Joint Diseases and Related Surgery

Jt Dis Relat Surg
2023;34(2):413-424

ORIGINAL ARTICLE

The effects of cephalexin on fracture healing in a rat femur fracture model

Muhammed Uslu, MD[1], Bilal Yılmaz, MD[2], Hamisi Mwarindano Mraja, MD[3], Mustafa Fatih Daşcı, MD[1], Elif Yaprak Saraç, PhD[4], Bedri Onur Küçükyıldırım, PhD[5], Mehmet Akif Güleç, MD[1], Serdar Yüksel, MD[1]

[1]Department of Orthopedics and Traumatology, Health Sciences University, Bağcılar Training and Research Hospital, Istanbul, Türkiye
[2]Department of Orthopedics and Traumatology, Birecik State Hospital, Şanlıurfa, Türkiye
[3]Department of Orthopedics and Traumatology, Istanbul Spine Center & Orthopedics, Group Florence Nightingale Hospitals, Istanbul, Türkiye
[4]Department of Molecular Biology-Genetics and Biotechnology, Istanbul Technical University, Istanbul, Türkiye
[5]Department of Mechanical Engineering, Yıldız Technical University, Istanbul, Türkiye

Open fractures are fractures in which the fracture and/or fracture hematoma contact with the external environment and are open to complications including an infection, nonunion, delayed union of fracture, and loss of the extremity. The main goals of treatment in these fractures are to assess the patient's general condition, to classify the injury, provide wound management, fracture stabilization and bone regeneration, when necessary.[1]

Surgical site infections (SSIs) can cause serious comorbidities in the practice of orthopedic surgery. Prevention and treatment of SSIs are still challenging for patients and healthcare

Received: December 20, 2022
Accepted: March 08, 2023
Published online: April 27, 2023

Correspondence: Muhammed Uslu, MD. SBÜ Bağcılar Eğitim ve Araştırma Hastanesi Ortopedi ve Travmatoloji Kliniği, 34200 Bağcılar, İstanbul, Türkiye.

E-mail: dr.muhammeduslu@gmail.com

Doi: 10.52312/jdrs.2023.994

Citation: Uslu M, Yılmaz B, Mraja HM, Daşcı MF, Yaprak Saraç E, Küçükyıldırım BO, et al. The effects of cephalexin on fracture healing in a rat femur fracture model. Jt Dis Relat Surg 2023;34(2):413-424. doi: 10.52312/jdrs.2023.994

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ABSTRACT

Objectives: The aim of this study was to examine the effects of cephalexin on the fracture union histomorphometrically, radiologically, biomechanically, immunohistochemically, and histopathologically in a rat femur fracture model and to evaluate the effects of the antibiotics to be used in the prophylaxis of fracture infection on the union of the fracture.

Materials and methods: A total of 48 male Wistar rats were divided into four groups as two-week control (C2) and cephalexin (CEP2) and four-week control (C4) and cephalexin (CEP4). After establishment of standard fracture model on right femurs, 60 mg/kg/day of cephalexin was applied to CEP2 and CEP4 by oral gavage. Radiological, biomechanical, histopathological, immunohistochemical, and histomorphometric examinations were performed on amputated femurs.

Results: Callus volume of CEP4 group significantly increased compared to CEP2 group (p=0.005), while no significant difference was found in the bone mineral density and callus/bone volume among the groups (p>0.05). There was no significant difference in flexural strength between the C4 and CEP4 groups (p=0.093). Histological healing scores increased from Week 2 to Week 4 (p=0.002) and inflammation scores decreased in both control and cephalexin groups (p=0.010 and p=0.008); however, no significant difference was found in healing and inflammation scores (p>0.05). The CD34+ immunoreactivity in the CEP2 group was significantly higher than the C2 group (p=0.029). Collagen type III level was significantly lower in the CEP2 and CEP4 groups compared to the corresponding control groups (p=0.008 and p=0.016, respectively).

Conclusion: Cephalexin did not exert any radiological, histopathological, histomorphometric, biomechanical, and immunohistochemical adverse effects on the femoral fracture healing model in rats; however, it showed positive effects on CD34 and Collagen type III levels. Based on these findings, antibiotherapy with cephalexin may be considered as a safe treatment for fracture union.

Keywords: Cephalexin, femur, fracture healing, rat.

professionals.[2] In recent years, there has been an increase in the number of studies on oral antibiotics owing to their lower costs and ease of use. Oral antibiotherapy can be applied in bone infections including the osteomyelitis, open fractures such as Seymour fracture and some gunshot injuries.[3,4] There are studies reporting that the long-term use of intravenous antibiotics in complex orthopedic infections does not have a significant advantage over the oral antibiotic use.[5] The significance and effectiveness of systemic antibiotic use in post-traumatic wound infections have been demonstrated in several randomized-controlled studies. Cephalosporins are now routinely used agents in the fracture healing due to their well distribution into the bone tissue and their cost-effectiveness. Despite the significance of cephalosporins in the fracture treatment, the number of studies on the effects of antibiotics on the fracture union is not sufficient and their impact on bone healing has not been clearly understood.[6,7] Cephalexin, an oral form of the first-generation cephalosporins, has been shown to have well distribution into the bone and soft tissues and is frequently used in the prophylaxis and treatment of bone infections,[8,9] but not in the fracture healing.

In the present study, we hypothesized that oral antibiotherapy with cephalexin would not have a negative effect on the fracture healing process. We, therefore, aimed to examine the effects of cephalexin on the fracture union histomorphometrically, radiologically, biomechanically, immunohistochemically, and histopathologically in a rat femur fracture model and to evaluate the effects of the antibiotics to be used in the prophylaxis of fracture infection on the union of the fracture.

MATERIALS AND METHODS

A total of 48 male Wistar young adult rats with a mean age of 10±1 weeks and a mean weight of 380±25 g were used in this study. All rats were randomly divided into four groups including 12 rats in each group, as the two- and four-week control groups [C2 and C4] and two- and four-week cephalexin-administered experimental group [CEP2 and CEP4]. The rats were kept in 12 h of light and 12 h of dark cycle at 22°C as 12 animals in each cage and fed with tap water *ad libitum* and standardized rodent chow.

No complications such as fracture fragmentation, fixation failure occurred, and all rats survived during the entire procedure, except for one subject in the control group at two weeks of the study which died due to the anesthesia complication on the first postoperative day.

Surgical procedures and sacrification

General anesthesia was performed intraperitoneally by administering 5 mg/kg of xylazine hydrochloride (HCL) (Rompun®; Bayer Pharmaceuticals, Istanbul, Türkiye) and 80 mg/kg of ketamine HCL (Ketalar®, Pfizer Pharmaceuticals, Istanbul, Türkiye). At the initiation of treatment, femoral osteotomy and fixation were performed in the same manner as previously reported.[10,11] The femoral diaphysis was exposed by an approximately 2-cm incision entering through the lateral right femur of each rat, and a transverse osteotomy was established in the middle of the femur with the help of a micro-cutter. A 1-mm Kirshner wire was inserted anterogradely from the fracture line and removed from the knee joint. Following the reduction, the fixation was achieved by inserting the wire retrogradely in the proximal fragment (Figure 1). The fragmentation and distraction at the fracture line was avoided by a careful fixation which was confirmed by X-ray images. Then, the skin and fascia were sutured and closed.

After the surgical procedures, cephalexin was orally applied to the CEP2 and CEP4 groups at a dose of 60 mg/kg per day by oral gavage until Days 14 and 28, respectively. The same dose of tap water was applied to the C2 and C4 groups until Days 14 and 28, respectively. The C2 and CEP2 rats at the end of the second week and C4 and CEP4 rats at the end of the fourth week were sacrificed by the cervical dislocation method under general anesthesia by applying high-dose ketamine HCL and xylazine HCL. The right femurs of each rat were removed and fixed in formalin for the analysis given below.

Micro-computed tomography (CT) analysis

Forty-seven samples were fixed in the falcon tube for micro-CT scanning (SkyScan™ 1174v2; Bruker Belgium SA, Kontich, Belgium). For monitoring the femoral fracture, each sample was scanned 360° with 1.00° rotation in approximately 45 min. Bone volume (BV) and callus volume (CV) was calculated in an area of 15 mm by marking 7.5 mm above and below the fracture line on the Micro-CT scanogram images obtained for each femur sample. Bone mineral density (BMD) was analyzed using a calcium hydroxyapatite calibration bar in the calcium densities of 0.25 g/mm^3 and 0.75 g/mm^3. Three-dimensional (3D) modeling of the images of samples was performed in CTAn version 1.16.4.1+ and CTVol version 2.3.2.0 software and BMD (g/cm^3), CV (mm^3) and BV (mm^3) were analyzed in each femur (Figure 2).

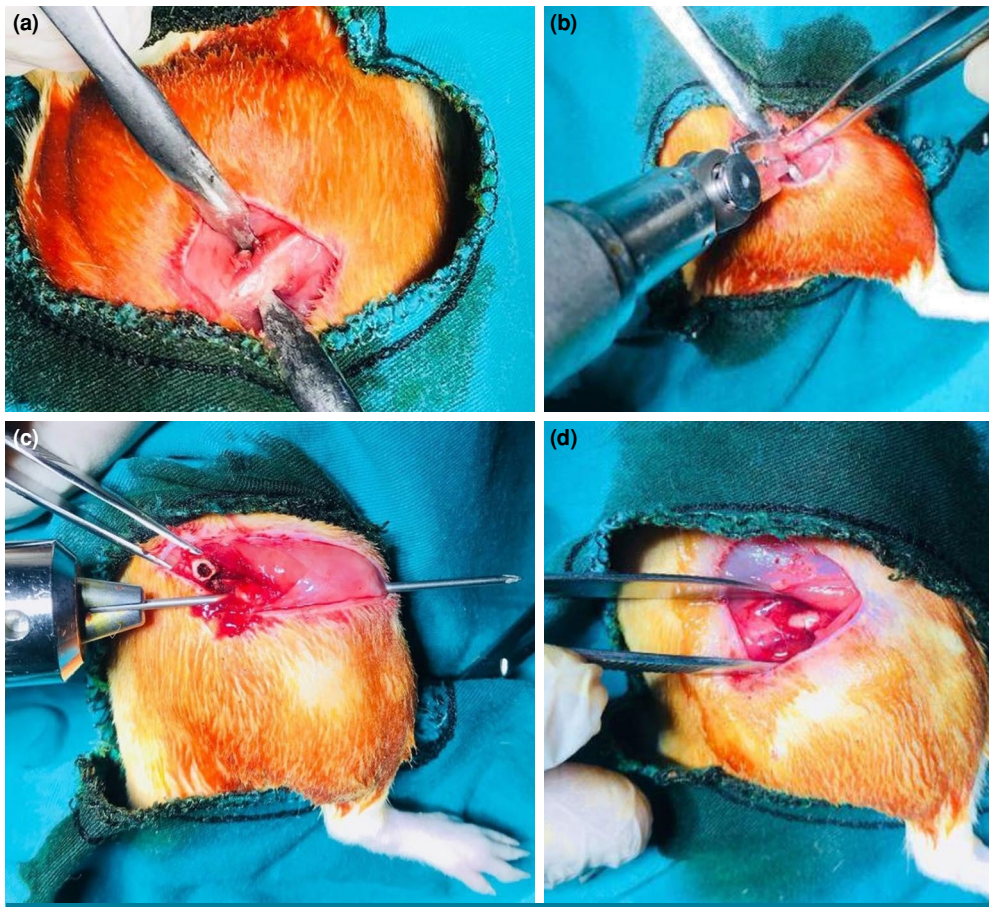


FIGURE 1. Operational procedures for osteotomy. **(a)** Exposure of the femoral body; **(b)** Osteotomization of the femoral body; **(c)** Intramedullary antegrade inserted Kirschner wire; **(d)** Evaluation of stability after reduction.

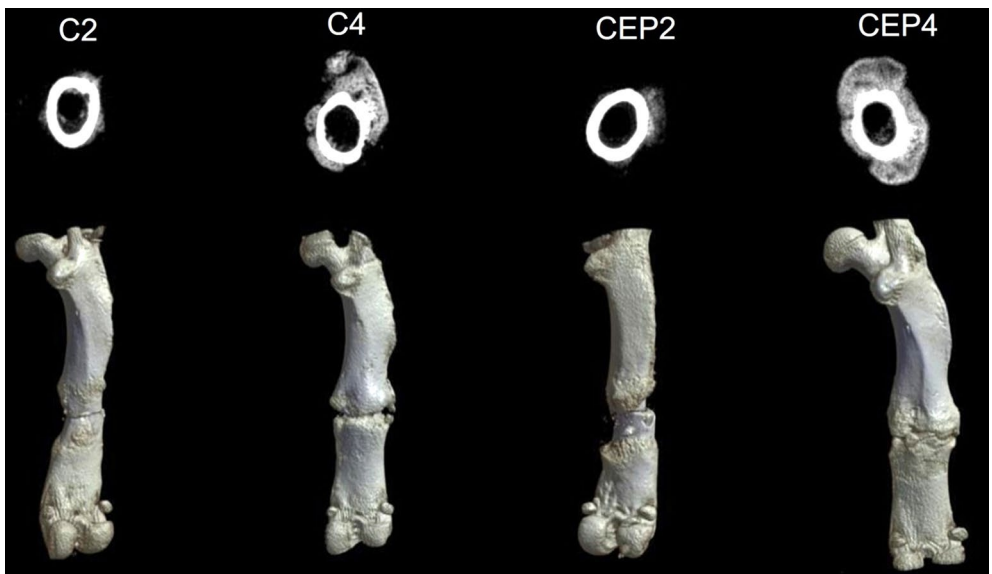


FIGURE 2. Reconstructed 3D radiological images of rat femurs of control groups (C2 and C4) and cephalexin groups (CEP2 and CEP4).

After radiological examination, all samples of C2 and CEP2 groups were prepared for the histopathological examination. The CEP4 and C4 groups were randomly divided into equal number of samples for the biomechanical and histopathological examination.

Biomechanical examination

Six randomized fractured samples from each of the CEP4 and C4 groups were subjected to biomechanical analysis. The samples from C2 and CEP2 collected at two weeks were not included in the biomechanical analysis due to the short duration for fracture union. A three-point bending test was performed with a Class 1 calibrated mechanical test device (Alsa Laboratory Devices Ltd., Istanbul, Türkiye). The test was performed by applying a force perpendicular to the fracture on the samples placed on the measuring device by means of their front faces up. The distance between the supports on which the samples were placed on the device was set as 10 mm. A force was applied to the samples at a rate of 5 mm/sec until a fracture occurred. The maximum bending force and the bending strength (σ_bend) applied, when the samples were broken and the results were calculated.

Histopathological examination

Twelve fractured femurs of CEP2 and eleven femurs of C2 groups (one died due to anesthesia) and six randomized samples of CEP4 and C4 groups were fixed in 10% formalin and, then, decalcified in ethylenediaminetetraacetic acid (EDTA) solution. After a routine histological tissue preparation, 3-μm longitudinal sections were prepared from paraffin-embedded blocks and hematoxylin & eosin (H&E) and Masson trichrome staining were performed. Light microscope (Olympus BX61; Olympus Corp., Tokyo, Japan) and camera (Olympus DP72; Olympus Corp., Tokyo, Japan) were used for the histopathological examination and photographing. Five sections were evaluated in each randomly selected sample and scored between 1 and 10 according to the histological scoring system of Huo et al.[12]

To determine inflammation in the fracture region, the leukocyte infiltration was scored as follows: 0, no inflammation; 1, mild; 2, moderate; and 3, severe inflammation.

Histomorphometry

Histomorphometric examination was performed in all fractured femurs, except for 12 randomized fractured samples that underwent biomechanical analysis. For the quantitative analysis of the histological changes, the area measurements were made on 3-μm paraffin sections. The bone cross-sectional area and callus area consisting of the fibrous tissue, cartilage and ossifying tissue were determined for the measurements. The ratio of the cartilaginous callus area to the total callus area and the ratio of total callus diameter to the femoral bone diameter were calculated in each sample at the end of the second week and defined as percentages. Since the soft callus disappeared in the samples at the end of the fourth week, only the ratio of the total callus diameter to femoral bone diameter was calculated as a percentage for C4 and CEP4 groups.

Immunohistochemical analysis

Immunohistochemical examination was performed in all fractured femurs, except for 12 randomized fractured samples that underwent biomechanical analysis. To determine the levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), bone morphogenic protein-4 and 7 (BMP-4 and BMP-7), collagen (COL) type I and III, CD34+ localization in the fracture healing area of femur sections, an immunohistochemical staining was performed with the streptavidin-biotin-peroxidase method as previously described.[13] Anti-BMP7 polyclonal antibody (ab56023, Abcam, UK), anti-BMP4 polyclonal antibody (ab39973, Abcam, UK), anti-CD34 monoclonal antibody (EP373Y) (ab81289, Abcam, UK), anti-IL-6 monoclonal antibody (1.2-2B11-2G10) (ab9324, Abcam, UK), anti-TNF-α polyclonal antibody (ab6671, Abcam, UK), anti-COL-I monoclonal antibody (EPR7785) (ab138492, Abcam, UK), and anti-COL-III monoclonal antibody (EPR17673) (ab184993, Abcam, UK) were used. Five regions showing positive immunoreactivity with the relevant antigens were scored by two researchers for staining intensity between 0 and 300 according to the modified H-score scoring according to the literature,[14] and the scores were averaged. Thus, the femoral localizations of these proteins were determined, and changes in the expression of the proteins and regional differences were determined semi-quantitatively.

Statistical analysis

Statistical analysis was performed using the GraphPad Instat version 3.06 software (GraphPad Inc., San Diego, CA, USA). Descriptive data were expressed in median (min-max) or number and frequency. The distribution of the variables was tested using the Shapiro-Wilk test. Continuous variables were compared using the Mann-Whitney U test. A p value of <0.05 was considered statistically significant.

TABLE I
Radiological findings of bone fracture healing of Weeks 2 and 4 control and cephalexin groups

Variables	Week 2 Median	Week 2 Min-Max	Week 4 Median	Week 4 Min-Max	*p* value
Callus BMD (g/cm³)					
Control	2.01	1.96-2.05	2.01	1.97-2.99	0.872
Cephalexin	2.02	2.00-2.03	2.02	2.00-2.03	0.604
p value		0.075		0.247	
Callus volume (mm³)					
Control	100.7	86.6-154.0	126.9	87.1-172.9	0.189
Cephalexin	111.4	72.9-131.4	136.1	103.7-166.4	**0.005**
p value		0.976		0.478	
Bone volume (mm³)					
Control	61.46	46.39-92.49	74.35	51.26-104.37	0.239
Cephalexin	70.96	51.56-76.50	58.05	51.72-91.64	0.224
p value		0.703		0.061	
Callus volume/bone volume					
Control	1.84	1.03-2.82	1.88	0.83-2.30	0.971
Cephalexin	1.69	1.37-2.24	1.70	1.30-3.12	0.574
p value		0.897		0.678	

BMD: Bone mineral density.

RESULTS

Radiological findings

Reconstructed 3D images are presented in Figure 2 and the measurements are given in Table I. Bridging bony callus was observed radiologically in all samples. In the radiological examinations, the median values of BMD, CV, and BV and the ratios measured at Week 4 increased compared to the Week 2, although the statistical significance was only found in the increased CV of the cephalexin groups (p=0.005). There was no significant difference in any of the radiological findings between the control and cephalexin groups (p>0.05).

Biomechanical findings

In the biomechanical examination, the median σ_bend flexural strength of the femurs of C4 group was 9.48 (range, 6.86 to 11.03) N/mm³, and 12.12 (range, 6.74 to 14.02) N/mm³ in the CEP4 group, indicating no statistically significant difference (p=0.093).

Histopathological and histomorphometric findings

Light microscopic images of two-week and four-week bone fracture healing are presented in Figure 3. Bridging bony callus was observed macroscopically and microscopically in all samples. The histopathological examinations of two-week images of femur fractures showed large soft callus areas with cartilaginous callus, inflammation and infiltration, while four-week images showed slightly reduced size of cartilaginous callus and local primary bone formations (Figure 3).

The histopathological and histomorphometric findings of two-week and four-week control and cephalexin groups are presented in Table 2. The four-week histological healing scores significantly increased in both groups compared to two-week scores (p=0.002); however, there was no significant difference between the healing scores of the control and cephalexin groups (p=0.517 and p=0.677, respectively).

The four-week inflammation scores significantly decreased in both groups compared to the two-week scores (p=0.010 and p=0.008, respectively); however, the inflammation scores in the cephalexin groups did not significantly differ from the scores of control group (p=0.933 and p=0.680, respectively) (Table II).

Histomorphometric analysis showed that the cartilaginous callus/total callus ratios of the cephalexin groups at Weeks 2 and 4 were higher than those the ratios of control group, although it did not reach statistical significance (p=0.099 and p=0.953, respectively). Again, no significant difference was found in the ratios of total callus diameter/femur diameter between the control and cephalexin groups and between the Weeks 2 and 4 (p>0.05) (Table II).

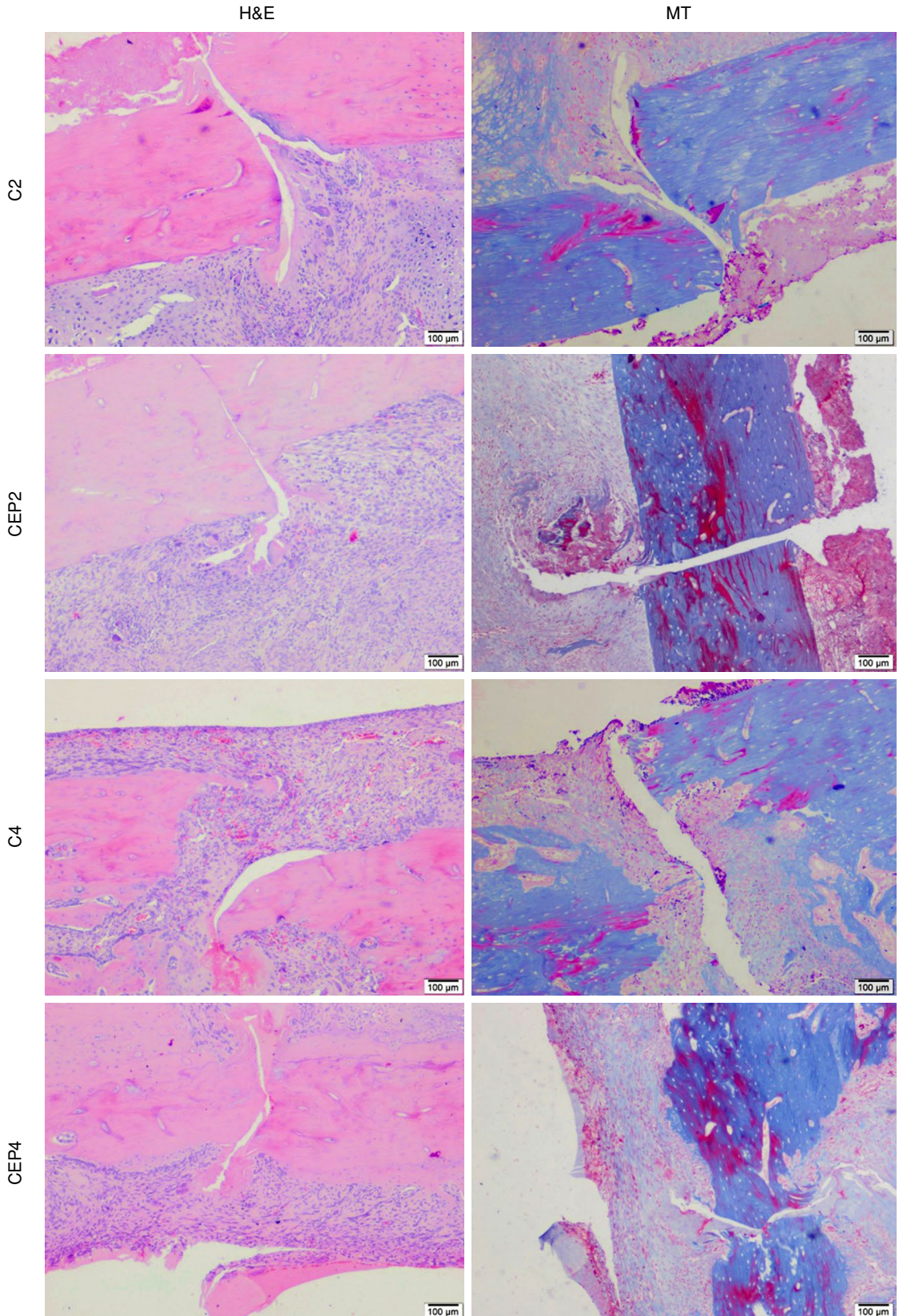


FIGURE 3. Light microscopic images of 2-week and 4-week bone fracture healing of control groups (C2 and C4) and cephalexin groups of rats (CEP2 and CEP4). H&E, ×40 and Masson trichrome stainings, ×40.

TABLE II
The histopathological and histomorphometric findings of Weeks 2 and 4 control and cephalexin groups

Variables	Week 2 Median	Week 2 Min-Max	Week 4 Median	Week 4 Min-Max	*p* value
Histological healing score					
Control	4.0	3.0-4.0	7.0	6.0-7.0	**0.002**
Cephalexin	3.5	2.0-5.0	7.0	6.0-7.0	**0.002**
p value	0.517		0.677		
Inflammation					
Control	3.0	2.0-3.0	1.5	1.0 - 2.0	**0.010**
Cephalexin	3.0	2.0-3.0	1.0	1.0-2.0	**0.008**
p value	0.933		0.680		
Cartilaginous callus area/total callus area (%)					
Control	29.37	24.92-48.78	50.36	24.68-75.09	0.096
Cephalexin	42.17	25.84-52.30	50.64	30.79-95.05	0.385
p value	0.099		0.953		
Total callus diameter/femoral diameter (%)					
Control	59.42	46.71-71.35	53.44	45.18-66.88	0.353
Cephalexin	53.77	42.51-73.37	51.24	42.81-58.38	0.325
p value	0.277		0.438		

BMD: Bone mineral density.

Immunohistochemical findings

Light microscopic images of immunoreactivities for BMP-4, BMP-7, CD34, TNF-α, IL-6, COL-I, and COL-III in two-week and four-week femurs are presented in Figure 4 and the H-scores are given in Table III.

The BMP-4 immunoreactivity did not significantly differ both between the control and cephalexin groups and between the weeks (p>0.05). Although there was an increase in the BMP-7 immunoreactivity of four-week femurs of the cephalexin groups compared to the control groups, the difference was not statistically significant (p=0.124) (Table III).

In addition, CD34 immunoreactivities differed slightly in both four-week groups compared to the two-week groups (p=0.057). However, CD34

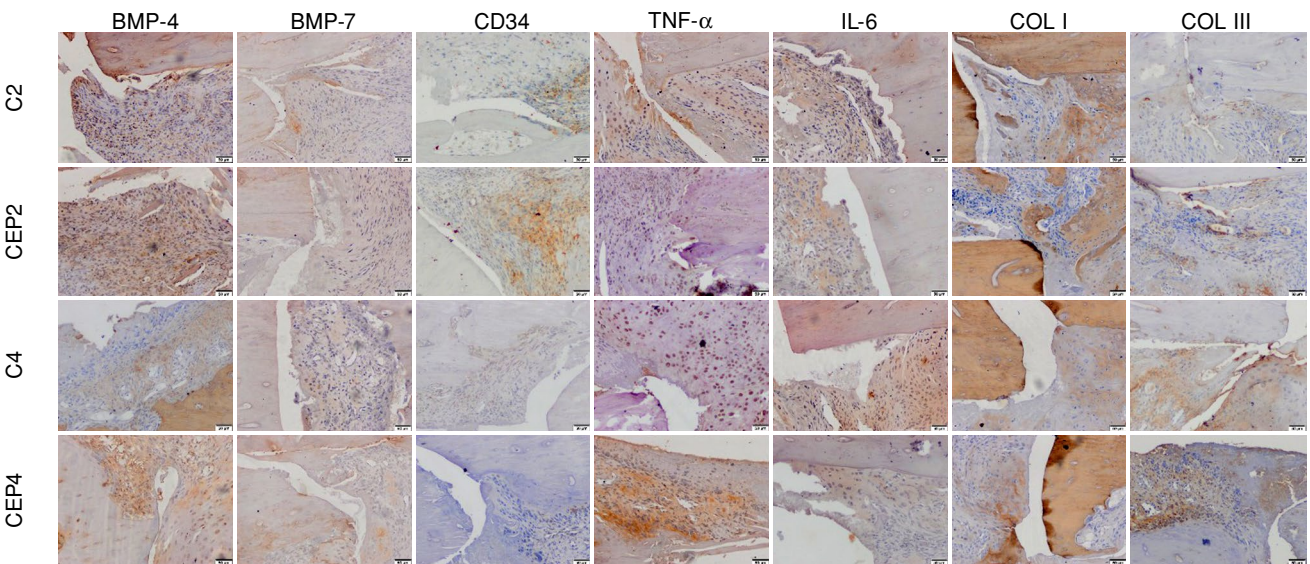


FIGURE 4. Light microscopic images of immunoreactivities against tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), bone morphogenic protein-4 and 7 (BMP-4, BMP-7), collagen I and III (COL I and COL III), CD34 in 2-week and 4-week femurs of control groups (C2 and C4) and cephalexin groups of rats (CEP2 and CEP4). Total magnification: ×100.

TABLE III
Immunoreactivities of the proteins in Weeks 2 and 4 control and cephalexin groups

Variables	Week 2 Median	Week 2 Min-Max	Week 4 Median	Week 4 Min-Max	*p* value
BMP-4					
Control	116.67	113.3-183.3	216.67	208.3-225	0.100
Cephalexin	150	125-158.3	183.33	183.33-191.67	0.100
p value		0.700		0.100	
BMP-7					
Control	70	60 -130	70	55-140	0.826
Cephalexin	75	50-160	180	140-200	0.200
p value		>0.999		0.124	
CD34					
Control	110	85-150	27	25-75	0.057
Cephalexin	215	190-230	20	15-25	0.057
p value		**0.029**		0.124	
TNF-α					
Control	230	190-230	200	160 - 260	>0.999
Cephalexin	230	180-250	200	140-270	>0.999
p value		>0.999	0.826		
IL-6					
Control	110	20-170	150	105-180	0.700
Cephalexin	85	45-190	10	6-180	0.400
p value		>0.999		0.510	
COL I					
Control	215	150-250	260	230-280	0.070
Cephalexin	200	160-250	200	190-250	0.582
p value		0.574		0.100	
COL III					
Control	215	200-300	255	200-300	0.667
Cephalexin	137.5	102-200	120.0	60-180	0.662
p value		**0.008**		**0.016**	

BMP-4: Bone morphogenic protein-4; BMP-7: Bone morphogenic protein-7, TNF-α: Tumor necrosis factor-alpha; IL-6: Interleukin-6; COL-I: Collagen I; COL-III: Collagen III.

immunoreactivity significantly increased in the CEP2 group compared to that of C2 group (p=0.029), but there was no significant difference in CD34 immunoreactivities of C4 and CEP4 groups (p=0.124) (Table III).

No significant increases were observed in the TNF-α and IL-6 immunoreactivities of the control and cephalexin group (p>0.05) (Table III).

The COL-I and COL-III immunoreactivities did not significantly differ in both groups at Week 4, compared to Week 2 (p=0.667 and p=0.662, respectively). However, the COL-III immunoreactivity was significantly lower in the CEP2 and CEP4 groups compared to the corresponding control groups (p=0.008 and p=0.016, respectively) (Figure 4) (Table III).

DISCUSSION

The correct use and selection of antibiotics is of utmost importance for patients using antibiotics to prevent any infection in the orthopedic injuries or patients having orthopedic injuries while still using antibiotics, in order not to interrupt the bone fracture healing. Although there are several studies in the literature examining the effects of antibiotics such as ciprofloxacin, cefazolin, cefuroxime and also cephalexin on the bone fracture union,[8,15-18] there is a limited number of studies regarding cephalosporins, which are commonly used in the orthopedic practice.[7] In the present study, we examined the effects of cephalexin, a first-generation cephalosporin, on fracture union in a rat femur fracture model and found that cephalexin treatment increased the CV by increasing the CD34+ expression and

decreasing COL-III production. No other adverse effects were observed in terms of radiological, histopathological, histomorphometric, biomechanical and immunohistochemical features of fracture union.

There are many studies planned for four and six weeks of fracture healing in the literature. In one of these, Özbay et al.[10] showed sufficient hard callus formation at four weeks; therefore, we chose the time interval as four weeks as previously described.[19] There are also reports showing that the quinolones adversely affect the fracture healing, particularly in the early period of process; therefore, we examined inflammation in two- and four-week groups to observe the effects of cephalexin on inflammation in the early phase of fracture healing as previously reported.[17]

In the animal studies on fracture healing, the outcomes are usually evaluated by histopathology, biomechanical, and radiological examinations.[17,18,20] To keep the number of animals to a minimum from an ethical point of view, it is critical to make a detailed evaluation of the current results as much as possible to increase the reliability of the results.[21] Therefore, we practiced the histopathology, histomorphometry, immunohistochemistry, micro-CT, and biomechanical analysis in the present study. Although there are studies on the use of cephalosporins such as cefazolin and cefuroxime in orthopedic studies in the literature,[18,22] to the best our knowledge, this study is the first in which all of all detailed examination methods used in a rat femur fracture model to examine the effects of cephalexin. Similar to the limited number of cephalexin studies in the literature,[23] we chose a dose of 60 mg/kg of cephalexin in this rat model of fracture healing.

There are studies in the literature in which only plain radiographs were evaluated as in a study conducted by Perry et al.,[24] whereas there are also studies using only 3D micro-CT for assessing the fracture union[10] The advantages of micro-CT is to be non-invasive technique used in small samples, to be able to show the trabecular structure of bone, to evaluate the BMD, and to allow the volumetric evaluation of bone and callus.[25-27] In our study, the definition of fracture union was determined by micro-CT and bridging bony callus was observed radiologically in all samples. In an experimental study by Bissinger et al.[22] investigating cefuroxime, another cephalosporin, by micro-CT analysis, no significant difference was found in the BMD and BV measurements between the control and cefuroxime groups. In our study, although the BMD values measured at Week 4 increased compared to Week 2, no statistically significant difference was found between the control and cephalexin groups in both time points. These findings suggest that cephalexin does not positively or negatively affect the bone mineralization in the fracture healing. Also, there was no significant difference in the CV/BV ratios between the control and cephalexin groups at Weeks 2 and 4. Considering that the fracture healing period was completed at Week 6 in rats, the CV/BV ratios could be higher over time, since the development of hard callus increases at Week 4 compared to Week 2.[28]

In studies on the fracture union, the biomechanical examinations are particularly valuable to test the ability of the fracture healing to perform its primary task of providing mechanical support to the body. Indeed, Delgado-Martinez et al.[29] examined the effects and safety of four different antibiotics, cefazolin, cefuroxime, vancomycin, and clindamycin on the femur fracture healing by only using the biomechanical tests. They found that the mechanical strength of fracture callus was similar between the cefazolin and clindamycin groups, but lower in vancomycin and probably cefuroxime groups and concluded that cefazolin and clindamycin were safe drugs to use during fracture healing. Prodinger et al.[30] performed systematic characterization of the mechanical properties of different rodent bones available for rat fracture models by comparing the radiological and biomechanical examinations and found the highest radiological and biomechanical consistency in the rat femur. They emphasized the superiority of the three-point bending test due to its reproducible and easy to apply features and found that the ideal rat bone for the three-point bending test was the femur bone with a long length and thick cortex. Therefore, in the present study, we focused on the biomechanical consistency of study by applying the three-point bending test on the rat femurs to examine the effects of cephalexin.

In a study by Natividad-Pedreño et al.,[18] cefuroxime significantly reduced the biomechanical resistance of the bone, while cefazolin did not make a significant difference compared to the control group. On the other hand, Bissinger et al.[22] did not detect a significant biomechanical difference in their *in vivo* study using cefuroxime in a rat fracture model. As in these studies in which different types of cephalosporines were studied, we found no significant difference between the control and cephalexin groups in terms of σ_bend flexural strengths as evidenced by the three-point bending test.

To support our radiological and biomechanical results, we used the histomorphometric criteria determined by Gerstenfeld et al.[31] These criteria included the ratio of cartilaginous callus diameter-to-total callus diameter and ratio of total callus diameter-to-femur diameter which were examined in two-week and four-week control and cephalexin-administered rats. Our micro-CT examinations showed that the CV increased significantly in the cephalexin groups and CV/BV ratios increased non-significantly in the femurs at Week 4 compared to Week 2; however, this increase was not observed in the histomorphometric examination. This difference was assumed to occur since micro-CT could not detect completely soft uncalcified tissue callus, but the histomorphometric examination could detect uncalcified soft tissue. While the ratio of cartilaginous callus-to-total callus increased in the femurs at Week 4 compared to Week 2, there was no statistically significant difference between the control and cephalexin groups in both time points, supporting the radiological and biomechanical findings.

In the present study, we used the scoring system between 1 to 10 defined by Huo et al.[12] and found a statistically significant increase in histological healing scores in all control and cephalexin groups between the second and fourth weeks, as expected, since the fracture healing is a progressive process over time. Histopathological evaluation was also used by several researchers to investigate the effects of antibiotics on the bone fracture healing.[24] One of these studies examined the first generation cephalosporins which were found to reduce the recovery scores compared to the control group, although this decrease was statistically significant only in the treatment with cefuroxime.[18] Consistent with this study, we also found lower recovery scores in the cephalexin group at Week 2 compared to the control group, but it did not reach statistical significance. On the contrary, Akkaya et al.[20] examined the effects of cefazolin, ciprofloxacin, and various vitamins on the fracture, found a better histological recovery in the group treated with cefazolin sodium, and emphasized the safety of cefazolin sodium. In this study, a plaster application was performed for the fracture fixation. However, we preferred intramedullary nailing for better standardization of the fracture fixation. Supporting some the studies in the literature, we found that cephalexin did not change the histological healing scores compared to the control group; thus, it may be a reliable antimicrobial agent in terms of safe effects on the fracture union.

Inflammation is the key step for the induction of healing in the first phase of fracture healing, and its development and continuation at an appropriate level is vital for the fracture healing, and there are studies examining this step in the literature.[32] One of these studies showed that the quinolones negatively affected the fracture healing, particularly in the early period of healing.[17] Our study examined the effects of cephalexin on the inflammation at two- and four-week fracture healing. As a result, the inflammation scores in the early phase of fracture healing in the control and cephalexin groups were higher than the scores in late phase due to the nature of the healing process. However, there was no statistically significant difference between the inflammation scores of the control and cephalexin groups at two and four weeks. This finding is critical, as it demonstrates that cephalexin does not have a negative effect on the cells and cytokines involved in the inflammation period.

Review of the literature reveals a number of studies investigating the effectiveness of various substances in the healing process of the fracture.[33,34] In the study by Kuroda et al.,[35] CD34+ cells were used in delayed union and nonunion cases and their effectiveness was evaluated. In the inflammatory phase of fracture healing, these cells at the fracture line were suggested to function by leading to revascularization with their capacity to differentiate into both osteoblasts and endothelial cells. In our study, we found that cephalexin provided a significant increase in the number of CD34+ cells, particularly after the second week of fracture healing. In both control and cephalexin groups, the number of CD34+ cells at Week 4 slightly reduced after the inflammation phase. We suggest that the number of these cells decreases due to differentiation of these cells into various endothelial cells and osteoblasts during the fracture healing, but further studies are needed to explain how cephalexin increases the number of CD34+ cells in the early period, but reduces those in late period and to elucidate the underlying biochemical mechanisms of this process.

Bone morphogenetic proteins (BMPs) initiate and regulate a series of cellular signaling pathways for embryological bone formation, which are expressed at various stages of fracture healing. After BMP-2 is expressed in the first 24 h of the fracture, it initiates a cascade in which other BMPs are expressed and, thus, stem cells in the environment differentiate into the chondroblasts and osteoblasts. The BMP-4 and BMP-7 increase in the osteogenic phase of the fracture, where the soft callus turns into hard callus.[36] Yu et al.[33] also

showed that BMP-2, -4, and -7 increased and peaked up to the sixth week after the tibia fracture in rats. As a result of the immunohistochemical analysis, we observed that BMP-4 and BMP-7 increased in the fourth-week callus of the control and cephalexin groups compared to Week 2, indicating no significant difference. In addition, we found a statistically non-significant increase in BMP-7 at Week 4 in cephalexin group compared to the control group. The reason for this may be the lack of information in fracture healing at Week 6, when the BMPs show the highest level in rats.

There are also reports that COL-I peaks at the fourth week immunohistochemically in a rat femur fracture model[37] and may have an accelerating and supportive effect on the fracture healing.[38] In the present study, the immunoreactivity of COL-I increased non-significantly in the fourth week of fracture healing in both groups, compared to Week 2; however, cephalexin did not cause a significant change in the amount of COL-I in the early and late periods compared to the control group. This finding suggests that cephalexin may be safe in preventing the integrity of extracellular matrix of bone.

The modulatory effect of COL-III on the fracture union is via the osteoblast differentiation and trabecular bone formation.[39] Lawton et al.[40] reported an increase in the amount of COL-III in the callus biopsies of the fractures in nonunion cases and suggested that the increased amount of COL-III in the fracture line might be related to the nonunion of fracture. In our study, COL-III level was significantly lower in cephalexin treated rats in the CEP2 and CEP4 groups, compared to corresponding control groups. We suggest that this finding related to possible positive effects of cephalexin on fracture healing.

The main limitations to the present study are its limited sample size due to the ethical concerns, the inability to simulate different fracture and infection models such as the infected open fracture model, inability to compare different antibiotics, the lack of dose and time-dependent experiments, the lack of determination of drug concentration at the fracture line or in the plasma, and not using the contralateral femur as a control for biomechanical analysis of each specimen. As a result, the findings of our study should be cautiously interpreted, although they indicate that cephalexin has no effects on the radiological, histopathological, histomorphometric and biomechanical fracture healing model in rats.

In conclusion, immunohistochemically, cephalexin significantly increased the number of CD34+cells in the early phase of the fracture healing, suggesting a significant positive effect of cephalexin. If further *in vivo* studies can provide comparable results in humans, cephalexin may be considered as an oral alternative drug that would not negatively affect the fracture union in the treatment of orthopedic infections leading to serious morbidity in the health system and serious morbidity in the patients and in the fractures that can be treated on an outpatient basis, and cephalexin may be evaluated in the antibiotic combinations. We believe that our findings are valuable in terms of showing the effects of cephalexin, one of the oral antibiotics, on fracture union in the management of orthopedic infections, which have a great place in daily orthopedic practice.

Ethics Committee Approval: The study protocol was approved by the Local Ethics Committee for Animal Experiments of Bagcilar Education and Research Hospital (date: 27.12.2019, no: 2019-217).

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

Author Contributions: Writing, editing: M.U.; Analysis and interpretation of data: B.Y.; Literature review: H.M.M.; Revised drafted article: M.F.D.; Data processing and statistic: E.Y.S.; Interpretation of data: B.K.; Critical review, supervision: M.A.G.; Supervision, proofreading: S.Y.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding: The authors received no financial support for the research and/or authorship of this article.

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