

Controlled release of nerve growth factor from heparin-conjugated fibrin gel within the nerve growth factor-delivering implant

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Abstract (J Korean Assoc Oral Maxillofac Surg 2014;40:3-10)

Objectives: Although nerve growth factor (NGF) could promote the functional regeneration of an injured peripheral nerve, it is very difficult for NGF to sustain the therapeutic dose in the defect due to its short half-life. In this study, we loaded the NGF-bound heparin-conjugated fibrin (HCF) gel in the NGF-delivering implants and analyzed the time-dependent release of NGF and its bioactivity to evaluate the clinical effectiveness.

Materials and Methods: NGF solution was made of 1.0 mg of NGF and 1.0 mL of phosphate buffered saline (PBS). Experimental group A consisted of three implants, in which 0.25 μ L of NGF solution, 0.75 μ L of HCF, 1.0 μ L of fibrinogen and 2.0 μ L of thrombin was injected via apex hole with micropipette and gelled, were put into the centrifuge tube. Three implants of experimental group B were prepared with the mixture of 0.5 μ L of NGF solution, 0.5 μ L HCF, 1.0 μ L of fibrinogen and 2.0 μ L of thrombin. These six centrifuge tubes were filled with 1.0 mL of PBS and stirred in the water-filled beaker at 50 rpm. At 1, 3, 5, 7, 10, and 14 days, 1.0 mL of solution in each tubes was collected and preserved at -20°C with adding same amount of fresh PBS. Enzyme-linked immunosorbent assay (ELISA) was done to determine *in vitro* release profile of NGF and its bioactivity was evaluated with neural differentiation of pheochromocytoma (PC12) cells.

Results: The average concentration of released NGF in the group A and B increased for the first 5 days and then gradually decreased. Almost all of NGF was released during 10 days. Released NGF from two groups could promote neural differentiation and neurite outgrowth of PC12 cells and these bioactivity was maintained over 14 days.

Conclusion: Controlled release system using NGF-HCF gel via NGF-delivering implant could be an another vehicle of delivering NGF to promote the nerve regeneration of dental implant related nerve damage.

Key words: Nerve growth factor-delivering implant, Nerve growth factor, Controlled release, Nerve regeneration

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I. Introduction

Over the recent decades, neurosensory impairment of trigeminal nerve associated with dental implant surgery, which has become a major medico-legal issue, has been continuously increased¹ and its incidence is reported to be from 6.5%

to 43%²⁻⁵. The inferior alveolar nerve (64.4%) and the lingual nerve (28.8%) are mostly damaged⁶. Current treatment modalities of peripheral nerve injury consist of pharmacologic therapy, physical rehabilitation and surgical management using direct repair or nerve graft. However, complete regeneration takes a long time and function rarely returns to the pre-injury level because of endogenous and exogenous factors in the nervous system. Many researchers and clinicians have tried to promote the nerve regeneration with exogenous neurotrophic factors⁷⁻⁹.

Nerve growth factor (NGF) was discovered about sixty years ago¹⁰ and it has been known to play a critical role in the regulation of peripheral and central nervous systems¹¹. However, clinical application of NGF poses a great challenge because of its rapid *in vivo* degradation¹² and non-specific,

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multifunctional activities in the non-neuronal tissues¹³. Therefore, it is necessary for NGF to be delivered with a preset, constant rate to the injury site to sustain its neural activities without undesired side-effect. Although many kinds of controlled NGF delivery systems using collagen¹⁴, polymer¹⁵, and fibrin¹⁶ have been introduced, heparin-conjugated fibrin (HCF) systems released NGF with a controlled, sustainable manner^{8,17}.

In this study, we loaded the NGF-conjugated HCF gel in NGF-delivering implant and analyzed the time-dependent release of NGF to evaluate its degradation kinetics. The bio-activity of NGF released from HCF gel was evaluated with neural differentiation and neurite extension of pheochromocytoma (PC12) cells.

II. Materials and Methods

1. Preparation of nerve growth factor controlled delivering system

1) Fabrication of nerve growth factor delivering implant

Based on the commercially available SimpleLine II implant (Dentium Inc., Seoul, Korea), NGF delivering implant was designed as an internal type fixture and it has the lumen whose diameter was 1.3 mm (Dentium Inc.; Korean patent No. 10-2011-0103682). Surface modification was done with sandblasting with large grit and acid etching (SLA) technique. The length of body was 8.0 mm and that of the collar was 2.2 mm with the internal design of the octagonal shape and 8-degree morse taper.(Fig. 1) This implant was qualified and approved by Ministry of Food and Drug Safety, Republic of Korea (approval No. 10-177).

2) Preparation of heparin-conjugated fibrin

Heparin-conjugated fibrinogen was prepared according to previous protocol¹⁸. Briefly, total 100 mg of heparin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mL buffer solution (pH 6.0) of 0.05 M 2-morpholinoethanesulfonic acid (Sigma-Aldrich). For activation of the carboxylic acid groups of the heparin, N-hydroxysuccinimide (0.04 mM; Sigma-Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.08 mM; Sigma-Aldrich) were added and incubated at 4°C for 12 hours. After that, this activated heparin solution was vigorously stirred, precipitated with excessive anhydrous acetone and lyophilized for 24 hours. Next, 100 mg of fibrinogen was dissolved in 20 mL phosphate buffered saline (PBS, pH 7.4) without bubbles at 4°C. This solution was reacted with 60 mg of lyophilized heparin for 3 hours at 4°C. After precipitation and lyophilization under the same conditions, white powder was prepared and dissolved in PBS. Dialysis was done with a porous membrane bag (12,000-14,000 Da molecular weight cutoff; Spectrum Lab, Rancho Dominguez, CA, USA) at 4°C for 24 hours and resultant solution was lyophilized for 48 hours to yield heparin-conjugated fibrinogen. HCF was fabricated by mixing heparin-conjugated fibrinogen, normal fibrinogen, aprotinin, calcium chloride and thrombin.

2. Determination of *in vitro* kinetics of nerve growth factor release

From fibrin sealant (2.0 mL syringe, Tisseel; Baxter, Deerfield, IL, USA), fibrinogen solution with aprotinin (3,000 KIU/mL) and thrombin solution with calcium chloride (5.88 mg/mL) were prepared. NGF solution was formulated with

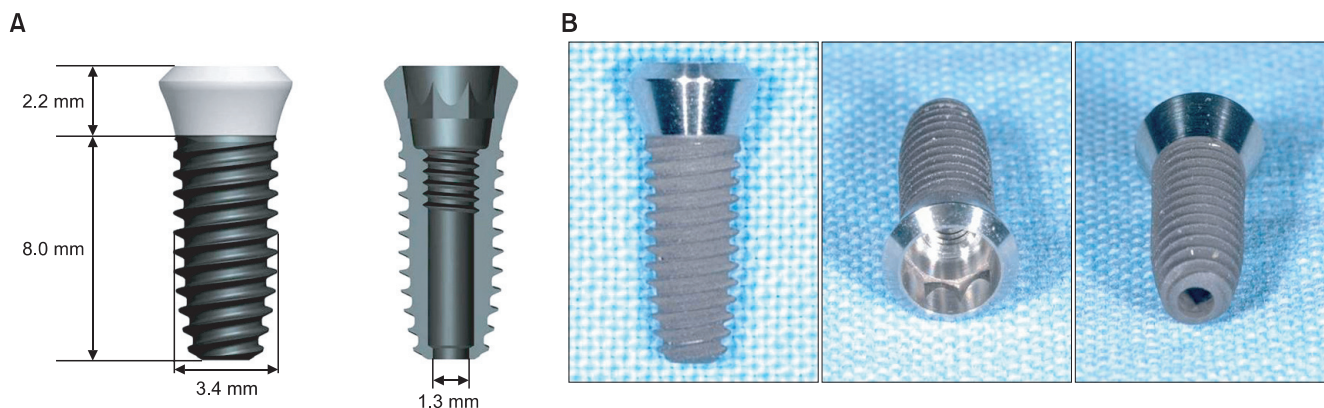


Fig. 1. Design of nerve growth factor (NGF) delivering implant. A. Schematic design. B. Photographic images of NGF delivering implant. Jin-Yong Lee et al: Controlled release of nerve growth factor from heparin-conjugated fibrin gel within the nerve growth factor-delivering implant. J Korean Assoc Oral Maxillofac Surg 2014

1 mg of NGF (Peprotech, Rocky Hill, NJ, USA) dissolved in 1 mL PBS. The NGF-delivering implants were sterilized and connected with cover screws. To load NGF-HCF gel inside these implants, two kinds of solutions were mixed with micropipette via apical hole as described in Table 1. After that, these implants were placed at room temperature for 2 hours to promote the gelation.

The implants loaded with NGF-HCF gel were incubated in 2 mL centrifuge tubes filled with 1 mL PBS at room temperature with continuous stirring (50 rpm). At prescheduled time points (1, 3, 5, 7, 10, and 14 days after NGF-HCF gel loading), the solution in each tube was completely collected and the 1 mL of fresh PBS was replaced. Each collected solution was evenly divided into five aliquots (200 μ L) and stored at -20°C . One of them was used for enzyme-linked immunosorbent assay (ELISA) with Human β -NGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. With Synergy H1 microplate reader (BioTeK Instrument, Winooski, VT, USA), the absorbance of the each sample was read at both 450 and 540 nm. The amount of NGF was calculated from the calibrated standard curve using Gen5 data analysis software version 2.0 (BioTeK Instrument).

3. *In vitro* assessment of bioactivity of released nerve growth factor

The biological activity of the released NGF was evaluated by its ability of neural differentiation and neurite extension of PC12 cells cultured in medium containing released NGF. Culture medium for PC12 cells were prepared with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 5% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco) at 37°C in 5% CO_2 . The cells were cultured for 3 days. The

cells were placed in 6-well plates from culture at (5,000 cells/mL), which were allowed to adhere to the cell dish for 2 days. Aliquot of harvested NGF solution (200 μ L) was mixed with 3 mL of culture medium. The PC12 cells were cultured for 3 days with this medium and neurites outgrowth was evaluated. Control group 1 was cultured for 3 days with same amount of medium without NGF. After medium was changed, another 3 day-culture was done for control group 2.

Samples in 6-well plates were analyzed via light microscopy and their images were digitally converted. Image analysis was done with Image J version 1.46 (National Institute of Health, Bethesda, MD, USA) to calculate the percentage of differentiated cells and average length of neurites. PC12 cells with neurites longer than the length of cell body was defined as differentiated one. Depending on the growth characteristics, 3 microscope fields were randomly selected in each well and assessed for each parameter.

4. Statistics

After the normality of data was verified with Lilliefors' modified Kolmogorov-Smirnov test, comparison of the released NGF was done with Mann-Whitney U-test and differentiation ratio and length of neurite were compared with one-way analysis of variance and post hoc analysis was done with Bonferroni's method. The level of significance was set

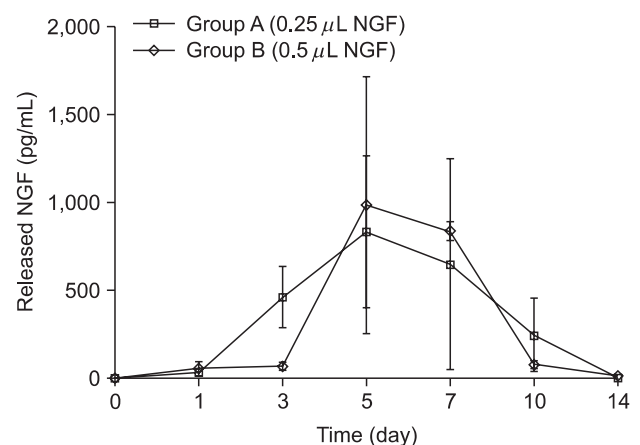


Fig. 2. *In vitro* profile of NGF release from NGF-HCF gel loaded in NGF delivering implant at each time point of sampling (pg/mL). The amount of released NGF of two groups were increased and reached peak at days 5 and decreased. At day 3, released NGF of group A (459.666 ± 302.739 pg/mL) was higher than that of group B (66.856 ± 42.352 pg/mL), but there was no significance (Mann-Whitney U-test). (NGF: nerve growth factor, HCF: heparin-conjugated fibrin)

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Table 1. Composition of NGF-HCF gel

Ingredient	Volume (μ L)	
	Group A	Group B
NGF	0.25	0.5
HCF	0.75	0.5
Fibrinogen	1.0	1.0
Thrombin	2.0	2.0
Total	4.0	4.0

(NGF: nerve growth factor, HCF: heparin-conjugated fibrin)

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at $P < 0.05$. All statistical analyses were done with IBM SPSS Statistics 20.0 (IBM Co., Armonk, NY, USA).

III. Results

1. *In vitro* kinetics of nerve growth factor release

At each time point of sample harvesting, the average amount of released NGF of two groups were increased and reached peak at day 5 and decreased thereafter. Except day 3 (459.666 ± 302.739 pg/mL in group A and 66.856 ± 42.352 pg/mL in group B; $P < 0.05$), when the amount of released NGF in group A was significantly higher than that of group B, there were no significant differences in NGF release between two groups. (Fig. 2) The cumulative release profile of NGF of two groups also showed a similar pattern over 14 days. For 10 days, almost all of NGF loaded with the implant was released. (Fig. 3)

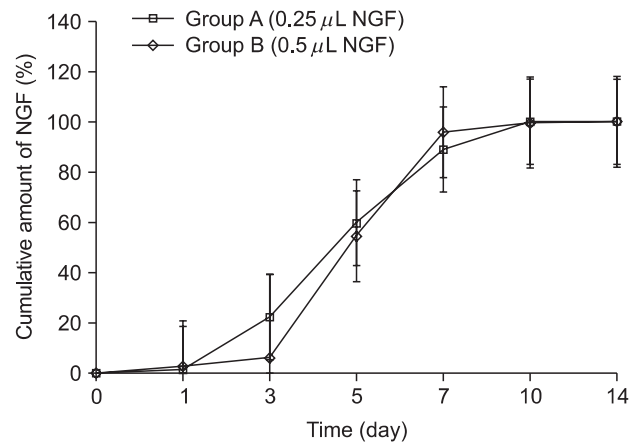


Fig. 3. Cumulative profile of released NGF from NGF-HCF gel loaded in NGF delivering implant (%). The cumulative release profile of NGF showed a similar pattern between two experimental groups over 14 days. For 10 days, almost all of NGF from NGF-HCF gel loaded in the NGF delivering implant was released. (NGF: nerve growth factor, HCF: heparin-conjugated fibrin)

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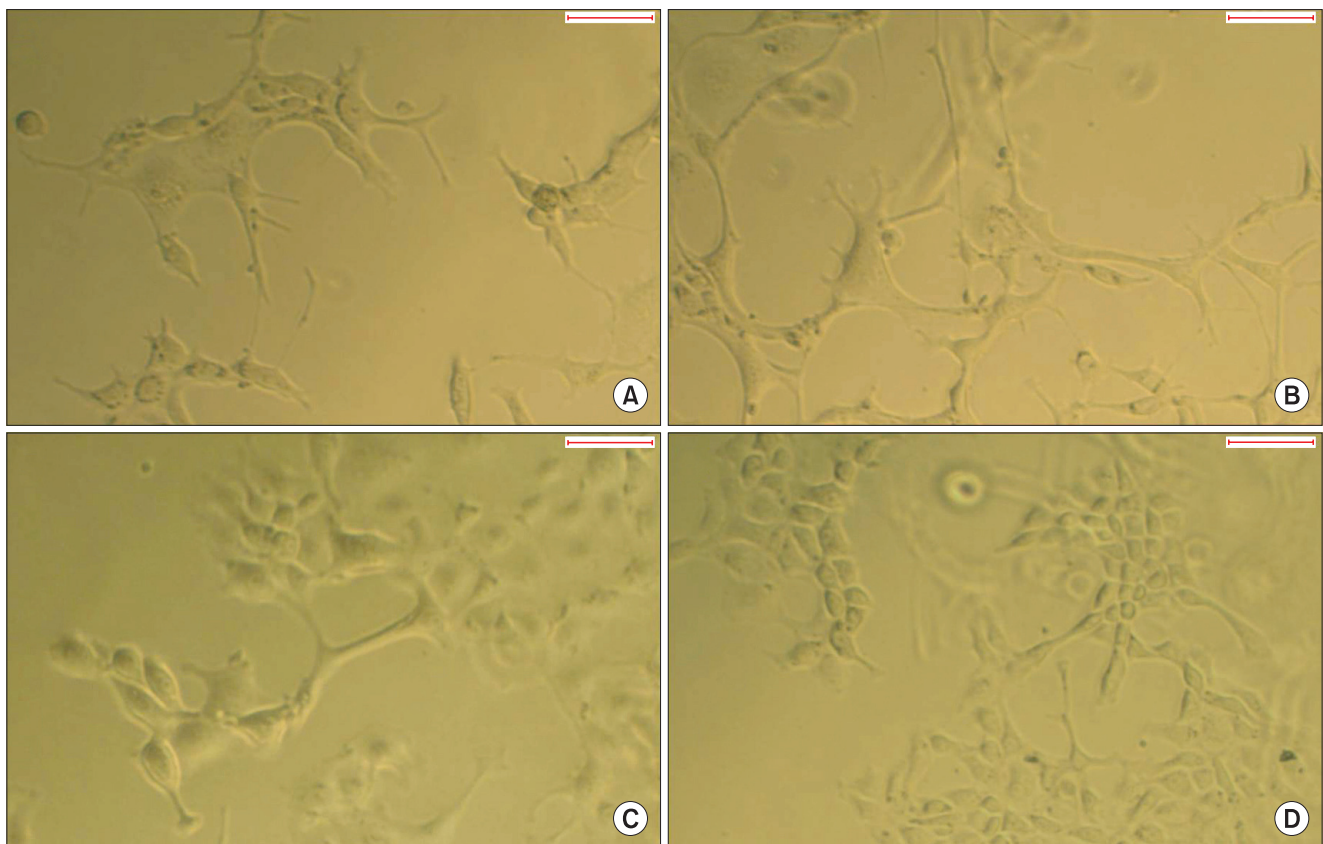


Fig. 4. Microscopic images of the differentiated PC12 cells (x100). A. PC12 cells cultured with medium containing group A sample harvested at day 5. B. PC12 cells cultured with medium containing group B sample harvested at day 5. C. PC12 cells cultured with medium without NGF for 3 days (control group 1). D. PC12 cells cultured with medium without nerve growth factor for 6 days (control group 2). Scale bars=50 μm.

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2. *In vitro* bioactivity of released nerve growth factor

The microscopic images on the neural differentiation of PC12 cells with culture medium were shown in Fig. 4. The ratio of differentiated PC12 cells in group A at days 3 and 5 were significantly higher than that of control group 1 (Fig. 5. A) and those of group A at days 1, 3, 5, and 7 were also higher than that of control group 2 with significance.(Fig. 5. B) In group B, the differentiation ratio of PC12 cells at days 3, 5, and 7 were significantly higher than those of control groups. (Fig. 6)

There were no significant differences between the average lengths of neurite in group A and those of control groups (Fig. 7), but not in group B, where the average length of neurite at day 1 was higher than those of control groups with significance.(Fig. 8)

IV. Discussion

In our previous pilot study (unpublished), total 20 μg of NGF was mixed in 1 mL PBS and NGF-HCF gel was prepared with the composition of group A. The loading of NGF-

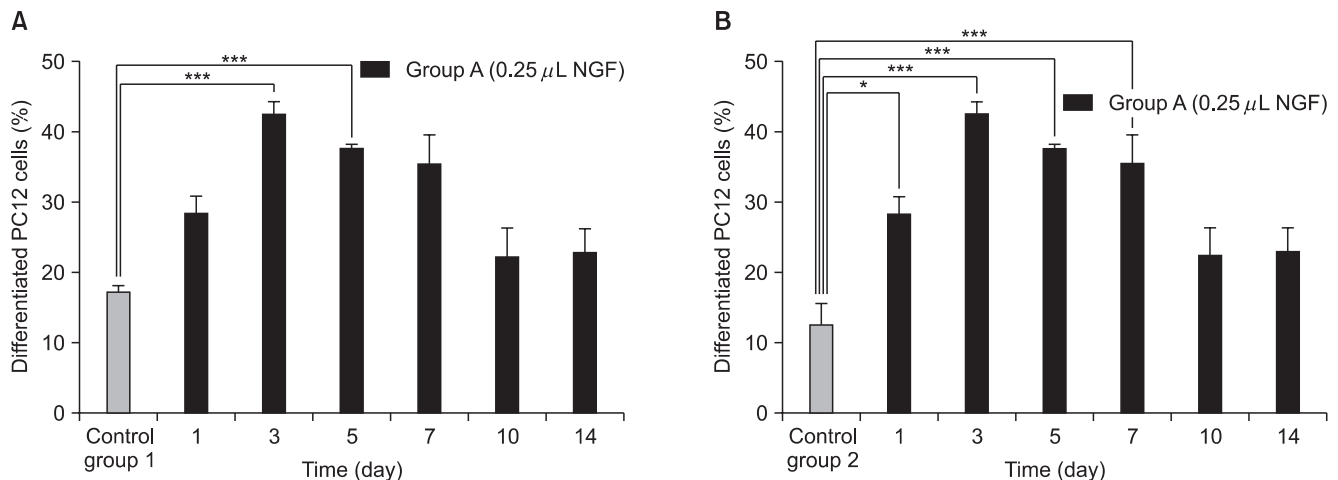


Fig. 5. Comparison of the ratio of differentiated PC12 cells in group A. A. Comparison with control group 1. The differentiation ratio at day 3 and 5 were significantly higher than that of control group 1. B. Comparison with control group 2. The ratio days 1, 3, 5, and 7 were higher than that of control group 2 with significance (* $P < 0.05$, *** $P < 0.001$; one-way ANOVA with Bonferroni's post hoc test). (NGF: nerve growth factor)

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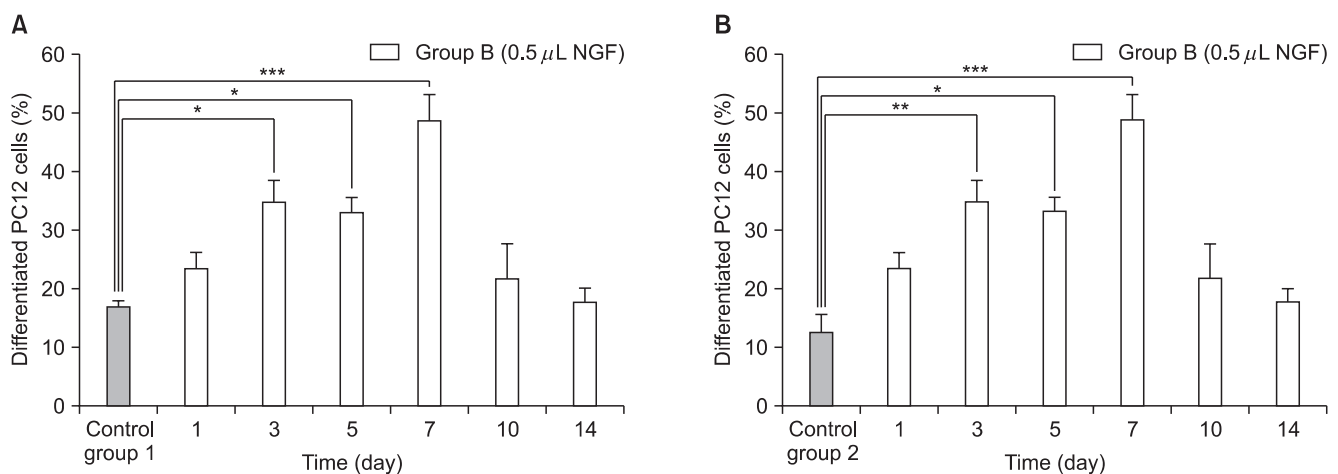


Fig. 6. Comparison of the ratio of differentiated PC12 cells in group B. A. Comparison with control group 1. B. Comparison with control group 2. The differentiation ratio of PC12 cells at days 3, 5, and 7 were significantly higher than those of control group 1 and 2 (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA with Bonferroni's post hoc test). (NGF: nerve growth factor)

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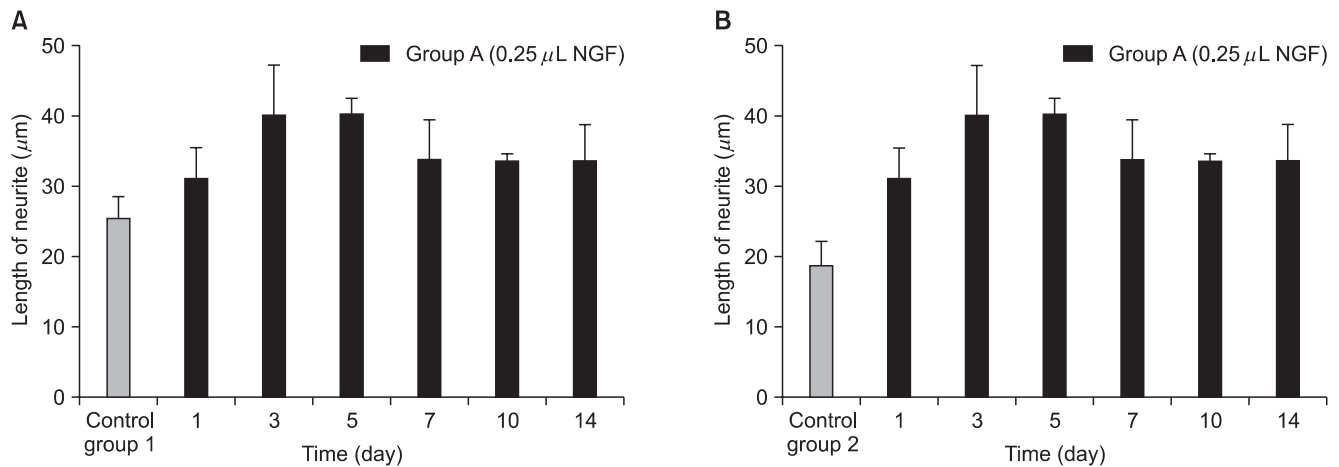


Fig. 7. Comparison of the neurite length of differentiated PC12 cells in group A. A. Comparison with control group 1. B. Comparison with control group 2. No significant differences were found between group A, control group 1 and 2 (one-way ANOVA with Bonferroni's post hoc test). (NGF: nerve growth factor)

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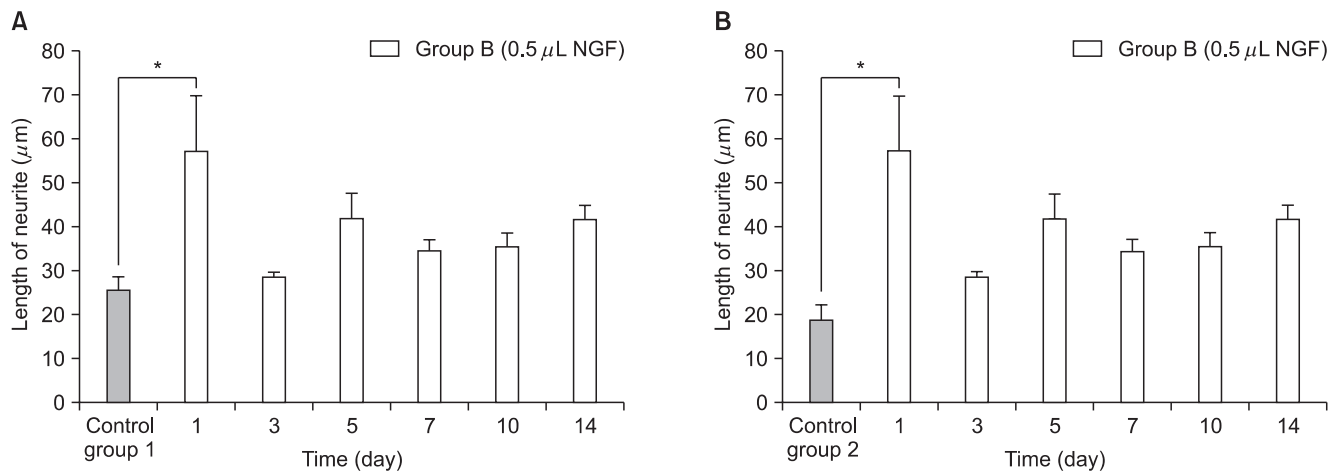


Fig. 8. Comparison of the neurite length of differentiated PC12 cells in group B. A. Comparison with control group 1. B. Comparison with control group 2. The average length of neurite of day 1 was significantly higher than those of control group 1 and 2 (* $P < 0.05$; one-way ANOVA with Bonferroni's post hoc test). (NGF: nerve growth factor)

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HCF gel, sample harvesting and ELISA was performed with the same protocol of this study. However, released amount of NGF ranged from 1 to 5 pg/mL, which was so small that it couldn't be useful in the clinical application. In order to elevate the amount of released NGF, two hypothetic strategies were planned: First, the increased concentration of NGF solution would result in the increased total volume of released NGF. Second, the decreased volume of HCF would weaken the cross-linking activity and result in the increased release of NGF. Therefore, in this study, NGF solution was enriched fiftyfold (1.0 mg/mL) and two kinds of NGF-HCF gel, in which the volume of HCF solution were different, were pre-

pared and assessed.

Our results showed that daily amount of released NGF in group B was very small until 3 day, sharply increased from 5 to 7 day and dramatically decreased. However, those of group A showed relatively gradual changes for 14 days. This difference would be explained by the different composition of NGF-HCF gel. Thrombin is known to reinforce the cross-linking of fibrin and lower the diffusion rate of NGF from fibrin gel¹⁹. In addition, heparin is known to be involved in the interaction between NGF and fibrin²⁰. As HCF was decreased in group B, therefore, relatively increased fraction ratio of thrombin to HCF would strengthen the cross-linking the de-

layed initial release of NGF in group B. Limited contact area in which diffusion through the PBS could be possible only via implant hole might also contribute to this tendency.

Physiological activity of released NGF from HCF gel was evaluated with the PC12 cell differentiation and neurite outgrowth. Overall, these parameters of group A and B were higher than those of control groups and this tendency was maintained throughout the experimental period. Therefore, it was shown that released NGF from two experimental groups remained as bioactive for 14 days.

The NGF-HCF carrier system has some advantages in clinical applications. First, it can be applied as an injectable material and easily manipulated as desired. Second, modulation of HCF gel can allow the loading dose and degradation rate of NGF¹⁹. Third, HCF gel can deliver the various kinds of growth factors including fibroblast growth factor²¹ and vascular endothelial growth factor²² because these growth factors have heparin-binding domains. Multiple neurotrophic factors mixed in HCF gel could be a treatment option for peripheral nerve injury. Moreover, NGF-delivering implant in this study can permit prosthetic rehabilitation without additional invasive procedures as well as loading of NGF-HCF gel.

Our study presented the possibility of controlled release using NGF-delivering implant. Nevertheless, further researches should be warranted for the following reasons. First, only two kinds of NGF-HCF compositions were assessed in this study and more optimal composition of NGF-HCF gel with ideal *in vitro* release kinetics should be explored. Second, it should be evaluated whether this ideal NGF-HCF system could show clinically acceptable *in vivo* release kinetics and nerve regeneration effect. Finally, this NGF-HCF gel system would be developed as a clinically available form which is easy to storage and handle.

V. Conclusion

In conclusion, NGF which was conjugated with HCF gel in NGF-delivering implant showed a controlled releasing kinetics and enhanced neural differentiation of PC12 cells. This system may have a potential as a new vehicle of delivering NGF to promote the nerve regeneration of dental implant related nerve damage.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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