Locally Applied Nerve Growth Factor Enhances Bone Consolidation in a Rabbit Model of Mandibular Distraction Osteogenesis

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ABSTRACT: Distraction osteogenesis is widely used in treating deformities, defects, and fractures of both long bones and craniofacial bones. Demands for acceleration of bone consolidation are increased in distraction osteogenesis. Nerve growth factor (NGF) can enhance innervation and bone regeneration in a fracture model and stimulate differentiation of osteoblastic cells. In this study, we tested the ability of locally applied NGF to enhance bone regeneration in a rabbit model of mandibular distraction osteogenesis. Twenty rabbits underwent bilateral distraction osteogenesis with a rate of 0.5 mm per 12 h. Two times 0.04 mg human NGF (hNGF) in buffer was injected into the callus after distraction. The contralateral side received placebo injections. Rabbits were euthanized at consolidation times of 14 and 28 days. Specimens were subjected to radiography, callus dimensions measurement, mechanical testing, and bone histological and histomorphometric analysis. The maximum load, bone volume/total volume, mineral apposition rate of the 1st to 11th day, and mineralized bone percentage were significantly higher in the hNGF side at 14 and 28 days (p < 0.05). The data indicate that locally applied hNGF can accelerate callus maturation and may be an option to shorten the consolidation period in distraction osteogenesis.


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

INTRODUCTION

Distraction osteogenesis (DO) has become a widely accepted method in orthopedic surgery and has been applied to resolve clinical problems such as deformities, defects, and fracture nonunions of long bones.1,2 DO has also been applied to craniofacial deformities, including hemifacial microsomia,3 retrognathia,4 and pediatric craniofacial syndromes, such as sleep apnea.5 However, the long period of bone consolidation can contribute to complications, such as bone infection, pain, and fracture of the distraction device. Consequently, the need exists for accelerated bone consolidation during DO.

Sensory and sympathetic nerves play an important role in normal skeletal metabolism and fracture repair.6,7 In sympathectomized animal models, bone resorption is increased, and bone deposition and mineralization are decreased.8,9 Additionally, sympathectomy can also result in osteoporosis in mandibular bone.10 Nerve growth factor (NGF) is an important neurotrophin involved in development, maintenance, and regeneration of sensory and sympathetic nerves.11,12 In vitro studies showed that NGF can stimulate differentiation and inhibit apoptosis of an osteoblastic cell line.13,14 Therefore, NGF may play a crucial role in bone regeneration. In a rabbit inferior alveolar nerve defect model, an incidental observation revealed that administration of NGF stimulated bone formation around the induced regenerating axons.15 In a rat fracture model, local administration of NGF increased the quality and rate of fracture repair.16 Additionally, NGF enhanced new bone ingrowth to a collagen/hydroxyapatite composite.17 In light of these findings, local administration of NGF is likely to be useful for bone regeneration in DO.
Expression of growth factors is closely related to mechanical strain in DO. For example, strong expression of angiogenic factors such as VEGF and bFGF and BMPs and their receptors was found during the phase of active distraction, and then much weaker expression was observed when distraction was discontinued.\textsuperscript{18–20} Expression of NGF in distracted inferior alveolar nerve was stimulated by strain and decreased in the consolidation period.\textsuperscript{21,22} In a fracture healing model, the expression level of NGF mRNA was increased right after fracture, peaked at 2 days, and began to decrease thereafter.\textsuperscript{23} The issue of NGF expression in distracted bone tissue has not been settled yet, but it is very likely to mimic the expression profile of BMPs. Exogenous NGF may therefore play a role in acceleration of callus maturation at the beginning of the consolidation period.

To our knowledge, no one has investigated the effects of NGF on bone formation during distraction osteogenesis. Therefore, the objective of this study was to determine the ability of locally applied NGF to enhance bone regeneration during mandibular DO in a rabbit model.

MATERIALS AND METHODS

Experimental Animal Model

Twenty skeletally mature (3.1–3.5 kg), male, New Zealand White rabbits were studied. The animals were housed and cared for in accordance with the guidelines established by the Animal Center for Medical Experiment at Fourth Military Medical University. Rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride and an intravenous injection of 40 mg/kg phenobarbital sodium. One percent lidocaine with 1:200,000 epinephrine was administered subcutaneously in the submental triangle. The incision was made in the midline, and periosteal flaps were reflected laterally to visualize mental nerve anterior to the first premolar teeth. A vertical osteotomy was performed bilaterally between the premolar teeth and mental foramen using a fissure bur. A custom-made distraction device (Zhongbang Titanium Biomaterials Corporation, Xi’an, P.R. China) was fixed with the distraction rod emerged into the labial vestibule (Fig. 1). The titanium device traveled 0.4 mm per complete rotation. Four microplates were secured to the mandible 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, parallel to each other and across both cortices. The clap-shaped microplates provided additional support for retention of the device. The osteotomy was completed with a fine chisel. Great care was taken to avoid injury to the inferior alveolar nerve, and the lingual periosteum was left intact. The wound was then closed in layers. The upper incisor teeth were burred to approximately half of their projecting distance, to reduce the impact on the lower gingival due to induced malocclusion. Postoperatively, the rabbits’ clinical condition, dietary habits, and weight were monitored. After

![Figure 1](image_url)
a latency period of 3.5 days, gradual distraction was performed at a rate of 0.5 mm per 12 h for 10 days. The experiment was approved by the Fourth Military Medical University Animal Care and Use Committee.

**NGF Administration and Fluorochrome Labeling**

On the first and third days after the end of distraction, two times 40 μg/hNGFβ (Laboratory of Biochemistry, Fourth Military Medical University, Xi’an, P.R. China) in 0.15 mL isotonic saline was injected into the palpable callus at the treatment side percutaneously. The contralateral side served as control and received placebo (isotonic saline) simultaneously in an identical manner. A sequential intravital fluorochrome labeling was performed in all animals by intramuscular injection according to the following scheme: in the 14 day consolidation experiment, 30 mg/kg tetracycline hydrochloride on the first and 11th days after the end of distraction; in the 28 day consolidation experiment, 30 mg/kg tetracycline hydrochloride on the first and 22nd days and 90 mg/kg calcein green on the 11th day after the end of distraction.

**Specimen Processing and Mechanical Testing**

The rabbits were euthanized at a consolidation time of either 14 (n = 8) or 28 (n = 8) days. 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/L PBS (pH 7.4) for the internal fixation of the tissue. The entire mandible was harvested, and the soft tissue was excised. The distances achieved were measured using a sliding caliper (mean of superior and inferior boundaries). Total regenerate callus dimension measurements were performed by measuring the external labial-lingual and superior-inferior dimensions at the middle of the distraction callus, again using a sliding caliper. Lateral X-rays were performed, and the radiographs of regenerated callus were graded by comparing them with the radiographs of normal bone, was cut from each hemimandible, and distracted region, including 1–3 mm of neighboring maximum load was obtained for all specimens. The materials testing machine (Shimadzu Corporation, Kyoto, Japan) at a displacement rate of 0.5 mm/s. The related bone histomorphometric parameters included bone volume/total volume (BV/TV, %, ratio of mineralized and unmineralized bone volume to the total tissue volume of distracted region), number of osteoblasts (per mm²), number of active osteoblasts per area, excluding lining cells), and mineral apposition rate (μm/ day, distance between labels per labeling period).

**Histology and Histomorphometry**

After fixation, the specimen was cut into two parts in the axial plane. The upper half was dehydrated and embedded in methylmethacrylate. Thirty-micron thick sections were cut for fluorescence measurements using a DM IRB fluorescence microscope (Leica, Germany), and the mineral apposition rates of the 1st–11th and 12th–22nd days of consolidation were determined by measuring the distance between the labeled zones. Undecalcified sections were also stained with von Kossa for evaluation of mineralized bone percentage. The lower half of the specimen was decalcified in buffered 14.5% EDTA (pH 7.3) for 20–30 days, dehydrated, and paraffin embedded. Each block was cut in 5 μm thick sections in the axial plane and stained with hematoxylin and eosin. Bone histomorphometric analysis was performed on four sections for each sample using NIH Image Analysis. Eight images were randomly selected from each section and measured twice with a 3 day interval by a single, unbiased examiner who was blinded to the experimental sides. Large basophilic cytoplasm and an eccentrically placed nucleus were recognized as osteoblastic morphology. The related bone histomorphometric parameters included bone volume/total volume (BV/TV, %, ratio of mineralized and unmineralized bone volume to the total tissue volume of distracted region), number of osteoblasts (per mm²), number of active osteoblasts per area, excluding lining cells), and mineral apposition rate (μm/ day, distance between labels per labeling period).

**Statistical Analysis**

Results are presented as mean and standard error of the mean. A paired t test was used to calculate differences between the treatment and control sides in callus dimensions, maximum load, and bone histomorphometric parameters. p < 0.05 was considered significant.

**RESULTS**

Four of the 20 rabbits had to be excluded from the study. One animal died during surgery because of an anesthesia problem. Two animals developed wound infections and one a respiratory infection. The remaining 16 rabbits showed good tolerance to the experimental procedure and were divided into two separate groups with consolidation times of 14 (n = 8) and 28 (n = 8) days, respectively. All specimens underwent radiographic evaluation, callus dimension measurements, mechanical testing, and decalcified and undecalcified histomorphometric analysis. No difference in the amount of distance achieved or regenerate callus dimensions was observed between the treatment and control sides (Table 1).

**Radiographic Evaluation and Mechanical Testing**

In the 14 day consolidation experiment, X-rays showed that the radiodensity at the treatment side
was greater than that at the control side in most cases (6/8, Fig. 2), and the maximum load in the treatment side was significantly higher than in the control side ($p < 0.01$, Fig. 3). Similarly, hNGFβ increased the maximum load by 23% in the 28 day consolidation experiment ($p < 0.01$, Fig. 3).

**Bone Histology and Histomorphometry**

In the 14 day consolidation experiment, the distraction gaps of both treatment and control sides were completely united mainly with woven bone and there was initial replacement of woven bone by lamellar bone. The bony trabeculae rimmed by osteoblasts showed orientation along the axis of mechanical force. In comparison, the bony trabeculae were thicker in the treatment side (Fig. 4). In the 28 day consolidation experiment, the bony trabeculae became thicker in both sides, also rimmed by osteoblasts. The structure of bony trabeculae was a mixture of woven and lamellar bone in the control side and mostly lamellar bone in the treatment side.

hNGFβ increased BV/TV by 51 ($p < 0.01$) and 26% ($p < 0.01$) in the 14 and 28 day consolidation experiments, respectively (Table 1). Similarly, hNGFβ increased mineralized bone percentage by 37 ($p < 0.01$) and 19% ($p < 0.01$) in the two consolidation experiments, respectively (Table 1). The number of osteoblasts/mm$^2$ was not significantly different between the treatment and control sides. In both the 14 and 28 day consolidation experiments, the mineral apposition rate of the 1st–11th day after distraction was significantly higher in the

![Image](https://example.com/image1.png)

**Table 1.** Callus Dimensions and Histomorphometric Analysis$^a$

<table>
<thead>
<tr>
<th></th>
<th>14 Days of Consolidation</th>
<th>28 Days of Consolidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Distance achieved (mm)</td>
<td>9.42 ± 0.21</td>
<td>9.35 ± 0.25</td>
</tr>
<tr>
<td>Labial-lingual dimension (mm)</td>
<td>6.99 ± 0.44</td>
<td>6.92 ± 0.91</td>
</tr>
<tr>
<td>Superior-inferior dimension (mm)</td>
<td>12.72 ± 0.78</td>
<td>12.59 ± 1.05</td>
</tr>
<tr>
<td>Bone volume/total volume (%)</td>
<td>33.0 ± 4.9</td>
<td>49.8 ± 11.6$^*$</td>
</tr>
<tr>
<td>Number of osteoblasts (mm$^2$)</td>
<td>208.3 ± 43.3</td>
<td>214.8 ± 36.5</td>
</tr>
<tr>
<td>Mineralized bone percentage (%)</td>
<td>30.7 ± 4.5</td>
<td>42.1 ± 8.6$^*$</td>
</tr>
</tbody>
</table>

$^a$Mean and standard error of the mean.

$^p < 0.01$ compared with the control side after the same days of consolidation.

![Image](https://example.com/image2.png)

**Figure 2.** Representative radiograph showing the effect of hNGFβ on bone regeneration (14 days consolidation experiment).
treatment side compared with the control side ($p < 0.01$), but the rate of the 12th–22nd day after distraction was not significantly different between the treatment and control sides in the 28 day consolidation experiment (Table 2).

**DISCUSSION**

Many attempts have been made to shorten the bone consolidation period during DO, including low-intensity pulsed ultrasound, recombinant growth hormone, intermittent parathyroid hormone, calcitonin and alendronate, calcium sulfate and growth factors such as BMPs and IGF. In the present study, locally applied hNGFβ accelerated callus maturation of a distraction gap in a rabbit mandibular distraction model.

Differences exist between distraction of the mandible and that of a long bone such as the tibia in rabbits. The mandible is mainly composed of cancellous bone with much thinner cortex than in tibia. Therefore, the callus formed in the mandible distraction is more uniform in cancellous bone and cortex than in a long bone distraction, making for easier histological evaluation. Besides, the more abundant blood supply in the mandible makes a shorter latency period possible. In this distraction model, we applied a distractor suitable for simultaneous bilateral distraction of the body of rabbit mandibles without the need for two separate devices. Another advantage of this model is the establishment of an intraindividual placebo control. The clap-shaped distraction device may also be adapted to a future model of simultaneous distraction in parallel long bones, such as radius/ulna and tibia/fibula. The possible advantages include providing a precise simultaneous distraction of both long bones and the smaller size, which causes less inconvenience. When using this model in previous studies, despite injection of antibiotics, we observed many local infections with subcutaneous abscesses as a major type (unpublished data). The high infection rate could be attributed to a less meticulous surgical technique and contamination of the midline incision during postoperative eating or drinking by the animal. The incidence of local infection in the present study was reduced to 10.5% (2/19) by improving surgical techniques and applying an antimicrobial ointment to the wound.

The rabbit models of leg or mandible lengthening are well-established and have been used extensively to perform callus stimulation studies. In the present study, locally applied hNGFβ accelerated callus maturation of a distraction gap in a rabbit mandibular distraction model.

**Figure 3.** The effect of hNGFβ on the maximum load in a three-point bending test. * $p < 0.01$ compared with the control side after the same days of consolidation.

**Figure 4.** The effect of hNGFβ on the histology of the distracted callus in the 14 day consolidation experiment. (A) Treatment side; (B) control side (hematoxylin and eosin staining, bar length 0.5 mm).
faster rates (>1.3 mm/day) can result in a poor quality of bone formation. Although fast rates (1.5 or 2 mm/day) were deliberately chosen to establish models of poor bone formation in many previous callus stimulation studies, we chose a slow rate of 0.5 mm per 12 h to mimic normal clinical situations of DO. We believe that this slow rate with the application of the NGF along with the latency period can be of importance clinically. Based on the understanding of a quicker healing process in rabbits than in humans, different latency periods such as 5 days and 3 days were recommended in previous studies on rabbit models of mandibular DO. If the latency period is too short, released soft tissue and periosteum can not recover, and there are not enough osteogenitor cells ready for the strain. But if the latency period is too long, premature consolidation may occur and distraction is consequently difficult. In the present model, we observed that an average of 2.5 days was needed for rabbits to adapt to the distraction device and regain full masticatory abilities. Thus, we assumed that soft tissue and periosteum could gain their recovery after 3.5 days, and a 3.5 day latency period was suitable.

In DO, mechanical strain is the key stimulator of the osteogenic cells, in which expression of growth factors and signaling factors is increased as long as the distraction is maintained and quickly down-regulated as soon as the distraction completes. Therefore, the beginning of consolidation period is likely to be a key period needing exogenous growth factors. Based on BMP expression profiles, single or repeated injections of BMPs applied at the beginning of the consolidation period resulted in enhanced bone consolidation in animal models of DO. Because the expression pattern of NGF likely mimics that of BMPs during DO, NGF injections were carried out at the beginning of the consolidation period (about 2 weeks after osteotomy). To reduce error in the injection procedure, we chose repeated injections instead of a single injection. Besides, an interval period of 2 days was also designed to allow the rabbits to recover from any injury from the puncture. Accelerated callus maturation of the distraction gap demonstrates that the beginning of the consolidation period is likely to be an important period needing exogenous NGF.

In this study, mechanical testing showed that the maximum load on the treatment side was significantly higher than that on the control side in both 14 and 28 day consolidation experiments. Because no difference in the callus dimensions was observed between treatment and control sides, the major reason for the increased load should be the improved arrangement and dimension of each trabecula and better bone mineralization. Further, bone histological and histomorphometric analysis demonstrated that hNGFβ improved the density and quality of regenerate trabeculae. Interestingly, no significant difference was detected in the number of osteoblasts between the treatment and control sides. This can be partly explained by the previous findings that NGF can enhance differentiation of the existent population of osteoblasts without enhancing their proliferation. Besides, we detected a significant difference in mineral apposition rates between the treatment and control sides. Such a difference reflects the effect of hNGFβ on individual osteoblasts.

Our study has limitations, and a variety of problems await further studies. First, we chose the dose of 40 µg hNGFβ only on data of a preliminary study, not on that of release kinetics or clearance rate. Second, we did not follow the whole healing process until the end of optimal consolidation, and did not test the influence of different distraction speeds, distances, and doses of hNGFβ on callus maturation. Third, the use of carrier materials is important to protect the locally applied soluble protein factors from inactivation. Although the distraction tissue that formed after 10 days of distraction may be considered as a matrix for the injected hNGFβ, and a clear effect on callus maturation was observed, the administration needs to be improved, for example by applying injectable or degradable carrier materials. Fourth, the potential ability of promoting the recovery of inferior alveolar nerve injury, one of the most important characteristics of NGF, was not evaluated. Moreover, many issues such as proper dose and adequate frequency of injections remain unanswered for future clinical application of hNGFβ.

Nonetheless, we demonstrated that locally applied hNGFβ accelerated callus maturation in a

| Table 2. Mineral Apposition Rate (µm/Day) Analysisa |
|-----------------|-----------------|-----------------|
|                 | 14 Days of     | 28 Days of     |
|                 | Consolidation   | Consolidation   |
| Day 1–11        | Day 1–11        | Day 12–22       |
| Control         | 2.41 ± 0.27     | 2.43 ± 0.21     | 1.71 ± 0.16     |
| Treatment       | 3.20 ± 0.69a    | 3.15 ± 0.56a    | 1.79 ± 0.38b    |

Mean and standard error of the mean.

*p < 0.01 compared with the control side after the same days of consolidation.

rabbit model of mandibular DO. It may therefore play a role in shortening the consolidation period and lowering the number of complications in mandibular or long bone DO clinically.

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