

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

Sen Yang

affiliated stomatological hospital of zunyi medical university

Jiao Cheng (✉ cjjuse2020@163.com)

affiliated stomatological hospital of zunyi medical university

Cheng Man

affiliated stomatological hospital of zunyi medical university

Lian Jiang

affiliated stomatological hospital of zunyi medical university

Guogeng Long

affiliated stomatological hospital of zunyi medical university

Wenjun Zhao

affiliated stomatological hospital of zunyi medical university

Dexin Zheng

affiliated stomatological hospital of zunyi medical university

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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 the intricate regulatory network to stimulate other growth factors such as bone morphogenic protein (BMP) and vascular endothelial growth factor (VEGF), which promote together essential osteogenesis and angiogenesis to physiological bone formation, growth and fracture healing. Therefore, it is necessary to analyze the combination of nerve growth factor (NGF), bone morphogenic protein-9 (BMP-9), and VEGF to accelerate the 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 hematoxylin and eosin (HE) staining observation, and quantitative real-time PCR (qRT-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 the NGF group and the amount of callus formation and reconstruction was better than GS group at 2nd weeks after the operation. QRT-PCR result indicated that the expression levels of BMP-9 mRNA and VEGF mRNA in the four groups reached the highest value at 2nd week, and then decreased with time. At the same time, the content of BMP-9 and VEGF in callus tissue in the mandibular fracture area increased significantly in the NGF group than the GS group.

Conclusion: The exogenous NGF could improve the expressions of BMP-9 and VEGF at the early stage of mandibular fracture to accelerate the healing of mandible fracture. This work will provide a new foundation and theoretical basis to make a 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% to 36% and remains one of the most facial fractures due to the increasing number of populations with motor vehicle accidents [1, 2]. 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, falls, and sports [6]. As the only movable bone of the skull, the mandible is connected to the temporal bone by the temporomandibular joint and rich with the inferior alveolar vessels and nerve bundle. The special nature of the mandible anatomy determines the mandibular fractures often accompanied damage of nerve bundles. Healing of fracture is an instinctive

physiological response, which requires coordination and control by nerve regulation and humoral regulation [7]. Many studies have investigated the effect of cytokines and nerve factors on bone healing. Chisalita *et al* showed that nerve growth factor (NGF) and insulin-like growth factor 1 (IGF1) played an important role in accelerating the rate of bone healing in patients with brain injury combined with a fracture [8]. A high concentration of NGF could promote the growth of bone callus in fractures and improve the rate of fracture repair [9]. Besides, the local application of NGF promoted bone morphogenic protein-2 (BMP-2) expression in mandibular fracture [10]. Explicating the molecular mechanism of fracture healing and then finding proper methods to treat mandibular fracture become even more important.

Fracture with mandible with the constantly moving bone damaged functions of the bone and increased challenges of fracture healing. Mandible fracture healing refers to the regeneration and repair reactions of local bone tissue at a fracture site and is mainly regulated by factors including cell proliferation and differentiation, matrix synthesis, and calcification [11-13]. These factors are jointly affected by nerve regulation and humoral regulation, in which a large number of cytokines and nerve factors are expressed in the process of fracture healing [7]. As a neurotrophic substance, NGF is not only important for maintaining the growth of sympathetic neurites [14] and increasing the activities of enzymes associated with catecholamine synthesis [15], but it is also participated in accelerating the rate of fracture healing with brain injury and developing skeleton [8, 16]. Eppley *et al* initially reported that through the application of NGF in mandibular defect repairs in experimental rabbits, NGF not only could promote axonal regeneration but also found that newborn osteoid was generated around the regenerated axons [17]. NGF has been demonstrated to stimulate axonal growth in sensory nerves as well as sympathetic fibres [18]. Some studies showed that after fracture compared with controls, NGF stimulated sympathetic neurite growth and influencing neurite extension [19-22]. It has been reported that NGF could promote the proliferation and differentiation of bone cells and inhibit the formation of osteoclasts through endocrine factors, such as other neuropeptides and hormones, thereby promoting fracture healing [23]. NGF promoted the expression of vascular endothelial growth factor in the fracture healing process [13]. Lv *et al* found that NGF might promote the expression of BMP-2 in the healing of rabbits' mandibular fracture with partial nerve injury [10].

Belonging to the transforming growth factor- β (TGF- β) superfamily, more than 14 Bone morphogenic proteins (BMPs) had been identified for their ability to induce ectopic bone growth and cartilage formation [24, 25]. Bone morphogenic protein-9 (BMP-9), a secreted protein [26], is known as growth differentiation factor 2 (GDF2) and expressed in the liver [27]. BMP-9 had 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 [28-30]. Osteogenesis and angiogenesis were essential to physiological bone formation, growth, and fracture healing. As a signal protein produced by cells, vascular endothelial growth factor (VEGF) was a powerful regulating factor of angiogenesis and vasculogenesis [31]. Skeleton was a highly vascularized tissue that relies on blood vessels and VEGF could promote the regeneration of blood vessels as an angiogenic factor, which is as important as in osteogenesis [32]. However, single growth factors

influencing bone regeneration were a limited degree and the concentration of cytokines and growth factors could accelerate the process of fracture repair [33].

To analyze the combination of NGF, BMP-9, and VEGF to accelerate the healing rate of mandible fracture, the rabbit models of mandible fracture with local nerve injury were constructed in this study. The exogenous NGF with gelatin sponge as a vector was used to investigate whether the concentration of BMP-9 and VEGF was highly expressed within the fracture site. This study further elucidates the possible mechanism of fracture healing via exogenous NGF, which presents an experimental foundation for clinical medication that the exogenous NGF could promote the high expressions of BMP-9 and VEGF at the early stage of mandibular fracture healing after neurovascular injury.

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 to help them adapt to the new environment as well as to ensure their health. This study was performed by the regulations of the Animal Management Regulations and Administrative Measures on Experimental Animal and approved by the medical ethics committee of Zunyi Medical University (Approval No. 2018. 246).

Establishment of rabbit fracture model

Forty-eight healthy rabbits were used to establish fracture models by the following surgery under aseptic conditions. 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% pentobarbitalum 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, both sides of the mandible anterior to the masseter muscle was exposed by blunt and sharp dissection, and the neurovascular bundle of the mandible of 36 rabbits 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 the 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 1 ml nerve growth factor (10 µg/ml) with gelatin sponge as the carrier. The mental neurovascular bundle was cut off and implanted with normal saline equivalent to NGF 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 2nd, 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 whether the callus formed in the fracture area was observed visually, to collect the callus immediately for subsequent hematoxylin and eosin (HE) staining observation and qRT-PCR analysis.

Histological observation

Callus tissue in the 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% ethylenediaminetetraacetic acid (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 phosphate buffered saline (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 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 the 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 ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA, USA). QRT-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 glyceraldehyde-3-phosphate dehydrogenase (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 a 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 was expressed as (Δ Ct), and $\Delta\Delta$ Ct equals the difference between the Δ Ct and the Ct value of the calibrator sample. The $2^{-\Delta\Delta$ Ct} 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 the mandible were successfully prepared (Fig. 1). The incisions healed well after the operation, without obvious swelling and bleeding, infection or death. The mental neurovascular bundle was successfully severed in the NGF group, GS group, and blank group, and remained intact in the 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 a contraction reaction of the lower lip in the intact group, but no reaction in the NGF group, GS group, and blank group, which proved that the nerve dissection operation was successful. At the 2nd, 3rd and 4th weeks after the operation, the number of animals with nerve reflex recovery in the NGF group was noticeably higher than that in the GS group and blank group, indicating that early nerve regeneration in the 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 recovered, with no significant difference (Table 2).

Radiographic and histological appearance

1 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).

2 weeks after the operation

Macroscopic observation and X-ray examination demonstrated that callus was formed in the fracture area of each group, and the osteotomy line was clear (Fig. 3A-D). The amount of callus formation in the intact group and NGF group was more than that in the GS group and blank group, but the osteotomy line was relatively fuzzy compared with the GS group and blank group. In the 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 the 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.

4 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 2nd weeks (Fig. 4A-D). Compared with the GS group and blank group, the osteotomy line in the fracture area of the NGF group and intact group became blurred, the amount of callus formation and the degree of fusion were better, and the bone gap became less obvious (Fig. 4A-B). The histological section showed that the trabecular arrangement of the 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 the trabecular bone (Fig. 4E-F). In the 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).

6 weeks after the operation

Macroscopic observation and X-ray examination determined that callus at both ends of the osteotomy line in the NGF group and intact group had fused and calcified, and the fracture line disappeared (Fig. 5A-B). Fusion calcification appeared in the callus at both ends of the osteotomy line in the GS group and blank group, and there were still ambiguous fracture lines. The regeneration of the ruptured neurovascular

bundle was also basically completed in the 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 the NGF group and intact group, while remained active in the GS group and blank group (Fig. 5E-H).

8 weeks after the operation

The combined results of macroscopic observation, X-ray examination, and histological section showed that the fractures in the NGF group, intact group, GS group, and blank group had completely healed, the osteotomy line had disappeared, and the degree of callus reconstruction was 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 2nd, 4th, 6th, and 8th weeks, the expression 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 2nd weeks, and then decreased with time (Fig. 7, Table S1-S2). At these four time points, the expression levels of BMP-9 mRNA and VEGF mRNA in the intact group were significantly higher than the blank group ($P < 0.05$). The expression level of BMP-9 mRNA in the NGF group was significantly higher than that in the GS group except for the 8th week ($P < 0.05$) (Fig. 7A, Table S1). At the 2nd weeks, the expression of VEGF mRNA in the NGF group was markedly higher than that in the GS group ($P < 0.05$), but there was no statistically significant difference at the other three time points (Fig. 7B, Table S2).

Discussion

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 [34]. NGF is widely distributed in central and peripheral nervous systems that not only regulate growth, development, differentiation, regeneration, and functional protein expression of neurons [35] but also stimulate osteoblasts to promote bone cell growth by NGF receptors' phosphorylation [36]. In this study, rabbit models of incomplete mandible fracture with mental neurovascular bundle were created in the NGF group, GS group, and blank group. At the 2nd, 3rd and 4th weeks after the operation, needling reaction in lower lip showed the number of animals with nerve reflex recovery in the NGF group was significantly higher than that in the GS group and blank group, implying that NGF contributed to early nerve regeneration (Table 2). Besides, the osteotomy line in the fracture area of the NGF group gradually fused and calcified, and the fracture line disappeared, while the situation in the GS group and blank group was delayed correspondingly at the same times after the operation, suggesting that NGF promoted healing of mandible fracture (Fig. 3, 4 and 5). The result came to the point that higher serum levels of NGF content were detected in fracture combined with brain injury

group than the control group [35, 37]. Besides, Zhuang *et al* [9] showed that a high concentration of NGF could promote the growth of bone callus in fractures and improve the rate of fracture repair, which is consistent with the results of this study. Therefore, NGF, as important bridge transmitters, could affect the metabolism of bone tissues in the nervous system and then plays an important role in the healing of mandible fracture.

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 [13]. In this study, there was obvious adhesion at the broken ends of angiogenesis in the NGF group than the GS group at the 2nd 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 was better, and the distribution of collagen in bone matrix was more uniform depending on the effect of exogenous NGF, implying that NGF could 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 abnormal growth of calluses in local fracture and improve the rate of fracture repair [9]. However, the mechanism of NGF for promoting the expression of related neuropeptides in peripheral nerves to affect the healing of mandibular fractures needs to be studied further.

As proteins are secreted by cells, growth factors act on critical functions like cell division, matrix synthesis, and tissue differentiation [33, 38], and play important roles in the healing of bone fractures [39]. These bone growth factors include bone morphogenetic protein, vascular endothelial growth factor, fibroblast growth factor, transforming growth factor- β , and so on. Among them, BMP-9 and VEGF are responsible for osteogenesis and angiogenesis, respectively [40-43]. In this paper, a large number of osteoblasts and some vascular endothelial cells were found around the trabecular bone in the NGF group at 2nd 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). Hayami *et al* indicated that VEGF induced the ingrowth of vessels in cartilage, leading to endochondral bone augmentation, which is very favorable for fracture healing [44]. Song *et al*. reported that BMP-2 used alone could induce a surplus of callus formation (heterotopic ossification) [45]. After a fracture, systemic or local regulation of nerves and body fluids were presented of fracture locate, and multiple cytokines and nerve regulation participated in the process of bone healing, especially BMP and VEGF. Lv *et al* reported a study to observe the effect of NGF on the expression of BMP-2 in rabbit models [10]. The local application of NGF promoted BMP-2 peaked in two weeks and decreased later with time, which builds the base of our study. Comparing with the using BMP-2 alone, our study found that NGF stimulated the concentration of growth factors such as BMP-9 and VEGF increased simultaneously, leading to binding to the receptors on the cell surface of the fracture or damaged site and accelerating the process of fracture repair. A limitation of this study is that the promotion of fracture healing by NGF might be achieved by regulating a variety of bone growth factors instead of BMP-9 and VEGF only. In the

future, we will experimentally investigate the regulating effects of NGF on other cytokines and the interacting mechanisms among the various factors. This work will provide a new foundation and theoretical basis to make a 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 at 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; HE staining: Hematoxylin and eosin staining; TGF- β : Transforming growth factor- β ; IGF1: Insulin-like growth factor 1

Declarations

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Availability of data and materials

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

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.

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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Tables

Table 1. Sequence of primers

Gene	Genebank No.	Forward prime (5'-3')	Reverse prime (5'-3')
BMP-9	XM_017339607	ACCCTGGTGCATCTCAAGTT	GTAGAGGATGGAGATGGGGC
VEGF	XM_017345155	AACGAACGTACTTGCAGATGT	GCTCACGCAGTCTCCTCTTC
GAPDH	NM_001082253	AGAGCACCCAGAGGAGGACGA	TGGGATGGAACTGTGAAGAGG

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

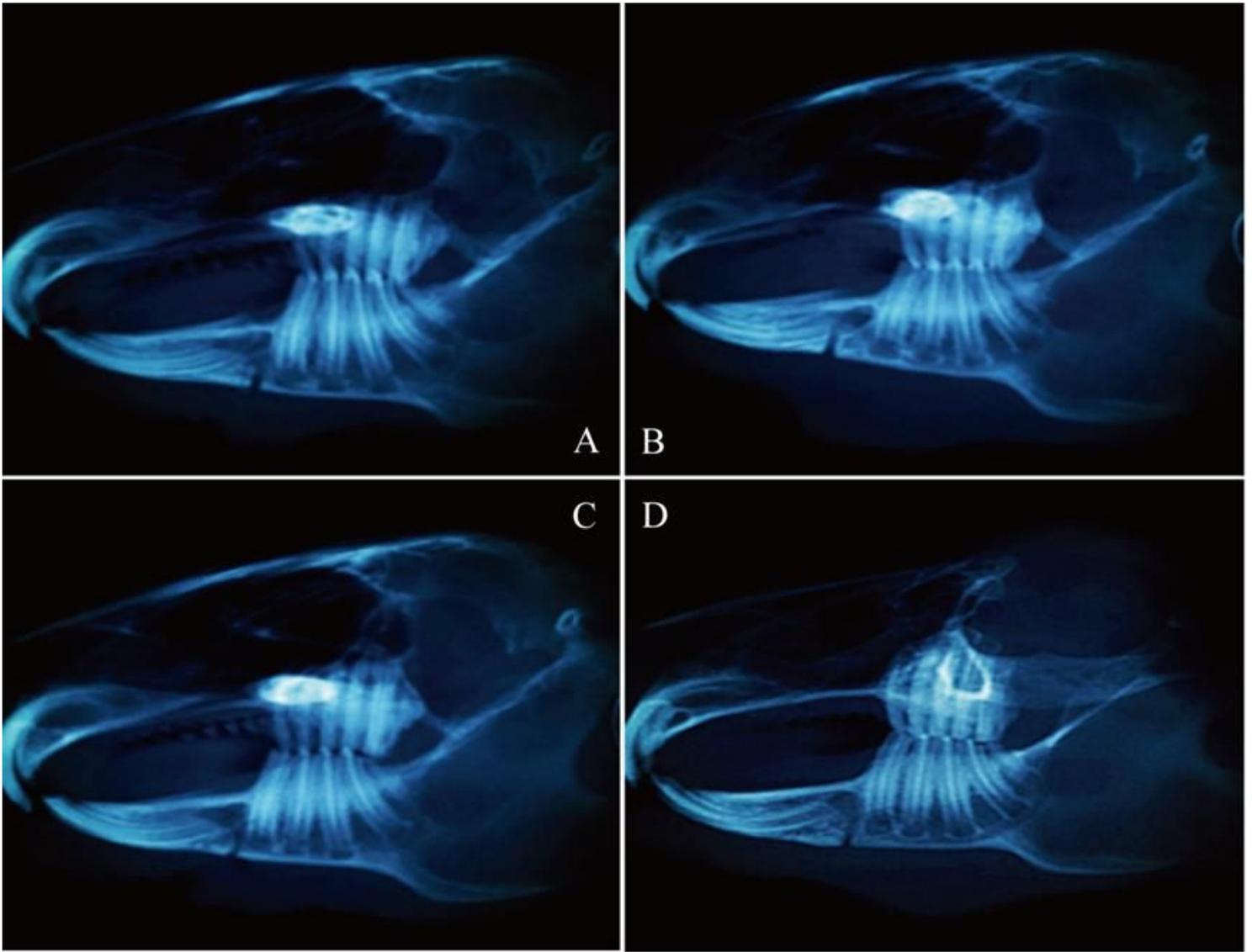


Figure 2

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

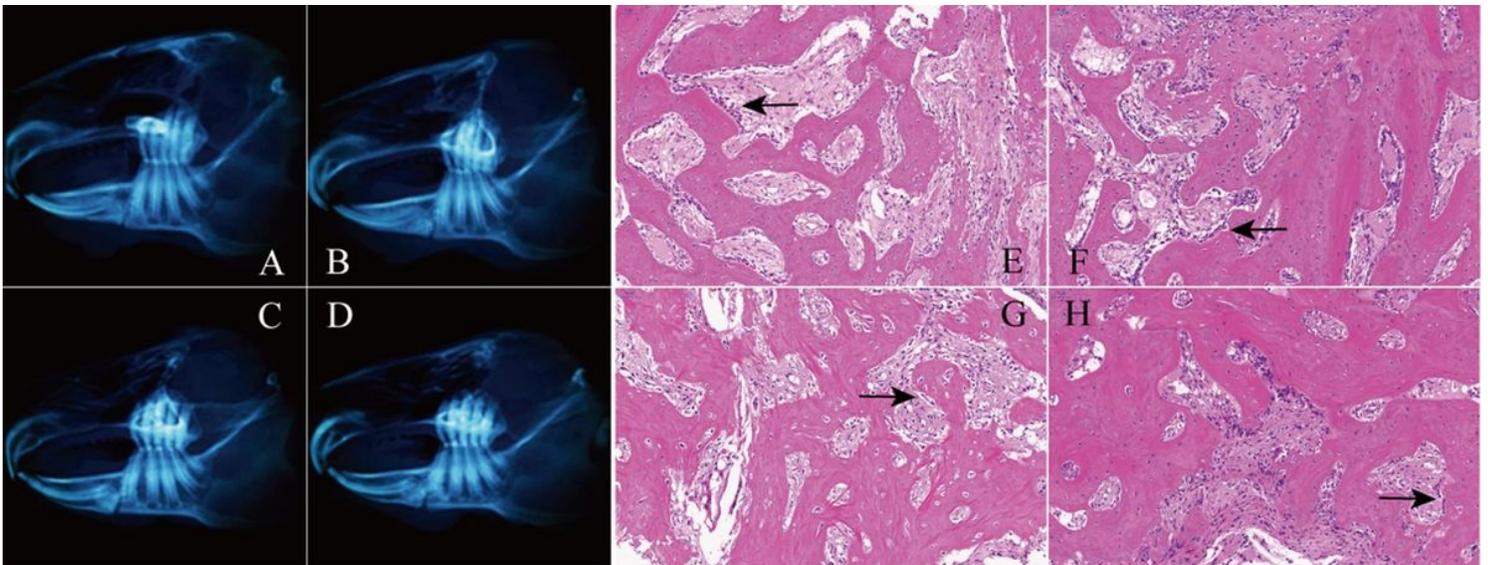


Figure 3

X-ray results and histological section at 2nd weeks after surgery. (A-D) The X-ray results of the intact group, NGF group, GS group, and blank group were shown. (E-H) Histological sections of the intact group, NGF group, GS group, and blank group were detected

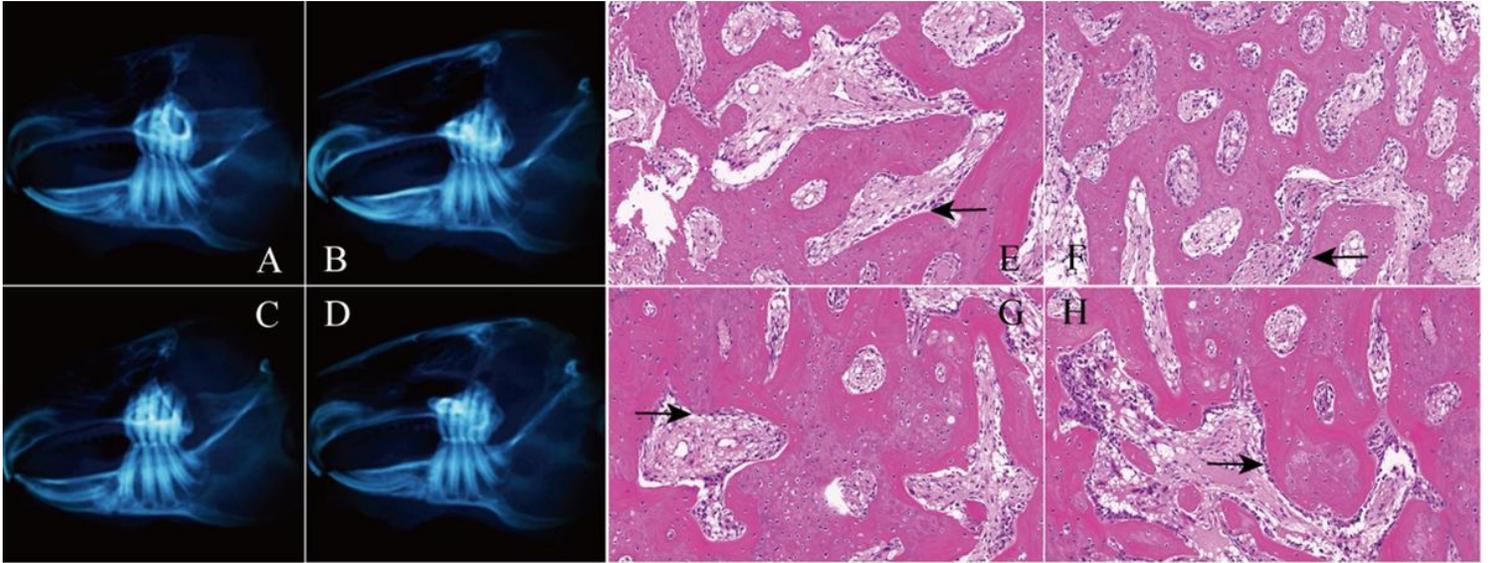


Figure 4

X-ray results and histological section at 4th weeks after surgery. (A-D) The X-ray results of the intact group, NGF group, GS group, and blank group were shown. (E-H) Histological sections of the intact group, NGF group, GS group, and blank group were detected

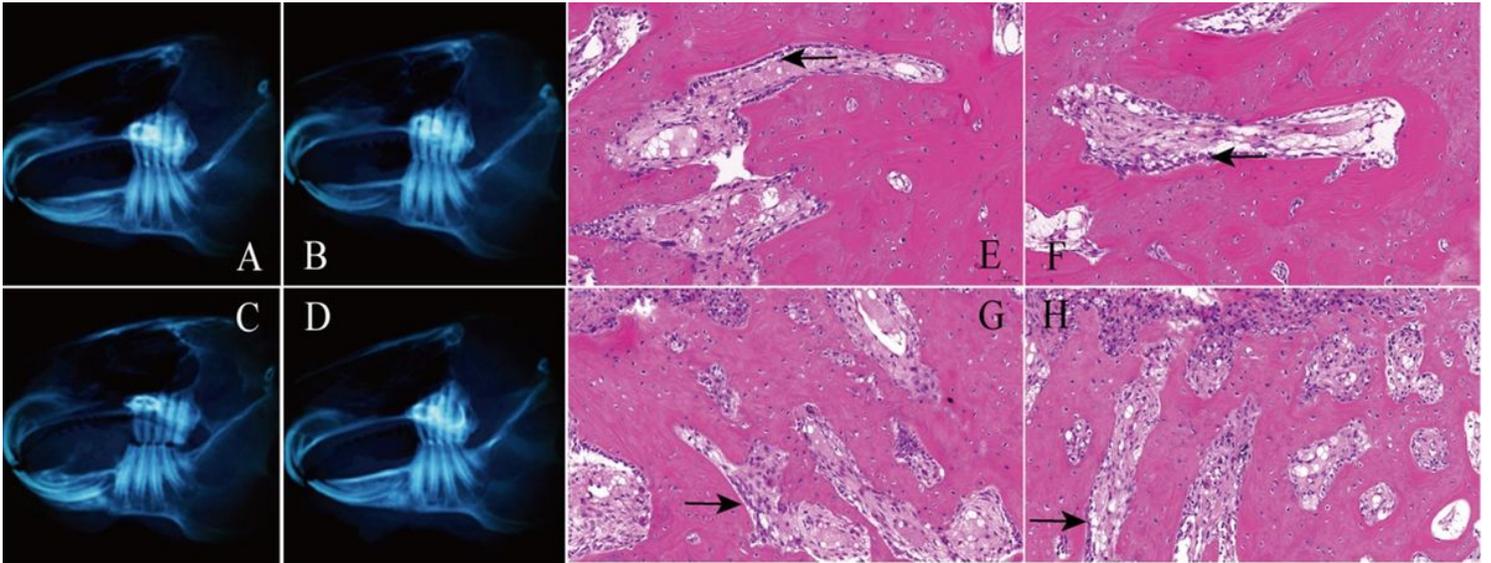


Figure 5

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

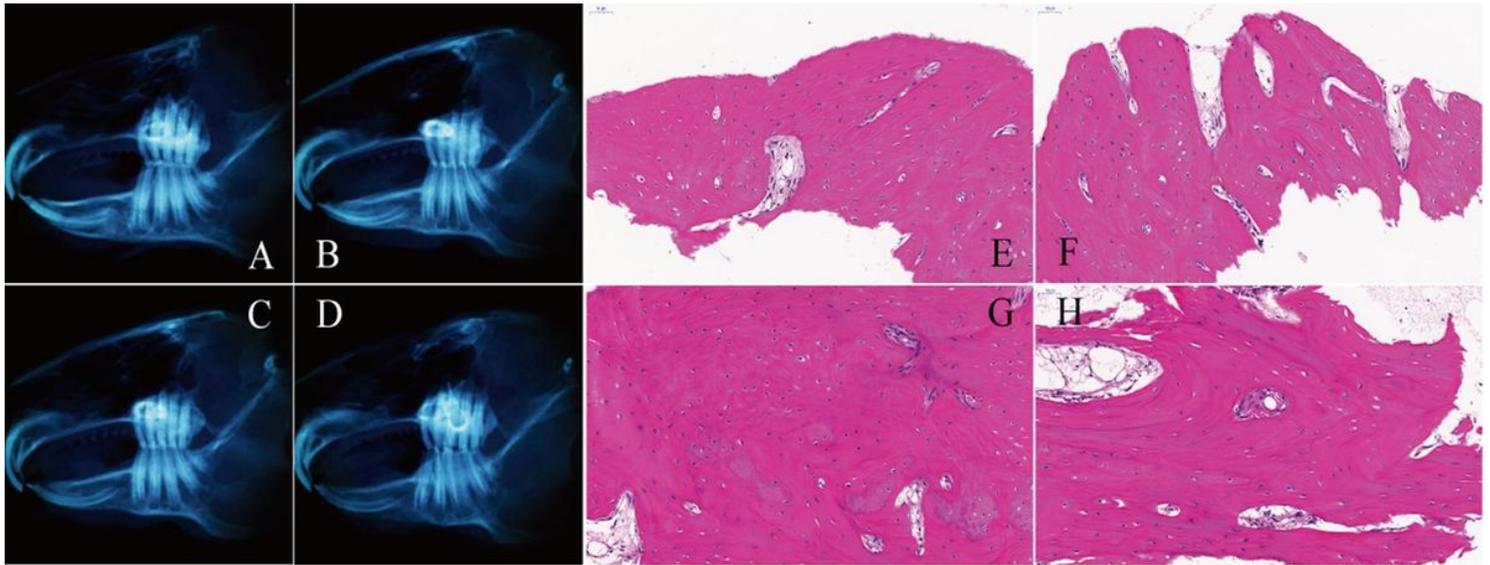


Figure 6

X-ray results and histological section at 8th weeks after surgery. (A-D) The X-ray results of the intact group, NGF group, GS group, and blank group were shown. (E-H) Histological sections of the intact group, NGF group, GS group, and blank group were detected

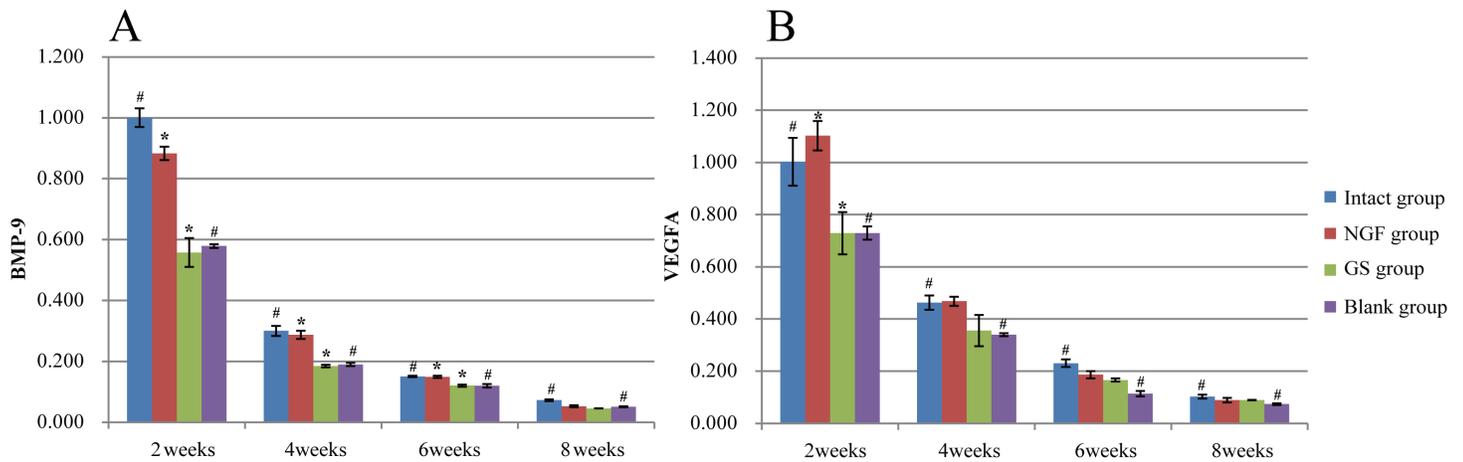


Figure 7

The expression level of BMP-9 mRNA and EGF mRNA in callus tissues at four stages. (A) The expression levels of BMP-9 mRNA in the intact group, NGF group, GS group, and blank group at the 2nd, 4th, 6th, and 8th weeks were evaluated. (B) The expression levels of VEGF mRNA in the intact group, NGF group, GS group, and blank group at the 2nd, 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 the intact group and blank group, $P < 0.05$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.docx](#)
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