Effects of exogenous nerve growth factor on the expression of BMP-9 and VEGF in the healing of rabbit mandible fracture with local nerve injury

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Research article

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Abstract

Background

The fracture of mandible remains one of the most facial fractures and its healing is a complex process, involving nerve and growth factors. Currently, nerve growth factor not only benefits maintenance of sympathetic neurite growth, but also takes part in intricate regulatory network to stimulate other growth factors such as bone growth protein and vascular endothelial growth factor, which promote together essential osteogenesis and angiogenesis to physiological bone formation, growth and fracture healing. Therefore, it is necessary to analysis the combination of nerve growth factor, bone growth protein-9 and vascular endothelial growth factor to accelerate healing rate of mandible fracture.

Methods

The models of mandible fracture with local nerve injury established in forty-eight rabbits were randomly divided into nerve growth factor group (NGF group), gelatin sponge group (GS group), blank group and intact group with 12 rabbits in each group. The fracture healing was observed with visual and X-ray after the operation, then callus tissue in mandibular fracture area were collected for HE staining observation, and quantitative RT-PCR was used to detect the expression of BMP-9 and VEGF in callus at different stages.

Results

The combined results of macroscopic observation, X-ray examination and histological section showed that a large number of osteoblasts and some vascular endothelial cells were found around the trabecular bone in NGF group and the amount of callus formation and reconstruction were better than GS group at 2th weeks after the operation. Quantitative RT-PCR result indicated that the expression levels of BMP-9 mRNA and VEGF mRNA in the four groups reached the highest value at the second week, and then decreased with time. At the same time, the content of BMP-9 and VEGF in callus tissue in mandibular fracture area increased significantly in NGF group than GS group.

Conclusion

The exogenous NGF could improve the expressions of BMP-9 and VEGF in the early stage of mandibular fracture to accelerate healing of mandible fracture. This work provides a new foundation and theoretical basis to make clear mechanism of fracture healing, thereby promoting patients’ fracture healing and reducing their disability rate.

Background
Among all the maxillofacial fractures, the fracture of mandible accounts for almost 29% with a range of 11–36% and remains one of the most facial fractures due to the increasing number of population [1, 2]. Fracture of the mandible occurs especially in addition with fasciculus damage as a result of prominent position. Many studies have reported that mandibular fracture patterns were usually from developing countries [3–5], and vehicular accidents were described as the most common cause of all etiology factors, followed by assaults, fall and sports [6]. Therefore, explicating molecular mechanism of fracture healing and then finding proper methods to treat mandibular fracture become even more important.

As the only movable bone of the skull, mandible is connected to the temporal bone by the temporomandibular joint and rich with the inferior alveolar vessels and nerve. Mandible fracture healing is very complex process that refers to the regeneration and repair reactions of local bone tissue at a fracture site and is mainly regulated by the phase of cell proliferation and differentiation, matrix synthesis, and calcification [7–9]. The factors are jointly effected by nerve regulation and humoral regulation, which a large number of cytokines and nerve factors are expressed in the process of fracture healing [10]. As a neurotrophic substance, nerve growth factor (NGF) not only is important for maintenance of sympathetic neurite growth [11], but also participates in accelerating the rate of fracture healing with brain injury and developing skeleton [12, 13]. Eppley et al initially reported that NGF stimulated nerve axon regeneration to induce mandibular defect repairs [14]. NGF and its receptor were expression in fracture callus, which had no function with little or no ingrowth of nerve fibers in bone tissues with nonunion and heterotopic ossications [15, 16]. In various regulating pathways in healing processing, NGF could promote angiogenesis in the callus and osteoblast proliferation and differentiation referring to bone morphogenetic protein and vascular endothelial growth factor.

Belonging to TGF-β superfamily, more than 14 BMPs had been identified for their ability to induce ectopic bone growth and cartilage formation [17, 18]. BMP-9, a secreted protein [19], is known as growth differentiation factor 2 (GDF2) and expressed in the liver [20]. BMP-9 has the most potent bone-forming capability and maybe participate in multiple physiological and pathological functions via complicated networks of signaling pathways, such as osteogenesis, chondrogenesis, angiogenesis and tumorigenesis [21–23]. Osteogenesis and angiogenesis are essential to physiological bone formation, growth and fracture healing. As a signal protein produced by cells, VEGF is a powerful regulating factor of angiogenesis and vasculogenesis [24]. Skeleton is a highly vascularized tissue that relies on blood vessels and VEGF could promote the regeneration of blood vessels as an angiogenic factor, which is more important than in osteogenesis [25]. However, single growth factors influencing bone regeneration is a limited degree and the concentration of cytokines and growth factors could accelerate the process of fracture repair.

In order to analyze the combination of NGF, BMP-9 and VEGF to accelerate healing rate of mandible fracture, the rabbit models of mandible fracture with local nerve injury was constructed in this study. The exogenous NGF with gelatin sponge as vector was used to investigate whether the concentration of BMP-9 and VEGF was highly expressed within the fracture site. Histological assessment was performed to explicit effect of exogenous NGF promoting mandibular fracture healing. The results provide co-
expression pattern of BMP-9 and VEGF under the effect of exogenous NGF in the process of mandibular fracture healing. Besides, this study further elucidates the possible mechanism of fracture healing via exogenous NGF, which presents experimental foundation for clinical medication.

**Materials And Methods**

**Animals**

Forty-eight skeletally-mature, male or female New Zealand white rabbits, weighing 2.5-3.0 kg (Mean 2.7±0.2), were included in the study. The animals were transferred to the experimental animal center of Zunyi medical University at least a week before surgery and kept in separate cages in order to help them adapt to the new environment as well as to ensure their health. This study was performed in accordance with the regulations of the Animal Management Regulations and Administrative Measures on Experimental Animal.

**Establishment of rabbit fracture model**

Forty-eight healthy rabbits were used to establish fracture models by the following surgery under aseptic condition. All rabbits were randomly selected and placed in the supine position, and their submandibular region was prepared individually. They were anesthetized with an ear vein injection of 3% pelltobarbitalum natricum (1 ml/kg body weight), and 2% lidocaine (2 mL) was then injected for intensive local anesthesia. After submandibular incision and dissection of the periosteum, the both side of the mandible anterior to the masseter muscle was exposed by blunt and sharp dissection, and neurovascular bundle of mandible was transected. Then, incomplete fractures (about 5 mm×2 mm) were made in front of the mental foramen of the mandible through the buccal and lingual using diamond burs, and the zone was fully rinsed and cooled by physiological saline at the same time. All fracture models need no reduction and fixation.

**Experimental groups**

Forty-eight New Zealand white rabbits were randomly assigned to nerve growth factor group (NGF group), gelatin sponge group (GS group), blank group and intact group with 12 rabbits in each group. In the NGF group, the mental neurovascular bundle was cut off and implanted nerve growth factor with gelatin sponge as the carrier. The mental neurovascular bundle was cut off and implanted normal saline with gelatin sponge as the carrier in the GS group. The mental neurovascular bundle was cut off without implanting material serving as the blank group. The intact group retained the neurovascular bundle intact and no material was implanted.

**Postoperative care**

After the rabbits were fully awakened from the operation, they were returned to separate cages and acupuncture lower lip response was performed on the same day. Penicillin (0.4 million units) was
administered to each rabbit intramuscularly twice a day for three days. Before the stitches were removed on the 7th day, the wound was cleaned and the healing status was observed every day.

**Visual observation and X-ray inspection of the fracture zone**

At the 2th, 4th, 6th and 8th weeks after the operation, three animals in each group were sacrificed and performed to observe and compare fracture healing using Cone-beam computed tomography systems (CBCT). The operated mandible of each executed rabbit was dissected subperiosteally and observed the formation of callus in the fracture area using visual.

**Histological observation**

Callus tissue in mandibular fracture area was collected using rongeur, rinsed in physiological saline and then immediately put in 10% neutral buffered formalin fixative for 48 hours. Subsequently, samples were rinsed in running tap water for ten minutes and incubated with 10% EDTA (pH=7.2). The decalcifying solutions were changed every three days until the decalcification was completed. The decalcification process was finished when the specimen was easily penetrated through by a needle without any force. Next, samples were washed in 0.01 M PBS for ten minutes and then followed by routine dehydration and paraffin embedding. The paraffin wrapped tissues were cut into 4 µm sections using a Leica microtome (Leica, Germany). The tissue sections were soaked by xylene solution for two times with twenty minutes to dewax the sections, and then soaked in 100%, 95%, 80% and 75% alcohol for 5 min, and finally rinsed in running tap water.

After deparaffinization and rehydration, the sections were stained with hematoxylin dye for five minutes and then rinsed in running tap water. Soak the sections with 100%, 95%, 80% and 75% alcohol to dehydrate the sections. The sections were soaked in xylene until the sections were clear, and then the tablet was sealed. Finally, the pathological changes of callus tissue were observed and photographed using an Olympus BX53 fluorescence microscope (Tokyo, Japan).

**RNA extraction and quantitative real-time PCR**

For RNA extraction, callus tissue in mandibular fracture area was collected using rongeur, and immediately frozen in liquid nitrogen. The frozen tissue was ground to a fine powder in liquid nitrogen using a freezer mill (Bone Mill; SPEX CertiPrep, Metuchen, NJ, USA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. The quantity, degradation and contamination of total RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis, respectively.

RNAs were reverse-transcribed by oligo (dT) primer using the ThermoScriptTM RT–PCR system (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR analysis was carried out using an ABI PRISM7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The published sequences of BMP-9, VEGF and GAPDH were obtained from GeneBank and these oligonucleotide primers for the rabbit-specific genes were designed using the Primer Express Software (Applied Biosystems), as shown in Table
1. GAPDH, a constitutively-expressed housekeeping gene, was used as control gene, and all gene expression data was calibrated to those for GAPDH. Gene expression quantitation was calculated with the comparative cross-threshold (Ct) method. The difference between the average Ct value of the gene of target and the GAPDH were expressed as (ΔCt), and ΔΔCt equals to the difference between the ΔCt and the Ct value of the calibrator sample. The $2^{-\Delta\Delta C_t}$ gave the relative quantitation value of gene expression.

**Statistical analysis**

The descriptive values for BMP-9 and VEGF in different periods in the NGF group, GS group, blank group and intact group were presented in mean and standard deviations. The statistical analysis was analyzed with SPSS 20.0, (IBM, Armonk, NY, USA). Statistical differences among groups were detected by one-way ANOVA followed by Bonferroni’s multiple comparison test. A $p$-value of <0.05 was considered statistically significant.

**Results**

**Model establishment and evaluation**

A total of 48 New Zealand white rabbits were included in this study. CBCT examination showed that all models of incomplete fracture of mandible were successfully prepared (Fig. 1). The incisions healed well after operation, without obvious swelling and bleeding, infection or death. The mental neurovascular bundle was successfully severed in NGF group, GS group and blank group, and remained intact in intact group.

**Needling reaction in lower lip**

Acupuncture of the lower lip was performed on the first day after the operation. The results showed that there was contraction reaction of the lower lip in intact group, but no reaction in NGF group, GS group and blank group, which proved that the nerve dissection operation was successful. At the 2th, 3th and 4th weeks after the operation, the number of animals with nerve reflex recovery in NGF group was noticeably higher than that in GS group and blank group, indicating that early nerve regeneration in NGF group was superior to GS group and blank group. The results of acupuncture reaction at the 5th, 6th, 7th and 8th weeks after the operation indicated that the nerve reaction of each group basically recovered, with no significant difference (Table 2).

**Radiographic and histological appearance**

**1th day after the operation**

The results of X-ray examination showed that the fracture situation of each group was roughly the same, with clear bone incision lines and obvious bone gap (Fig. 2).

**2th weeks after the operation**
Macroscopic observation and X-ray examination demonstrated that callus was formed in fracture area of each group, and osteotomy line was clear (Fig. 3A-D). The amount of callus formation in intact group and NGF group was more than that in GS group and blank group, but the osteotomy line was relatively fuzzy compared with GS group and blank group. In NGF group, there was adhesion at the broken ends of nerve vessels (Fig. 3B), but there was no obvious change in the broken neurovascular bundles between GS group and blank group (Fig. 3C-D). Histological section showed that compared with GS group and blank group (Fig. 3G-H), the distribution of collagen in bone matrix of NGF group and intact group was more uniform, the process of callus reconstruction was more obvious, and a large number of osteoblasts and some vascular endothelial cells were found around the trabecular bone (Fig. 3E-F). However, the arrangement of trabeculae was irregular in all four groups at the point after the operation.

4th weeks after the operation

Macroscopic observation and X-ray examination indicated that the hyperplasia of callus was obvious in all the four groups, and the bone gap became smaller than that at 2th week (Fig. 4A-D). Compared with GS group and blank group, osteotomy line in fracture area of NGF group and intact group became blurred, the amount of callus formation and the degree of fusion were better, and bone gap became less obvious (Fig. 4A-B). Histological section showed that the trabecular arrangement of NGF group and intact group became more regular, the process of callus reconstruction remained obvious, and a large number of osteoblasts were still visible around trabecular bone (Fig. 4E-F). In GS group and blank group, the arrangement of local bone trabeculae was still irregular, the distribution of collagen in bone matrix became more uniform than before, the reconstruction process of osteocytes and callus became more obvious, and more osteoblasts began to appear around the trabeculae (Fig. 4G-H).

6th weeks after the operation

Macroscopic observation and X-ray examination determined that callus at both ends of osteotomy line in NGF group and intact group had fused and calcified, and fracture line basically disappeared (Fig. 5A-B). Fusion calcification appeared in callus at both ends of osteotomy line in GS group and blank group, and there were still ambiguous fracture lines. The regeneration of the ruptured neurovascular bundle was also basically completed in GS group and blank group (Fig. 5C-D). Histological sections showed that in the four groups, the trabecular arrangement was relatively regular, the distribution of bone matrix collagen tended to be uniform, and there were more columnar osteoblasts around the trabeculae. However, the process of callus reconstruction was not obvious in NGF group and intact group, while remained active in GS group and blank group (Fig. 5E-H).

8th weeks after the operation

The combined results of macroscopic observation, X-ray examination and histological section showed that the fractures in NGF group, intact group, GS group and blank group had completely healed, the osteotomy line had disappeared, and the degree of callus reconstruction was basically similar, tending to
normal bone tissue structure (Fig. 6A-D). There was no significant difference in the visual observation of neurovascular bundles in each group (Fig. 6E-H).

Quantitative real-time reverse transcription polymerase chain reaction

At the 2th, 4th, 6th and 8th weeks, the expressions levels of BMP-9 mRNA and VEGF mRNA in the four groups were detected respectively. The results showed that the expression levels of BMP-9 mRNA and VEGF mRNA in the four groups reached the highest value at the second week, and then decreased with time (Fig. 7). At these four time points, the expression levels of BMP-9 mRNA and VEGF mRNA in intact group were significantly higher than blank group \((P < 0.05)\). The expression level of BMP-9 mRNA in NGF group was significantly higher than that in GS group except for the 8th week \((P < 0.05)\) (Fig. 7A). At the second week, the expression of VEGF mRNA in NGF group was markedly higher than that in GS group \((P < 0.05)\), but there was no statistically significant difference at the other three time points (Fig. 7B).

Discussion

NGF accelerates healing of mandible fracture

Fracture healing especially to mandible fracture due to special physiology and facial parts refers to the process of repair, which is jointly affected by nerve regulation and humoral regulation after the physiological results and functions of the bone are damaged [26]. Nerve growth factor (NGF) is widely distributed in central and peripheral nervous systems that not only regulate growth, development, differentiation, regeneration, and functional protein expression of neurons [27], but also stimulate osteoblasts to promote bone cell growth by NGF receptors’ phosphorylation [28]. In this study, rabbit models of incomplete mandible fracture with mental neurovascular bundle were created in NGF group, GS group and blank group. At the 2th, 3th and 4th weeks after the operation, needling reaction in lower lip showed the number of animals with nerve reflex recovery in NGF group was significantly higher than that in GS group and blank group, implying that NGF contributed to early nerve regeneration (Table 2). Besides, osteotomy line in fracture area of NGF group gradually fused and calcified, and fracture line basically disappeared, while the situation in GS group and blank group was delayed correspondingly at the same times after the operation, suggesting that NGF promoters healing of mandible fracture (Figs. 3, 4 and 5). The result came to the point that higher serum levels of NGF content was detected in fracture combined with brain injury group than control group [27, 29]. Therefore, NGF, as important bridge transmitters, affects the metabolism of bone tissues in the nervous system and then plays an important role in healing of mandible fracture.

NGF improved the expression of BMP-9 and VEGF in fracture healing.

Fracture healing is a very complex process of tissue repair and NGF is key impact factor in the way. NGF promotes fracture healing as follows: inducing nerve growth in bone callus, interaction with neuropeptide substance, regulating bone growth factors, promoting angiogenesis factors in callus and inhibiting osteoclast function [9]. In this study, there was obvious adhesion at the broken ends of angiogenesis in
NGF group than GS group at 2th and 4th weeks (Fig. 3B, 4B). Macroscopic observation and X-ray examination showed that a large number of some vascular endothelial cells were found around the trabecular bone, suggesting that NGF would facilitate angiogenesis at the position of fracture at the early stage of healing (Fig. 3F). Besides, the amount of callus formation and reconstruction were better, and the distribution of collagen in bone matrix was more uniform depending on the effect of exogenous NGF, implying that NGF can promote the proliferation and differentiation of bone cells to reconstruct mandible (Fig. 3B, 4B, 5B). It comes to the point that accumulating NGF could promote an abnormal growth of calluses in local fracture and improve the rate of fracture repair [30].

As proteins secreted by cells, growth factors act on critical functions as cell division, matrix synthesis and tissue differentiation [31, 32], and play important roles in healing of bone fractures [33]. These bone growth factors include bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) and so on. Among them, BMP-9 and VEGF are responsible for osteogenesis and angiogenesis, respectively [34–37]. In this paper, a large number of osteoblasts and some vascular endothelial cells were found around the trabecular bone in NGF group at 2th weeks after the operation (Fig. 3F), while the expression levels of BMP-9 mRNA and VEGF mRNA in the four groups reached the highest value, and the content of BMP-9 and VEGF in callus tissue in mandibular fracture area increased significantly in NGF group than GS group at the same time (P < 0.05) (Fig. 7). After fracture, systemic or local regulation of nerves and body fluids are presented of fracture locate, and multiple cytokines and nerve regulation participate in the process of bone healing, especially BMP and VEGF. Though endocrine factors, NGF stimulates the concentration of growth factors such as BMP-9 and VEGF increased, leading to bind to the receptors on the cell surface of the fracture or damaged site and accelerating the process of fracture repair. This work provides a new foundation and theoretical basis to make clear mechanism of fracture healing, thereby accelerating patients’ fracture healing and reducing their disability rate.

**Conclusions**

The results of this study show that the exogenous NGF would facilitate angiogenesis at the position of fracture at the early stage of healing and could improve the expressions of BMP-9 and VEGF in the early stage of mandibular fracture to accelerate healing of mandible fracture.

**Abbreviations**

BMP-9: Bone morphogenic protein-9; QRT-PCR:Quantitative real-time PCR; NGF:Nerve growth factor; VEGF:Vascular endothelial growth factor; GS:gelatin sponge; GDF2: growth differentiation factor 2; CBCT:Cone-beam computed tomography systems; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase

**Declarations**
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This project was approved by the medical ethics committee of Zunyi Medical University (Approval No. 2018. 246).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

SY and JC designed the study, analyzed the experiments, and wrote the paper. GGL, LJ, CM, WJZ and DXZ carried out the data collection and data analysis and revised the paper. All authors read and approved the final version of the manuscript.

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Tables

Table 1. Sequence of primers

| Gene   | Genebank No. | Forward prime (5'-3')          | Reverse prime (5'-3')          |
|--------|--------------|--------------------------------|--------------------------------|
| BMP-9  | XM_017339607 | ACCCTGGTGCACTCTCAAGTT         | GTAGAGATGGAGATGGGAC           |
| VEGF   | XM_017345155 | AACGAACGTACTTGCAGATGT         | GCTCACGCAGTCTCCTCTTT          |
| GAPDH  | NM_001082253 | AGAGCACAGAGAGAGAGAGCAGA       | TGGGATGGAACACTGTGAAGAGG        |

BMP-9, bone morphogenetic protein-9; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase
Table 2. Needling reaction in lower lip (number of responding animals/number of remaining animals)

| Group | 1 day | 1 week | 2 weeks | 3 weeks | 4 weeks | 5 weeks | 6 weeks | 7 weeks | 8 weeks |
|-------|-------|--------|---------|---------|---------|---------|---------|---------|---------|
| intact| 12/12 | 12/12  | 12/12   | 9/9     | 9/9     | 6/6     | 6/6     | 3/3     | 3/3     |
| NGF   | 0/12  | 0/12   | 3/12    | 7/9     | 9/9     | 6/6     | 6/6     | 3/3     | 3/3     |
| GS    | 0/12  | 0/12   | 0/12    | 4/9     | 7/9     | 6/6     | 6/6     | 3/3     | 3/3     |
| blank | 0/12  | 0/12   | 0/12    | 3/9     | 6/9     | 6/6     | 6/6     | 3/3     | 3/3     |

Figures

Figure 1

The result of CBCT examination after the operation
Figure 2

X-ray results at the first day after surgery. (A) Intact group. (B) NGF group. (C) GS group. (D) Blank group
Figure 3

X-ray results at the first day after surgery. (A) Intact group. (B) NGF group. (C) GS group. (D) Blank group

Figure 4

X-ray results and histological section at four weeks after surgery. (A-D) The X-ray results of intact group, NGF group, GS group and blank group were shown. (E-H) Histological sections of intact group, NGF group, GS group and blank group were detected

Figure 5

X-ray results and histological section at six weeks after surgery. (A-D) The X-ray results of intact group, NGF group, GS group and blank group were shown. (E-H) Histological sections of intact group, NGF group, GS group and blank group were detected
Figure 6

X-ray results and histological section at eight weeks after surgery. (A-D) The X-ray results of intact group, NGF group, GS group and blank group were shown. (E-H) Histological sections of intact group, NGF group, GS group and blank group were detected.

Figure 7

The expression level of BMP-9 mRNA and EGF mRNA in callus tissues in four stages. (A) The expression levels of BMP-9 mRNA in intact group, NGF group, GS group and blank group at the 2th, 4th, 6th and 8th weeks were evaluated. (B) The expression levels of VEGF mRNA in intact group, NGF group, GS group and blank group at the 2th, 4th, 6th and 8th weeks were detected. Asterisk represents a significant difference between NGF group and GS group, P <0.05; number sign represents a significant difference between intact group and blank group, P <0.05.