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Original research article

Does vanillic acid affect fracture healing? An experimental study in a rat model of femur fracture

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Abstract
Background and objectives: We aimed to determine the effects of vanillic acid (VA) on fracture healing radiologically, histologically, immunohistochemically, and biomechanically using a rat femur open fracture injury model.
Methods: 32 male Wistar-Albino rats were used and divided into two groups: the study group (VA) and the control group. From the time they were operated on until they were sacrificed, the rats in the study group were given 100 mg/kg/day VA by oral gavage. After sacrification, the femurs were analyzed.
Results: It was observed that the Huo histological scoring was significantly higher in the VA group (p = 0.001), and the ratio of the amount of callus tissue compared to intact bone tissue was significantly higher. While no significant difference was observed in immunohistochemical H-scores in ColI antibody staining (p = 1.000), a borderline significant difference in favor of VA was observed in ColIII antibody staining (p = 0.078). In biomechanical analysis, failure load (N), total energy (J), maximum stress (MPa), and stiffness (N/mm) measurements were significantly higher in the VA group (p = 0.040, p = 0.021, p = 0.015, and p = 0.035, respectively).
Conclusion: It has been observed that VA, with its antioxidative properties, increases fracture healing in rats, in which an open fracture model was created. We are hopeful that such an antioxidant, which is common in nature, will increase fracture healing. Since this study is the first to examine the effect of VA on fracture healing, further studies are needed.

Keywords: *Angelica sinensis*; Fracture union; Open fracture; Phenolic compounds; Vanillic acid

Highlights:
- The rats were divided into two groups: the study group (VA) and the control group, with 16 rats in each group.
- Radiologically, the amount of callus tissue formation was significantly higher in the VA group.
- It was observed that histological scores were significantly higher in the VA group.
- Biomechanically, VA group was more stable.
- VA may be an adjunct agent in the treatment of nonunion, but this study is the first to examine its effect on fracture healing.

Introduction

Fracture healing represents a unique remodeling process with complex interactions (Claes et al. 2012; Szczęsny, 2015). This process starts with inflammation after damage to the bone tissue and ends with osteogenesis (Einhorn and Gerstenfeld, 2015; Maruyama et al., 2020). Various systemic and local factors can affect the course of this process, which re-establishes the physical and mechanical properties of the tissue. The balance between free radicals and antioxidants is critical in the fracture healing process (DePhillipo et al., 2018; Sheweita and Khoshhal, 2007). However, in this balance, insufficiency of anti-oxidative mechanisms and an increase in reactive oxygen species (ROS) can be observed for various reasons. This condition is called oxidative stress and is usually characterized by a lack of anti-oxidant factors and high levels of signs of oxidative damage. These reactive oxygen radicals are known to affect the pathogenesis of bone loss because they can inhibit angiogenesis and bone turnover (Amanvermez et al., 2013; Topak et al., 2023).

Vanillic acid (4-hydroxy-3-methoxybenzoic acid), which is formed as an intermediate compound during the conversion of ferulic acid to vanillin, is a phenolic compound found in high concentration in the roots of *Angelica sinensis*, a Chinese plant, and is used in the food industry due to its odor (Calixto-Campos et al., 2015; Circosta et al., 2006). Açaí oil, extracted from the fruit of the Açaí palm (*Euterpe oleracea*), is also rich in van-

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illic acid (VA) (Pacheco-Palencia et al., 2008). Additionally, VA is one of the main natural phenols in argan oil, and it is also found in wine and vinegar (Charrouf and Guillaume, 2007; Gálvez et al., 1994). There have been studies reporting that VA is a powerful anti-oxidant due to the presence of hydroxyl and carboxyl groups and its osteoprotective effects in cases of increased oxidative stress, such as osteoporosis (Wang et al., 2017).

In this context, our hypothesis is that compounds such as VA, which is a powerful antioxidant, may play a potential role in accelerating the healing process of bone fractures. To the best of our knowledge, there is no study investigating the effect of VA on fracture healing yet, and this study stands out as the first study in this field.

Materials and methods

In this study, 32 male Wistar-Albino rats, 12 weeks old and weighing between 423 and 512 g, were used. The experimental design and all procedures were approved by the Selçuk University Animal Research and Ethics Committee (protocol number 2023/51). To perform radiological, histological, immunohistochemical, and biomechanical analyses of bone healing, the rats were divided into two groups: the study group (VA) and the control group, with 16 rats in each group.

Surgical technique

All surgical procedures were performed under general anesthesia following injections of 50 mg/kg ketamine hydrochloride (Ketalar® 500 mg; Pfizer Pharmaceuticals, Istanbul, Turkey) and 10 mg/kg xylazine (Rompun® 2% Bayer Pharmaceuticals, Istanbul, Turkey). In order to ensure that all rats were operated on painlessly, support was provided with additional doses of anesthetics when necessary. Before surgical intervention, the operating area was shaved and prepared with povidone-iodine (Batticon®, Adeka Pharmaceuticals, Samsun, Turkey). To expose the femurs, a 2 cm longitudinal incision was made on the anterolateral side of the thigh, and subcutaneous tissue was excluded. The vastus lateralis and rectus femoris muscles were separated by blunt dissection, and the femoral diaphysis was reached. A transverse femur shaft fracture model was created using a microcircular saw. Then, a 1 mm Kirschner wire (K-wire) was entered into the knee joint from the medial aspect of the patella, and the fracture was stabilized by passing through the intercondylar fossa and advancing to the trochanteric region. The K-wire is placed in the femoral trochanteric cortex proximally and is embedded in the intercondylar fossa in the distal part without disturbing the knee joint. After bleeding control was achieved, the skin and subcutaneous tissues were closed.

No bandage or cast was applied to the rats after surgery. All rats were allowed to perform lower extremity joint movements and bear weight in the early period. The rats in the study group were given 100 mg/kg/day vanillic acid (Sigma-Aldrich Inc., MO, USA), which was dissolved in a 0.5 ccs 50/50 ethanol-water solution and given to each rat by oral gavage at the same time every day, from the time they were operated on until they were sacrificed.

The rats in the control group were given only 0.5 ccs of a 50/50 ethanol-water solution via oral gavage. The amount of VA administered to rats was calculated based on previous studies (Wang et al., 2017). At the end of the 6th week, all rats were euthanized by cervical dislocation under high-dose anesthesia. The operated femurs of sacrificed rats were excised, separating them from other soft tissues and leaving only the broken callus tissue, and then subjected to radiological, histological, immunhistochemical, and biomechanical analysis.

Radiological evaluation

A total of 256-slice 2-section dual source computed tomography (CT) (SOMATOM Definition Flash, Siemens, Germany) was used for radiological imaging, and CT images of all operated femurs were obtained. For measurements of CT images, RadiAnt DiCOM Viewer version 2020.2.3 software (Medixant, Poznań, Poland) was used (Fig. 1). Low-density bone areas showing callus tissue and high-density bone areas showing intact bone tissue were calculated in square centimeters (cm^2). Radiological analysis was performed on the callus tissue by comparing the ratio of low-density bone areas (LDBA) (callus) to high-density bone areas (HDBA) (fractured femur) (Dempster et al., 2013; Recker et al., 2011).

Histological evaluation

Following the CT scans of 16 rats in each group obtained on the same day, 8 of them were subjected to histological evaluation and 8 were subjected to biomechanical evaluation. Femurs subjected to histological study were placed in a 10% formalin solution for fixation and then in a 10% acetic acid solution for decalcification. Then, 3 μm sections per block were taken

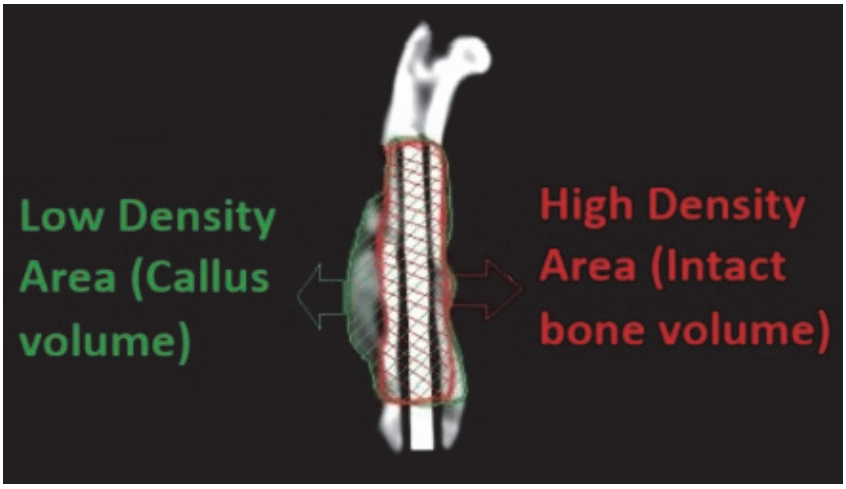


Fig. 1. Measurement of micro-CT images. Longitudinal sections of newly formed callus tissue in the femur.

from the paraffin-embedded blocks and stained with hematoxylin-eosin (H&E). In the histological examination, fracture healing was scored according to the histological scale developed by Huo et al. (1991), and the study and control groups were compared with each other in this respect. This classification allows us to evaluate the stage of healing of the fracture by grading the process from fibrous tissue formation to mature bone tissue on a scale from 1 to 10.

Immunohistochemical analysis

5 µm-thick sections taken from paraffin blocks with the assistance of a microtome were then placed on polylysine slides (Sigma, St. Louis, MO, USA). After the dehydration phase, microwave application was made in citrate solution with a pH of 6 (Carlo Erba, 368057). Sections were kept in 3% hydrogen peroxide (Dako, Glastrup, Denmark) to prevent endogenous peroxidase activity. After blocking with normal goat serum, the sections were incubated with primary antibodies, which are type I collagen (ColI) and type III collagen (ColIII) antibodies (1:100, Sigma, USA). After the biotin-labeled secondary antibody (Abcam, UK) step, the tissues were treated with the streptavidin-HRP kit (Santa Cruz, USA) and were finally treated with 0.05% diaminobenzidine (Zymed Histostain Plus CA, USA). All rinses during the staining process were done with phosphate-buffered saline (PBS). Immunohistochemical staining results were evaluated with an H-score. The staining ratio was graded semi-quantitatively [0 = staining in less than 1% of cells, 1+ = staining in (1–10) % of cells, 2+ = staining in (11–50) % of cells, 3+ = staining in (51–80) % of cells, 4+ = staining in more than 80% of cells]. Staining intensity was also determined by the blind method (0 = no staining, 1 = pale, 2 = moderate, 3 = intense). Then, the total score was calculated with the formula "(1+ staining intensity / 3) × staining ratio".

Biomechanical evaluation

Biomechanical testing was conducted utilizing an Elista TST 2500 material testing machine (Elista, Istanbul, Turkey). A 3-point bending test was applied to a total of 16 femur bones (8 of them in the study group and 8 of them in the control group). The applied force was increased until refracture occurred (Fig. 2). The load (N) at the time of refracture, the total energy (J) applied until refracture, the maximum stresses (MPa) on the femurs, and the stiffness (N/mm) values of the femurs were recorded.

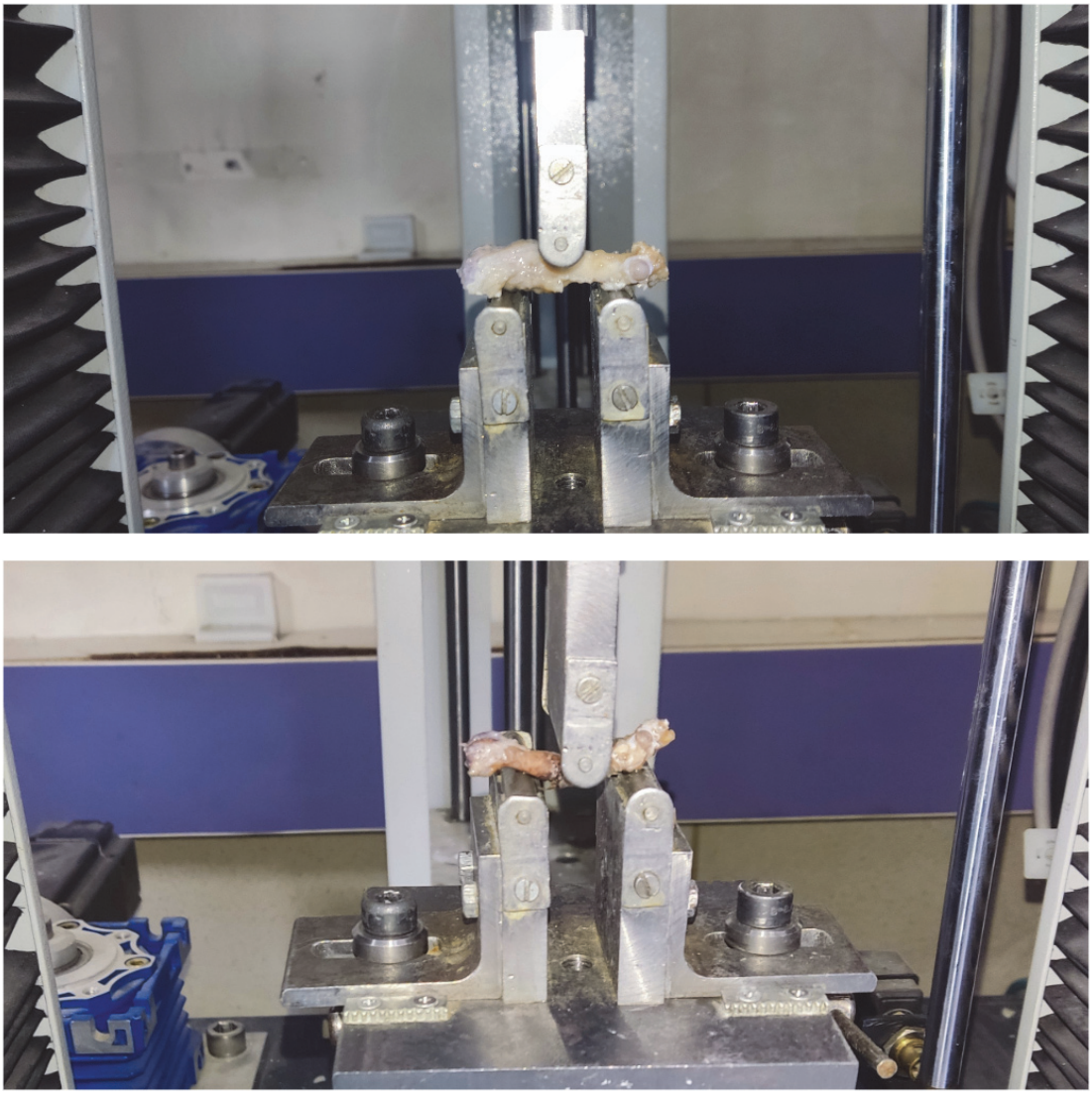


Fig. 2. Application of the 3-point bending test and subsequent refracture in the femur

Statistical analysis

The statistical package program utilized for the calculations was IBM SPSS version 22.0 (IBM Corporation, New York, USA). The descriptive statistics were presented as the mean plus or minus the standard deviation (SD), or as the median for continuous variables. The Shapiro–Wilk test was employed to assess the normality of continuous variables. After confirming that the assumption of normality was not met, the Mann–Whitney *U* test was used to compare the study and control groups. *P*-values of <0.05 and <0.10 were considered statistically significant and borderline statistically significant, respectively.

Results

The ratios of low-density bone areas (LDBA) showing callus tissue and high-density bone areas (HDBA) showing intact bone tissue obtained on CT images are given in Table 1. This ratio was, on average, 2.89 ± 0.58 in the VA group and 2.19 ± 0.53 in the control group. It was observed that the LDBA/HDBA ratio was significantly higher in the VA group (p = 0.031). In addition, CT images show that the callus tissue in the VA group is more radiopaque, as in Fig. 1. This image shows that the VA group was ahead in fracture healing compared to the experimental group.

Histological scores according to the Huo classification are given in Table 1. According to this scoring, the average score of the VA group was 8.12 ± 0.64, and the average score of the control group was 3.12 ± 1.45. It was observed that histological scores were significantly higher in the VA group compared to the control group (p = 0.001) – Fig. 3.

H-score values for ColI and ColIII antibody stainings obtained from immunohistochemical imaging are given in Table 1. The average H-score in ColI antibody staining was 115.62 ± 3.34 in the VA group and 115.62 ± 3.34 in the control group, and there was no significant difference between the two groups in this respect (p = 1.000). The average H-score in ColIII antibody staining was 20.41 ± 16.08 in the VA group and 54.99 ± 38.62 in the control group, and a borderline significant difference was observed between the two groups in this respect (p = 0.078). As seen in Fig. 4, widespread immunopositive staining was observed in the callus area with ColI antibody in both the VA group and the control group. As seen in Fig. 5, in the immunohistochemical ColIII antibody staining, an immunonegatively stained callus area was seen in the VA group, while focal immunopositive staining was seen in the callus area in the control group (the lower right part of the callus area is immunopositive, the lower left part is immunonegative).

In biomechanical analyses, the values obtained with the three-point test until refracture occurs are shown in Table 1. The force required to be applied for refracture (failure load) was an average of 50.25 ± 6.06 N in the VA group and an average of 41.75 ± 7.92 N in the control group. It was observed that the amount of failure load was significantly higher in the VA group compared to the control group (p = 0.040). The average total energy required for refracture was 189.00 ± 14.38 joules in the VA group and 169.87 ± 15.80 joules in the control group. It was observed that the amount of total energy required for refracture was significantly higher in the VA group compared to the control group (p = 0.021). The maximum stress occurring in the femurs was an average of 180.25 ± 13.99 megapascals in the VA group and an average of 160.12 ± 13.03 megapascals in the control group. It was observed that the maximum stress on the femurs was significantly higher in the VA group compared to the control group (p = 0.015). The stiffness values of the femurs were an average of 175.25 ± 10.30 N/mm in the VA group and an average of 163.25 ± 9.67 N/mm in the control group. It was observed that the stiffness values of the femurs were significantly higher in the VA group compared to the control group (p = 0.035).

Table 1. The results of histological, radiological, and biomechanical parameters

	Control group		VA group		*p*-value
	Mean ± SD	Min–Max	Mean ± SD	Min–Max	
Histological analysis					
Huo score	3.12 ± 1.45	2–6	8.12 ± 0.64	7–9	**0.001**
Immunohistochemical analysis					
ColI	115.62 ± 3.34	112.50–118.75	115.62 ± 3.34	112.50–118.75	1.000
ColIII	54.99 ± 38.62	6.66–113.33	20.41 ± 16.08	3.33–40.00	**0.078**
Radiological analysis					
LDBA/ HDBA ratio	2.19 ± 0.53	1.42–3.20	2.89 ± 0.58	1.85–3.62	**0.031**
Biomechanical analysis					
Failure load (N)	41.75 ± 7.92	35–56	50.25 ± 6.06	41–58	**0.040**
Total energy (J)	169.87 ± 15.80	142–156	189.00 ± 14.38	156–202	**0.021**
Maximum stress (MPa)	160.12 ± 13.03	142–178	180.25 ± 13.99	154–194	**0.015**
Stiffness (N/mm)	163.25 ± 9.67	148–176	175.25 ± 10.30	156–188	**0.035**

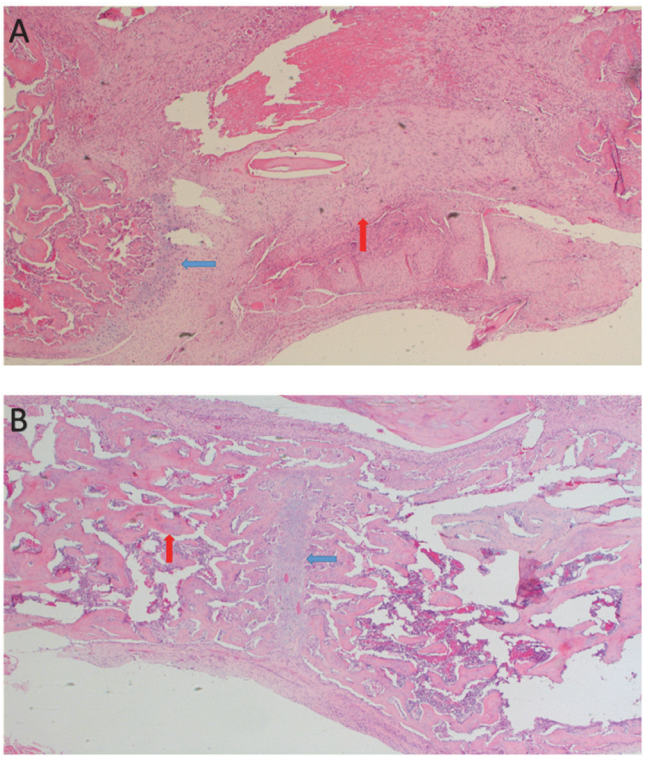
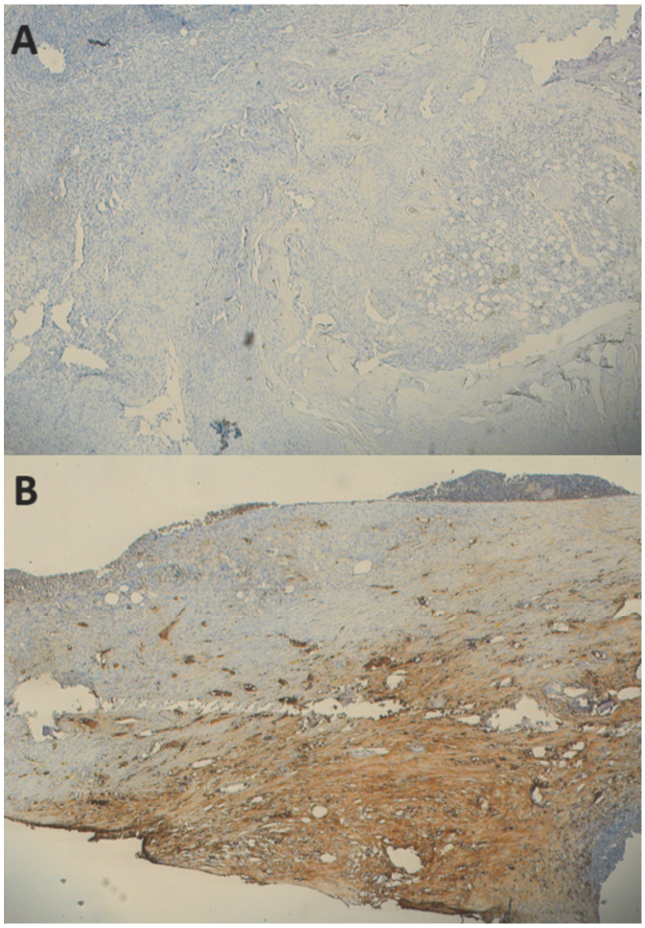


Fig. 3. (**A**) Predominantly fibrous tissue with a small amount of cartilage (red arrow: fibrous tissue, blue arrow: cartilage; H&E ×100); (**B**) Predominantly immature bone with a small amount of cartilage (red arrow: immature bone, blue arrow: cartilage; H&E ×100).



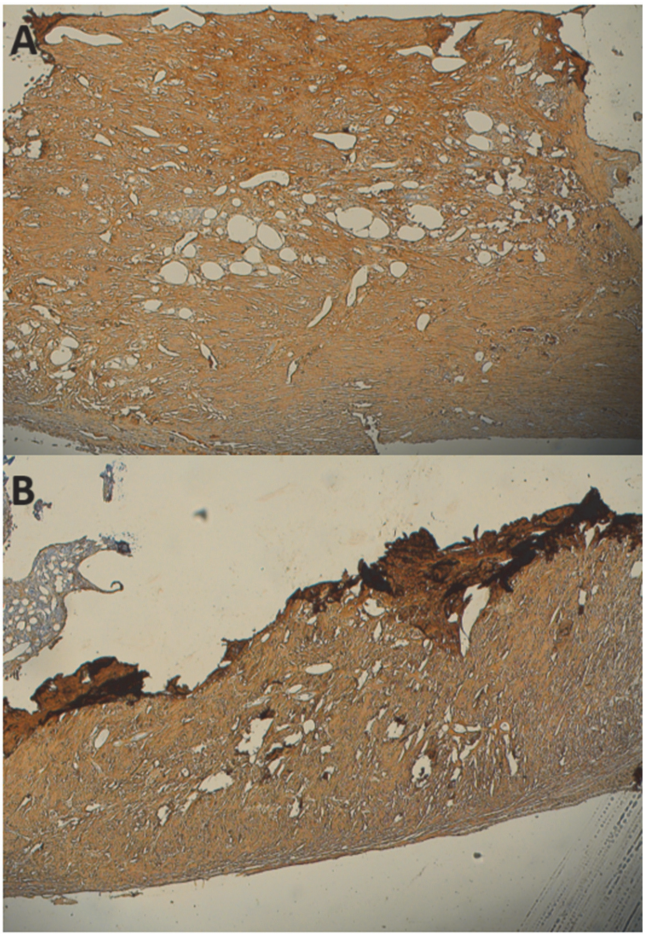


Fig. 4. (**A**) In the VA group, widespread immunopositive staining in the callus area with immunohistochemical ColI; (**B**) In the control group, widespread immunopositive staining in the callus area with immunohistochemical ColI.

Fig. 5. (**A**) In the VA group, the callus area stained immunonegatively with immunohistochemical ColIII; (**B**) In the control group, focal immunopositive staining in the callus area with immunohistochemical ColIII (in the callus area, the lower right part is immunopositive, the lower left part is immunonegative).

Discussion

Our study was conducted based on the hypothesis that VA, which is a powerful antioxidant, would strengthen fracture healing by reducing oxidative stress in rats with an open femur fracture model. Using the histological scoring of fracture healing as defined by Huo et al. (1991), increased fracture healing was observed in the VA group compared to the control group. In immunohistochemical analysis, no significant difference was observed in ColI antibody staining between the two groups. While there was no staining with ColIII antibody in the VA group, localized immunopositive staining was observed in the control group.

Radiologically, the amount of callus tissue formation was significantly higher in the VA group compared to the control group. In femurs subjected to a biomechanical three-point bending test, the forces required for refracture were found to be significantly higher in the VA group compared to the control group. Although we found that VA increases fracture healing in all three parameters (radiologically, histologically, and biomechanically), we do not currently know the mechanism of this situation at the molecular level. Although this study is the first to examine the effect of VA on fracture healing, the findings that it has a positive effect in terms of these three parameters (radiologically, histologically, and biomechanically) give hope for its use in comorbidity situations where fracture healing is difficult, or in treatments for fracture nonunion.

In our study, we observed that the mean values obtained according to the classification developed by Huo et al. (1991) were significantly higher in the VA group compared to the control group. These results suggest that VA accelerates the fracture healing process. We think that VA shows activity against oxidative stress that occurs after the open fracture model through the hydroxyl group it contains. Another finding that supports this situation are the ColIII antibody staining results. We found that ColIII antibody staining showed an immunonegative reaction in the VA group, while it showed a localized immunopositive reaction in the control group. Because type III collagen is densely found in the mesenchymal cell cluster or bone marrow stroma at the beginning of chondrogenesis and osteogenesis (Apaja-Sarkkinen et al., 1986; Bentley et al., 1981; Volk et al., 2014), it is stated in the literature that type I collagen is abundant in mature bone, while type III collagen is absent (Miller, 1973; Müller et al., 1977).

Additionally, in biomechanical tests and radiological analyses, we found that the fracture healing in the VA group was more stable compared to the fracture healing in the control group. Based on these findings, we can say that VA application increases fracture healing and accelerates this process. We can say that the correlation between all these parameters may indicate the positive effects of VA in cases where oxidative stress is high and fracture union is difficult (such as diabetes or malnutrition).

There are studies in the literature indicating that VA shows estrogenic activity (Circosta et al., 2006; Zhang et al., 2011). In their study on a cell model, Xiao et al. (2014) stated that VA, as an estrogen analogue, showed an osteoprotective effect by inducing osteoblastic activity. Since the estrogen hormone plays a critical role in maintaining bone mineralization and the remodeling (turnover) process by regulating the osteoclastic-osteoblastic balance, compounds that act as estrogen analogues, such as VA, can be considered to have an osteoprotective effect. We believe that further studies are needed to elucidate the effectiveness of VA on bone tissue at the molecular level.

Wang et al. (2017) examined the antiosteoporotic activity of VA in ovariectomized rats. They stated that, after ovariectomy, the decrease in body weight observed in the rats in the control group was not seen in the VA-supplemented study group, and that there was a decrease in bone turnover markers [calcium (Ca), phosphorus (P), osteocalcin (OC), alkaline phosphatase (ALP), and deoxypyridinoline (DPD)] and inflammatory markers (IL-1β, IL-6, and TNF-α) indicating osteoclastic activity in the VA-supplemented study group. They also stated that there was an increase in the biomechanical stability of bone tissue and bone densitometry in the VA-supplemented study group. Although this study, unlike ours, was examining osteoporosis, the common conclusion of both is that VA has an osteoprotective effect on bone tissue. The fact that the biomechanical stability of the bone in the VA-supplemented groups was significantly higher compared to the control group in both studies can be considered an indication of this situation. Osteoporosis and open fracture are both pathological conditions that create oxidative stress in bone tissue, and we need antioxidant agents to combat this pathophysiology.

There are some limitations in our study. First, we do not have the opportunity to determine the absorption or concentration in the bloodstream of VA administered to rats by gavage. Therefore, it is not possible to estimate precisely how much VA has an effect on fracture healing. One of the shortcomings of our study is that components such as osteoblasts and CD34+ cells, which are important components for examining fracture healing at the molecular level, are not examined in detail. The small sample size for both the control and study groups (eight for each group) is a factor that may affect the results of the study. However, these numbers have been limited, considering animal rights and ethical concerns. On the other hand, although it contributes to the literature as the first study examining the effect of VA on fracture healing, our study cannot be compared with another study on this subject.

Conclusion

We observed histologically, immunohistochemically, radiologically, and biomechanically that VA, with its antioxidative properties, increased fracture healing in rats with an open fracture model. We are hopeful that such an antioxidant, which is common in nature, will increase fracture healing. VA may be an adjunct agent in the treatment of nonunion, but this study is the first to examine its effect on fracture healing. Larger studies are needed to examine the effect of VA on fracture healing and to investigate the mechanisms related to its activity at the molecular level.

IRB ethics statement

The study protocol was approved by the Selçuk University Experimental Medicine Application and Research Center Animal Experiments Ethics Committee with protocol number 2023/51.

Conflict of interest

The authors have no conflict of interest to declare.

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