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Original Research Article

Gefitinib enhances healing of long bone fractures in rats via inhibition of the epidermal growth factor receptor signal pathway

Aofei Shen[1]*, Mengwei Wang[2], Xiangheng Li[2]
[1]Department of Orthopedics, Baoji Hospital of Traditional Chinese Medicine, Baoji 721000, Shaanxi Province, [2]The Third Clinical Medical College, Guangzhou University of Chinese Medicine, Guangzhou 510000, Guangdong Province, China

For correspondence:* ***Email:* *yingqiaopan522247@163.com*

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Abstract

Purpose: *To investigate the influence of gefitinib on healing of long bone fractures in rats, and the involvement of epidermal growth factor receptor (EGFR) signal route in the process.*
Methods: *A model of long bone fracture was established in 30 Sprague-Dawley (SD) rats which were allocated to control and study groups (n = 15/group). Rats in study group received gefitinib (100 mg/kg per day) via gavage, while control rats were given 0.55 % methylcellulose daily for 7 - 42 days. Fracture healing, maximum callus diameter, serum levels of bone markers and mRNA levels of bone turnover indices in the callus, were determined.*
Results: *Following 1 week of therapy, fracture lines were clear in both groups, and callus was produced. On days 7, 14, 21, and 28, maximum callus diameter of study group was significantly higher than that of control group ($p < 0.05$). However, there was no significant difference in the maximum callus diameter between the two groups after 42 days ($p > 0.05$). On days 7, 14 and 21, there were significantly higher BALP and PINP levels, and TRACP-5b and CTX values in study group than in control ($p < 0.05$). On days 7 and 14, study group COLa1 mRNA and osteocalcin (OC) mRNA was significantly raised, relative to control values. On days 7, 14 and 21, COL10 mRNA expression was significantly up-regulated in study group relative to controls ($p < 0.05$). However, mRNA expression levels of COLa1, OC, COL10, and COL2A1 were similar in both groups at other time points.*
Conclusion: *Gefitinib enhances the healing of long bone fractures and callus formation in rats, probably through inhibition of EGFR signaling pathway. Therefore, gefitinib is beneficial in bone formation in rats.*

Keywords: *Gefitinib, EGFR signal pathway, Long bone fracture, Fracture healing*

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INTRODUCTION

The process of fracture healing involves many cytokines and signal routes such as Mitogen-Activated Protein Kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and Wnt pathway. These factors such as Transforming growth factor-β (TGF-β) and other pathways are closely monitored at different periods of fracture healing and in different

positions of the fracture. There are two types of fracture healing: direct and indirect healing. However direct healing is not common in clinical practice [1,2]. As different biomechanics lead to different fracture positions in different environments, the healing types will also be different [3]. With the continuous improvement in medical technology, the fracture healing rate is gradually increasing, although the healing time of fracture is long. Indeed, most patients take 6 - 12 weeks to heal [4,5]. If the fracture continues to heal slowly or does not heal, it may cause a decline in limb function, resulting in serious effects on the patient's daily life and psychology [6]. Therefore, there is need to identify methods that can effectively enhance fracture healing and reduce healing time. Clinical studies have shown that the epidermal growth factor receptor (EGFR) signal pathway plays an important positive role in fracture healing by significantly enhancing osteoblast proliferation while inhibiting its differentiation, and also suppressing chondrocyte maturation. However, its relationship with bone fracture healing in rats has not been studied [7,8]. Gefitinib belongs to the family of EGFR inhibitors, and it has become the first-line therapeutic drug for non-small cell lung cancer [9,10]. Therefore, this work was focused on investigating the influence of gefitinib on long bone fracture healing in rats, because it is an EGFR inhibitor and the implication of the EGFR signaling pathway in the process.

EXPERIMENTAL

Animals

Thirty male Sprague-Dawley (SD) rats were obtained from Shatianqin Biotechnology Co. Ltd. (production batch no. scxk (Xiang) 21-170029). The rats weighed 120 ± 20 g and were aged 4 weeks. They were housed in a diurnal rhythm environment. The SD rats were fed complete, high-quality and safe diet, and were provided filtered water ad libitum. The rats were housed in a clean, dry, temperature-controlled room maintained at a temperature of 22 ± 3 °C and a relative humidity of 40 to 70 %. The rats were allowed to acclimatize to the environment for at least three days before the start of experiments. This research received approval from the Animal Ethics Authority of Baoji Hospital of TCM in line with NIH directives (approval no. BJHTCM2022003) [12], and complied with international guidelines for animal studies.

Main reagents

The major reagents used, and their sources were: Gefitinib (Beijing Kairuiji Biotechnology Co. Ltd.); methylcellulose (Nanjing Daosifu Biotechnology Co. Ltd.); ELISA Kit (Shanghai Jingkang Bioengineering Co. Ltd.), and reverse transcription Kit (Beijing Biolabs Technology Co. Ltd.).

Surgical procedures and treatments

The rats were anesthetized with intraperitoneal injection of sodium pentobarbital at a dose of 30 - 40 mg/kg [13]. Then, the left lower limb was shaved and disinfected. A longitudinal incision was made on the tibial side under aseptic conditions, and the skin and muscles were carefully layered open to expose the tibia. A 3-mm wide strip of periosteum was removed using a surgical blade at the junction of the middle and lower third of the tibia, in order to expose the subperiosteal bone. A bone clamp was used to create a fracture. A Kirschner wire with a diameter of 0.8 mm was inserted through the knee joint and drilled into the distal end of the fracture under direct vision. Then, the fracture ends were reduced. The wire was firmly secured against the proximal end of the knee joint, and the muscle and skin layers were closed without the need for any fixation. The movements and appearance of the rat were observed to confirm whether there was a fracture. A preliminary diagnosis of a long bone fracture in the rat was made if it exhibited a clear pain response, reduced mobility, and a swollen appearance.

Thereafter, the 30 rats were randomly divided into control and study groups, with 15 rats in each group. The rats in study group were treated with gefitinib dissolved in 0.5 % methylcellulose (100 mg/kg daily) via gavage, while control rats received 0.55 % methylcellulose, once a day orally, for 7, 14, 21, 28 and 42 days. The rate of fracture healing and maximum callus diameter of rats in each group were measured using X-rays on days 7, 14, 21, 28 and 42 following treatments [13].

The rats in each group were sacrificed via cervical dislocation on the 7th (15 rats), 14th (15 rats), 21st (15 rats), 28th (15 rats) and 42nd (15 rats) days. Then, iodine was used to disinfect the rat's abdominal hair. Longitudinal incisions were made on the abdominal region of each rat, and using syringes, blood samples were taken in pre-prepared sterile centrifuge tubes. The blood samples were centrifuged to obtain sera. Finally, the sera were put into different centrifuge tubes and stored. Bone alkaline phosphatase (BALP), PINP, TRACP-5b and crosslinked C-telopeptide of type I collagen (CTX) were assayed using ELISA. The rats were sacrificed painlessly under anesthesia [13]. The rats in each group were

sacrificed on days 7, 14, 21 and 28. The mRNA expression levels of osteocalcin (OC), COLa1, COL2a1 and COL10 were determined using real-time fluorescence quantitative PCR method. Reverse transcription was carried out to synthesize cDNA according to the instructions of the reverse transcription kit. Then, qPCR was carried out using 2 × SYBR Green PCR Master Mix. The cDNA was used as the template, with primer concentration of 0.4 μmol/L, and a 15 μL system was used for amplification. Each test sample was set up with 3 parallel samples. The corresponding upstream and downstream primers for OC, COLa1, COL2a1 and COL10 were designed for PCR amplification. The reaction conditions were: 95 ℃10 min, (95 ℃ for 15 sec, 60 ℃ for 30 sec, and 72 ℃ for 30 sec) in 40 cycles. The internal standard gene was GAPDH. The relative mRNA expression levels were calculated using the 2-ΔΔCt procedure. Each assay was done in triplicate.

Statistical analysis

Data on maximum callus diameter and serum levels of bone turnover markers are presented as mean ± standard deviation (SD) and t-test was used to compare both groups. The SPSS19.0 was used for all data analyses. Values of $p < 0.05$ were taken as significant.

RESULTS

Effect of gefitinib on fracture healing

After 7 days of treatment, fracture line was visible in both groups, and callus was produced. On the 14th day, the fracture line was indistinct in rats in study group, while it was visible in control rats. On day 21, the fracture line of study group had almost disappeared, while the fracture line of control group was clear. On the 28th day, the rats in study group were in the plastic stage, while the fracture location density in control group was reduced. At 42 days, the rats in study and control groups were similar.

Effect of gefitinib on maximum callus diameter

After 7, 14, 21 and 28 days of treatment, values of maximum callus diameter in gefitinib group were significantly higher than in control, but the maximum callus diameter did not differ between the two groups after 42 days. These results are shown in Table 1.

Table 1: Effect of gefitinib on maximum callus diameter (n = 15)

Group	Time (days)	Maximum callus diameter (mm)
Study	7	5.22±0.94[a]
	14	7.18±0.78[a]
	21	6.38±1.01[a]
	28	5.80±0.72[a]
	42	4.43±0.23
Control	7	4.58±0.77
	14	6.37±1.25
	21	5.59±1.00
	28	5.16±0.77
	42	4.47±0.19

Note: aP < 0.05, vs. control rats at the same time point

Effect of gefitinib on serum bone turnover markers

At days 7, 14 and 21 following treatment gefitinib significantly raised BALP and PINP levels. However, on days 28 and 42, BALP and PINP levels in both groups were comparable. At day 7, TRACP-5b and CTX values in study group were significantly higher than the control values, but BALP and PINP levels were similar in the two groups at other time points. These data are shown in Table 2.

Table 2: Effect of gefitinib on serum bone turnover markers

Group	Time (days)	BALP (ng/mL)	PINP (ng/mL)	TRACP-5b (ng/mL)	CTX (ng/mL)
Study	7	2.24±0.29[a]	2189.23±105.89[a]	11.88±0.29[a]	8.01±0.53[a]
	14	2.55±0.20[a]	2600.78±198.25[a]	10.99±0.28	6.53±0.52
	21	3.60±0.30[a]	3358.46±356.45[a]	7.56±0.19	5.02±0.50
	28	3.43±0.24	2986.54±356.78	7.99±0.20	4.59±0.49
	42	3.01±0.11	2602.69±315.89	7.52±0.21	4.55±0.48
Control	7	1.88±0.12	2000.45±111.45	10.55±0.25	7.05±0.49
	14	2.01±0.11	2236.51±156.45	9.03±0.19	6.52±0.54
	21	2.73±0.23	2500.12±299.91	8.14±0.21	5.26±0.49
	28	3.25±0.31	2899.99±345.46	7.95±0.20	4.55±0.47
	42	3.00±0.09	2563.43±311.25	7.52±0.23	4.56±0.47

[a]P < 0.5

Table 3: Effect of gefitinib on mRNA expressions of bone turnover markers in callus

Group	Time (days)	COL1A1	COL10	COL2A1	OC
Study	7	1.25±0.14	1.25±0.13	1.27±0.21	1.30±0.25
	14	1.46 ±0.18	1.49 ±0.20	1.49 ±0.22	1.52± 0.26
	21	1.63±0.25	1.78±0.21	1.78±0.21	1.76±0.25
	28	1.64±0.26	1.80±0.20	1.89±0.30	1.92±0.30
Control	7	1.12±0.10	1.11±0.09	1.13±0.12	1.12±0.13
	14	1.32±0.08	1.23±0.11	1.26±0.14	1.19±0.20
	21	1.40±0.20	1.46±0.15	1.45±0.15	1.45±0.21
	28	1.51±0.20	1.52±0.16	1.60±0.20	1.50± 0.22

Effect of gefitinib on mRNA expression levels of bone turnover markers in callus

On days 7 and 14 following treatment, there were higher relative amounts of COLa1 mRNA and OC mRNA in gefitinib group. Again, on the 7th, 14th and 21st days, the COL10 mRNA expression in rats in study group was also significantly higher than that in control group ($p < 0.05$). Moreover, on days 14 and 21, COL2A1 mRNA expression was significantly up-regulated in the gefitinib group. However, mRNA expression levels of COLa1, OC, COL10 and COL2A1 were similar in both groups at other time points not indicated above. These results are presented in Table 3.

DISCUSSION

Monoclonal antibodies and tyrosinase inhibitors are epidermal growth factor receptor (EGFR) inhibitors which have been widely used in the clinical treatment of tumors. Gefitinib, a tyrosine kinase inhibitor, blocks the EGFR signal pathway which has been implicated in bone fracture healing [11].

Secondary healing is the most common type of clinical fracture healing at present, and its main feature is callus formation. Thus, callus plays an important role in fracture healing such that the better the formation of callus, the faster the healing process [11]. Clinical research has indicated the crucial function of EGFR in promoting osteoblast proliferation and hindering differentiation, which ultimately inhibits bone fracture healing [13]. Therefore, in this study, gefitinib was used to inhibit this signal pathway to ascertain its role in antagonizing the negative effect that EGFR has on bone fracture healing and X-ray technique was used to assess the healing and maximum diameter of callus in rats with long bone fracture. The results showed that, initially, the fracture line was clear in both groups, and callus was produced after 7 days of treatment. On the 14th day, the fracture line was indistinct in study group, while it was visible in control rats. On day 21, the fracture line of study group had almost disappeared, while that of control group was visibly clear. On the 28th day, rats in study group were in the plastic stage, while the fracture location density in control group had reduced. At 42 days, the rats in the study and control groups were about the same. At 7, 14, 21 and 28 days, the values of maximum callus diameter were significantly raised in the gefitinib group. These results indicate that gefitinib effectively enhanced bone healing in rats as typified by the higher callus diameter produced by it.

Clinical studies have shown that many members of the EGRF signal pathway such as TGF-β, Insulin-like Growth Factor and Bone Morphogenetic Proteins, are expressed in skeletal cells, and they also affect growth and development of the body. Bone turnover markers reflect the activity of osteoblasts and the state of bone formation. Indeed, bone alkaline phosphatase (BALP) is currently used as a key marker for evaluation of bone formation, and it is also an early marker of osteoblast differentiation [14]. In contrast, cross-linked C-telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase 5b (TRACP-5p) are used as markers of bone resorption, and TRACP-5p reflects the number of osteoclasts. Changes in serum levels of PINP reflect the capacity of bone cells to synthesize bone collagen to a certain extent. The function of CoL1A1 is similar to that of BLAP, with respect to early bone formation. Clinical studies have shown that regulating CoL1A1 expression up-regulates type I collagen levels, which is beneficial to bone fracture healing in rats [15].

One of the key markers of osteoblast differentiation is osteocalcin (OC), and its sensitivity is strong. The degradation of COL2A1 initiates cartilage damage and osteoarthritis, and inflammatory mediators in cells have been shown to promote COL2AI degradation. The expression

of COL10 peaks at the end of cell differentiation which reflects hypertrophic chondrocyte maturation [16]. The results of this study showed significantly higher BALP and PINP levels in study group than in control rats at days 7, 14 and 21 following treatments. At day 7, TRACP-5b and CTX levels were significantly higher in study group than in control rats. On days 7 and 14, mRNA expression amounts of COLa1 and OC in study group were significantly higher than those in control group. On days 7, 14 and 21, the COL10 mRNA level was raised in gefitinib group, and on days 14 and 21, COL2A1 mRNA level was also significantly higher in study group. These data indicate that gefitinib significantly promoted the differentiation and maturation of chondrocytes and osteoblasts, which has significant effect on bone healing.

CONCLUSION

Gefitinib enhances fracture healing and callus formation of long bone in rats, probably through inhibition of EGFR signal pathway. Thus, gefitinib is beneficial in bone formation. Future studies will involve cytological experiments to further validate these findings.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aofei Shen designed the study, supervised the data collection, and analyzed the data. Mengwei Wang and Xiangheng Li interpreted the data and prepared the manuscript for publication. Mengwei Wang and Xiangheng supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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