

Impact of Gefitinib on Traumatic Fracture Healing in Rats via the OPG/RANKL/RANK Signaling Pathway

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ABSTRACT

This study investigates how gefitinib (Gef), an epidermal growth factor receptor (EGFR) inhibitor, affects traumatic fracture healing in rats, particularly through the OPG/RANKL/RANK signaling pathway, which regulates osteoclast and osteoblast activity. Sixty Sprague-Dawley rats were divided into different treatment groups, including fracture only, fracture plus gefitinib, fracture plus functional OPG antagonist (i.e., recombinant mouse RANKL protein), and combinations of both. After 28 days of treatment, bone healing was evaluated using techniques such as micro-CT, histological staining, and molecular analysis. Additionally, *in vitro* experiments on bone marrow mesenchymal stem cells (BMSCs) were conducted. The results showed that rats treated with gefitinib had significantly higher bone density and better bone structure compared to the fracture-only group. The effects were diminished when the functional OPG antagonist was added. Histological analysis revealed that gefitinib promoted better callus formation and more organized trabecular bone, effects that were reversed by the functional OPG antagonist. Further analysis indicated that gefitinib inhibited osteoclast activity and enhanced osteoblast function. At the molecular level, gefitinib increased the expression of OPG and bone-related proteins while reducing the levels of RANKL and osteoclast markers. In contrast, the functional OPG antagonist had the opposite effect. *In vitro*, gefitinib increased osteogenic activity and gene expression in BMSCs, but this was blocked by RANKL. The findings suggest that gefitinib promotes fracture healing by upregulating OPG and suppressing RANKL/RANK signaling, reducing inflammation, and this effect can be completely negated by exogenous RANKL.

Keywords: Gef, fracture healing, OPG/RANKL/RANK pathway, rat model, inflammatory factors.

INTRODUCTION

Traumatic fractures, commonly caused by traffic accidents, falls, or high-altitude incidents, can lead to severe functional impairment and reduced quality of life¹. Around 5-10% of fractures may experience delayed union or nonunion, complicating recovery². Fracture healing is a multi-stage process involving inflammation, chondrogenesis, angiogenesis, and bone remodeling, with the balance between osteoclasts and osteoblasts being crucial for bone repair³.

The OPG/RANKL/RANK pathway plays a key role in regulating bone metabolism⁴. RANKL binding to RANK on osteoclast precursors activates signaling pathways that promote osteoclast differentiation and survival⁵. OPG, secreted by osteoblasts, binds RANKL to inhibit osteoclast activity and reduce bone resorption^{5,6}. This pathway is dynamically regulated

during fracture healing and significantly impacts callus formation and remodeling⁷.

Recent studies highlight the involvement of the epidermal growth factor receptor (EGFR) pathway in both tumorigenesis and bone metabolism⁸. Gefitinib (Gef), an EGFR inhibitor, is used to treat non-small cell lung cancer and has been linked to bone sclerosis in EGFR-mutant lung cancer patients⁹. Animal studies suggest that Gef enhances callus formation and promotes bone repair by inhibiting the EGFR signaling pathway¹⁰. Additionally, a connection exists between the EGFR and RANKL/RANK pathways, with high EGFR expression in tumor tissues correlating with increased RANKL levels¹¹. Despite the above findings, to date, only one study has reported that Gef (100 mg/kg) increases callus volume and the expression of osteogenesis-related genes in a rat model of long bone fracture, but its

molecular mechanism was not explored¹². Therefore, whether Gef promotes traumatic fracture healing through the OPG/RANKL/RANK pathway remains to be established.

In summary, this study aimed to: (1) evaluate the effect of Gef on femoral fracture healing in rats; (2) determine whether these effects are mediated by the OPG/RANKL/RANK signaling axis; and (3) verify the direct effect of Gef on the osteogenic differentiation of BMSCs in vitro. By combining in vivo experiments with functional OPG antagonist intervention and in vitro mechanistic validation, we provide the first evidence of a mechanism by which Gef modulates the OPG/RANKL/RANK pathway in traumatic fracture repair. The findings of this study may provide a theoretical basis for the potential application of gefitinib in promoting fracture healing, particularly in patients with delayed union.

MATERIALS AND METHODS

Experimental animals

Sixty healthy male specific pathogen-free (SPF)-grade Sprague-Dawley (SD) rats, aged 8 weeks and weighing 250 ± 20 g, were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Production License No.: SCXK (Jing) 2021-0010). All rats were housed in the SPF-grade animal facility of the Peking University Laboratory Animal Center, with ambient temperature maintained at 22-25°C, relative humidity at 50%-60%, and a 12-hour light/dark cycle. The rats had ad libitum access to a standard laboratory diet and drinking water. All animal experimental procedures were strictly conducted in accordance with the Regulations on the Management of Laboratory Animals of the People's Republic of China and national guidelines for laboratory animal welfare ethics, and were reviewed and approved by the First Affiliated Hospital of Wannan Medical University. All experiments were conducted in accordance with relevant guidelines and regulations. (Ethical number:2024-231). Before experimentation, the rats were acclimatized for seven days to ensure health status and experimental reproducibility. Among them, 50 rats were used for in vivo experiments and 10 for in vitro experiments.

Sample Size Calculation Description: The sample size for this study was determined based on a priori power analysis for the primary outcome measure—bone volume fraction (BV/TV) on postoperative day 28¹³. Referring to the preliminary experimental data from our research group ($n=3$ per group),

the mean BV/TV in the fracture model group was approximately $14.8\% \pm 2.9\%$. Based on the effect size criteria from previous studies on fracture healing in rats¹⁴, Gef intervention was expected to increase BV/TV by approximately 30%. With a two-sided $\alpha = 0.05$ and power $(1-\beta) = 0.80$, the minimum required sample size per group was calculated to be 4 rats using G*Power software (version 3.1, Heinrich Heine University Düsseldorf, Germany). Considering that half of the animals needed to be sacrificed at each time point, as well as potential risks of exclusion due to intraoperative complications, infection, dislocation, etc., the final sample size was set at 10 rats per group.

Fracture model establishment

A standardized femoral midshaft transverse fracture model was established in rats following the method of Kaur et al. (2020)¹⁵. After 12 h of preoperative fasting, anesthesia was induced via intraperitoneal injection of 2% pentobarbital sodium (Sigma-Aldrich, USA) at 40 mg/kg. The right hind limb was shaved, disinfected with iodine, and draped sterilely. A 1.5 cm longitudinal incision was made along the femoral axis on the right lateral thigh. The vastus lateralis muscle was bluntly dissected to expose the midshaft of the right femur. Subsequently, a standardized transverse fracture was created at the femoral midshaft using a portable electric micro-oscillating saw (Nouvag AG, Switzerland; blade thickness: 0.5 mm), with continuous irrigation of sterile saline during the procedure to prevent thermal injury. A 1.2 mm stainless steel intramedullary pin (Fine Science Tools, Germany) was inserted through the knee joint to stabilize the fracture fragments and restore longitudinal stability. After confirming satisfactory fracture alignment, layered closure was performed using 4-0 absorbable sutures (Ethicon, USA). Postoperatively, rats were housed individually and administered penicillin sodium (40,000 U/d, North China Pharmaceutical Group, China) intramuscularly for 24 h to prevent infection. Rats were monitored daily for complications (e.g., infection, severe displacement) before inclusion in subsequent studies.

Grouping and drug intervention

Fifty healthy male SD rats were grouped at random ($n=10$ per group). The random allocation sequence was generated by an independent statistician using Excel software and was sealed in sequentially numbered opaque envelopes. The envelopes were opened to reveal the group assignments only after all rats had undergone surgery and baseline assessments, ensuring

allocation concealment. Researchers responsible for the surgical procedures and subsequent outcome assessments remained blinded to group allocation throughout the entire experiment until the final data analysis was completed.

Sham Group: rats were anesthetized, and a 1.5 cm incision was made on the right thigh followed by immediate suturing without femoral exposure or intramedullary nail fixation. Daily gavage with an equivalent volume of saline was administered. This procedure aimed to simulate surgical trauma and stress while excluding the potential influence of intramedullary nail implantation on experimental outcomes.

Fracture Group (Fx Group): standardized femoral midshaft transverse fracture model of the right femur with daily gavage of saline (0.9% NaCl, 1 mL/100 g).

Fracture + Gef Group (Fx + Gef Group): Fracture model with daily gavage of Gef (20 mg/kg/d).

Fracture + functional OPG antagonist Group (Fx + OPG-inh Group): Fracture model with intraperitoneal injection of functional OPG antagonist (recombinant mouse RANKL protein, 2 mg/kg, every other day).

Fracture + Gef + functional OPG antagonist Group (Fx + Gef + OPG-inh Group): Based on the fracture model, recombinant mouse RANKL protein was administered via intraperitoneal injection (2 mg/kg every other day). This recombinant RANKL competitively binds to the RANK receptor and functionally antagonizes the effect of endogenous OPG; therefore, it is subsequently referred to as a functional OPG antagonist (based on its functional effect, rather than directly inhibiting the OPG protein).

All interventions lasted 28 days. Gef (Sigma-Aldrich, catalog no. I4409) was administered via gavage as a suspension in 0.5% CMC-Na. The functional OPG antagonist (recombinant mouse RANKL, PeproTech, catalog no. 315-11) was dissolved in sterile saline for intraperitoneal injection.

Blinding Design: During the experimental phases, including animal housing, surgical modeling, drug intervention, sample collection, Micro-CT scanning, histology section staining, and data analysis, the personnel performing the specific operations were unaware of the group allocations. The group codes were only unblinded by an independent statistician during the final statistical analysis stage. Specimen numbers were assigned using a unified numerical coding system that contained no group information, thereby ensuring objectivity in outcome assessment.

Pre-specification and definition of outcome measures

This study pre-specified the following outcome measures in accordance with the ARRIVE 2.0 guidelines¹⁶.

Primary outcome measure: Bone volume fraction (BV/TV) measured by Micro-CT on postoperative day 28. BV/TV is a core indicator for quantifying trabecular bone structural integrity and bone healing progression, and is widely adopted as the primary efficacy endpoint in fracture healing studies in rats. BV/TV data on postoperative day 7 (the peak inflammatory phase) were used as a secondary reference to assess early callus formation.

Secondary outcome measures: (1) Micro-CT quantitative parameters: trabecular bone density (Tb. Dn), trabecular thickness (Tb.Th). (2) Histological assessments: Hematoxylin-eosin (HE) staining to evaluate callus morphology and trabecular bone arrangement; tartrate-resistant acid phosphatase (TRAP) staining to quantify osteoclast number; osteocalcin (OCN) immunohistochemistry for semi-quantitative evaluation of osteoblast activity. (3) Serum biochemical markers: Bone metabolism markers: bone gla protein (BGP), alkaline phosphatase (ALP); Inflammatory factors: inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6). (4) Molecular biological indicators: Expression levels of genes and proteins including osteoprotegerin (OPG), receptor activator of nuclear factor- κ B ligand (RANKL), receptor activator of nuclear factor- κ B (RANK), tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase (ALP), and bone morphogenetic protein 2 (BMP2), in bone tissue and bone marrow mesenchymal stem cells (BMSCs).

The detection methods for all outcome measures are detailed below. Outcome assessments were performed by researchers who remained blinded to group allocation.

Sample collection and bone tissue analysis

To comprehensively evaluate the fracture healing process, encompassing both the early inflammatory phase and the late bone remodeling phase, sample collection was conducted at two time points: postoperative day 7 (peak inflammation) and day 28 (callus remodeling phase). At each time point, five rats per group were randomly selected and anesthetized via intraperitoneal injection of 10% chloral hydrate (40 mg/kg). Blood (3 mL) was collected via the abdominal

aorta, and serum was isolated and cryopreserved until biochemical assays. Rats were then euthanized, and fracture-adjacent cartilage tissues were subjected to fixation in 4% paraformaldehyde (24–48 h), dehydration, paraffin embedding, and sectioning were carried out. The fractured right femur was subjected to scanning, adopting micro-CT (Skyscan 1176, Bruker) for 3D reconstruction and quantitative analysis. Trabecular bone density (Tb.Dn), trabecular thickness (Tb.Th), and bone volume fraction (BV/TV) were measured.

Histological observation

The prepared right femur sections from the fracture site were deparaffinized and rehydrated. HE staining was performed by staining with hematoxylin (5–8 min), followed by rinsing, differentiation in 1% hydrochloric acid alcohol, bluing, and counterstaining with 0.5% eosin (2–3 min). After dehydration and clearing, the sections were mounted. An optical microscope (Olympus BX53, Japan) was adopted for observation at magnifications of $\times 100$ and $\times 400$. Images were captured and saved using an image analysis system.

Tartrate-resistant acid phosphatase (TRAP) staining

To evaluate osteoclast activity, bone tissue sections were subjected to TRAP staining. After deparaffinization and rehydration, the sections were processed strictly according to the instructions of the TRAP staining kit (Sigma-Aldrich, USA, Cat. No. 387A). The sections were incubated in a staining solution containing tartrate (37°C, protected from light) for 60 minutes. After rinsing with distilled water, the nuclei were counterstained with hematoxylin for 2 minutes. Following another rinse, the sections were dehydrated, cleared, and mounted. Under an optical microscope (Olympus BX53, Japan), multinucleated cells (≥ 3 nuclei) with red or purplish-red cytoplasm were identified as osteoclasts. Five non-overlapping high-power fields ($\times 400$) were randomly selected per section, and TRAP-positive multinucleated osteoclasts were counted using Image-Pro Plus 6.0.

Osteocalcin (OCN) immunohistochemical staining

To assess osteoblast activity, the expression of OCN was detected using immunohistochemistry. After deparaffinization and rehydration, paraffin sections were subjected to antigen retrieval in citrate repair buffer (pH 6.0). Subsequently, endogenous

peroxidase activity was quenched by incubation with 3% H_2O_2 solution, and nonspecific binding sites were blocked with 5% bovine serum albumin (BSA). The sections were incubated overnight at 4°C with rabbit anti-rat OCN primary antibody (Cat. No.: ab93876, Abcam, UK; dilution ratio: 1:200). After PBS rinsing, HRP-labeled goat anti-rabbit IgG secondary antibody (Cat. No.: ZB-2301, ZSGB-BIO, China; dilution ratio: 1:1000) was applied and incubated at room temperature for 1 hour. Color development was performed using a DAB substrate kit (ZSGB-BIO, China), followed by counterstaining of nuclei with hematoxylin. Finally, the sections were dehydrated, cleared, and mounted. Under an optical microscope, OCN positivity was identified as brownish-yellow or brown granular deposits in the cytoplasm. Five high-power fields ($\times 400$) were randomly selected per section, and the mean optical density value (IOD/Area) was calculated using ImageJ for semi-quantitative analysis of OCN expression levels.

ELISA

Serum samples were analyzed using commercial ELISA kits (Abcam, UK) for BGP, ALP, iNOS, TNF- α , and IL-6. Assays were conducted based on manufacturer instructions, and the OD value at 450 nm was subjected to measurement using a microplate reader (BioTek Synergy H1, USA). Triplicate wells were adopted for each sample, and concentrations were calculated via standard curves.

qRT-PCR

Bone tissue (callus and new bone regions) was homogenized in liquid nitrogen. Extraction of total RNA was carried out adopting Trizol (Invitrogen, USA), and purity/concentration were determined via NanoDrop 2000 (Thermo Fisher Scientific, USA). The A260/A280 ratio was maintained between 1.8 and 2.0. Reverse transcription was performed using the PrimeScript™ RT reagent kit (Takara, Japan) to obtain cDNA. Subsequently, amplification was carried out on an ABI 7500 qRT-PCR instrument (Applied Biosystems, USA) with SYBR® Premix Ex Taq™ II (Takara, Japan) as the reaction system. Triplicate wells were adopted per sample. Target genes included OPG, RANKL, RANK, TRAP, Alkaline phosphatase (ALP), and bone morphogenetic protein 2 (BMP2), with GAPDH as the reference. All primers were synthesized by Sangon Biotech (Shanghai, China). The primer sequences for each gene are summarized in Table I. Relative expression (RE) was computed adopting the $2^{-\Delta\Delta Ct}$ approach. Primer sequences were as follows.

Table I. — qRT PCR primer sequence (5' → 3').

Gene	Forward	Reverse
OPG	AGAGTGGAGATGAACGAGAG	CCGTGGTGAGGTAGTCTTGA
RANKL	TGCTTTGGGCCAAGATCTAA	GGCTGTTGGCACTTGAGATA
RANK	CGAGGTCATGTTTGAGTGGA	TTGAGGCAGAGTCACAGTGA
TRAP	CAGACCGACATGGAAAGAGC	GCTGGTAGGAGGTTGAGGAG
ALP	GGACCTGCTGACTACCAAGG	TCCACGAGTCACTTCCACAG
BMP2	TGGAGGAGTGCAGAGACGAC	CCTCAGTGGCTGGAGTGTAG
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Western blot (WB)

Fracture site tissues were minced and homogenized in RIPA lysis buffer (Beyotime, China) with PMSF protease inhibitor (Beyotime, China). After centrifugation (4°C), protein quantification was achieved by adopting a BCA kit (Thermo Fisher Scientific, USA). Equal protein aliquots (30 µg) were processed by SDS-PAGE (10% gel), and PVDF membranes (Millipore, USA) were adopted (overnight, 4°C). The following first antibodies (Ab) were applied: OPG (Cat. No.: ab73400, 1:1000, Abcam, UK), RANKL (Cat. No.: ab45039, 1:1000, Abcam, UK), RANK (Cat. No.: sc-52953, 1:500, Santa Cruz, USA), TRAP (Cat. No.: ab185716, 1:1000, Abcam, UK), ALP (Cat. No.: ab108337, 1:1000, Abcam, UK), BMP2 (Cat. No.: ab14933, 1:500, Abcam, UK), β -actin (Cat. No.: 4970S, 1:3000, Cell Signaling Technology, USA). HRP-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG, Cat. No.: ZB-2301, 1:5000, ZSGB-BIO, China) was applied (1 h, 25°C). ECL substrate (Thermo Fisher Scientific, USA) and ChemiDoc™ MP imaging system (Bio-Rad, USA) were employed. Band intensities were recorded by ImageJ software (NIH, USA), with β -actin as the control.

In vitro experiment: effect of Gef on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs)

To determine whether Gef directly acts on osteoblast precursor cells and regulates their function, rat primary BMSCs were isolated and cultured for in vitro osteogenic induction experiments to observe the direct effects of Gef on BMSC osteogenic differentiation.

I. Isolation and culture of BMSCs

Ten healthy male SD rats were euthanized by cervical dislocation and immersed in 75% ethanol for 5 minutes. Under aseptic conditions, the femurs and tibiae were harvested, and both epiphyses were excised. The bone marrow cavity was flushed with

α -MEM complete medium (HyClone, USA, Cat. No. SH30265.01) supplemented with fetal bovine serum (ExCell Bio, China, Cat. No. FSP500) and penicillin/streptomycin (Beyotime Biotechnology, China, Cat. No. C0222) to collect the bone marrow suspension. The suspension was centrifuged at 1000 rpm for 5 minutes, and the pellet was resuspended and seeded into culture dishes. Cells were cultured at 37°C in a 5% CO₂ incubator. The medium was first changed after 72 hours to remove non-adherent cells, and subsequently refreshed every 3 days. When cells reached 80%–90% confluence, they were passaged using 0.25% trypsin. Third-passage cells were used for subsequent experiments.

II. Experimental grouping and drug treatment

BMSCs were randomly divided into four groups: Control group: conventional osteogenic induction medium (OIM: α -MEM + 10% FBS + 50 µg/mL ascorbic acid + 10 mmol/L β -glycerophosphate + 100 nmol/L dexamethasone);

Gef-L group: OIM + 1 µmol/L Gef;

Gef-H group: OIM + 10 µmol/L Gef;

Gef-H + RANKL group: OIM + 10 µmol/L Gef + 50 ng/mL recombinant RANKL protein.

The drug-containing medium was replaced every 3 days, and the cells were cultured for 14 days.

III. ALP activity assay

On day 7 of culture, the medium was discarded, and cells were washed twice with PBS. Cells were lysed with 0.1% Triton X-100, and protein concentration was determined using the BCA method. ALP activity was measured strictly according to the instructions of the ALP assay kit (Beyotime, China). ALP activity was expressed as the amount of p-nitrophenol (pNP) catalyzed by ALP per milligram of protein (U/mg prot).

IV. Alizarin red S staining

On day 14 of culture, the medium was discarded, and cells were washed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 minutes. Subsequently, 1% Alizarin Red S staining solution (Solarbio, China, Cat. No. G8550) (pH 4.2) was added and incubated at room temperature for 20 minutes. Cells were gently rinsed with distilled water until no unbound dye remained. Calcium nodule formation was observed under a microscope. Stained products were extracted with 10% cetylpyridinium chloride (CPC) solution, and absorbance was measured at 562 nm for semi-quantitative analysis of calcium deposition.

V. qRT-PCR analysis of osteogenic-related gene expression

On day 7 of culture, total RNA was extracted from each group of cells and reverse-transcribed into cDNA. qRT-PCR was performed to detect the mRNA expression levels of osteogenic marker genes, including ALP, OCN, Runt-related transcription factor 2 (Runx2), as well as OPG and RANKL. GAPDH was used as the internal reference, and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical processing

SPSS 26.0 was employed and data were represented as mean $\pm$ SD ($\bar{x} \pm SD$). Normality and homogeneity of variance were tested. One-way ANOVA was performed for contrasts among three or more groups when conditions were met. If ANOVA results were significant, the LSD method was adopted for pairwise contrasts. For data violating normality or homogeneity of variance, the Kruskal-Wallis test was employed instead. All tests were two-tailed, with $P < 0.05$ considered statistically significant.

RESULTS

Establishment of rat fracture model and general observations

All 50 male SD rats successfully underwent fracture modeling or sham surgery. No surgery-related deaths occurred within 24 h postoperatively. Wounds healed well without infection or severe displacement. Postoperative activity was restricted in the Fx Group and drug-treated groups, with slight weight loss (no visible distinctions, all $P > 0.05$) (Table II). The stable fracture model and good animal condition supported subsequent experiments.

Trabecular bone parameters (Tb.Dn, Tb.Th)

On postoperative day 7, compared with the Sham Group, the Fx Group showed significant decreases in Tb.Dn, Tb.Th, and BV/TV. Compared with the Fx Group, all parameters were significantly increased in the Fx + Gef Group, while the Fx + OPG-inh Group exhibited further decreases (all $P < 0.05$). The Fx + Gef + OPG-inh Group showed values similar to the Fx Group ($P > 0.05$), indicating that the pro-repair effect of Gef was blocked by the functional OPG antagonist. On postoperative day 28, the Fx Group still exhibited low trabecular parameters, while the Fx + Gef Group demonstrated significant increases in Tb. Dn, Tb.Th, and BV/TV, indicating enhanced bone remodeling activity. The Fx + OPG-inh Group showed further decreases (all $P < 0.05$), and the Fx + Gef + OPG-inh Group remained comparable to the Fx Group ($P > 0.05$) (Figure 1).

Observation of micro-CT three-dimensional reconstruction images revealed that the Sham group exhibited intact bone structure, no fracture line, and continuous cortical bone. In the Fx group, the fracture line remained clearly visible, with thin and unevenly distributed callus at the fracture ends, and sparse, disorganized trabeculae. The Fx + Gef group showed significantly thickened and continuous callus, blurred or absent fracture lines, and markedly improved trabecular quantity and arrangement. The

Table II. — Preoperative and postoperative body weight and survival in rats ($n=10$, $\bar{x} \pm s$).

Grouping	Preoperative weight (g)	Weight at postoperative day 1 (g)	Weight at postoperative day 7 (g)	Survival rate (%)
Sham Group	320.5 $\pm$ 12.3	318.2 $\pm$ 11.9	325.0 $\pm$ 13.0	100
Fx Group	318.9 $\pm$ 13.0	305.4 $\pm$ 12.8	315.8 $\pm$ 12.5	100
Fx + Gef Group	321.7 $\pm$ 11.7	307.2 $\pm$ 12.4	317.4 $\pm$ 12.2	100
Fx + OPG-inh Group	319.4 $\pm$ 12.1	304.7 $\pm$ 11.5	314.5 $\pm$ 11.9	100
Fx + Gef + OPG-inh Group	320.1 $\pm$ 12.0	306.0 $\pm$ 11.7	316.7 $\pm$ 12.1	100

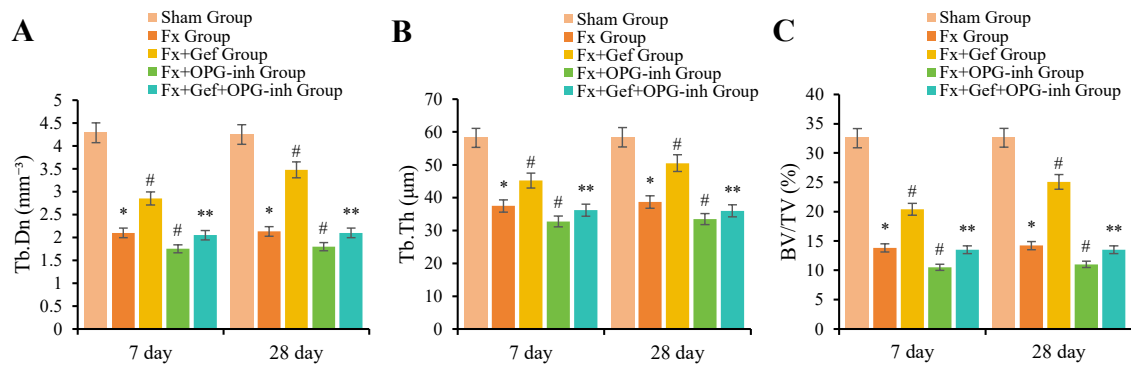


Fig. 1 — Quantitative analysis of trabecular parameters in different groups at postoperative days 7 and 28. Data are presented as mean $\pm$ standard deviation ($n = 5$ independent rats/group). (A) Trabecular density (Tb.Dn); (B) Trabecular thickness (Tb.Th); (C) Bone volume fraction (BV/TV). Note: * vs. Sham Group, # vs. Fx Group, ** vs. Fx + Gef Group, $P < 0.05$.

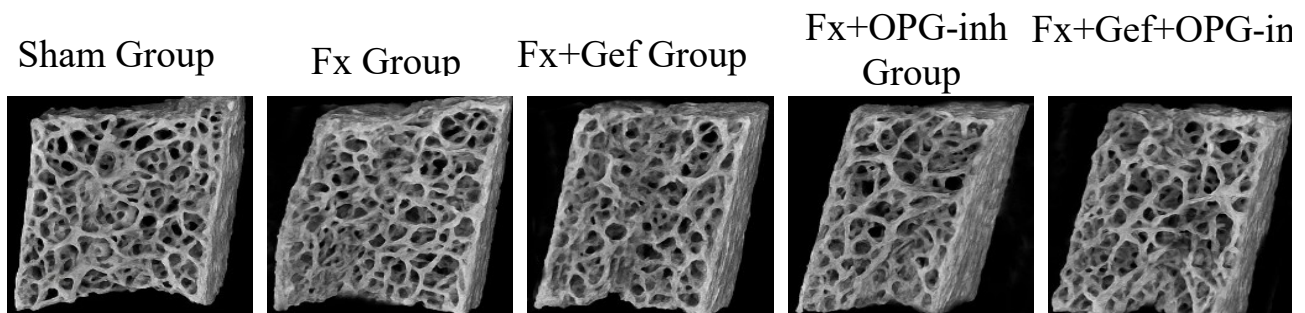


Fig. 2 — Comparative micro-CT three-dimensional reconstruction images (representative results at day 28).

Fx + OPG-inh group exhibited insufficient callus formation, persistent fracture lines, and disorganized structure. The Fx + Gef + OPG-inh group displayed thin callus and persistent fracture lines, similar to the Fx group, indicating that the functional OPG antagonist counteracted the bone repair effects of Gef (Figure 2).

Histological observations

The HE staining results showed that the Sham Group exhibited intact bone structure, dense cortical bone, and regularly arranged trabeculae at both postoperative day 7 and day 28, with no signs of fracture. In the Fx Group, on day 7, new bone formation was delayed, with thin callus, sparse and disorganized trabeculae, indicating impaired early bone repair. By day 28, the callus was slightly thickened, but trabeculae remained sparse, suggesting suboptimal overall bone repair. The Fx + Gef Group demonstrated thickened callus and improved trabecular quantity and arrangement by day 7. By day 28, the callus was significantly thickened, with regularly arranged and dense trabeculae, indicating that Gef markedly promoted bone repair. The Fx + OPG-inh Group exhibited poor callus formation, reduced trabecular number, and loose structure on day 7. By day 28, the callus remained thin with sparse trabeculae, reflecting aggravated impairment of bone repair.

The Fx + Gef + OPG-inh Group showed callus and trabecular characteristics similar to the Fx Group on day 7. By day 28, no significant improvement in callus thickness or trabecular arrangement was observed, and the outcomes were significantly inferior to those of the Fx + Gef Group, suggesting that the functional OPG antagonist reversed the promotive effects of Gef (Figure 3, representative results at day 28).

Comparison of osteoclast and osteoblast activity (TRAP and OCN staining)

TRAP staining results revealed that on postoperative day 7, the number of osteoclasts in the Fx Group was significantly higher than that in the Sham Group ($P < 0.01$), while the Fx + Gef Group showed a marked reduction ($P < 0.01$). The Fx + OPG-inh Group exhibited an increase ($P < 0.01$), and the Fx + Gef + OPG-inh Group showed no significant difference compared to the Fx Group ($P > 0.05$). By day 28, the Fx + Gef Group maintained the lowest osteoclast count, significantly lower than all other groups (all $P < 0.01$), while no significant differences were observed among the Fx Group, Fx + OPG-inh Group, and Fx + Gef + OPG-inh Group ($P > 0.05$). OCN immunohistochemistry results demonstrated that on day 7, the Fx Group exhibited lower expression ($P < 0.05$), the Fx + Gef Group

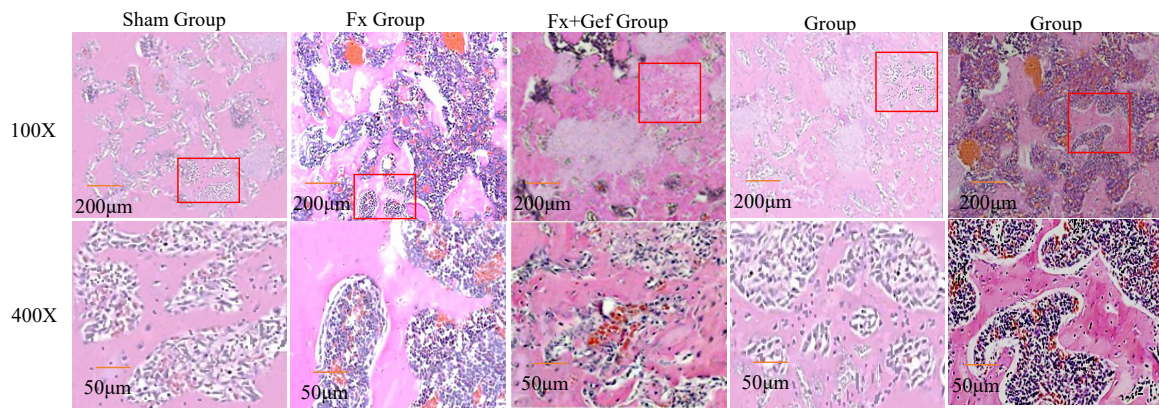


Fig. 3 — HE staining results of fractured bone tissue. (Representative images of each group on the 28th day after surgery ($n=5$ independent rats/group)).

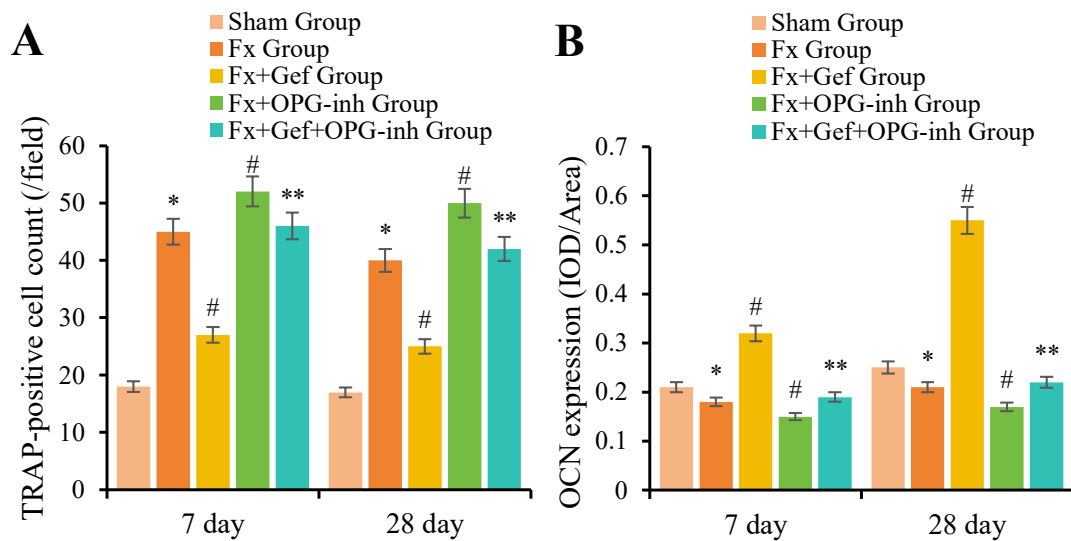


Fig. 4 — Number of TRAP-positive osteoclasts and OCN expression levels in rats of each group at different time points.

Data are presented as mean $\pm$ standard deviation ($n = 5$ independent rats/group). (A) Number of TRAP-positive multinucleated osteoclasts (per high-power field); (B) OCN expression level (integrated optical density value, IOD/Area). Note: * as against Sham Group, # as against Fx Group, ** as against Fx + Gef Group, $P < 0.05$.

showed significantly enhanced expression ($P < 0.01$), and the Fx + OPG-inh Group had the lowest expression ($P < 0.01$). The Fx + Gef + OPG-inh Group showed no difference compared to the Fx Group ($P > 0.05$). By day 28, the Fx + Gef Group displayed significantly higher OCN expression than all other groups ($P < 0.001$), while the remaining three groups remained at low levels with no significant differences among them ($P > 0.05$) (Figure 4).

Contrast of serum bone metabolism and inflammation-related indicators

On postoperative day 7, compared with the Sham Group, the Fx Group and Fx + OPG-inh Group exhibited significantly decreased serum BGP and ALP levels, along with markedly elevated iNOS, TNF- α , and IL-6 levels ($P < 0.05$). In contrast, the Fx + Gef

Group showed increased bone metabolism markers and reduced inflammatory cytokine levels ($P < 0.05$). No significant differences were observed between the Fx + Gef + OPG-inh Group and the Fx Group ($P > 0.05$). By postoperative day 28, the Fx Group and Fx + OPG-inh Group maintained elevated inflammatory cytokine levels and persistently low BGP and ALP levels ($P < 0.05$ vs. Sham Group). The Fx + Gef Group demonstrated significantly increased BGP and ALP levels alongside a continued reduction in inflammatory cytokines ($P < 0.05$ vs. Fx Group). The Fx + Gef + OPG-inh Group showed results comparable to the Fx Group ($P > 0.05$) (Figure 5).

Contrast of gene expression at the fracture site

On postoperative day 7, compared with the Sham Group, the Fx Group and Fx + OPG-inh Group

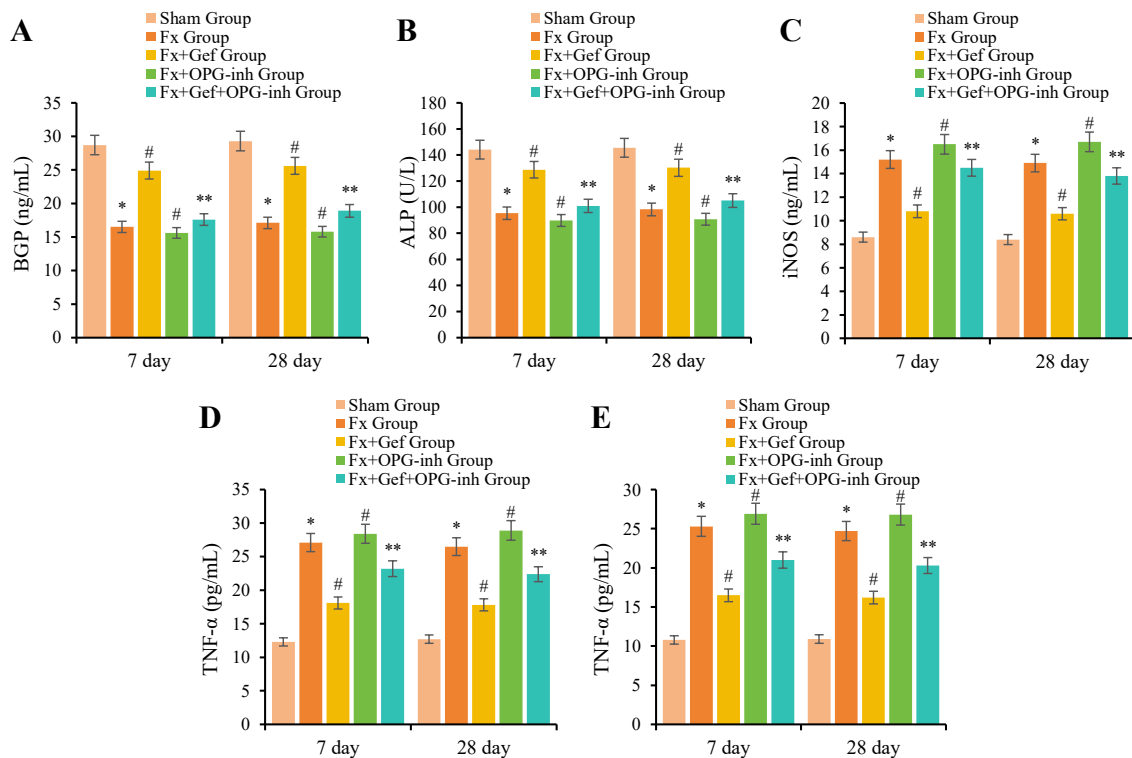


Fig. 5 — Levels of bone metabolism and inflammatory factors in rat serum.
Data are presented as mean $\pm$ standard deviation ($n = 5$ independent rats/group). (A) Bone gla protein (BGP); (B) Alkaline phosphatase (ALP); (C) Inducible nitric oxide synthase (iNOS); (D) Tumor necrosis factor- α (TNF- α); (E) Interleukin-6 (IL-6). Note: * as against Sham Group, # as against Fx Group, ** as against Fx + Gef Group, $P < 0.05$.

exhibited significantly decreased mRNA expression of OPG, ALP, and BMP2, along with increased expression of RANKL, RANK, and TRAP ($P < 0.05$). The Fx + Gef Group demonstrated markedly up-regulated mRNA expression of OPG, ALP, and BMP2, alongside reduced expression of RANKL, RANK, and TRAP ($P < 0.05$ vs. Fx Group). The Fx + Gef + OPG-inh Group showed gene expression levels comparable to those of the Fx Group ($P > 0.05$). By postoperative day 28, the Fx + Gef Group maintained significantly up-regulated mRNA expression of OPG, ALP, and BMP2, with down-regulated expression of RANKL, RANK, and TRAP ($P < 0.05$ vs. Fx Group). The Fx + OPG-inh Group continued to exhibit low expression of OPG, ALP, and BMP2, while RANKL, RANK, and TRAP remained highly expressed ($P < 0.05$ vs. Sham Group). The Fx + Gef + OPG-inh Group displayed gene expression levels similar to the Fx Group ($P > 0.05$ vs. Fx Group; $P < 0.01$ vs. Fx + Gef Group) (Figure 6).

Contrast of protein expression levels in rat bone tissue

On postoperative day 7, compared with the Sham Group, the Fx Group and Fx + OPG-inh Group exhibited significantly decreased levels of OPG, ALP,

and BMP2, along with markedly elevated levels of RANKL, RANK, and TRAP ($P < 0.05$). The Fx + Gef Group showed partial restoration of OPG, ALP, and BMP2 levels, accompanied by reduced expression of RANKL, RANK, and TRAP ($P < 0.05$ vs. Fx Group). The Fx + Gef + OPG-inh Group demonstrated protein expression profiles similar to the Fx Group, characterized by decreased OPG, ALP, and BMP2 and increased RANKL, RANK, and TRAP ($P < 0.05$ vs. Fx + Gef Group; $P > 0.05$ vs. Fx Group). By postoperative day 28, the Fx + Gef Group displayed significantly higher levels of OPG, ALP, and BMP2 compared to all other groups, alongside markedly reduced levels of RANKL, RANK, and TRAP ($P < 0.01$). In contrast, the Fx + OPG-inh Group and Fx + Gef + OPG-inh Group maintained low protein levels, comparable to those of the Fx Group ($P > 0.05$) (Figure 7).

In vitro effects of Gef on BMSCs

Gef enhances ALP activity and mineralization capacity of BMSCs

After 7 days of culture, both low-concentration (Gef-L) and high-concentration (Gef-H) Gef treatment groups exhibited significantly increased ALP activity compared to the Control group ($P < 0.05$), with the Gef-H group showing the highest activity. The

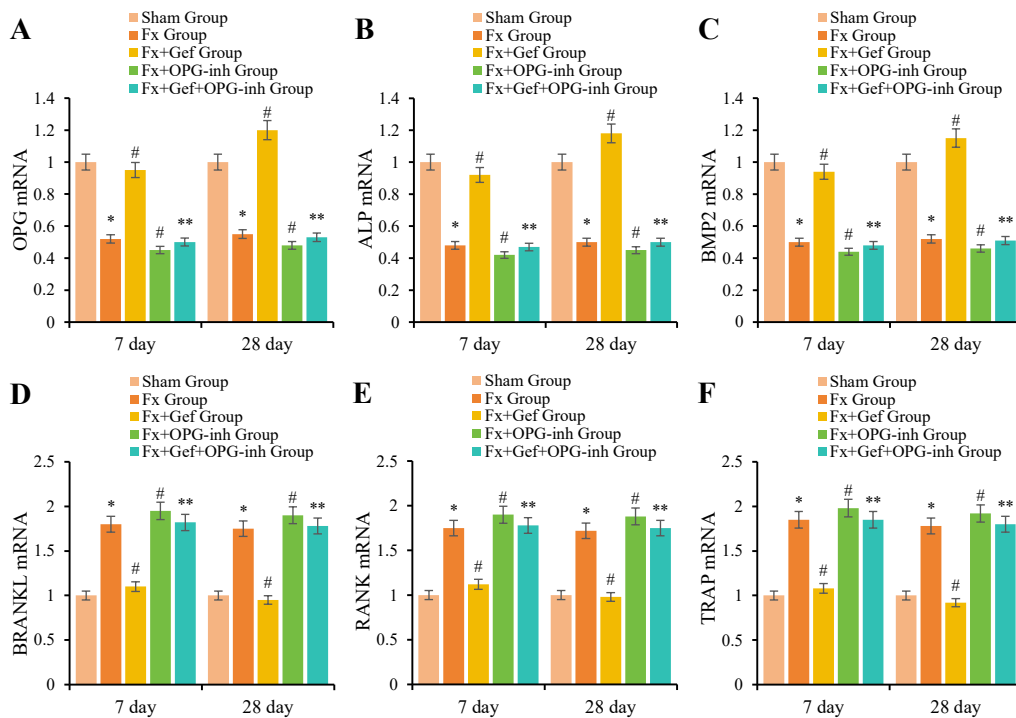


Fig. 6 — RE of OPG/RANKL/RANK mRNA in bone tissue.

Data are presented as mean ± standard deviation (n = 5 independent rats/group). (A) OPG, (B) ALP, (C) BMP2, (D) RANKL, (E) RANK, (F) TRAP. Note: * as against Sham Group, # as against Fx Group, ** as against Fx + Gef Group, $P < 0.05$.

addition of RANKL significantly suppressed the Gef-induced increase in ALP activity ($P < 0.05$, Gef-H + RANKL vs. Gef-H). After 14 days of culture, Alizarin Red S staining demonstrated that Gef treatment concentration-dependently promoted mineralized nodule formation, with the Gef-H group exhibiting the highest mineralization level. Co-treatment with RANKL markedly attenuated this effect ($\#P < 0.05$) (Figure 8).

Gef upregulates osteogenesis-related and OPG gene expression in BMSCs

qRT-PCR analysis on day 7 demonstrated that Gef treatment significantly and concentration-dependently upregulated the mRNA expression of osteogenic marker genes (ALP, OCN, Runx2) and OPG ($P < 0.05$ vs. Control). Concurrently, Gef downregulated RANKL mRNA expression, resulting in a drastic increase in the OPG/RANKL ratio ($P < 0.05$). These effects were significantly reversed by the addition of exogenous RANKL ($P < 0.05$, Gef-H + RANKL vs. Gef-H) (Figure 9).

DISCUSSION

Previous studies have demonstrated that promoting fracture healing through pharmacological modulation is feasible. A systematic review pointed out that

multiple drugs can accelerate bone repair by regulating the osteoblast-osteoclast coupling mechanism¹⁷. Jing et al. (2024) (18) further proved that enhancing osteoblast activity by downregulating miR-656-3p and upregulating BMP2 promoted fracture healing in rats. In contrast, how Gef, as an EGFR inhibitor, regulates the OPG/RANKL/RANK signaling axis during traumatic fracture healing has not been explored. Therefore, this study systematically observed the effects of Gef for the first time in a rat model, and the results showed that Gef improved Tb.Dn, Tb.Th, BV/TV, BGP, and ALP levels, which was consistent with previous findings on the promotion of fracture healing. These results supported that Gef may have an osteogenic-promoting role during fracture repair.

At the molecular level, Gef was found to upregulate OPG expression. During bone repair, osteoblasts not only synthesize bone matrix but also dynamically regulate osteoclastogenesis through the secretion of OPG/RANKL. OPG competitively binds to RANKL, blocking the differentiation signals of osteoclast precursors and thereby reducing bone resorption. Studies on bone resorption and osteoclast formation models have shown that inhibiting EGFR signaling directly downregulates RANKL expression and suppresses bone resorption pathways, thereby improving microstructure and function^{19,20}. These findings are highly consistent with the molecular

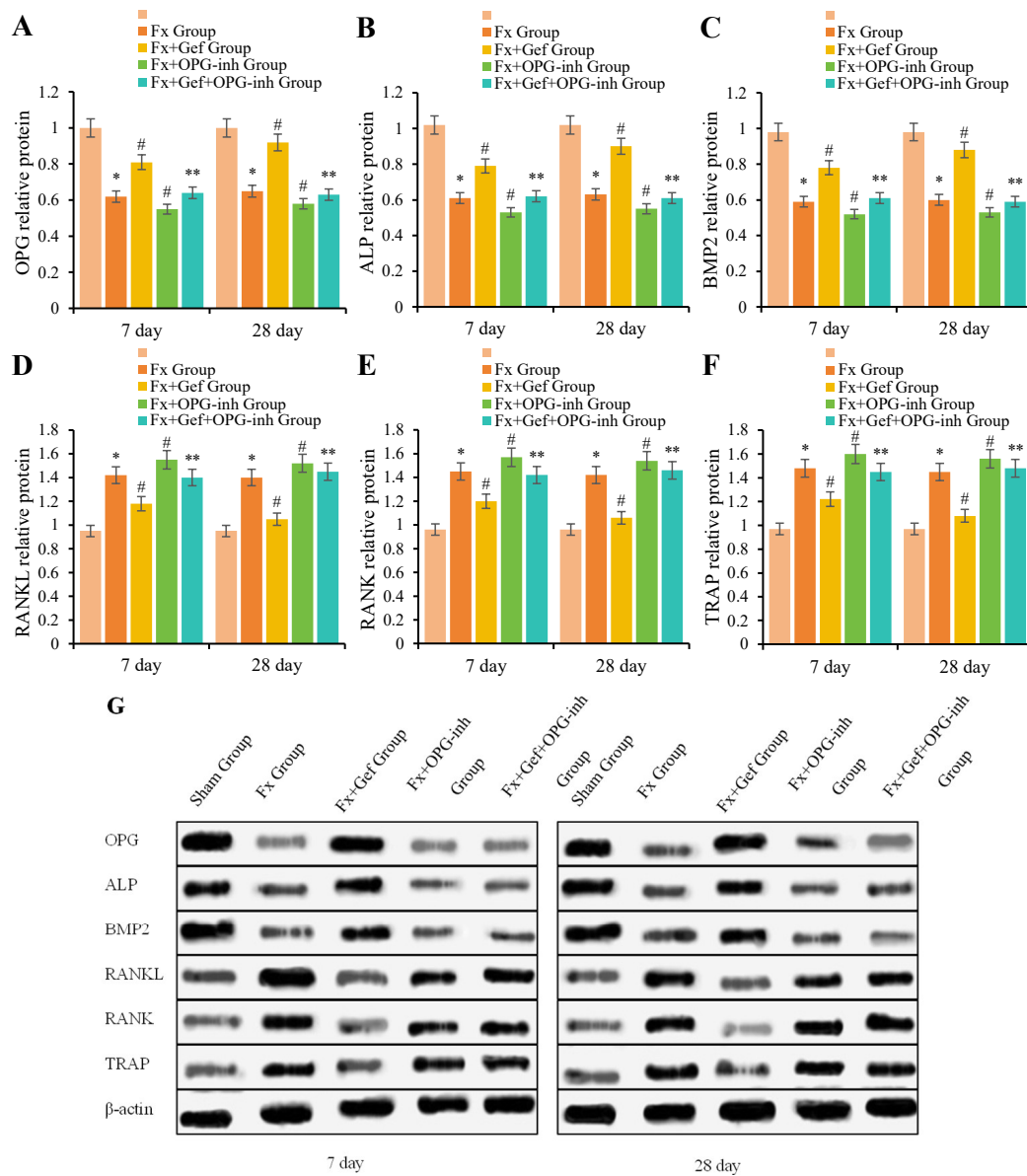


Fig. 7 — Protein expression levels of OPG/RANKL/RANK in bone tissue.
Data are presented as mean ± standard deviation (n = 5 independent rats/group). Band intensity values were quantified after normalization to β-actin. (A) OPG, (B) ALP, (C) BMP2, (D) RANKL, (E) RANK, (F) TRAP. Note: * as against Sham Group, # as against Fx Group, ** as against Fx + Gef Group, P < 0.05).

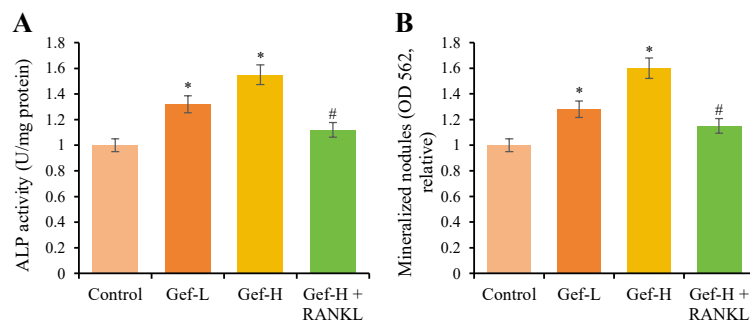


Fig. 8 — In vitro effects of Gef on ALP activity and mineralized nodule formation in BMSCs.
(A) ALP activity assay results on day 7 of culture; (B) Alizarin Red S staining and semi-quantitative analysis results on day 14 of culture. Data are presented as mean ± standard deviation (n = 5 independent cell experiments). Note: *P < 0.05 vs. Control, #P < 0.05 vs. Gef-H.

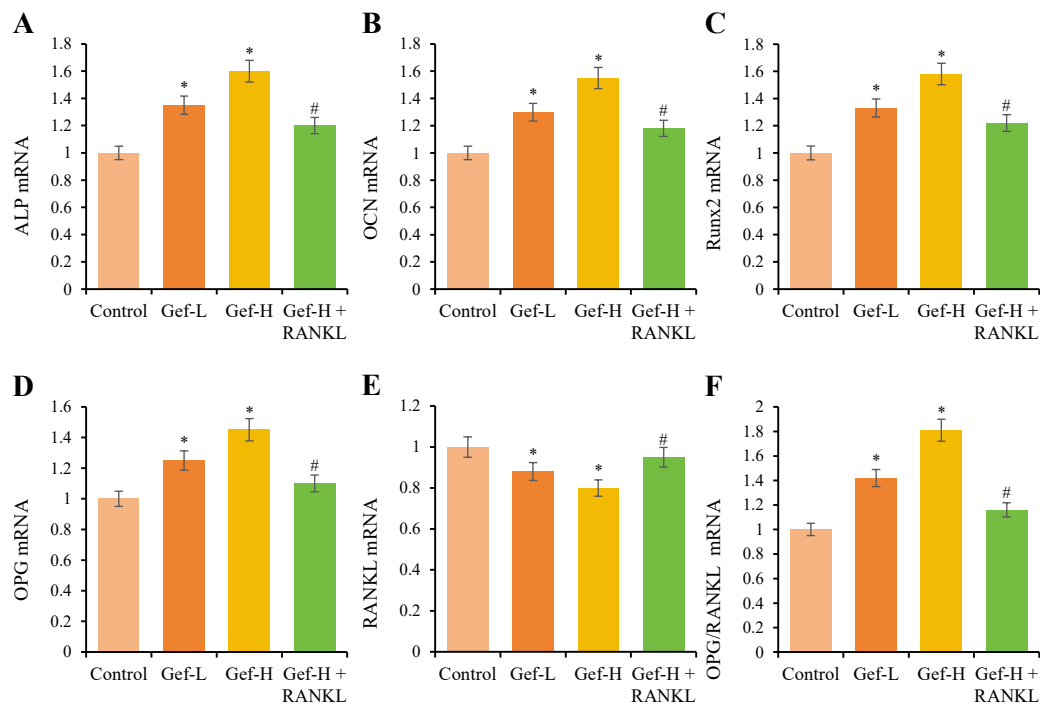


Fig. 9 — Regulatory effects of Gef on osteogenesis-related and OPG/RANKL gene expression in BMSCs. Data are presented as mean $\pm$ standard deviation ($n = 5$ independent cell experiments). (A) ALP, (B) OCN, (C) Runx2, (D) OPG, (E) RANKL, (F) OPG/RANKL ratio. Note: * $P < 0.05$ vs. Control, # $P < 0.05$ vs. Gef-H.

results of this study. Additionally, Gef has been shown to reduce RANKL expression and osteoclast activity by inhibiting EGFR signaling in particle-induced bone resorption models²¹, further supporting the mechanisms. The TRAP staining results of this study showed that the number of osteoclasts in the Gef-treated group was reduced at both day 7 and day 28, while OCN immunohistochemistry revealed enhanced osteoblast activity, suggesting that Gef may be involved in the regulation of the osteoclast-osteoblast balance.

Moreover, Western blot results showed that Gef intervention not only upregulated OPG protein expression but also increased the expression levels of the osteogenic markers ALP and BMP2, while reducing the expression of RANK, RANKL, and the osteoclast activity marker TRAP. These results suggested that Gef might exert a dual effect of both inhibiting osteoclast formation/bone resorption and promoting osteoblast differentiation/matrix mineralization. The reversal of these effects by combined functional OPG antagonist treatment further underscores the central role of the OPG/RANKL/RANK signaling axis in mediating Gef's actions. The functional OPG antagonist alone worsened bone microstructure (lowest Tb.Th and BV/TV), and when combined with Gef, it completely abolished the bone repair effects of Gef (parameters returned to levels comparable to those in the Fx group). OPG upregulation occurred following Gef

intervention, while the functional OPG antagonist reversed this effect. These results align with previous studies showing that enhanced RANKL/reduced OPG weakens osteogenesis and can even lead to a sharp decline in bone strength²².

The in vitro results provided cytological evidence for the effects of Gef: Gef concentration-dependently enhanced ALP activity and mineralized nodule formation in BMSCs, up-regulated mRNA expression of ALP, OCN, Runx2, and OPG, while down-regulating RANKL and significantly increasing the OPG/RANKL ratio. Since osteoclast formation depends on RANKL secreted by osteoblasts, this suggests that Gef promotes a pro-osteogenic rebalancing in the bone repair microenvironment by modulating osteoblast-osteoclast coupling signals. The significant reversal of these effects upon exogenous RANKL addition further confirms the critical role of this coupling pathway.

This study confirmed the anti-inflammatory effects of Gef in fracture models. The EGFR inhibitor's suppression of inflammation synergized with its regulation of osteogenic-osteoclastic balance, enhancing overall fracture repair efficacy. This aligns with reports that EGFR inhibitors mitigate bone inflammation and modulate osteoblast-osteoclast activity. Gef inhibited NF- κ B signaling, blocking inflammation-induced RANKL expression at the source²³. Other studies also emphasized that inhibiting

inflammation during bone repair can directly weaken RANKL-mediated osteoclastic activity, thereby preserving bone formation²⁴.

In the clinical setting, several clinical reports showed that EGFR-TKIs (e.g., Gef or osimertinib) induced osteoblastic changes in bone metastases, which provided clinical clues regarding the role of EGFR inhibitors in bone metabolism regulation²⁵. The present study extended the observation of this mechanism to a traumatic fracture model, suggesting that the EGFR signaling pathway may play a complex and context-dependent role in acute fracture repair. However, the animal dose used in this study was 20 mg/kg/day (administered orally). Based on body surface area conversion²⁶, this dose corresponded to a human equivalent dose of approximately 3.2 mg/kg/day. Nevertheless, no reference standard for the human dose of Gef for promoting fracture healing currently exists. Therefore, the relationship between this animal dose and the potential clinically effective dose remained unclear, and future systematic dose-response studies in multiple animal models were needed to determine the therapeutic window and safety range. In addition, the potential adverse effects associated with long-term use of EGFR inhibitors, such as skin and gastrointestinal toxicities, also require careful evaluation when used for fracture healing. In addition to the well-known cutaneous and gastrointestinal toxicities, the safety profile of gefitinib in the context of bone repair requires further consideration. The EGFR signaling pathway is involved in multiple aspects of tissue healing, including angiogenesis, chondrogenesis, and mesenchymal stem cell function. Therefore, in future preclinical studies, the potential off-target effects of gefitinib on soft tissue healing (e.g., muscle and skin incisions) as well as on newly formed bone quality (e.g., matrix organization, biomechanical strength) should be systematically evaluated through extended observation periods. Furthermore, the timing and duration of gefitinib administration may critically influence the risk–benefit balance: early administration during the inflammatory phase could theoretically suppress necessary EGFR dependent regenerative signals, whereas delayed administration or a short course regimen may mitigate adverse effects while preserving the pro healing actions of the OPG/RANKL/RANK axis. Before considering clinical translation, a comprehensive toxicological evaluation must be performed, including liver and kidney function tests, hematological monitoring, and histopathological examination of major organs.

Regarding translational feasibility, several steps are required to move from the current rat model to

potential human application. First, dose finding and pharmacokinetic/pharmacodynamic studies in large animal models (e.g., rabbits or dogs) with clinically relevant fracture types (e.g., delayed union or nonunion models) would help determine the effective dose range and optimal route of administration (systemic vs. local delivery). Second, the human equivalent dose estimated based on body surface area (approximately 3.2 mg/kg/day) is significantly higher than the standard oncology dose of gefitinib (250 mg once daily, which is approximately 3.6 mg/kg/day for a 70 kg adult). Whether such doses are tolerable in non cancer fracture patients, and whether intermittent or local administration could reduce systemic exposure, remain to be determined. Third, the ideal target population needs to be defined—for example, patients at high risk of delayed union (such as those with diabetes, smoking history, or open fractures) or trauma fracture patients with concomitant EGFR related diseases. Finally, comparative effectiveness studies against existing pro healing agents (such as teriparatide, BMP2, or icariin) are necessary to determine the positioning of gefitinib among therapeutic options. Before these preclinical and early clinical data are obtained, it is premature to directly extrapolate the current findings to human fracture treatment. Other drugs that promote fracture healing (e.g., raloxifene, icariin, organic gallium) have been more extensively reported. For example, icariin enhances the OPG/RANKL ratio and increases Runx2/COL1A2 expression, promoting fracture healing in ovariectomized rats²⁷. Organic gallium increases BV/TV, regulates the OPG/RANKL ratio, and reduces TNF- α /IL-6 levels^{25,28}, providing mechanistic references for Gef's effects.

Despite comprehensively validating Gef's pro-healing effects at tissue, molecular, and functional levels, this study has limitations. First, efficacy observations were limited to the 28-day healing phase, without examining late-stage bone remodeling or long-term bone strength recovery, necessitating extended observation periods. Second, the dose-response relationship of different Gef doses and administration frequencies was not systematically investigated. Finally, experiments were confined to traumatic fracture models, and the drug's efficacy and safety in osteoporosis or chronic fracture environments require further validation^{29,30}.

CONCLUSION

The results of this study indicated that, in a traumatic fracture model of SD rats, Gef may promote the

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fracture healing process by upregulating OPG expression, inhibiting the RANKL/RANK signaling pathway, and reducing local inflammation. The OPG/RANKL/RANK signaling axis was identified as one of the important molecular pathways through which Gef exerted its effects in this study, and the antagonistic effect of a functional OPG antagonist on Gef efficacy provided direct evidence for this mechanism. The findings of this study suggested that EGFR inhibitors may serve as candidate drugs for promoting fracture healing, warranting further exploration, particularly in models of delayed healing or nonunion. However, it should be noted that this study was a preclinical animal experiment, and the conclusions cannot be directly extrapolated to humans. Before clinical translation can be considered, further studies with larger sample sizes, safety evaluations, and dose optimization are still needed.

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