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ORIGINAL ARTICLE

Effect of N-acetylcysteine on fracture healing in rat femoral diaphysis: A histopathological, radiological, and biomechanical analysis

Burak Yildirim, MD[1], Cemil Erturk, MD[2], Halil Buyukdogan, MD[3], Tahir Burak Saritas, MD[4], Nilsen Yildirim Erdogan, MD[5], Fatih Ertem, PhD[6]

[1]Department of Orthopedics and Traumatology, Kırklareli Training and Research Hospital, Kırklareli, Türkiye
[2]Department of Orthopedics and Traumatology, Health Sciences University, Kanuni Sultan Süleyman Training and Research Hospital, İstanbul, Türkiye
[3]Department of Orthopedics and Traumatology, Beykent University Hospital, İstanbul, Türkiye
[4]Department of Orthopedics and Traumatology, Tuzla State Hospital, İstanbul, Türkiye
[5]Department of Pathology, Health Sciences University, Bağcılar Training and Research Hospital, İstanbul, Türkiye
[6]Department of Biomechanics, Dokuz Eylül University Institute of Health Sciences, İzmir, Türkiye

Despite significant advancements in fracture surgery, issues such as fracture healing delays and nonunions persist.[1] This prompts the testing of numerous medical and surgical techniques for fracture healing, and ongoing intensive research persists.[2,3]

One of the most important factors which negatively affect fracture healing is oxidative stress. Oxidative stress causes reactive oxygen species (ROS) to form at the fracture site.[4]

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Correspondence: Burak Yildirim, MD. Kırklareli Eğitim ve Araştırma Hastanesi, Ortopedi ve Travmatoloji Kliniği, 39010 Kırklareli, Türkiye.

E-mail: brkyld-92@hotmail.com

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ABSTRACT

Objectives: The aim of this study was to examine the effect of N-acetylcysteine (NAC), which has antioxidant properties, on healing in a rat femoral diaphysis fracture model.

Materials and methods: Twenty-four male Wistar-Hannover rats were randomly divided into two groups: experimental (n=12) and control groups (n=12). An open femur fracture model (osteotomy) was applied to the right femora of both groups. Fixation was performed with Kirschner wire. While intraperitoneal NAC treatment was given to the experimental group for 21 days after surgery, an equal volume of intraperitoneal saline injection was administered to the control group. At the end of this period, the femurs obtained from the sacrificed animals were examined histopathologically, radiologically, and biomechanically. Huo scoring was used for histopathological examination. The samples were examined radiologically according to the Radiographic Union Scale in Tibial Fractures (RUST) scoring system. The three-point bending test was used for the biomechanical examination.

Results: According to the third-week results, NAC could histopathologically contribute positively to fracture healing in rats ($p=0.003$ and $p<0.05$, respectively). Considering radiological and biomechanical parameters, no significant difference was observed between the groups in terms of healing ($p>0.05$). However, a positive significant correlation (67.7%) was found between histopathological results and radiological findings ($p=0.016$ and $p<0.05$, respectively).

Conclusion: Our study results indicate that NAC may have a histopathologically positive effect on the healing process in rat traumatic fractures. Based on these findings, NAC preparations may be used as a supportive agent in the treatment of fractures. Further clinical studies are needed.

Keywords: Bone healing, N-acetyl cysteine, open fracture, reactive oxygen species.

Post-traumatic, physiological, and environmental factors increase the production of ROS, which occurs continuously during normal metabolism and enzymatic reactions. Consequently, tissue proteins, lipids, enzymes, and deoxyribonucleic acid (DNA) can be damaged.[5,6]

It is reported that increased ROS levels negatively impact the function of osteoblast and osteoclast cells.[7] The ROS binding competitively to β-catenin leads to the activation of a family of transcription factors known as Forkhead box O (FoxO). FoxOs antagonize Wnt/Tcf-mediated transcription after activation by ROS. Weakening of the Wnt/β-catenin/Tcf pathway, which has an important role in bone formation, will lead to a decrease in osteogenesis.[8,9] The ROS also play a critical role in osteoclast differentiation and function. Increased production of nuclear factor-Kappa B ligand (RANKL) and activation of the ERK/NF-κB/tumor necrosis factor (TNF)/interleukin (IL)-6 pathways, osteoclast apoptosis is prevented, thereby leading to osteoclastogenesis.[10] Changes in mitochondrial dynamics, endoplasmic reticulum stress pathway, and autophagy are other factors contributing to oxidative stress-induced bone loss.[11,12]

N-acetylcysteine (NAC) is an agent which is usually used in respiratory system disorders and to reduce the hepatotoxic effect of acetaminophen in overdose. In addition, NAC is an amino acid molecule and has antioxidant properties.[13] Its chemical structure consists of the sulfhydryl functional group (-SH) and the acetyl group (–COCH3) bonded to the amino group (NH2). This structure is associated with direct and indirect antioxidant and mucolytic effects.[14] It shows direct antioxidant activity thanks to the free thiol group's ability to interact with reactive oxygen and nitrogen.[15] In our previous study investigating the effect of NAC on sciatic nerve healing, significant differences were found in the levels of native thiol, native thiol/total thiol and disulfide/total thiol in the blood parameters of treated rats compared to the control group and we observed that the antioxidant effect in blood parameters was stronger in rats receiving NAC treatment.[16] Recent *in vitro* studies have demonstrated that NAC decreases ROS in cell culture, boosts alkaline phosphatase levels, and enhances osteoblast activities by reducing cytotoxicity.[17,18] On the other hand, experimental animal and clinical studies of NAC are limited in the literature.

In the present study, we hypothesized that NAC would promote fracture healing. We, therefore, aimed to evaluate the effect of NAC on fracture healing histopathologically, radiologically, and biomechanically.

MATERIALS AND METHODS

In our experimental study, a total of 24 adult healthy male rats of Wistar-Hanover breed, 12-to 14-week-old and weighing 403 g (range, 369 to 455 g) were used. The study protocol was approved by the İstanbul Bağcılar Training and Research Hospital Animal Experiments Local Ethics Committee (date: 22.10.2021, no: 2021/18). All procedures performed with live experimental animals were carried out at Experimental Animal Laboratory of İstanbul Bağcılar Training and Research Hospital. The rats were provided with water and standard pellet feed ad libitum and were kept in a room with 50 to 60% humidity and a temperature range of 18 to 22°C, under a 12-h light/12-h dark cycle. Our study was designed according to the ARRIVE guide for experimental animal studies.[19] The rats were randomly divided into two groups as control and experimental groups.

Surgical technique

The surgeon performed all surgical procedures in a single session. Following the preparation, 90 mg/kg of ketamine hydrochloric acid (HCl) and 10 mg/kg of xylazine HCl were administered intraperitoneally to the animals. A single dose of antibiotic prophylaxis was administered with 8 mg/kg of gentamicin subcutaneously before the operation. A 2-cm longitudinal incision was made on the lateral thigh. With the help of retractors, the femoral shaft was accessed between the biceps femoris and vastus lateralis muscles. The periosteum was carefully preserved and not detached. Using a micro-circular saw with a blade thickness of 0.6 mm, a transverse osteotomy of 1/3 midline was applied to the femoral diaphysis. Antegrade intramedullary Kirschner wire (K-wire) was sent from the distal osteotomy line. The skin incision was shifted to the knee region, allowing the wire to exit the knee. After the fracture was properly reduced, the wire was sent intramedullary to the proximal part in the retrograde direction up to the trochanter major. The K-wire was cut. The fascia and skin were, then, sutured and closed separately (Figure 1).[20] No anesthesia or procedure-related complications were observed in any animal during and after the surgical procedure.

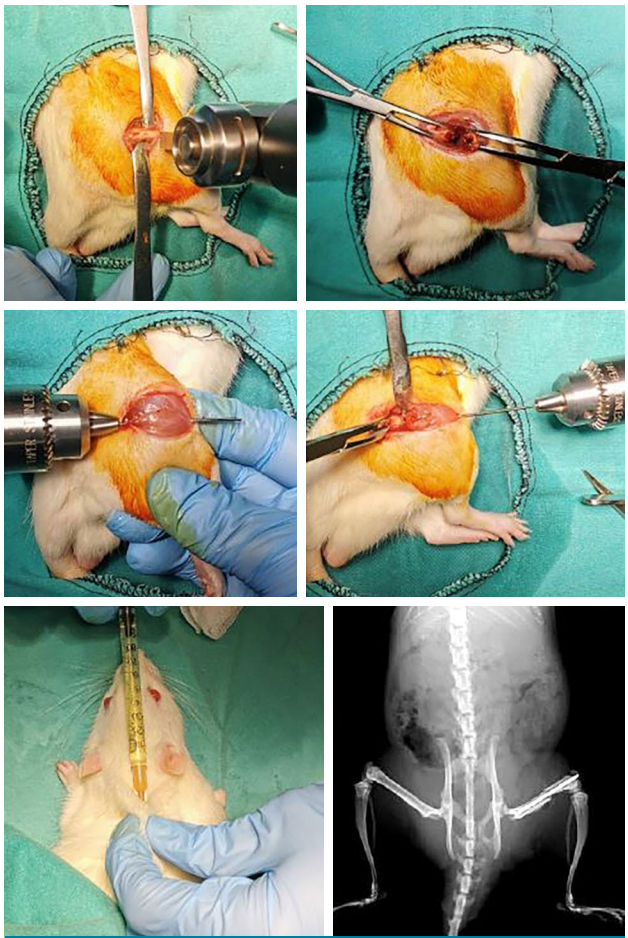


FIGURE 1. Surgical procedure and radiological confirmation.

Treatment/placebo administration and follow-up procedure

The rats in the experimental group were given 150 mg/kg/day NAC (Asist®, 300 mg/3 mL, Hüsnü Arsan İlaçları A.Ş., Istanbul, Türkiye) as a single daily intraperitoneal injection for 21 days. The dose of NAC applied in the treatment protocol was determined as the antioxidant activity of NAC has been shown in previous studies.[20,21] For 21 days, the rats in the control group received 0.9% NaCl saline in the same volume (mL) as a placebo.

During the postoperative follow-up, the possibility of complications such as wound site problems (infection, wound site deterioration), skin changes, limitation of movement, systemic problems and death were taken into account. Daily nutritional monitoring (water and food) was planned at least twice a day. The criteria which led to the removal of the animal from the experimental protocol were the development of the mentioned complications or the approval of the veterinarian.

Euthanasia and obtaining samples

Both groups were euthanized after 21 days of follow-up and treatment. The information that histopathological examination and biomechanical studies planned in the very early period of fracture healing may not be performed with sufficient reliability and that remodeling may begin in the late period and negatively affect the results in terms of significance was effective in our study plan.[22-24]

Intraperitoneal injection of high-dose ketamine and xylazine was performed. Animals were sacrificed by cervical dislocation method after the disappearance of grimace reflexes. Surgical preparations were made to obtain a femur sample from the right thigh of the subgroups which would be subjected to histopathological examination. Surgical preparations were made to obtain femur samples from both the previously broken right femur and the healthy left femur of the subgroups which would be subjected to biomechanical examination. All femurs, compartments and soft tissues were removed by gently disarticulating from the hip and knee joints without stripping, without damaging the callus tissue of the fractured sides. Afterwards, intramedullary K-wire extraction was performed carefully without interfering with the callus area. All the samples obtained were wrapped in saline-soaked cloths and placed in containers.

All femur samples, as experimental (n=12) and control groups (n=12), were first subjected to radiological examination. Subsequently, the experimental and control groups were further divided into two subgroups (n=6) and subjected to histopathological and biomechanical examinations.[25]

Histopathological evaluation

Samples taken from the experimental and control subgroups for histopathological examination were preserved in 10% formaldehyde solution and sent to Pathology Laboratory of İstanbul Bağcılar Training and Research Hospital. After the samples were fixed with 10% buffered formaldehyde for 24 h, they were decalcified with 10% hydrochloric acid prepared with distilled water. Routine tissue observation was performed for 12 h in the Thermo Fisher Scientific™ Shandon™ (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) closed system. Tissues were embedded in paraffin blocks. Sections of 4-μm thickness obtained from paraffin blocks by microtome were taken on slides and all sections

were stained with hematoxylin and eosin (H&E) after deparaffinization. Histologically, fracture maturity was assessed by a single pathologist unaware of the groups using an Olympus BX51 (Olympus, Tokyo, Japan) light microscope. In histological evaluation, Huo et al.'s[25] histological scoring system, in which 10 stages were determined in fracture repair, was used (Table I). In this scoring system, at least five areas of each sample were examined to evaluate fracture healing. Ten points represent the most mature healing and 1 point the most immature healing, and each sample was assigned a score corresponding to each stage of fracture healing with these histological scoring parameters.

Radiological evaluation

All groups were examined radiographically after euthanasia. The distance between the samples and the device (Toshiba-Rotanode®, Model no: E7869X, 150kV, Japan) was set to 100 cm. The radiation dose to be given to the samples was determined as 52 kV, 6.4 mA/400 ms. Anteroposterior (AP) and lateral radiographic images of all fracture specimens were taken. The captured images were transferred to digital media using the VXvue® software (Vieworks Co., Ltd., Gyeonggi-do, Republic of Korea). Images were scored according to the Radiographic Union Score for Tibial Fractures (RUST) system by two different orthopedists who were blinded to the sample codes (Table II). The RUST is a scoring system based on the visibility of callus tissue and fracture line, in which four cortices are evaluated separately on AP and lateral radiographs. Accordingly, a fracture with no improvement is scored 4 points in total, while a fully healed fracture receives 12 points in total.[26]

TABLE I
Numerical scoring for histological assessment of fracture healing[12]

Score	
1	Fibrous tissue
2	Containing a small amount of cartilage, predominantly fibrous tissue
3	Equal distribution of fibrous tissue and cartilage tissue
4	Small amount of fibrous tissue, predominantly cartilage
5	Cartilage
6	Small amount of immature bone, predominantly cartilage
7	Equal distribution of cartilage and immature bone
8	Less cartilage, predominantly immature (woven) bone
9	Healing of the fracture with immature (woven) bone
10	Healing of the fracture with mature (mature-lamellar) bone

Biomechanical evaluation

The examination was carried out in the biomechanics laboratory of Dokuz Eylül University. Biomechanical examination of the samples was performed by three-point bending method using an axial test device (AG-I 10 kN, Shimadzu®, Tokyo, Japan). The distance between the support bars was set to 15 mm. The distal and proximal ends of the femur were placed with the anterior surface facing up. Load aligned to center 1/3 of the body (Figure 2). Calibration was achieved by taking the load gap, until it reached a power of 1 N for each sample loaded into the system. The bending load was applied with a blunt tip load applicator at a constant speed of 5 mm/min, until the sample failed. Every 0.001 mm was recorded until callus fracture occurred. A decrease in strength of more than 20% was determined as the termination criterion. With the help of the computer program to which the device is connected, the displacement curve against the force was recorded instantly and the sustainable maximum load (N), displacement (mm) and stiffness (N/mm) data were obtained.[27,28]

The intact femurs of the biomechanical subgroups were also planned to be included in the biomechanical study to compare the biomechanical and structural features of both groups. If there was a difference, the results could be affected positively or negatively.

Statistical analysis

Based on the power analysis conducted in a similar studies utilizing a femur fracture model in

TABLE II
Radiographic union score for tibial fractures scoring[13]

	Radiographic criteria	
Score/cortex*	Callus	Fracture line
1	Absent	Visible
2	Present	Visible
3	Present	Invisible

* Anterior, posterior, lateral and medial cortex scores are summed and the final score is obtained.

rats, the subgroup size was calculated to require six subjects.[25]

Statistical analysis was performed using the SPSS version 22.0 software (IBM Corp, Armonk, NY, USA). The conformity of the parameters to the normal distribution was evaluated using the Shapiro Wilks test. Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The Mann-Whitney U test was used for the comparison of the parameters that did not show normal distribution in the comparison of quantitative data between two groups. Spearman correlation analysis was used to examine the relationships between parameters that did not conform to the normal distribution. A p value of <0.05 was considered statistically significant.

RESULTS

All animals were observed until the end of the protocol in accordance with the parameters specified in the exclusion criteria. During the three-week follow-up, bursitis was observed in the anterior of the knee joint in two rats in the experimental group and one rat in the control group in the last week. Treatment with the aspiration method was applied. No death, wound infection, or serious complication necessitating exclusion was observed in any of the rats.

Histopathological findings

According to the results of the examination of the H&E-stained samples made under the light microscope, the cartilage structure was found to begin to replace the immature bone structure in the experimental group. Predominantly fibrous and cartilage structure was observed in the control group (Figure 3). When the histopathological scores were compared, the data of the experimental group were found to be statistically significantly higher than the control group (p=0.003 and p<0.05, respectively) (Table III).

Radiological findings

Callus formation was observed in almost all samples. Although cortical continuity was observed in some samples, no sample achieved the highest score of 12. When the results of the groups were analyzed statistically, there was no significant difference (p>0.05) (Table IV).

Biomechanical findings

The maximum load values of the intact left femur samples obtained from the experimental and control groups were similar to each other.

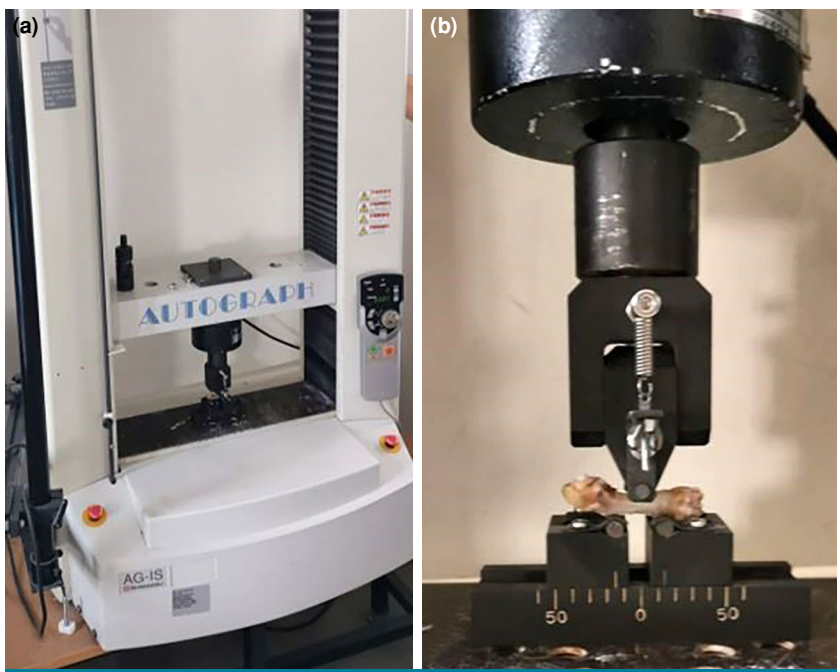


FIGURE 2. Biomechanical evaluation, **(a)** Axial three-point bending test machine, **(b)** Placement of femur specimen.

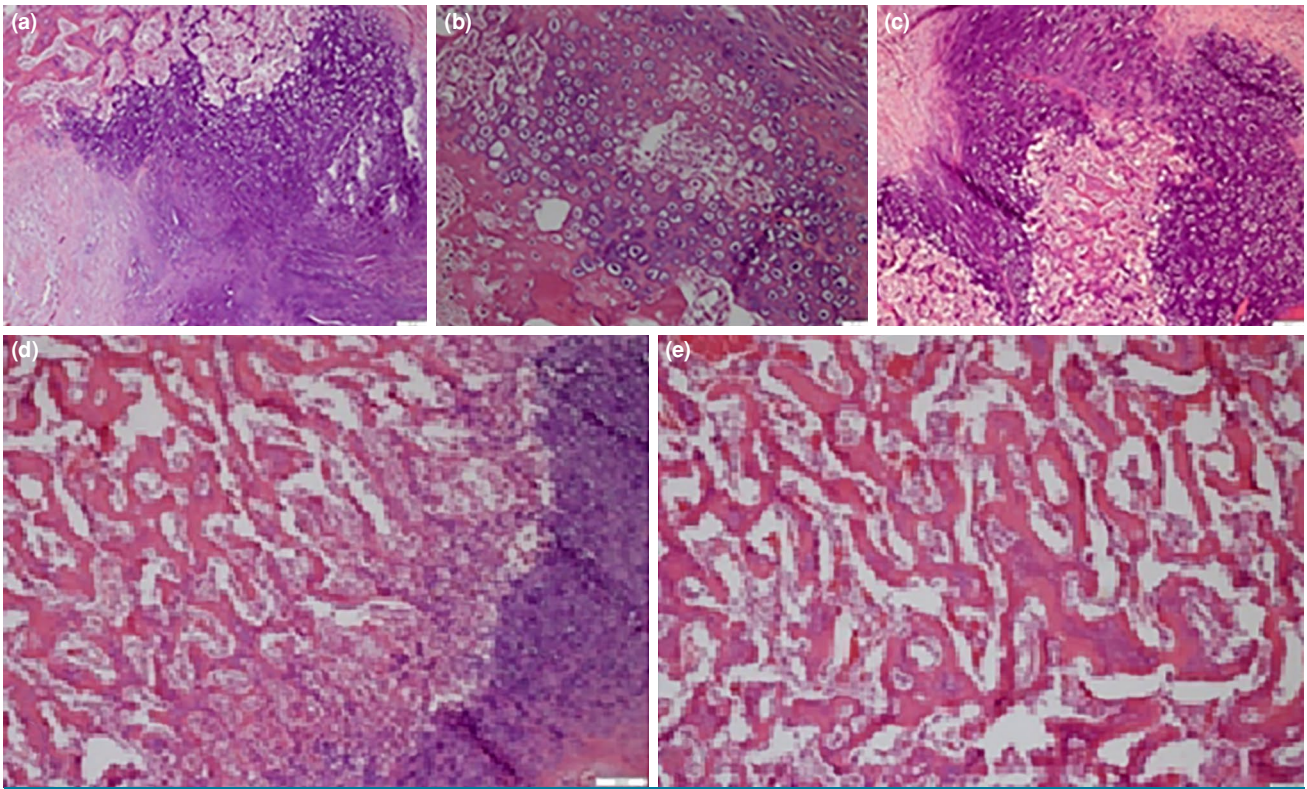


FIGURE 3. Histopathological findings. **(a)** Small amount of fibrous tissue, mainly cartilage tissue (H&E, ×100). **(b)** Entirely cartilage tissue (H&E, ×200). **(c)** Small amount of immature bone tissue, predominantly cartilage tissue (H&E, ×100). **(d)** Small amount of cartilage tissue, predominantly immature (woven) bone tissue (H&E, ×100). **(e)** Immature (immature-woven) bone tissue (H&E, ×100).

The maximum load values of the intact left femur did not show a statistically significant difference between the groups (p>0.05). The stiffness values of the intact left femur samples obtained from the experimental and control groups were similar to each other. Intact left femur stiffness values did not show a statistically significant difference between the groups (p>0.05) When the right femur maximum load results of the groups were examined statistically, there was no significant difference between the two groups (p>0.05). When the right femur stiffness results of the groups were examined statistically, there was no significant difference between the two groups (p>0.05) (Table V).

TABLE III
Evaluation of Huo histopathological scoring parameters between groups

	Experimental group (n=6)			Control group (n=6)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
Histopathological score	8.33±0.52	8	8-9	4.83±0.98	4.5	4-6	0.003*

SD: Standard deviation; * p<0.05; Mann-Whitney U test.

TABLE IV
Evaluation of RUST score parameters between groups

	Experimental group (n=12)			Control group (n=12)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
RUST score	8.5±0.8	8.5	7-10	7.92±1.17	8	6-9	0.271

RUST: Radiographic union scale in tibial fractures; SD: Standard deviation; * p<0.05; Mann-Whitney U test.

TABLE V
Evaluation of biomechanical parameters of right and left femur samples between groups

	Experimental group (n=6)			Control group (n=6)			
	Mean±SD	Median	Min-Max	Mean±SD	Median	Min-Max	*p*
Left max load (N)	230.08±48.04	180.9-305.8	223.8	230.17±51.02	179-303.9	221.2	0.873
Left stiffness (N/mm)	311.72±57.8	236.9-380.3	311	313.2±74.7	177.6-372.5	341.2	0.749
Right max load (N)	75.86±16.67	58.6-103.3	75.2	62.83±20.07	39.4-94	57.6	0.262
Right stiffness (N/mm)	46.73±13.6	32-70.3	46.4	35.44±8.57	27-48	33.5	0.078

SD: Standard deviation; * p<0.05; Mann-Whitney U test.

Correlation analysis

There was a positive, 67.7% and statistically significant relationship between RUST radiological score and histopathological score values in all subgroups in which radiological and histopathological examination was performed (p=0.016 and p<0.05, respectively). There was no statistically significant relationship between RUST radiological score and maximum load and stiffness values in all subgroups undergoing radiological and biomechanical examination (p>0.05) (Table VI, Figure 4).

TABLE VI
Evaluation of the correlation between radiological scores and biomechanical/histopathological scores

	RUST score	
	r	*p*
Biomechanic values		
Right femur maximum load (N)	0.147	0.648
Right femur stiffness (N/mm)	−0.162	0.615
Histopathological score	0.677	0.016*

Spearman's Rho Correlation analysis; * p<0.05.

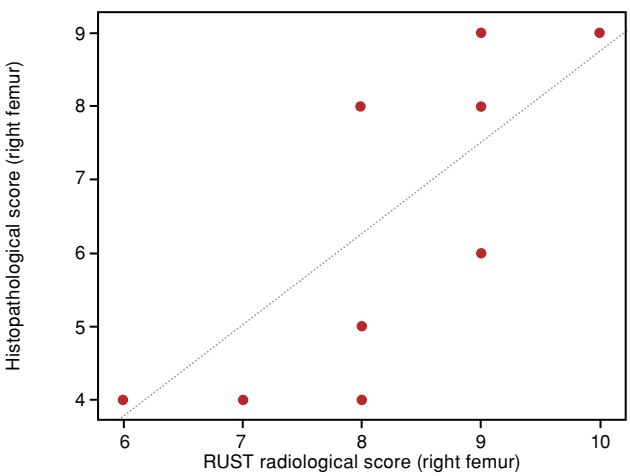


FIGURE 4. Correlation of RUST radiological score and histopathological score values.
RUST: Radiographic Union Score for Tibial Fractures.

DISCUSSION

In this experimental study, we evaluated the effect of NAC on fracture healing histopathologically, radiologically, and biomechanically. Our study results showed that NAC contributed positively to fracture healing histopathologically. In addition, radiological signs of healing were similar between the groups. Also, biomechanically similar results were obtained between the groups. Finally, our histopathological and radiological data were positively correlated. Based on these findings, we concluded that NAC could accelerate fracture healing histopathologically.

Considering ethanol and NAC studies of Volkmer et al.,[29] chondrocytes with hypertrophic and endochondral ossification activity were intensely observed in the second week histopathological examination of the control group that did not receive ethanol and treatment. Hypertrophic chondrocytes and endochondral ossification area were observed in the second week results of the animal group that received NAC and ethanol. At Week 4, immature bone areas were observed intensively in both the NAC and ethanol group and the control group.[30] In the study conducted by Safali et al.[27] investigating the effect of curcumin, an antioxidant molecule, on fractures, Huo scoring was used, but no significant results were found between the second and forth weeks between the groups.[25]

Furthermore, we found no significant difference in radiological results between the two groups in our study. In the literature, radiological studies on rat femur fracture healing are limited. The RUST

scale, defined by Kooistra et al.,[26] is a scoring system designed primarily for tibia fractures, and later studies indicated that it can be used safely in long bone fractures such as humerus, radius, ulna, and femur, and in animal experiments. The main advantage of this scale compared to other scales is that it provides the opportunity to evaluate each cortex separately and handles the healing model of the fracture more accurately.[26,30,31] The methodological methods used in these studies also differ greatly from each other. In the femur fracture study of Safali et al.,[27] animals in the experimental group were treated with curcumin, which has a potential antioxidant and anti-inflammatory effect, and no significant difference was observed in the micro-computed tomography (CT) results examined at second and forth weeks.

We found no significant difference in biomechanical results between the two groups in this study. Although they are siblings and animals of the same age, we included intact left femurs of the same animals in the biomechanical study, with the idea that bone sizes may vary and there may be possible differences in their structures. Thus, we attempted to see the relationship between the two groups under standard conditions. In the left femur analyses of the experimental and control groups, we found that there was no significant difference between the maximum load and stiffness values in the two groups. This showed us that the NAC molecule did not make a significant change in the biomechanical structure of the intact bone at the third week. In the closed fracture study of Volkmer et al.[29] in the first week biomechanical values obtained with the four-point bending test, significant maximum load and stiffness values were found in the ethanol group that received NAC treatment. They did not report a significant effect particularly in terms of stiffness in the second and forth weeks. Again, in the study of curcumin, which contains antioxidant properties, by Safali et al.,[27] biomechanical measurement could not be made in the second week due to soft callus, and no significant results were obtained in the forth-week maximum load values.

In the current study, we found a significant correlation between radiological and histopathological data. Radiographic and histopathological findings were compared in the study of femur fracture treated with peperomia pellucida extract by Florence et al.[32] The fracture line, which was observed to heal radiographically in animal groups treated with different doses of extract, was also histologically healed with compact callus tissue. The aforementioned authors confirmed the data obtained by both methods. In the cortex evaluations of the samples with excessive chondrocyte healing tissue, the fracture line was not closed. It is obvious that the samples, in which the mineralization increased and the immature bone tissue began to condense, began to appear more radiodense. As a result of our study, we also obtained histopathologically higher scores in samples with high radiological union findings and scores. In contrast, we found no significant relationship between radiological and biomechanical results. The fracture strength of healing bone is related to the inhomogeneous structural distribution of fibrous tissue, cartilage, new bone, and irregular cross-sectional area. Due to this complex structure, it is difficult to predict the fracture strength in the healing bone. Claes et al.,[33] in their study of cortical defect in sheep, reported that it took a long time for bones to reach full strength, and radiological healing may not have fully reflected structural restoration or normal strength.

Nonetheless, there are some limitations to this study. First, micro-CT analysis could provide more quantifiable results regarding callus formation due to its capability for three-dimensional imaging. Additionally, the collagen structure could be assessed using immunohistochemical methods. However, these techniques were not applied in the current study due to technical limitations. Second, due to ethical concerns, the number of rats was limited and there was no sham group. Furthermore, our planned study investigating NAC's effects on fracture healing does not include an examination of its underlying mechanisms of action. Further research on this subject is warranted.

In conclusion, NAC may be used safely in the experimental fracture model. In this experimental study, although we could not demonstrate that NAC supports radiological and biomechanical healing, we obtained histopathologically improved healing findings. Therefore, our study is a rare preliminary study showing the effect of NAC molecule on fracture healing with these methods in an experimental animal fracture model. Further prospective, randomized-clinical studies are needed to investigate the potential effects and mechanisms of NAC in the fracture healing phase.

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

Author Contributions: Idea/concept, design, writing the article, references and fundings: B.Y. C.E.; Control/supervision, analysis and/or interpretation, critical review: C.E., N.Y.E., F.E.; Data collection and/or processing, literature review: B.Y., H.B., T.B.S.; Materials: B.Y., T.B.S., N.Y.E., F.E.

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