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RESEARCH

Comparison of the effects of recombinant human epidermal growth factor (rhEGF) and alendronate sodium on tibial fracture healing in rats: an experimental study

Rekombinant insan epidermal büyüme faktörü (rhEGF) ve alendronat sodyumun sıçanlarda tibial kirik iyileşmesi üzerindeki etkilerinin karşılaştırılması: deneysel çalışma

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Abstract

Purpose: The objective of this study is to compare the effects of recombinant human epidermal growth factor (rhEGF) on bone healing with those of alendronate, a bisphosphonate widely used in practice.

Materials and Methods: An iatrogenic fracture was created in the tibial shaft of 24 Sprague-Dawley rats with osteotome and fixed with an intramedullary Kirschner wire (K-wire). After surgery, Group 1 was given 0.2 mg/kg/day of oral alendronate sodium on postoperative Day 1 to 28, Group 2 received a single dose of 0.5 mg/kg of intraosseous rhEGF on postoperative Days 1 and 14, and Group 3 was followed for a total of four weeks with oral saline. At the end of Week 4, the animals were euthanized and the lower extremities were removed by stripping the soft tissues without damaging the callus. Tissue samples of groups were prepared and and stained with hematoxylin-eosin. After staining, histological scoring was performed to evaluate the degree of union.

Results: Alendronate sodium group demonstrated a mean histological score of 6.95± 1.28. The rhEGF group had a lower mean score of 4.85±1.66. The placebo group exhibited the least progress in bone healing with a mean score of 4.10±1.68. The histological score was significantly higher in the alendronate sodium group compared to both the rhEGF and placebo groups. There was also a statistically significant difference between the rhEGF and placebo groups in terms of scores.

Öz

Amaç: Bu çalışmanın amacı rekombinant insan epidermal büyüme faktörünün (rhEGF) kemik iyileşmesi üzerindeki etkilerini pratikte yaygın olarak kullanılan bir bifosfonat olan alendronat ile karşılaştırmaktır.

Gereç ve Yöntem: Osteotom ile 24 Sprague-Dawley sıçanın tibial şaftında iyatrojenik kırık oluşturuldu ve intramedüller Kirschner teli (K-teli) ile tespit edildi. Operasyondan sonra, Grup 1'e postoperatif 1 günden 28. güne kadar 0,2 mg/kg/gün oral alendronat sodyum, Grup 2'ye postoperatif 1 ve 14. günlerde tek doz 0,5 mg/kg intraosseöz rhEGF verilmiş ve Grup 3 ise toplam dört hafta boyunca oral salin ile takip edilmiştir. Dördüncü haftanın sonunda hayvanlara ötenazi uygulandı ve alt ekstremiteler kallusa zarar vermeden yumuşak dokuları sıyrılarak çıkarıldı. Gruplara ait doku örnekleri hazırlandı ve hematoksilen-eozin ile boyandı. Boyama sonrasında kaynama derecesini değerlendirmek için histolojik skorlama yapıldı.

Bulgular: Alendronat sodyum grubu ortalama 6.95± 1.28 histolojik skor gösterdi. RhEGF grubunun ortalama skoru 4.85±1.66 ile daha düşüktü. Plasebo grubu 4.10±1.68 ortalama skor ile kemik iyileşmesinde en az ilerleme gösteren grup olmuştur. Alendronat sodyum grubunda histolojik skor hem rhEGF hem de plasebo gruplarına kıyasla anlamlı derecede yüksekti. Skorlar açısından rhEGF ve plasebo grupları arasında da istatistiksel olarak anlamlı bir fark vardı.

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Conclusion: Alendronate sodium enhanced fracture healing processes in rats. The role of rhEGF in bone healing requires further exploration. As the understanding of bisphosphonates and growth factors in bone healing evolves, the strategies for optimizing patient care in orthopedic settings are expected to be developed.

Keywords:. Bisphosphanate, rhEGF, fracture, nonunion, bone healing

Sonuç: Alendronat sodyum sıçanlarda kırık iyileşme süreçlerini geliştirmiştir. rhEGF'nin kemik iyileşmesindeki rolü daha fazla araştırma gerektirmektedir. Kemik iyileşmesinde bifosfonatlar ve büyüme faktörlerinin anlaşılması geliştikçe, ortopedik ortamlarda hasta bakımını optimize etmek için stratejilerin geliştirilmesi beklenmektedir.

Anahtar kelimeler: Bisfosfonat, rhEGF, kırık, kaynamama, kırık iyileşmesi

INTRODUCTION

Fractures are a common traumatic injury resulting from various causes such as high-energy incidents (e.g., falls, collisions, gunshot wounds) or conditions that weaken bone quality (e.g., osteoporosis, malignancies)[1]. The healing of fractures depends on complex biological processes and mechanical factors. Effective fracture healing requires assessing the fracture site, type, preservation of the bone blood supply, and selecting treatment methods that support the appropriate biological environment and provide ideal mechanical factors[2].

Fractures pose a significant healthcare burden worldwide. Although the specific annual incidence rate for fractures in Turkey has not been exactly cited, the study by Birinci et al.[3] analyzed 2.1 million patients with 2.2 million fractures between January 2021 and May 2023. Hip fractures are notably common among the elderly population and represent a significant global healthcare burden. Globally, the incidence of hip fractures was estimated as 14.2 million in 2019, with an incidence of 182/100,000 individuals[4]. It is also associated with a substantial economic strain on the healthcare system due to high treatment costs and lost productivity, as highlighted by the global burden of hip fractures and the anticipated increase in incidence and healthcare costs[5].

Delayed fracture healing or nonunion poses substantial economic and healthcare burdens, irrespective of the patient's age. Nonunion occurs in approximately 5 to 10% of long bone fractures and can significantly affect healthcare systems due to the need for prolonged treatment and multiple surgical interventions[6]. Although recent advancements in the field of surgical management have reduced the rates, delayed healing still remains prevalent in 10 to 15% of surgically managed fractures, thereby leading to increased healthcare costs and patient morbidity[7]. In severe cases, nonunion can result in the need for complex revision surgeries or even amputation, further exacerbating the economic burden[8].

Addressing these challenges requires ongoing advancements in treatment strategies to improve recovery outcomes and alleviate the associated societal costs[9]. In recent years, molecular biological agents have been explored to enhance fracture healing[10]. Numerous natural and synthetic compounds have been studied in the literature regarding their effects on fracture and defect bone healing. These include cortical autograft and allograft, platelet-rich plasma (PRP), bone morphogenic protein-2 (BMP-2), adipose tissue stem cells (ADSc), heparin sulfate, 5-acetyl salicylate (5-ASA), and the commonly used COX inhibitors, namely ibuprofen and rofecoxib[11]. Although the impact of some of these agents on bone healing has been demonstrated in animal studies[12], they have not been incorporated into clinical practice on a routine basis due to economic and practical considerations.

Adequate mineralization and stem cell transformation are of utmost importance during bone union. Nonunion is common in osteoporosis due to impaired mineralization and bone quality. Bisphosphonates are the most commonly used drugs in the treatment of osteoporosis and they exert their effects by inhibiting the osteoclast mechanism[13]. However, the effect of bisphosphonates on bone healing is still controversial. While some studies have reported that bisphosphonates contribute to bone healing in the short term, they inhibit the bone healing cycle and cause atypical fractures in the long term[14]. Conversely, other studies have indicated that it has no effect on short-term healing[15-17].

Of bisphosphonates, alendronate sodium (AS) are promising for enhancing fracture healing, due to short-term benefits of stabilizing bone density and reducing postoperative pain; Although its long-term use increases bone mineral density, it may delay healing in atypical fractures[18, 19].

Recombinant human epidermal growth factor, a growth factor used in non-healing wounds and diabetic ulcers, has been shown to stimulate bone healing in osteonecrosis of the femoral head, in addition to its effect on wound healing[20, 21]. However, the effect of rhEGF on fracture healing has not been fully elucidated, yet[22]. On the other hand, rhEGF can be cost-effective by reducing healing time and complications, although it has a higher initial cost. Alendronate is usually cost-effective due to its widespread availability and low cost.

In the present study, we hypothesized that AS and rhEGF administration could accelerate fracture healing, with rhEGF potentially having a greater effect on the healing rate. We, therefore, aimed to compare the effects of rhEGF, which acts by stimulating stem cell transformation and proliferation, on bone healing with AS, a bisphosphonate commonly used in clinical practice.

MATERIALS AND METHODS

This experimental study was conducted in accordance with the ethical standards of the institutional Ethics Committee and with the 1964 Helsinki Declaration and its later amendments. The study was approved by Gazi University Ethics Committee (Date: 08.06.2018, No: G.Ü.ET-18.36). and perfomed in Gazi University Laboratory Animal Breeding and Experimental Research Center (GÜDAM).

Animals

The study involved 24 Sprague-Dawley rats and the rats were divided into three groups of eight in each group: Group 1 received AS (Fosamax®, Merck Sharp Dohme Pharmaceuticals, Istanbul, Türkiye), Group 2 received rhEGF (Heberprot-p®, Hasbiotech İlaç San. ve Tic. A.Ş., Istanbul, Türkiye), and Group 3 received a placebo (oral saline). Prior to surgery, the experimental animals were administered 45 mg/kg of ketamine + 5 mg/kg of xylazine intravenously for anesthesia. The animals were, then, placed in a supine position on the operating table, and their upper and lower extremities and tail were appropriately secured. After shaving the surgical areas with a razor, a 3-cm longitudinal anterior skin incision was made in the tibial bone following appropriate surgical field cleaning and draping. The fascia was separated by passing through the skin-subcutaneously, and the tibia was brought into the surgical field of view. After completing the surgical opening, an iatrogenic fracture was created in the tibial shaft using an osteotome.

Surgical method

The fracture was fixed internally with an intramedullary Kirschner wire (K-wire) before beginning the closure procedure (Figure 1). The fascia and subcutaneous fascia were sutured with 4/0 absorbable (Vicryl) sutures, followed by individual suturing of the skin with 4/0 polypropylene (Prolene).

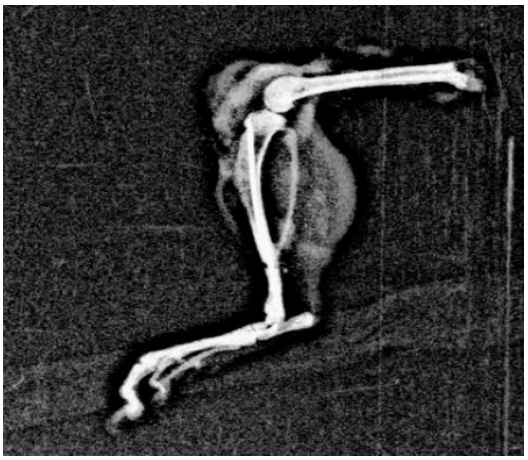

Figure 1. Fixation model of tibia fracture via intramedullary K-wire.

Chemical agents

After surgery, the animals were administered 4 mg/kg/ day of oral meloxicam (Melox®, Nobel İlaç San. ve Tic. A.Ş., Istanbul, Türkiye) as an analgesic started from postoperative Day 1 to postoperative Day 28. Group 1 was given 0.2 mg/kg/day of oral AS, Group 2 received a single dose of 0.5 mg/kg of intraosseous rhEGF on postoperative Days 1 and 14, and Group 3 was followed for a total of four weeks with oral saline treatment as placebo. The animals were not subjected to any restrictions or immobilization throughout the course of the follow-up, and no complications, such as infection, death, tissue necrosis, re-fracture, or implant failure, were observed. At the end of Week 4, the animals were euthanized by anesthesia. The lower extremities were, then, removed by stripping the soft tissues without damaging the callus after appropriate surgical field cleaning and covering.

Table 1. Histological scoring of bone healing.

Score	Histological Findings
1	Fibrous tissue
2	Mostly fibrous tissue, small amount of cartilage
3	Equal amounts of fibrous and cartilage tissue
4	Mostly cartilage, small amount of fibrous tissue
5	Cartilage tissue
6	Mostly cartilage, small amount of immature bone
7	Equal amounts of cartilage and immature bone tissue
8	Mostly immature bone, small amount of cartilage tissue
9	Immature bone with fracture healing
10	Mature bone with fracture healing

Histological preparation and grading

Tissue samples of the experimental groups were fixed in 10% formaldehyde for 48 hours and, then, decalcified in 10% formic acid solution. After deparaffinization, the sections of the experimental groups were kept in a decreasing series of ethyl alcohol (100%, 90%, 80%, 70%, and 50%) for 10 min each. After drying, the sections were washed with running tap water for 10 min to remove the alcohol. After washing, the sections were kept in Harris hematoxylin solution for 10 min and washed under running tap water for additional 10 min. After immersion in glacial acetic acid and alcohol solution, the sections were washed again for 10 min under running tap water. The sections were soaked in eosin dye solution for 10 min and rewashed for 10 min under running tap water. After washing, the sections were dehydrated by passing through a series of 50%, 70%, 80%, 80%, 90%, and 100% ethyl alcohol treatment, followed by a xylol treatment and the sections were sealed[23]. The images obtained from the stained sections in a Leica DM 4000B of 4 μm in thickness were taken with a microtome and stained with hematoxylin-eosin. After staining, histological scoring described by Han et. al. [24] was performed to evaluate the degree of union in the tissues (Table 1).

Statistical analysis

Statistical analysis was performed using the SPSS for MAC version 28.0 software (IBM Corp. Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median and min-max values (25^{th}-75^{th} percentiles) or number and frequency, where applicable. To assess the normality of the data distribution, the Shapiro-Wilk test was performed. Given the deviation from normality, non-parametric Mann-Whitney U test and Kruskal-Wallis H test were employed to compare the mean values across the different treatment groups. A p value of <0.05 was considered statistically significant. Power analysis was performed through the Power A&X application on input data

RESULTS

There were significant differences in histological scores among the treatment groups, indicating varying levels of effectiveness in promoting bone healing. The group treated with AS demonstrated the most promising results, with a mean histological score of 6.95±1.28 and a median of 7.00.

Table 2. Comparative analysis of histological scores: Alendronate sodium, rhEGF, and placebo groups.

Group	Comparison Group	P-Value
Alendronate Sodium	rhEGF	p=0.001
Alendronate Sodium	Placebo	p<0.001
rhEGF	Placebo	p=0.048

Histological scores ranged from 4.00 to 9.00, indicating a progression from tissues consisting mainly of cartilage with some fibrous content to more advanced stages of bone healing involving immature bone. The interquartile range of 6.00 to 8.00 showed this trend toward more mature bone formation. The standard error of the mean (SEM) for this group was 0.29.

In contrast, the rhEGF group had a lower mean histological score of 4.85±1.66 with a median score of 5.00. The score range of 2.00 to 7.00 indicated a higher prevalence of less mature tissue types in the healing process compared to the AS group. The placebo group exhibited the least progress in bone healing, as evidenced by a mean histological score of 4.10±1.68. In this group, the median score was 3.50, indicating a predominance of early-stage healing tissues ranging from 2.00 to 7.00. (Table 2).

The histological score was significantly higher in the AS group compared to both the rhEGF and placebo groups (p=0.001 and p<0.001, respectively). There was also a statistically significant difference between the rhEGF and placebo groups. The mean histological score was slightly lower in the placebo

group compared to the rhEGF group (p=0.048) (Table 3).

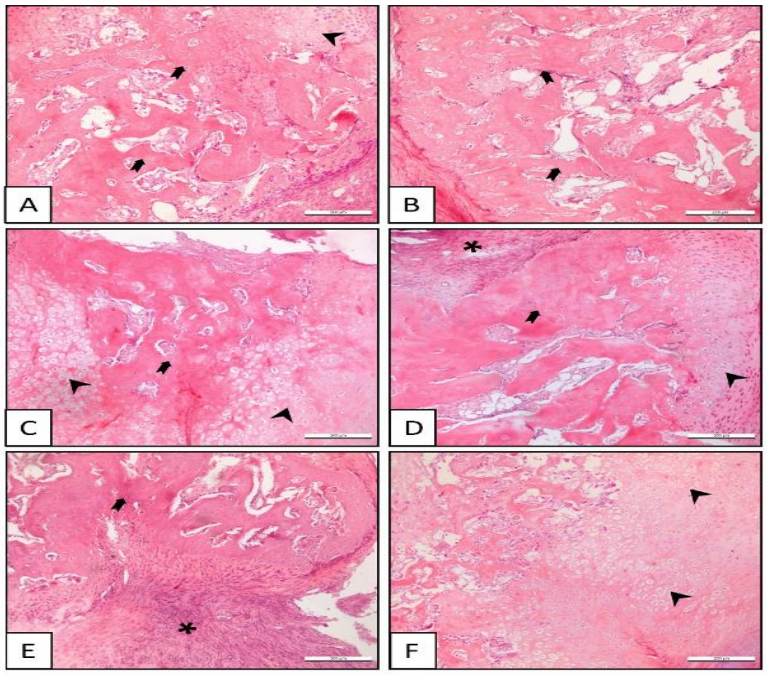

Figure 2. Histological images of the experimental groups showing fibrous tissue (*), cartilage tissue (^) and immature bone tissue (⇧) (A, B: Alendronate Sodium group; C, D: rhEGF group; E, F: Placebo group, x200, HE).

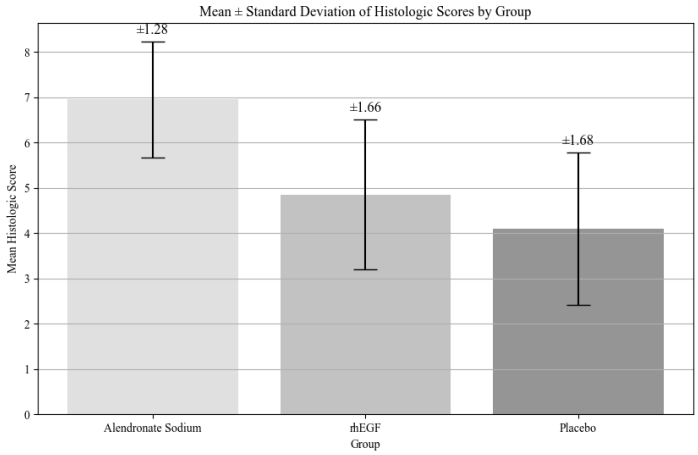

Figure 3. Summary of histological score outcomes for alendronate sodium, rhEGF, and placebo groups.

DISCUSSION

Fractures represent a significant public health issue worldwide, with the potential to affect the capacity of an individual to engage in work and social activities. Prolonged periods of fracture healing can impede an individual's ability to actively participate in work and social life. Additionally, the disability resulting from trauma can lead to an increase in healthcare utilization rates

It is estimated that, in about 10% of all cases, delayed repair of a bone fracture results in potential progression to nonunion. Nonunion is defined as a permanent failure of bone healing after a period of nine months, with no evidence of progressive repair observed over a consecutive three-month period[25]. The occurrence of delayed union or nonunion can be attributed to a number of factors: surgical complications such as inadequate stabilization or infection; intrinsic characteristics of the injury itself, such as the presence of tissue loss or an open fracture; and a multiple patient-specific factors, such as smoking, metabolic disorders; and the utilization of pharmaceutical agents that may influence tissue repair. Nevertheless, the successful repair of a nonunion fracture depends on the provision of support for the body's intrinsic mechanisms involved in tissue repair. The advent of osteoanabolic drugs for the treatment of osteoporosis has provided an alternative strategy for enhancing fracture repair. The use of antiresorptive agents, such as bisphosphonates, has been demonstrated to be effective in promoting fracture repair[26]. Recombinant human epidermal growth factor may also be considered an alternative therapeutic option to promote intrinsic mechanisms of bone healing. It has the potential to enhance defective bone healing through synergistic activation, which stimulates cellular growth and differentiation and a decrease in the social burden.

In the present study, we focused on the effects of AS and rhEGF on the healing of iatrogenic tibial fractures in living rat specimens. Our study results provide valuable insights and are consistent with the recent literature. In the current study, AS significantly increased bone healing compared to both rhEGF and placebo in the short term. This is consistent with the results of recent studies demonstrating the efficacy of bisphosphonates such as AS in improving bone quality and fracture healing. The study by Fink-Eriksen et al.[27] showed promising results in the use of bisphosphonates to promote healing of complicated stress fractures. Similarly, another study showed no significant delay in fracture healing in elderly treated with bisphosphonates, highlighting their potential role in the management of osteoporotic fractures[28]. Taken together, these findings suggest that bisphosphonates induce bone healing in the short term through their osteoclast inhibitory effect. Bisphosphonates and similar drugs are used in patients with osteoporosis and some other diseases. Apart from these conditions, there is no indication for their use in normal fractures[29]. However, we believe that showing an accelerated effect on fracture healing may help to start the rehabilitation process earlier and speed up the return to work and normal life.

On the other hand, bisphosphonates have also been shown to stagnate bone remodeling. A randomized-controlled study conducted by Lyles et al.[14] in 1,065 hip fracture patients demonstrated the adverse effects of zoledronic acid administration on fracture healing. Similarly, cases of fracture nonunion due to AS were shown to heal simply by discontinuing the drug in a case report[30].

Bisphosphonates may cause osteonecrosis of the jaw and atypical fractures in the long term which can be attributed to osteoblast inhibition. As aforementioned, zoledronic acid, which is given annually, shows that its effect is long term, but the concept of long term is still controversial and how long the drug should be given should be established. Alendronate, which is given orally once daily, is convenient for adjusting this period. Therefore, we believe that the use of AS and other short-acting bisphosphonates in the treatment of fracture healing may prevent adverse effects on fracture healing.

The presence of a significant difference in bone healing between the rhEGF and placebo groups in our study suggests a limited role of rhEGF in bone healing processes, at least in the context of mechanically induced tibial fractures in rats. This is an interesting finding considering the known efficacy of rhEGF in non-healing wounds and diabetic ulcers. The literature on the specific role of rhEGF in bone healing, particularly in comparison to bisphosphonates, is still evolving, and our study contributes to this field of research by highlighting the need for further investigation. In a study, Yüce et al.[31] investigated the efficacy of concentrated growth factors in osteoporotic patients with medication-related osteonecrosis of the jaws. Although this study

focuses on a different clinical scenario, it emphasizes the potential of growth factors in bone healing and tissue regeneration, which may provide valuable insights into the limited efficacy of rhEGF observed in our study compared to AS. In another study, the effect of epidermal growth factor (EGF) on osteonecrosis of the femoral head in a rat model was examined[32]. The results of this study showed that EGF promoted bone formation and microvascularization, positively affecting the preservation of the femoral head during healing. Although the focus was on osteonecrosis rather than fracture healing, the authors highlight the potential of EGF in bone regeneration, which is consistent with our findings observed with rhEGF.

In addition to their differences in efficacy, the two drugs exhibit separate cost-effectiveness in varying contexts. In a global context, the annual cost of AS treatment varies considerably, with figures ranging between US$49 and 289 per annum[33]. The cost of a single vial of rhEGF is US$762.87[34]. Although both pharmacological agents have been shown to facilitate bone healing, AS appears to be more cost-effective than rhEGF in the context of fracture healing. Nevertheless, considering the socioeconomic burden of fracture cases, it becomes evident that both have a favorable effect. The superior performance of AS observed in the present study is consistent with the accumulating body of evidence supporting the use of bisphosphonates in fracture healing and improvement of bone quality. However, it is essential to consider the potential long-term consequences of bisphosphonate therapy, particularly in the context of atypical femoral fractures. Adherence to treatment is also a crucial aspect, and while a single administration of rhEGF is sufficient, it is relatively more challenging for the patient to adhere to a daily regimen of one tablet.

Nonetheless, there are some limitations to this study. First, although the use of a rat model may have provided valuable physiological insights, it may not fully replicate the complex nature of human bone healing processes and, therefore, the findings may not be directly applicable to clinical settings in humans. Second, the specific doses and administration methods of AS and rhEGF were selected based on existing literature; however, variations in these factors could have potentially yielded different outcomes. Third, the study duration was limited to four weeks, which constrains the ability to assess long-term effects of the treatments and any associated adverse effects, particularly in the context of concerns about prolonged bisphosphonate use. Additionally, the timing and frequency of rhEGF administration were fixed at two specific postoperative time points, not exploring the potential benefits of alternative dosing schedules. Also, the focus on histological scoring as the primary assessment of bone healing may have overlooked other aspects of the healing process that could be revealed through different investigative methods, such as biomechanical testing or molecular analysis. Finally, the findings are specific to the models and conditions tested and cannot be generalized to other fracture types, different animal models, or human subjects.

Furthermore, the lack of a treatment group receiving a combination of AS and rhEGF indicates that the study does not provide insights into potential synergistic or antagonistic effects of combined treatment. Further large-scale, well-designed, long-term studies are warranted to confirm these results and draw more reliable conclusions on this subject.

In conclusion, our study results highlight the potential of AS in improving fracture healing processes in rat models. The role of rhEGF in bone healing, in contrast, requires further exploration. These insights are crucial for developing targeted treatment strategies for fracture management, particularly in the context of osteoporotic fractures and the need for rapid healing. As the understanding of bisphosphonates and growth factors in bone healing evolves, the strategies for optimizing patient care in orthopedic settings are expected to be developed.

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