Effects of locally applied nerve growth factor to the inferior alveolar nerve histology in a rabbit model of mandibular distraction osteogenesis

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Abstract. Distraction osteogenesis (DO) is widely used in deformities and defects of the craniofacial bone. Accelerating inferior alveolar nerve (IAN) recovery would aid the process. Nerve growth factor (NGF) plays a vital role in peripheral nerve regeneration. In this study, the ability of locally applied human NGF beta (hNGFβ) to enhance the morphological recovery of the IAN in a rabbit model of mandibular DO was studied. Rabbits underwent bilateral DO with a rate of 0.5 mm per 12 h. Two doses of 40 μg hNGFβ in buffer were injected into callus at the beginning the of consolidation time. The contralateral side received injections of placebo. Rabbits were killed at 14 and 28 days. IAN specimens were subjected to histological and histomorphometric analysis. In both 14 and 28 days consolidation experiments, nerve histological analysis showed less degeneration and more regeneration in nerve fibers on the hNGFβ treated side than the control side. Histomorphometric analysis showed that the myelinated fiber density on the hNGFβ treated side was significantly higher than on the control side (p < 0.01). The data indicate that locally applied hNGFβ can accelerate the morphological recovery of the IAN and may play a role in reducing nerve injury in mandibular DO clinically.

Keywords: nerve growth factor; distraction osteogenesis; nerve regeneration; rabbit.

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Distraction osteogenesis (DO) has been used in deformities, defects and fracture non-unions in craniofacial and orthopaedic surgery. The soft tissues, including skin, muscle and nerve, can be elongated together with the bone. Mandibular DO led to sensory disturbances in up to 52% of patients and degenerative changes were observed in the inferior alveolar nerve (IAN), even at a slow distraction rate. The two major types of nerve injuries in DO are direct injuries caused by surgical procedures and indirect injuries caused by increases in stretching tension. Through careful planning and meticulous surgical techniques, most of the direct injuries to the IAN can be avoided, but tension-related nerve injuries are harder to avoid. This type of indirect injury is reversible to some extent, owing to axon regeneration, but it takes a long time. Consequently, there is a need for acceleration in the recovery of nerve injuries during mandibular DO.

Nerve growth factor (NGF) is an important neurotrophin involved in the development, maintenance and regeneration of sensory and sympathetic nerves. In a rabbit IAN defect model, regenerating axons were stimulated by administration of NGF. In a rat fracture model, local administration of NGF increased the level of catecholamine concentrations in the fracture site, indicating increased adrenergic neural innervation of the callus. Exogenous NGF may play a role in facilitating Schwann cell proliferation and remyelination of the affected nerve in DO.

To the authors’ knowledge, no one has investigated the effects of exogenous NGF on nerve injuries during DO. The objective of this study was to determine the histological changes and the ability of locally applied NGF to enhance morphological recovery in the IAN during mandibular DO in a rabbit model.

**Materials and methods**

**Animals**

A total of 35 skeletally mature (2.9–3.5 kg), male, New Zealand White rabbits were included in this study. They were randomly allocated into seven groups. Group 1, distraction rate 0.5 mm/12 h, killed at a consolidation time of 14 days (n = 3); group 2, distraction rate 0.5 mm/12 h, killed at a consolidation time of 28 days (n = 3); group 3, underwent sham operation and were killed 27.5 days after operation (n = 3); group 4, underwent sham operation and were killed 41.5 days after operation (n = 3); group 5, normal rabbits with no surgery (n = 3); group 6, distraction rate 0.5 mm/12 h, NGF administered to one side of mandible, killed at a consolidation time of 14 days (n = 10); group 7, distraction rate 0.5 mm/12 h, NGF administration to one side of mandible, killed at a consolidation time of 28 days (n = 10).

In groups 3 and 4, sham-operated rabbits underwent identical osteotomy procedures and were killed at the same time as groups 1 and 2; no distraction was performed. Groups 1, 2, 3, 4 and 5 were designed to study the establishment of this animal model. Groups 6 and 7 were designed to study the effects of locally applied NGF, and used identical DO protocols as groups 1 and 2. The animals were raised under the regulations of the Experimental Animals Holding Center at Fourth Military Medical University.

**Surgical procedure**

The details of the animal model are described elsewhere. Rabbits were anesthetized and an incision was made in the midline of the submental triangle.

![Fig. 1. The animal model, note the anatomy of the IAN and the direction of hNGFβ or placebo injection.](image1)

![Fig. 2. Lateral view of the distraction device.](image2)
Periosteal flaps were reflected laterally to visualize the mental nerves anterior to the first premolar teeth. Vertical osteotomies were performed bilaterally between the premolar teeth and mental foramen using a fissure bur. A custom-made distraction device (Zhongbang Titanium Biomaterials Co., Xi’an, PR China) was fixed with the distraction rod emerging into the labial vestibule (Figs. 1 and 2). Four microplates were secured to the mandible bilaterally with self-tapping tapered screws (1.5 mm in diameter and 7 mm in length). All 10 screws were placed perpendicular to the lateral aspect of the mandible. Osteotomies were completed with a fine chisel. Great care was taken to avoid direct injuries to the IAN, and lingual periosteum was left intact. The wound was then closed in layers. After a latency period of 3.5 days, gradual distraction was performed at a rate of 0.5 mm per 12 h for 10 days in groups 1, 2, 6 and 7.

NGF administration

In groups 6 and 7, two doses of 40 μg human NGF beta (hNGFβ; Laboratory of Biochemistry, FMMU, PR China) in 0.15 ml isotonic saline were injected into the palpable callus at the treatment side percutaneously under general anesthesia on days 1 and 3 after the end of distraction. The contralateral side served as a control and received placebo (isotonic saline) simultaneously in an identical manner. In each injection, the mental foramen and the root apex of the first premolar tooth were identified. The acupuncture point was then chosen in the callus, 45° to the skin and 2.0 mm away from the IAN (Fig. 1).

Specimen processing

The rabbits were killed under general anesthesia. Both carotid arteries were perfused with normal saline to remove blood, followed by 2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PH7.4) for the internal fixation of the tissue. An 8 mm long sample of the IAN in each hemimandible was dissected out in the distracted region. These samples were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in serial ethanol solutions, and embedded in Epon.

Nerve histology and histomorphometry

Semi-thin (1 μm) transverse sections were stained with 1% toluidine blue. Two randomly selected sections at 4 mm intervals from each IAN sample were digitized using an Olympus light microscope (BX41) equipped with a 12.5 megapixel cooled CCD camera (DP71) at a final magnification of ×400. Nerve histomorphometry analysis was performed in 8 randomly selected fields of each sample (4 fields for each section) using a NIH Image Analysis System. The measurement was performed twice with a 3-day interval by an experienced, unbiased examiner (Yinghua Zhao) who was blinded to the experimental sides. Myelinated fiber density (number/mm²) and mean myelin thickness (μm) were calculated. All data were presented as the mean and standard error of the mean. Ultra-thin sections of the IAN were cut on an ultramicrotome (Leica Scientific Instruments, Nussloch, Germany). These sections were double stained with uranyl acetate and lead citrate.
and examined by electron microscopy (H-7100; Hitachi, Tokyo, Japan).

**Statistical analysis**

A box-and-whisker diagram was draw to compare the myelinated fiber density in groups 1, 2, 3, 4 and 5. In groups 6 and 7, a paired t-test was used to calculate the differences in myelinated fiber density and mean myelin thickness between the treatment and control sides; \( p < 0.05 \) was considered statistically significance.

**Results**

Five rabbits in groups 6 and 7 had to be excluded; one died during surgery because of anesthesia problems, one developed wound infections, one developed respiratory infection, and the IAN of one received an accidental injury from the chisel. The remaining rabbits in group 6 (n = 7) and group 7 (n = 8) and all the 15 rabbits in the groups 1, 2, 3, 4 and 5 showed good tolerance to the experimental procedure. All the IAN specimens of the 30 rabbits underwent nerve histology and histomorphometric analysis.

Nerve histology and histomorphometric analysis showed that myelinated and unmyelinated fibers were unchanged in both groups 3 and 4 when compared with group 5 (Fig. 3a). In group 1, many signs of moderate demyelination were seen, such as segmental demyelination and delamination (Fig. 3b). In group 2, mild to moderate demyelination and a few regenerating axons were observed (Fig. 3c). No difference was observed between both sides in the same animal in groups 1, 2, 3 and 4. Fig. 4 shows the results of myelinated fiber density in the five groups. When compared with group 5, groups 1 and 2 showed a trend toward a decrease in myelinated fiber density; degenerating fibers with dark axons were seen on the control side.

Nerve histology on the control side of groups 6 and 7 was identical with that on either side of groups 1 and 2 (Fig. 5a and c). When compared with the control side, mild demyelination and more regenerating axons were observed on the treatment side in group 6 (Fig. 5b), and more regenerating nerve fibers and active Schwann cells were observed on the treatment side in group 7 (Fig. 5d).

Transverse electron micrographs revealed signs of moderate demyelination in myelinated fibers on the control side (Fig. 6a and b). On the treatment side, bundles of regenerating nerve fibers and active Schwann cells were easily observed (Fig. 6c and d). Further analysis suggested that most of the thin myelin fibers were regenerating fibers on the treatment side; degenerating fibers with dark axons were seen on the control side.

**Discussion**

In this distraction model, the authors applied a distractor suitable for simultaneous bilateral distraction of the mandible without the need for two separate devices\(^1,2\). An advantage of this model was the establishment of an intraindividual placebo control. In groups 1, 2, 3, 4 and 5, the identical histology in the IAN of both sides showed that this intraindividual control was reliable. Previous reports have

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**Fig. 4.** The myelinated fiber density (number/mm\(^2\)) in groups 1–5.

**Fig. 5.** The effect of hNGFβ on the histology of the IAN (toluidine blue stain in transverse sections, ×400). After consolidation time of 14 days: (a) moderate demyelination and few regenerating axons on the control side; (b) mild demyelination and more regenerating axons on the treatment side. After consolidation time of 28 days: (c) mild to moderate demyelination on the control side; (d) more regenerating nerve fibers on the treatment side.
underwent histological analysis and the IAN during the surgery; the rabbits avoided to reduce disturbances to the IAN. The authors did not directly damage it to consider suitable.

A 3.5 day latency period was therefore considered suitable. The Schwann cells and NGF are crucially important to nerve regeneration. The degradation products of the nerve, together with macrophage secretion, can stimulate the activation and proliferation of Schwann cells, which can facilitate axon remyelination and guide the regenerating axons to their target. In this process, NGF plays a vital role. NGF can be secreted by Schwann cells in an autocrine and paracrine manner; it is thought that proper use of exogenous NGF can protect adult sensory neurons from cell death and enhance nerve regeneration. In DO, it has been demonstrated that the expression of NGF and many other growth factors and signaling factors is increased as long as the tension-stress is maintained and quickly down-regulated when the distraction completes. The beginning of the consolidation period is therefore likely to be a key period requiring exogenous NGF for regeneration of the nerves in DO. The hNGFβ injections were given at the beginning of the consolidation period in the present study and the IAN histological changes were studied. Eppley et al gave a single injection of 75 μg of NGF to the IAN. In the present study, the authors gave a total dose of 80 μg of hNGFβ. To reduce the error of the injection procedure, repeated injections were given instead of a single injection. An interval of 2 days was designed to allow the rabbits to recover from puncture injuries.

In the present study, nerve histological and histomorphometric analysis in the IAN showed that there was less degeneration and more regeneration of nerve fibers on the hNGFβ treated side compared with the intraindividual placebo control side at consolidation times of 14 and 28 days. It could be argued that the puncture and injection of isotonic saline might cause changes in the IAN, but the identical nerve histology between the control sides of groups 6 or 7 and both sides of groups 1 and 2 suggests that injections of isotonic saline have no effect on the morphology of the IAN. This indicates that the accelerated morphological recovery of the IAN was caused by exogenous hNGFβ, which might meet the increased need for neurotrophins from the Schwann cells when applied at the beginning of the consolidation period.

This study has limitations. It is a preliminary study with only morphological

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**Fig. 6.** Electron micrographs of transverse sections of the IAN (bar length 5 μm). The control side after consolidation time of 14 days: (a) segmental demyelination; (b) watery and dark axon. The treatment side after consolidation time of 28 days: (c) a bundle of regenerating fibers; (d) an active Schwann cell.

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**Table 1.** Nerve histomorphometric analysis in groups 6 and 7.

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<th>Group 6 (14 days of consolidation)</th>
<th>Group 7 (28 days of consolidation)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Myelinated fiber density (n/mm²)</td>
<td>8755 ± 1356</td>
<td>11283 ± 2175</td>
</tr>
<tr>
<td>Mean myelin thickness (μm)</td>
<td>1.72 ± 0.37</td>
<td>1.80 ± 0.44</td>
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* p < 0.01, compared with the control side after the same days of consolidation.
analysis, no conclusion on the improvement in nerve function can be made based on the histology. Future neurophysiological and behavioral studies are needed to test the effects of locally applied NGF to neurosensory function. The dose of 40 μg hNGFβ was based on data from a preliminary study, not on that of release kinetics or clearance rate. Carrier materials are important, to protect the locally applied soluble protein factors from inactivation. Although the distraction tissue formed after 10 days of distraction consisted of plenty of collagen and could be considered a matrix for the injected hNGFβ, the administration needs to be improved, for example by applying injectable carrier materials.

In conclusion, the authors have demonstrated that locally applied hNGFβ can accelerate the morphological recovery of IAN in this rabbit model of mandibular DO. It may play a role in reducing the major complications of nerve injury in mandibular DO clinically.

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