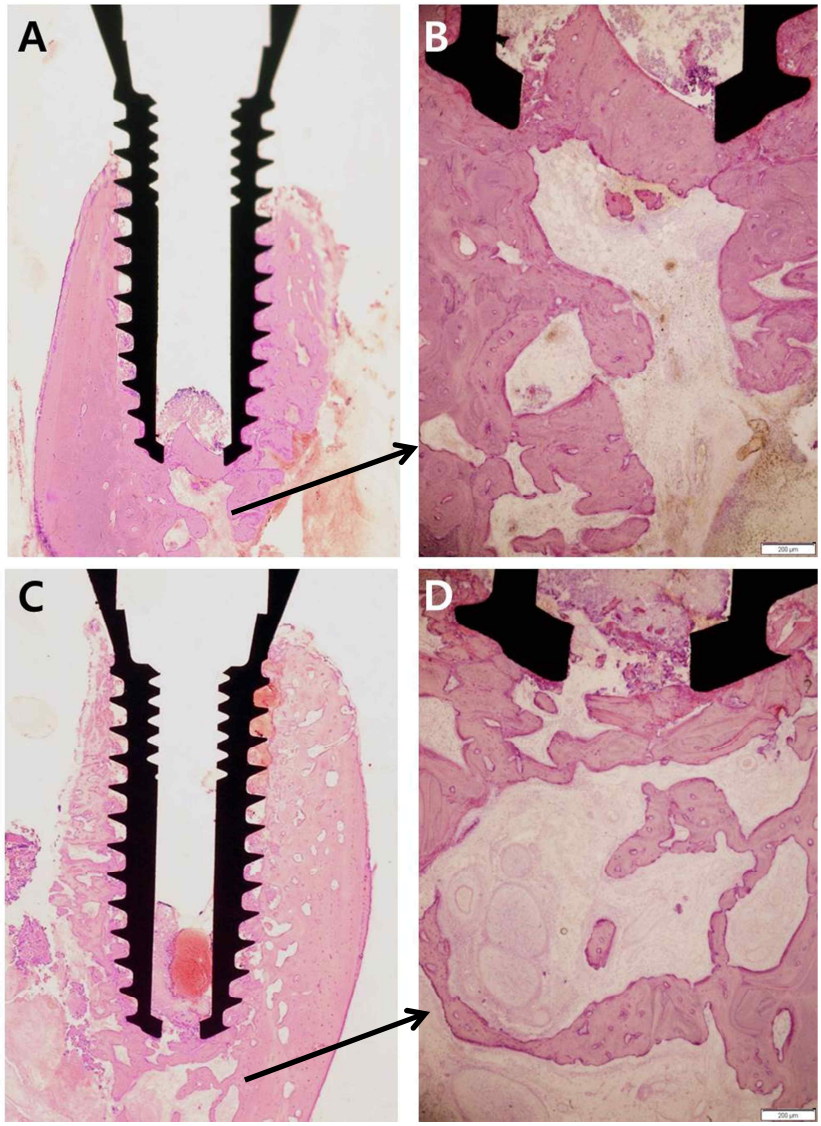

Fig 9. Histologic findings from a non-decalcified specimen (H&E staining, 40× magnification).

Microscopic images of the NGF-supplying implant in the alveolar bone **(A)** and tissue response around the implant apical hole **(B)** of the NGF group. Bone formation in the NGF group was significantly more prominent than in the PBS group.**(C, D)**

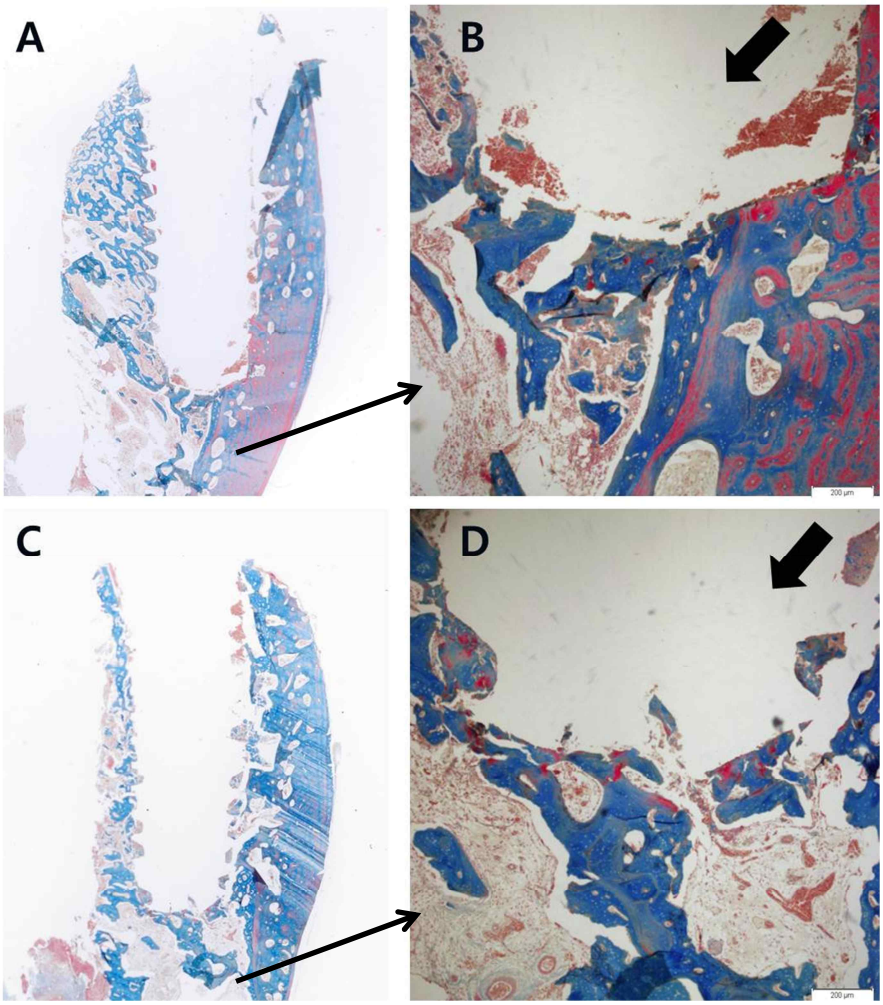

Fig 10. Histologic findings of decalcified specimens (MT staining, 40× magnification).

Microscopic images of the NGF-supplying implant in the alveolar bone **(A)** and tissue response around the implant apical hole **(B)** of NGF group. New bone formation was more prominent in the NGF group compared to the PBS group **(C, D)**. Block arrows indicate the area where the implant was placed, but removed during the decalcification procedures in the PBS group.

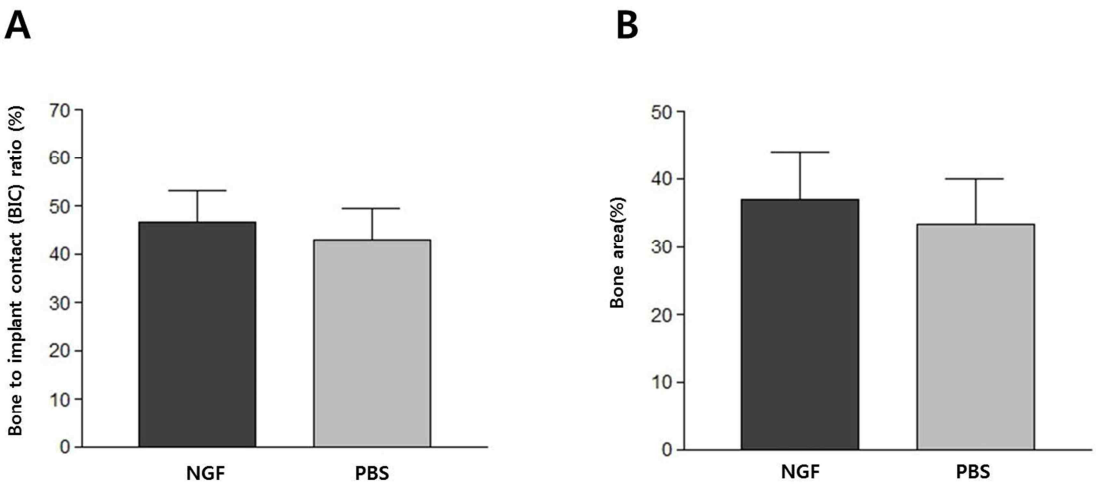

Fig 11. Comparison of BIC and BA between groups.

(A) BIC was higher in the NGF group (46.609±18.445 %) than the PBS group (42.884±17.168 %), but this difference was not significant. **(B)** BA was higher in the NGF group (36.993±19.923 %) than the PBS group (33.327±17.332 %), but this difference was not significant.

국문초록

신경성장인자 공급 임플란트를 이용한 하치조신경 손상 치료에서 신경 재생 및 골유착에 관한 연구

이 진 용

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목적

임플란트 시술이 대중화되면서 하치조신경 손상에 의한 감각 이상도 급격히 증가하고 있으며 그 중요성도 점점 부각되고 있다. 본 연구에서는 하치조신경 손상 수복 부위에 신경성장인자(NGF)를 공급할 수 있도록 특별히 제작된 치과 임플란트를 이용하여 NGF 를 공급했을 때 신경 재생에 대한 효과를 평가하였으며, NGF 공급과 개조된

임플란트의 디자인이 임플란트의 골유착에 미치는 영향을 검증하고자 하였다.

재료 및 방법

18 주령 성견 (beagle dog, n=9)을 전신 마취시킨 후 양측 하치조신경을 노출시키고 하악 제 3 소구치를 발거한 후 NGF 공급 임플란트를 즉시 식립하였다. 임플란트에 근접한 부위의 하치조신경을 절단한 후 단단 문합하고 mini osmotic pump 카테터를 임플란트에 연결하였다. 우측 하치조신경에는 2mL 의 NGF 용액 (150μg/mL)이 그리고 좌측 하치조신경에는 동량의 PBS 용액이 담긴 pump 를 연결하였다. 신경재생 평가를 위해 술전 및 술후 3, 6 주에 needle electrode 를 이용해 진폭과 잠복시간을 측정하였고 신경절단 전후 및 술후 6 주에 hook electrode 를 이용해 신경전도속도와 최대전압을 측정하였다. 술후 6 주에 손상부를 중심으로 신경표본을 채취한 후 시편 중앙부를 횡분절 (cross section)하여 epon 포매 절편 toluidine blue 로 염색 후 전체 축삭 수, 축삭밀도를 측정하고 대표 부위를 TEM 표본 제작하여 축삭 수초의 두께 및 G-ratio 를 측정하였다. 임상적으로는 식립된

임플란트의 염증 등 부작용을 관찰하였으며, 술후 6 주째 임플란트 안정도 (ISQ)를 측정하였다. 그리고 6 주 후 희생 시 임플란트를 주변 조직과 같이 채취하여 탈회, 비탈회 표본을 제작하고 이를 통해 bone-implant contact (BIC) 비율과 bone area (BA) 비율을 측정하였다.

결과

전기신경생리측정에서 진폭과 잠복시간은 두 군 사이에 유의한 차이가 없었다. 신경전도속도에서는 6 주째 NGF 군의 중앙값(2.675 m/s)이 PBS 군(1.892 m/s)보다 유의하게 높았으며 ($p < 0.01$), 최대전압도 같은 결과를 보였다 (NGF vs. PBS : 1.940 μV vs. 1.300 μV, $p < 0.01$). 마찬가지로 NGF 군의 전체 축삭 수 및 축삭밀도 (4576.107 ± 270.413, 10707.458 ± 638.835/mm^2)가 모두 PBS 군 (3606.972 ± 242.876, 7899.781 ± 1063.625/mm^2)에 비해 유의하게 높았지만 ($p < 0.001$), G-ratio 는 두 군간 차이가 없었다. BIC 는 NGF 군이 46.609±6.521 %, PBS 군이 42.884±6.489 % 였으며, 두 군 간에 유의한 차이는 없었다. BA (NGF vs. PBS : 36.993±7.0434% vs. 33.327±6.551 %)와 6 주 후 ISQ (NGF vs. PBS : 42.375±3.017 vs. 38.714±2.533)에서도 두 군간 유의한 차이가 관찰되지

않았다. NGF 공급 임플란트의 전체 생존률은 83.33% 이었으며 NGF 군은 88.89%, PBS 군은 77.78%였다.

결론

본 연구에서 NGF 공급 임플란트를 이용한 NGF 공급은 하치조신경의 재생을 도모하였으며 이러한 처치가 골유착을 저해하지 않았음을 알 수 있었다. 이러한 치료 방식은 신경재생과 골유착을 동시에 도모할 수 있기 때문에 임플란트 식립과 관련된 하치조신경 손상치료의 새로운 가능성을 제시할 수 있다고 사료되었다.

주요어 : 신경성장인자, 신경성장인자 공급용 임플란트, 하치조신경 손상, 말초신경 재생

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