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Effect of curcumin on bone healing: An experimental study in a rat model of femur fracture

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ABSTRACT

Objective: To determine the radiologic, histologic and biomechanical effects of curcumin on bone healing using a total rat femur fracture injury model.
Materials and methods: Sixty four male Wistar–Albino rats weighing 170–210 g were used in this study. The animals were randomly divided into eight groups and 5 or 6 animals were placed in each cage. A transverse femur shaft fracture model used. The animals in study groups received oral curcumin at a dose of 200 mg/kg for 14 days or 28 days. Remaining animals received only saline solution by oral gavage for a period of 14 days and 28 days as control groups. After sacrification the left femurs used for radiological, histological and biomechanical evaluation.
Results: The groups treated with curcumin showed no significant difference in terms of radiological, histological and biomechanical evaluations in 14 days groups. Also there was no significant difference between curcumin and control groups for 28 days according to radiological, histological and biomechanical tests.
Conclusions: According to our results, curcumin has no positive effect on fracture healing not only histologically but also radiologically and biomechanically. Curcumin's antioxidant effect may be more noticeable with long term follow up investigation as it may have a positive effect in remodelling phase. Long term follow up designed studies may be planned to investigate its effect on remodelling phase of fracture healing.

Introduction

Fracture healing continues to be a significant challenge for orthopaedic surgeons. It leads to significant health care costs which result in restricted activity and individual morbidity [1]. Bone is a unique tissue with a strong healing capability in terms of restoring original tissue but not in terms of fibrosis repair. Healing is a complex and orderly regulated process that can be affected by multiple factors. However, previous publications have shown that in 5%–10% of bone fractures, healing cannot be achieved leading to impaired quality of life [1,2].

Curcumin, a natural compound extracted from turmeric (*Curcuma longa*), has strong antioxidant and anti-inflammatory activities. More than 10,000 articles have discussed the molecular basis of curcumin's potential antioxidant, anti-inflammatory, and anti-infectious activities during the last decade [3–7]. Many studies have shown curcumin's positive effects on tissue injuries of the dermis, nerve fibres, and burns are mediated via activation of cell migration and wound healing [8–10]. It has been reported that antioxidant therapy and inhibiting inflammatory processes could have positive effects on the healing of soft tissue injuries [1,11–13].

Many factors have been studied for their role in improving bone healing processes such as stem cells, growth factors, and haemostatic agents [14–17]. However, until now, there has been only one reported study in the English literature assessing curcumin's effect on bone healing via histologic and radiological evaluation but not biomechanical evaluation [18].

In the present study, we aimed to investigate the radiologic, histologic, and biomechanical effects of curcumin on bone healing using a rat femur total fracture injury model.

Materials and methods

Sixty four male Wistar albino rats weighing 170–210 g were used. The experimental design and all procedures were approved

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Table 1
The distrubution of groups, contents and sacrification times.

Group Name	Group Feature	Number of Rats	Sacrification Time
A	14 days Histological Assesment Curcumin Group	10	14 days
B	14 days Histological Assesment Control Group	6	14 dayd
C	14 days Biomechanical Assesment Curcumin Group	10	14 days
D	14 days Biomechanical Assesment Control Group	6	14 days
E	28 days Histological Assesment Curcumin Group	10	28 days
F	28 days Histological Assesment Control Group	6	28 days
G	28 days Biomechanical Assesment Curcumin Group	10	28 days
H	28 days Biomechanical Assesment Control Group	6	28 days

by XXX Animal Research and Ethics Committee (protocol number 2015/38). All the animals for this study were purchased from the Laboratory of Experimental Animals, XXX University, BB city, CCC Country.

The animals were randomly divided into eight groups and five or six animals were placed in each cage. The eight groups were designated as A, B, C, D, E, F, G, and H. The properties and the numbers of the animals of each group are shown in Table 1.

A well-known rat fracture model was used [18]. Surgical procedures were conducted under general anaesthesia with intraperitoneal injection of 50 mg/kg ketamine (Ketalar; Eczacıbaşı, İstanbul, Turkey) and 10 mg/kg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany). The left lower limbs in the rats were shaved and disinfected using povidone-iodine, and surgery was performed under sterile conditions. A 3-cm lateral longitidinal incisions were made to expose the femurs. A transverse femur shaft fracture was made using an ossilating microsaw. A 1-mm Kirschner wire (Hipokrat, İzmir, Turkey) was used for intramedullary fixation. The subcutaneous tissue and the skin were closed following standard procedures.

No bandage or casting was placed after the surgeries. All rats were allowed to move freely and were fed with standard laboratory feed and tap water.

The animals in groups A and C received curcumin (curcumin, Sigma-Aldrich, Germany suspended in saline at a dose of 200 mg/kg orally) via oral gavage for 14 days and the animals in Group E and F received curcumin for four weeks starting from the day of surgery. The remaining animals received only saline solution via oral gavage for a period of 14 days (in groups B and D) or 28 days (in groups E and F). The dosage of curcumin was based on previous similar studies [13,19].

High-dose ether vapour was used for euthanasia. After the rats were killed, their left femurs were disarticulated from their hip and knee joints. Soft tissues on the femoral bone were peeled off gently from the bone without any harm to the callus tissue. All of the left femurs were studied addressing clinical, radiological, and histological aspects.

All the left femurs were removed and taken immediately for radiologic analysis. The femurs obtained from groups A, B, E, and F were used for histological analysis. The femurs obtained from groups C, D, G, and H were used for biomechanical analysis.

Radiologic analysis

Sixty four femurs were used for radiological analysis. Computed tomography (CT) imaging was performed with a 128 × 2-slice dual-source CT (Somatom Definition Flash, Siemens, Germany). The samples were placed in the CT for obtaining micro–CT images by scanning along the long axis of the femoral samples and the region of interest, 10 mm around the fracture site, with a 0.6 mm collimation. The K wires were not removed because they did not cause any artefacts in radiological analysis.

The software Radiant Dicom Wiever 4.6.9 was used for radiological measurements. Total callus diameter, low-radiodensity bone measurements, high density bone measurements, length of callus tissue, and femoral diameter parameters were measured. For measuring callus diameter, low-radiodensity bone measurements, high density bone measurements, and femoral diameter, axial sections were used. Analysis after two weeks was done using the ratio of total callus diameter/femoral diameter, and analysis after four weeks was done using the ratio of low density bone/high density bone measurements [Fig. 1].

Histological analysis

A total 32 femurs from groups A, B, D, and E were used for histological analysis. A buffered formalin solution was used for fixation over two days. Next, 10% acetic acid solution was used for decalcification over four days. Next, the specimens were embedded in paraffin and 3 mm-thick serial sections were taken from the callus site of each femur. Hematoxylin and eosin staining was performed. Histological classification of the healing was done per the histological healing scale published by Huo et al. [20].

Biomechanical analysis

A total 32 left femurs of the rats from groups C, D, G, and H were used for biomechanical analysis. Biomechanical testing were

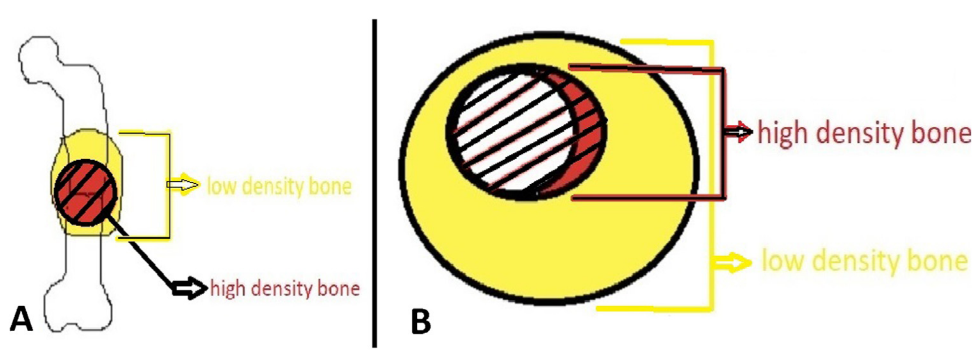

Fig. 1. Schematic drawings of measurements used for radiological evaluation. A, longitudinal section. B, Transverse section.

Table 2
Radiological results of curcumin and control groups.

Groups	Number of rats	Average radiological assesment score	P value
A + C	20	2,514	P >0.05
B + D	12	2,509	
E + G	20	1,314	P >0.05
F+H	12	1,281	

performed using a Elista TST 2500 material testing machine (Elista, Istanbul, Turkey). The femurs were mounted with mini clamps on the testing device. The clamps gripped each specimen at the distal and proximal metaphyseal parts of the femurs for measuring resistance to longitudinal distraction forces (in Newtons). A distraction force was applied at 3 mm/min. The distraction forced applied was increased until failure of the healing region. Load to failure parameters were recorded for each specimen. A three-point bending test was planned but could not be used because two-week specimens were not usable due to only soft callus formation. The recorded measurements (in Newtons) were used to compare the results of biomechanical evaluation.

Statistical analysis

Mann–Whitney U test was used for analysing radiological, histological, and biomechanical results to evaluate differences between the two groups. All the analyses were performed with IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA. A p value <0.05 was considered to be statistically significant).

Results

Radiological results based on CT images are shown in Table 2. There was no significant difference in radiological evaluation results between the curcumin 2nd week group and the control group (Mann–Whitney test; $U = 117.00$; $p > 0.05$) [Fig. 2]. For radiological evaluation after two weeks, only total callus diameter/femoral diameter measurement were used because hard callus formation was insufficient. Comparison of radiological evaluation results from the rats killed in the second week showed that the diameter of the callus in the curcumin group was smaller than that in the control group. At four weeks, the ratio of low density bone/high density bone was used to represent healing. Low density bone is a predictor of new bone formation and high density bone indicates the cortex of the intact (before fracture) bone. Radiological evaluation results of the rats killed in the fourth week showed that the low-radiodensity bone/high radiodensity bone ratio was higher in the curcumin group [Fig. 3]. However, statistical comparison of radiological evaluation results at two or four weeks did not show any statistical significance.

Histological scores per the Huo classification are shown in Table 3. There was no significant difference in histological examination results between the 2nd week curcumin group and the 2nd week control group (Mann–Whitney U test; $U = 30.00$; $p > 0.05$) [Fig. 4]. There was no significant difference in histological examination results between the 4th week curcumin group and 4th week control group (Mann–Whitney U test; $U = 21.00$; $p > 0.05$) [Fig. 5]. The mean scores of histological examination were 4.8 in the control group and 4.2 in the curcumin group at two weeks. The mean scores were 5.5 in the control group and 5.9 in the curcumin group at four weeks. The scores at two weeks were better in the control group, and those at four weeks were better in the curcumin group; however, these differences were not statistically significant.

The results of biomechanical analysis are shown in Table 4. There was no significant difference in biomechanical examination results between the 2nd week curcumin group and the 2nd week control group (Mann–Whitney U test; $U = 29.00$; $p > 0.05$). There was no significant difference in the biomechanical examination results between the 4th week curcumin group and the 4th week control group (Mann–Whitney U test; $U = 27.50$; $p > 0.05$). The mean load to failure was 8.3 N in the control group and 8.6 N in the curcumin group after two weeks; it was 8.9 in the control group and 8.2 in the curcumin group after four weeks.

Discussion

There are limited studies on curcumin's effect on fracture healing. Curcumin's positive effect on fracture healing has been reported in a recent study, which found that curcumin has potential in promoting bone healing by activating autophagy in a

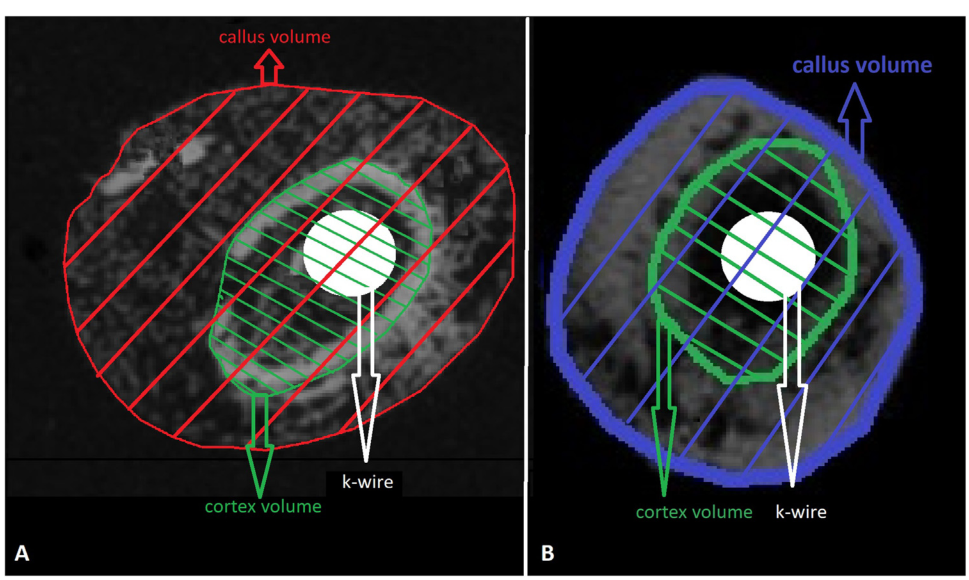

Fig. 2. A, Sample of transverse radiological imaging (micro-CT) after scarification at 14 days from Curcumin group. B, Sample of transverse radiological imaging after scarification at 14 days from control group.

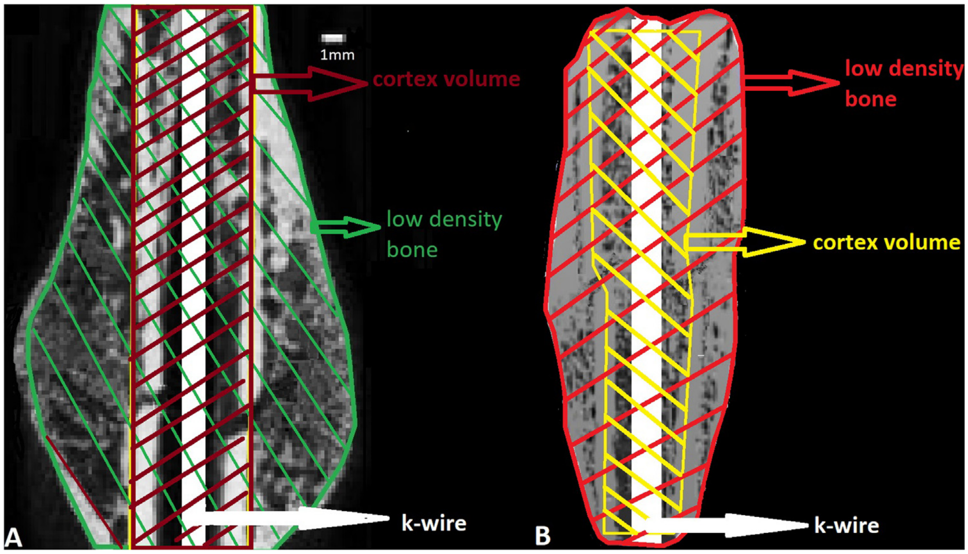

Fig. 3. A, Sample of longitudinal radiological imaging (micro-CT) after scarification at 28 days from curcumin group. B, Sample of longitudinal radiological imaging after scarification at 28 days from control group.

Table 3
Histological examination results of control group and curcumin group.

Group Name	Number of Rats	Average histological assesment score	P value
A	10	4,6	P >0.05
B	6	4,8	
E	10	5,9	P >0.05
F	6	5,5	

rat model [18]. Histological and radiological evaluation showed positive effects of curcumin on bone healing but biomechanical tests were not used in the above study. To the best of our knowledge, the present study is the first animal model study investigating curcumin's effect on fracture healing using radiological, histological, and biomechanical tests.

In the present study, we created a femur fracture injury model and evaluated the effects of oral curcumin. The histological results were better in the curcumin group at two and four weeks, but the differences were not statistically significant. This may be secondary to curcumin's anti-inflammatory effect. The first phase of bone healing is the inflammation phase and curcumin may inhibit this phase. A similar situation may exist at four weeks, representing the healing or callus phase in fracture healing. This is quite different from previously reported findings [18]. Their study design is quite different from our study. Firstly, they used a long K wire fixation technique penetrating the knee joint and ends out of the skin. This type of fixation can block the motion of the knee joint and load of the extremity which may be positive factor for fracture healing. In our study we used short K wires which were completely buried intramedullary for not limiting the early load and range of motion of the joints. Secondly the time period is for sacrifying the animals is different as they used two and six weeks results. Thirdly, Li et al. designed their study on blocking the autophagy of the rats by using 3-methyladenine and curcumin but they did not study the only effect of 3-methyladenine on fracture healing. Also the histological evaluation system in our study was different from that used in the above study. In our study, we evaluated healing using Huo's classification, which has 10 parameters, using only light microscopy with haematoxylin and eosin staining. The above study used osteoblast cell counting, immunohistochemical tissue staining, and western blotting. However, there is no reported objective

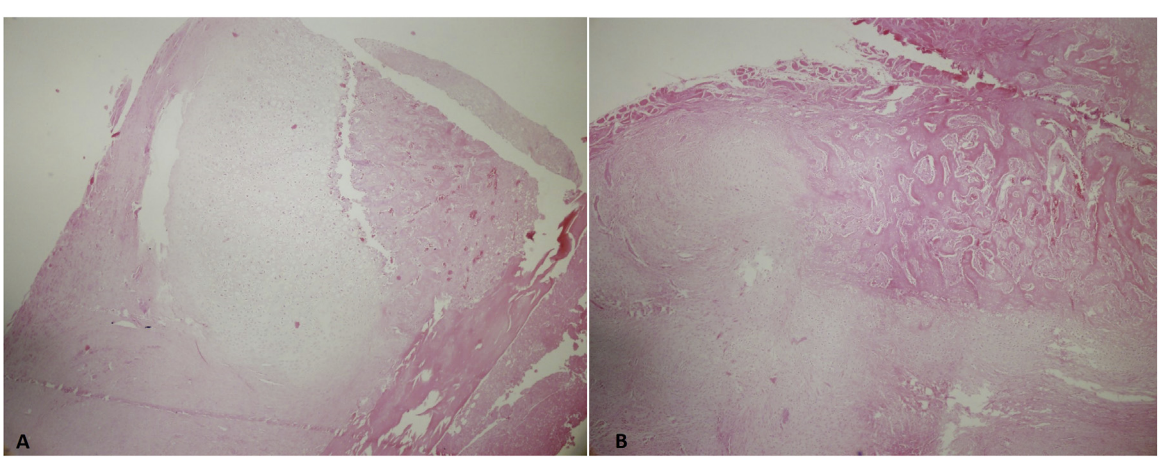

Fig. 4. A, Histological images of healing after two weeks (H&E staining X100). A, Curcumin group showing mainly chondroid tissue and some woven bone. B, Control group showing equal amount of chondroid tissue and woven bone.

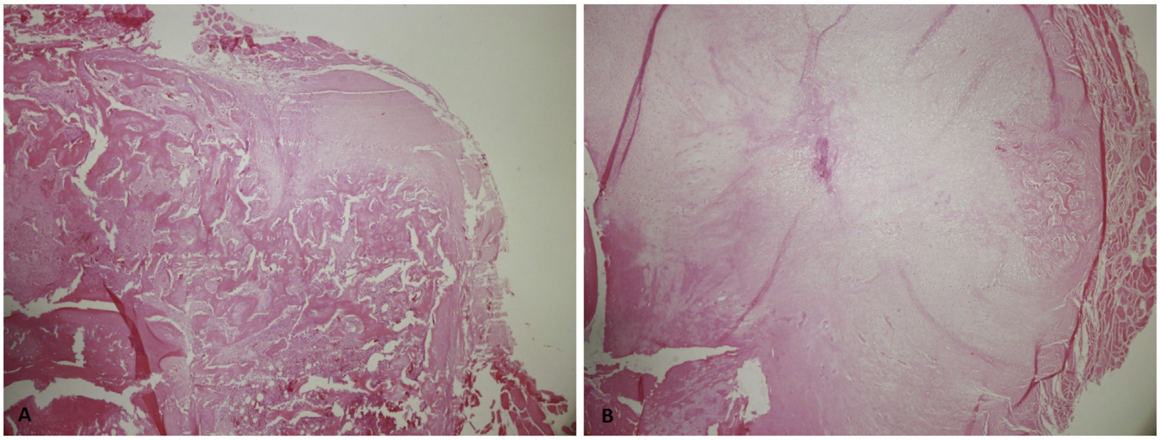

Fig. 5. A, Histological images of healing after four weeks (H&E staining X100). A, Curcumin group showing mainly woven bone and some chondroid tissue. B, Control group showing mainly chondroid tissue and some woven bone.

Table 4
Biomechanical examination results of curcumin and control groups.

Groups	Number of rats	Average load to failure	P value
C	10	37,7	P >0.05
D	6	31,9	
G	10	49,2	P >0.05
H	6	48,6	

classification system for this histologic examination. Indeed, the number of osteoblasts and other immunohistochemical parameters are important in fracture healing, but we could not find any reports comparing Huo's histologic classification method and the osteoblast counting method. It is known in some cases, although the number of blastic cells is high, it may not be functional (such as hypertrophic pseudoarthrosis). Therefore, the difference in histological results between these similar studies may be secondary to the usage of different histological evaluation methods.

The radiological evaluation results were better in the curcumin group, which also did not show statistically significant differences. CT evaluation was used for investigating bony union in the rat femurs. The diameter of callus formation and its ratio with intact bone diameter was used for radiologic examinations. Radiological evaluation results from similar reports have shown statistically better results with curcumin use in X-ray visualisation; only the callus diameter was used as a parameter in their CT evaluation. In our study, we used callus diameter plus callus diameter to intact bone ratio. The difference in our CT evaluation results may be secondary to this situation.

We also performed analysis of biomechanical evaluation; the maximum load to failure parameter was measured in all rats. Three-point bending tests could not be applied because the callus tissues were too soft. All the failures were at the fracture healing site; this is similar to that seen in human re-fractures. The two-week results were better in the curcumin group and the four-week results were better in the control group. However, when we compared the results, there was no statistically difference between the control and curcumin groups. There has been only one report on the biomechanical effects of curcumin on bone tissue [21]; in their study, the authors evaluated the effect of curcumin on intact bone tissue by using an ovariectomised rat model. They concluded that curcumin had no statistically positive effect on bone tissue via evaluation of biomechanical tests. Our results are similar to that of the above study, although we used a femur fracture model.

There are also some limitations in our study. First, we could not apply the three-point bending test for biomechanical evaluation. Although fracture-healed tissues were not applicable for this test, longer follow-up analysis can be performed to address this. Secondly, the number of rats used would need to be higher for more accurate results.

Curcumin has been used, especially in Asian countries, to treat different kinds of diseases due to its antioxidant, anti-inflammatory, immunomodulatory, and antimicrobial effects for many years [3–10]. In our study, we found that curcumin did not improve fracture healing based on biomechanical, radiological, and histological evaluation. We infer that curcumin's anti-inflammatory property may be responsible for its ineffectiveness in fracture healing.

Conclusion

Our results showed that curcumin has no positive effect on fracture healing, not only histologically but also radiologically and biomechanically. Curcumin's effect may be more noticeable in long-term follow-up investigations because of its potential positive effects in the remodelling phase. Long term follow up designed studies may be planned to investigate its effect on remodelling phase of fracture healing.

Statement of welfare of animals

All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures performed were in accordance with ethical standards of the institution (Selcuk University Experimental Research Centre, Konya, Turkey) where the study was conducted.

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