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ORIGINAL ARTICLE

The effect of quercetin on bone healing in an experimental rat model

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Fracture healing, which is a unique regenerative process including complex interactions between various anatomical, biomechanical and biochemical processes that begins with inflammation after injury and ends with osteogenesis,[1,2] repairs the physical and mechanical properties of the tissue and this process can be influenced by number of systemic and local factors.[3] During the bone healing process, free radicals and antioxidants are in balance. However, due to the weakening of antioxidant mechanisms for various reasons, an increase in reactive oxygen species (ROS) can be observed and this condition is characterized as oxidative stress and usually indicates a deficiency of antioxidant factors and a high level of oxidative damage markers. It is known

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ABSTRACT

Objectives: This study aims to evaluate the effect of quercetin on fracture healing in an open fracture model in rats.

Materials and methods: A total of 80 Wistar-Albino male rats were used in this study. The rats were divided into 10 groups. Daily oral treatment of 100 mg/kg of quercetin dissolved in corn oil were given to four groups, whereas the other four group of control rats were treated with corn oil only. Histopathological and radiological examinations of fracture healing were performed at the end of Weeks 2 and 4 in these rats, while biomechanical and biochemical examinations were performed at the end of Weeks 4 and 6, since harder callus was required. Among the rats in the last two group that were not subjected to the open fracture model, one group was given only quercetin for three weeks and the other for six weeks, and the biochemical markers in the blood were compared between these two groups. Computed tomography images were taken for radiological evaluation. The modified Lane and Sandhu scoring system was used for histological evaluation. The 3-point bending test was performed for biomechanical evaluation. For biochemical evaluation, plasma alkaline phosphatase (ALP), acid phosphatase (AP), total antioxidant status (TAS) and total oxidant status (TOS) levels were measured.

Results: Radiologically, there was no significant difference between the early-stage results of quercetin and control groups (p=0.247), while quercetin caused a significant increase in callus tissue in terms of late-stage results (p=0.012). Histopathologically, there was no significant difference in the early stage (p=0.584); however, in the late stage, a borderline significant increase was observed in the quercetin group compared to the control group (p=0.091). Biomechanical analysis showed that quercetin significantly increased the fracture strength in the healing bone both in the early period (p=0.036) and in the late period (p=0.027). Among biochemical markers, TOS and AP were found to be significantly decreased in the quercetin group. In the non-operated and quercetin given groups, TAS levels was significantly higher (p=0.001) and AP levels were borderline significantly lower at the end of Week 6 (p=0.063).

Conclusion: Quercetin did not have a significant effect on bone healing in the early period, but significantly promoted bone healing in the late period in rats. We recommend the use of quercetin, a strong antioxidant, in cases with high oxidative stress and conditions such as diabetes, smoking, and malnutrition which may inhibit fracture union, although further clinical studies are needed to confirm these findings.

Keywords: Bone healing, fracture union, open fracture, quercetin, rat.

that these reactive oxygen radicals have effects on the pathogenesis of bone loss by causing inhibition of angiogenesis, bone resorption, and osteoclast differentiation.[4,5]

Quercetin, on the other hand, is a yellow crystalline solid substance with a bitter taste and is the most abundant naturally occurring flavonoids. It has anti-inflammatory, anti-tumoral, anti-diabetic, and anti-microbial properties.[6] However, since its antioxidant feature is more prominent than other flavonoids, recent studies on quercetin have emphasized its antioxidant feature.[7]

Despite the incredible regeneration ability of the bone, complications such as nonunion, delayed union or malunion can be seen in 2 to 30% of the fracture union process.[8] Defective union secondary to the increased free radical formation during the fracture healing process also may occur. Oxidative stress, which causes an imbalance associated with osteoclastogenesis, inhibits fracture healing by increasing osteocyte apoptosis. Changes in ROS and/or antioxidant pathways are critical in the process of fracture union; therefore, antioxidants such as quercetin may help to enhance the healing of broken bones.

In the current study, we hypothesized that quercetin, a powerful antioxidant, had a positive effect on fracture healing. We, therefore, aimed to evaluate the effect of quercetin, a strong antioxidative flavonoid, on fracture healing in an experimental rat model.

MATERIALS AND METHODS

In this study, a total of 80 Wistar-Albino male rats weighing between 450 and 500 g which completed their sixth month were used. Ten groups, including eight rats, were formed to perform biomechanical, histological, radiological and biochemical analyses in the early and late phases of bone healing. The groups were given names from A to J and the procedures applied to the groups are summarized in Table I.

Surgical technique

The entire surgical procedure was performed under general anesthesia after injection of 50 mg/kg ketamine hydrochloride (Ketalar® 500 mg; Pfizer Pharmaceuticals, Istanbul, Türkiye) and 10 mg/kg xylazine (Rompun® 2% Bayer Pharmaceuticals, Istanbul, Türkiye). All rats were operated without pain, with additional doses of anesthetics when necessary. During surgery, the operation area was shaved and prepared with povidone-iodine (Batticon®, Adeka Pharmaceuticals, Samsun, Türkiye) under sterile conditions. A 2-cm longitudinal incision was made on the anterolateral thigh to expose the femurs and the subcutaneous tissue was retracted. After separating vastus lateralis and rectus femoris with blunt dissection, the femoral diaphysis was reached. A transverse femoral shaft fracture was performed using a micro circular saw. A 1-mm Kirschner wire (K-wire) was inserted into the knee joint from the medial side of the patella, and the fracture was stabilized by advancing it from the intercondylar fossa to the trochanteric region. The K-wire, which was placed proximally in the femoral trochanter cortex, was embedded into the intercondylar fossa distally without discomforting the knee joint. After providing bleeding control, the skin and subcutaneous tissues were closed (Figure 1).

Inadequate fracture reduction, improper positioning of the implant, and infection in the

TABLE I
Study groups (I and J Groups not operated, all other groups operated)

Group	Features	Number of rats	Sacrifice time (Week)
A	Early histology-radiology control group	8	2
B	Early histology-radiology quercetin group	8	2
C	Early biomechanics-biochemistry control group	8	3
D	Early biomechanics-biochemistry quercetin group	8	3
E	Late histology-radiology control group	8	4
F	Late histology-radiology quercetin group	8	4
G	Late biomechanics-biochemistry control group	8	6
H	Late biomechanics-biochemistry quercetin group	8	6
I	Non-operated group given only quercetin	8	3
J	Non-operated group given only quercetin	8	6

fractured extremity were considered exclusion criteria. No bandages or casts were applied to the rats postoperatively, and all rats were allowed to perform joint movement and early weight-bearing on the operated thigh side after surgery. The rats in Groups A, C, E, G, I, and J were given 100 mg/kg/day of quercetin (Sigma-Aldrich Inc., MO, USA) as a suspension in corn oil via oral gavage same time daily until sacrificed. Other control groups were given only corn oil via oral gavage. Quercetin dose was adjusted based on previous studies.[9]

The rats were euthanized performing cervical dislocation after high-dose anesthesia. The femurs of the sacrificed rats were separated from other soft tissues, leaving only the broken callus tissue.

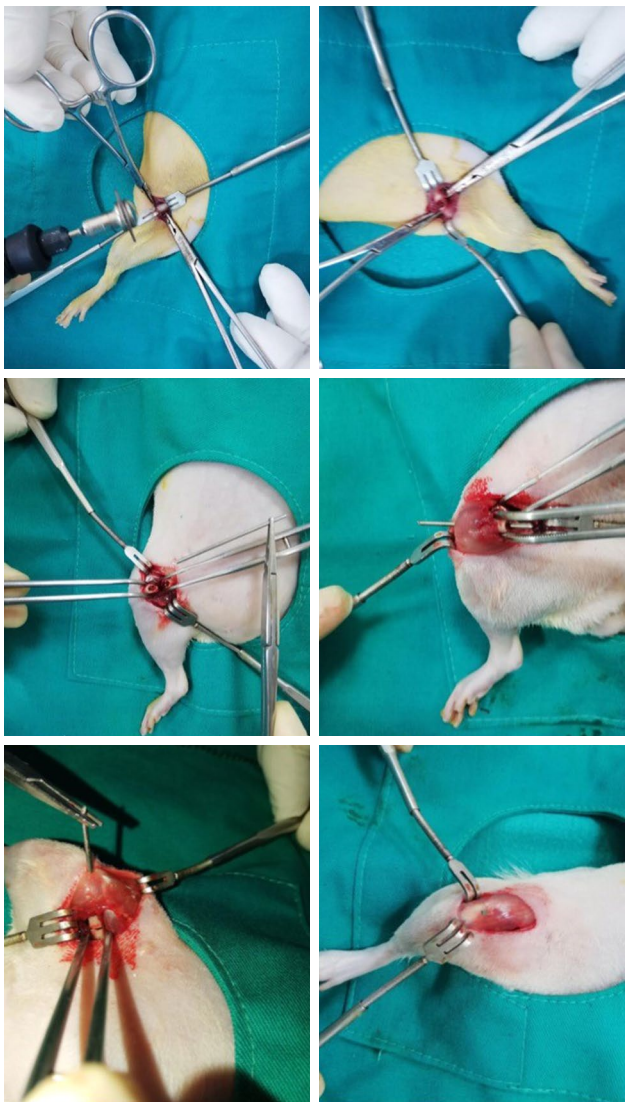

FIGURE 1. Steps taken while creating an open fracture model.

All right femurs were examined biomechanically, radiologically and histologically and, after sacrification, intracardiac blood samples were drawn to investigate biochemical markers.

Early and late evaluation of broken callus tissue for the histological and radiological examination groups were performed at the end of two weeks (early) and four weeks (late) after surgery. The groups used for biomechanical test and biochemical marker evaluation were investigated at the end of three weeks (early) and six weeks (late), since sufficient calcification was required to perform the 3-point bending test. The femurs of rats in Groups A, B, E, and F were utilized for radiological and histopathological evaluation, and blood and femurs of rats in Groups C, D, G, and H were utilized for biomechanical and biochemical evaluation. In the non-operated groups (Groups I and J), the markers were measured in the intracardiac blood taken after sacrification.

Radiological evaluation

Thirty-two femurs of rats in Groups A, B, E, and F were utilized for radiological evaluation. A total of 256-slice 2-section dual source computed tomography (CT) was used for radiological imaging and K-wires were left in the intramedullary area, as it did not affect the evaluation. RadiAnt DiCOM Viewer version 2020.2.3 software (Medixant, Poznań, Poland) was used for the measurements of the CT images (Figure 2).

Since hard callus formation in the femurs obtained from the rats sacrificed at the end of the second postoperative week was insufficient for radiological evaluation, callus diameter/femoral diameter measurements were performed for analysis. Since adequate calcification of the fracture callus was achieved in the femurs of the rats sacrificed at Week 4 after the operation, radiological analysis was performed based on the ratio of low radiodensity areas (callus)/high radiodensity areas (fractured femur).[10,11]

Histopathological evaluation

Thirty-two femurs of rats in Groups A, B, E, and F were utilized for histopathological evaluation following CT taken on the same day. Each of the 32 femurs was placed in 10% formalin solution for fixation and, then, 10% acetic acid for decalcification. Then, 3-μm sections per block were taken from the paraffin-embedded blocks followed by staining with hematoxylin-eosin. In the histological examination, fracture healing was scored for each section according to the histological scale of the modified Lane and Sandhu criteria (Figure 3). This scoring system includes

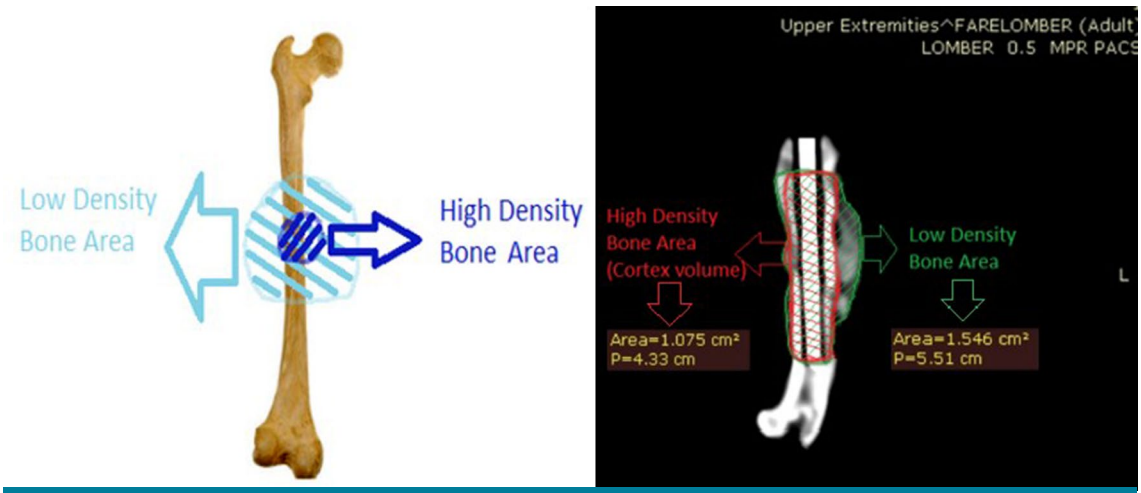

FIGURE 2. Measurement of micro-computed tomography longitudinal sections of newly formed callus tissue in the femur after sacrification.

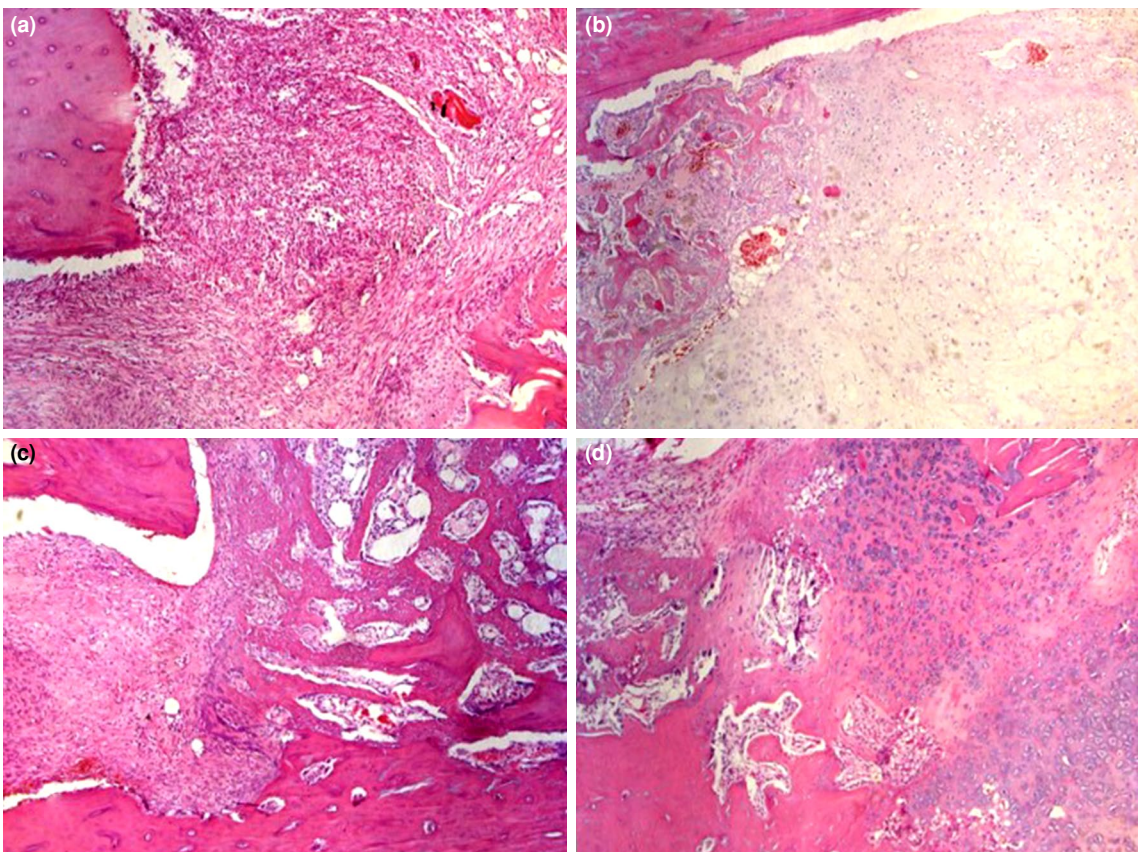

FIGURE 3. (a) Fibrous union findings at the control group fracture site in the second week (H&E, ×100). Connective tissue areas and lymphocytic cell infiltration are seen between bone trabeculae. **(b)** Findings of osteochondral union at the quercetin group fracture site in the second week (H&E, ×100). Enchondral ossification areas consisting of cartilage structures and woven type immature bone tissue are seen. **(c)** At the fourth week, bony union findings at the fracture site of the control group (H&E, ×100). Among the bone trabeculae, ossification areas formed in woven type immature bone tissue are seen. **(d)** At the fourth week, signs of osteochondral union at the quercetin group fracture site (H&E, ×100). Between the bone trabeculae, enchondral ossification areas consisting of woven type immature bone tissue and cartilage areas are seen.

comprehensive evaluation of callus tissue, cortex at the fracture line, and cancellous bone marrow, and it has been extensively used in previous fracture healing studies.[12,13]

Biomechanical evaluation

A total of 32 femurs obtained from rats in Groups C, D, G, and H were performed 3-point bending tests (Figure 4), and the measured values were recorded in Newton (N). The applied force was increased, until the fracture re-occurred at the healing tissue of the femur line with osteotomies. For each femoral specimen, the applied load parameters until the fracture occurred were recorded. In a previous study, the 3-point bending test was not found to be appropriate, as there was not enough calcification in the healing tissue for two weeks.[14] Therefore, we determined the timing of the biomechanical analysis of early bone healing as the third week and the late bone healing as the sixth week.

Biochemical evaluation

Intracardiac blood samples taken from the rats in the operated groups of C, D, G, and H and in the non-operated groups of I and J were centrifuged and, then, plasma alkaline phosphatase (ALP), acid phosphatase (AP), total antioxidant status (TAS) and total oxidant status (TOS) were measured. Alkaline phosphatase has been shown to be elevated during bone formation.[15] On the other hand, AP has been demonstrated to have a major role during bone resorption.[16] In the current study, the effects of quercetin on bone formation and resorption during bone healing were examined based on these two markers.

To investigate the effects of antioxidant properties of quercetin on fracture healing, instead of separate measurements of antioxidant molecules, after the blood samples from each rat were centrifuged, TAS and TOS levels were also measured using the Rel Assay Diagnostics kit (Mega Tip, Gaziantep, Türkiye).[17] Using these two markers, we evaluated the effects of quercetin on oxidative stress during bone healing in an open fracture model.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The conformity of the data to the normal distribution was evaluated using the Shapiro-Wilk ($n<50$) test. The Mann-Whitney U test was applied, as the data did not show a normal distribution. *P* values of <0.05 and <0.10 were considered statistically significant and borderline statistically significant, respectively.

FIGURE 4. Biomechanical evaluation, refraction of femur specimens by 3-point bending test.

RESULTS

Two weeks after the fracture model was created, there was no significant difference between the rats given quercetin and the control group in terms of radiological analysis results ($p=0.247$). At the end of Week 4, the low-density bone areas indicating the callus tissue in which calcification started to form and high-density bone areas indicating healthy bone tissue were calculated in cm^2 and their ratios were determined (Table II). The ratio of low radiodensity areas (calls)/high radiodensity areas (broken femur) was significantly higher in rats given quercetin than in rats that were not given quercetin ($p=0.012$). In Figure 5, the mean values obtained from the quercetin and control groups are shown graphically. Accordingly, no significant difference was observed between quercetin-treated and non-treated groups in the early period of bone healing (at the end of Week 2), while a larger amount of callus tissue was seen in the group given quercetin in the late period (at the end of Week 4).

TABLE II
Post-radiology, histology and biomechanical evaluation scoring of open fracture femurs between quercetin and control groups by week

		n	Mean±SD	Median	Max-Min	*p*
Radiological	Quercetin W: 2	8	2.99±0.34	2.01	2.41-1.48	0.247
	Control W: 2	8	1.83±0.20	1.82	2.07-1.46	
	Quercetin W: 4	8	2.81±0.38	2.70	3.64-2.42	0.012
	Control W: 4	8	2.13±0.60	2.09	3.33-1.37	
Histological	Quercetin W: 2	8	4.25±0.46	4	5-4	0.584
	Control W: 2	8	4.12±1.88	5	6-1	
	Quercetin W: 4	8	8.62±1.84	9	11-6	0.091
	Control W: 4	8	7.12±1.24	7	9-6	
Biomechanic	Quercetin W: 3	8	25.04±3.94	26.623	28.634-17.062	0.036
	Control W: 3	8	20.70±3.94	21.770	25.534-11.179	
	Quercetin W: 6	8	68.14±27.56	64.06	106.00-34.81	0.027
	Control W: 6	8	56.66±22.32	55.55	95.70-31.08	

D: Standard deviation; p: Statistical significance level; W: Week from fracture to sacrification.

All scores of histological examinations are given in Table II. The mean of the histological scorings of the groups is shown in the graph in Figure 5. There was no significant difference in the early stage (p=0.584); however, in the late stage, a borderline significant increase was observed in the quercetin group compared to the control group (p=0.091).

Maximum resistance to 3-point bending tests for biomechanical analysis is shown in Table II. The mean resistance graph of the groups is shown in Figure 5. Statistical analysis of 3-point bending test results between the rats given quercetin and those not given quercetin at both Week 3 (p=0.036) and Week 6 (p=0.027) showed that quercetin significantly increased resistance to refracture. Accordingly, quercetin increased union in both early and late stages in fracture healing.

The mean values of ALP, AP, TAS, and TOS levels of the non-operated group given quercetin for three weeks (NQ 3. W), the non-operated group given quercetin for six weeks (NQ 6. W), and the operated groups are given in Table III. Among these markers examined in blood taken from Groups C and D, only TOS level was found to be lower in Group D than in Group C (p=0.037), while no significant difference was observed in other markers. The TOS and AP levels were lower in Group G than in Group H (p=0.018 and p=0.013, respectively), while no significant difference was observed in the TAS and ALP levels. There was no significant difference in the TOS and ALP in the blood taken after sacrification between Group 1 and Group J (p>0.1). The TAS levels were found to be significantly higher (p=0.001) and AP levels was found to be borderline significantly lower (p=0.063) in Group J than Group 1.

DISCUSSION

During fracture healing, osteoclast and inflammatory cells cause ROS production and oxidative stress.[18,19] Antioxidant enzymes should eliminate these reactive oxygen products without damaging the cellular components to complete the fracture healing phases and maintain union, since the ROS interferes with the task of osteoblasts by reducing the activity of cells vital for fracture healing, such as osteoprogenitors and angioprogenitors.[20,21]

In the present study, we investigated the effects of quercetin on fracture healing in rats following an open model of femoral fracture injury. The reason for using this fracture model in our study is that immobilization is not required after fracture fixation, allowing early weight-bearing without limitation of joint movements. Our study results showed that quercetin has positive effects on fracture healing, which is the main subject of orthopedic surgeons, and can be added to the diet as a supplement.

In a study on osteoblasts exposed to cigarette smoke *in vitro* by Braun et al.,[22] quercetin reduced reactive oxygen derivatives. The authors reported that quercetin inhibited reactive oxygen derivatives that damaged osteoblasts by increasing superoxide dismutase and heme-oxygenase

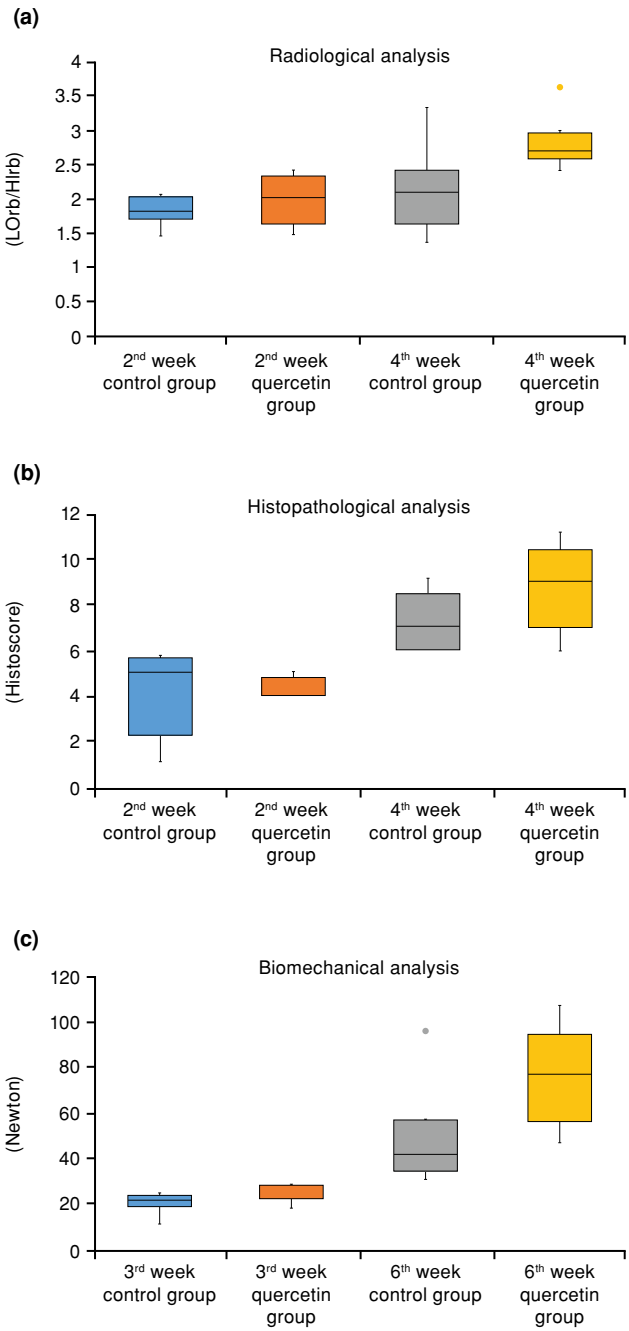

FIGURE 5. (a) Low radiodensity bone/High radiodensity bone ratio (LOrb/Hlrb). **(b)** Histoscore according to the histological scale of the modified Lane and Sandhu criteria. **(c)** Refraction with the 3-point bending test (Newton).

expression and concluded that dietary supplementation with quercetin could improve the bone quality, stability, and even fracture healing in individuals who smoke. Osteoblasts have a critical function in fracture healing and preservation of skeletal structure. Indeed, our study suggests that quercetin increases bone healing by reducing the impact of free oxygen metabolites formed in rats with a fracture model.

In their study, Liang et al.[23] examined the effect of different amounts of quercetin on streptozotocin injection-induced diabetic osteopenia. The 3-point bending test performed on the femurs of sacrificed rats at the end of Week 12 showed that diabetic rats given quercetin had a significantly higher resistance to fracture than diabetic rats given saline. The authors concluded that quercetin could relatively reverse the impaired microarchitecture and poor bone biomechanical quality of rat femurs due to osteopenia. Similarly, we found that the resistance strength against the 3-point bending test was significantly higher at the end of Weeks 3 and 6 in the operated femurs. However, while this study showed the efficacy of quercetin on intact bones, we examined the efficacy of quercetin in rats with a femoral fracture model.

The effects of oral quercetin on bone tissue were investigated in the osteoporosis animal model developed by Tsuji et al.[24] The authors compared the bone mineral density of the lumbar spine and femur and reported that the density was higher in the quercetin group. However, the lack of 3-point bending test for biomechanical analysis limited the effectiveness of this study. Similarly, in our study, the group given quercetin had positive effects on bone tissue in fracture healing. In addition, in our study, a 3-point bending test was performed and biomechanical analysis was also performed.

In another study by Wong and Rabie,[25] local influence of mixture of quercetin and collagen matrix and only collagen matrix administration were compared in a rabbit model of parietal bone defects. There was a total of 556% more new bone formation in bone defects treated with a mixture of quercetin and collagen matrix than in those treated with a collagen matrix alone. Accordingly, the authors concluded that quercetin with collagen matrix had an impact to increase new bone formation locally and could be used as a bone graft material. However, the aforementioned study includes only histological analysis and the absence of radiological, biomechanical and biochemical analyses limits the study. For histological analysis, instead of examining all of the new bone tissue formed in the area where the defect was created, sampling randomly from different parts of the defected region reduced the standardization of the study.

Studies on the antioxidant, anti-inflammatory, anti-diabetic and neuroprotective properties

TABLE III

P value after comparison of TOS, TAS, ALP, AP mean values between quercetin and control groups by week in blood samples taken from rats with and without open fracture

	TAS	TOS	ALP	AP
Control W: 3	108	14.63	1.43	40.81
Quercetin W: 3	150.75	8.8	1.71	38.78
Control W: 6	129.85	9	1.51	37.11
Quercetin W: 6	169.16	6.45	1.75	11.82
NQ W: 3	162.97	6.14	1.51	20.12
NQ W: 6	324	4.98	1.55	11.27

	Marker examined in blood	Compared groups	*p*
Rats with open fracture model	TOS	W: 3 Quercetin & control	0.037
		W: 6 Quercetin & control	0.018
	TAS	W: 3 Quercetin & control	0.734
		W: 6 Quercetin & control	0.116
	ALP	W: 3 Quercetin & control	0.317
		W: 6 Quercetin & control	0.247
	AP	W: 3 Quercetin & control	0.567
		W: 6 Quercetin & control	0.013
Rats that are not operated and only given Q	TOS	Quercetin W: 3 & W: 6	0.886
	TAS	Quercetin W: 3 & W: 6	0.001
	ALP	Quercetin W: 3 & W: 6	0.918
	AP	Quercetin W: 3 & W: 6	0.063

TOS: Total oxidant status; TAS: Total antioxidant status; ALP: Alkaline phosphatase; AP: Acid phosphatase; NQ 3.W: Non-operated group given only 3 weeks of quercetin; NQ 6.W: Non-operated group given only 6 weeks of quercetin.

of quercetin have been conducted and are still continuing. Studies investigating the effectiveness of quercetin on bone tissue have mostly focused on its effects on osteoporosis. Huang et al.,[26] reviewed 19 studies investigating the effects of quercetin on osteoporosis and reported that quercetin could reverse osteoporosis-related osteopenia and could be added to the diet in the treatment of clinical osteoporosis. Although a meta-analysis examining the effect of quercetin on osteoporosis has been found in the literature, there is no study to investigate its effect on bone fracture healing. In this respect, the present study is the first experimental study examining the effects of quercetin on fracture healing.

In the early-stage biochemical, histological and radiological analyses of quercetin in fracture healing, quercetin showed no significant effect. We believe that this is because the anti-inflammatory property of quercetin prevents inflammation, which is the initial stage of bone healing. In the late stage of fracture healing, quercetin increased union in all biomechanical, biochemical, histological, and radiological examinations performed in our study. Based on these findings, we can speculate that quercetin increases fracture healing by reducing oxidative stress.

Nonetheless, there are some limitations to our study. First, it is unclear that the bioavailability of quercetin is equal in all rats. Second, we examined the radiological and histological analyses at the end of Weeks 2 and 4 after the operation. Since hard callus tissue did not form, we examined the biochemical and biomechanical analyses at the end of Weeks 3 and 6 after the operation. It would be better to examine all operated rat femurs at the end of the same period in terms of standardization of the study. However, we already hypothesized that dietary quercetin might positively affect fracture healing; therefore, we believe that the effect of this limitation is low.

In conclusion, our study showed that quercetin did not have a significant effect on bone healing in the early period, but significantly promoted bone

healing in the late period in rats. We recommend the use of quercetin, a strong antioxidant, in cases with high oxidative stress and conditions such as diabetes, smoking, and malnutrition which may inhibit fracture union. However, there are no clinical studies investigating quercetin on bone tissue in humans. Therefore, further clinical studies are needed to examine the role of quercetin on fracture healing.

Ethics Committee Approval: The study protocol was approved by the Selçuk University Experimental Medicine Application and Research Center Animal Experiments Ethics Committee (date: 27.03.2020, no: 2020-16).

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Conceptualization, methodology,software, data curation, writing, original draft: A.Y.; Methodology, project administration: A.Y.; Histopathological analysis: Z.E.Ç.; Biochemical analysis: H.V.; Radiological analysis: M.S.D.

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