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ORIGINAL RESEARCH

An experimental study on the effects of splenectomy on bone fracture healing

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Abstract

Bone fracture healing is a complex process involving multiple mechanisms where immune system has vital role. The spleen is a part of this process and its effect on bone metabolism is not completely understood. Herein, the effects of simultaneous bone fractures and splenectomy were evaluated regarding early and late periods of fracture healing in rats. The closed fracture model was applied in sixty-four healthy male Wistar-albino rats. Rats were randomly selected and divided into four groups. The splenectomy was carried out in two groups. The early period was declared as three weeks and late period as five weeks for evaluating the fracture healing in rats, (Groups A: splenectomized 3rd week, Groups B: non-splenectomized 3rd week, Groups C: splenectomized 5th week, and Groups D: non-splenectomized 5th week). The groups were compared regarding radiological, biomechanical, and histopathological aspects at 3rd and 5th weeks of fracture healing. The callus/bone volume ratios of Groups C and D were lower than those of Groups A and B (A–C, $p \leq 0.001$; B–D, $p \leq 0.001$). Radiographic Union Score for Tibial Fracture scores of Groups C and D were higher than those for Groups A and B (A–C, $p \leq 0.001$; B–D, $p \leq 0.001$). Bone mineral density measurements of Groups C and D were higher than those for Groups A and B (A–C, $p \leq 0.001$; A–D, $p \leq 0.001$; B–C, $p \leq 0.001$ and B–D, $p \leq 0.001$). The histological scoring of Groups A was lower than Groups C and D (A–C, $p = 0.004$; A–D, $p = 0.001$). Splenectomy may thus adversely affect the early phase fracture healing.

Keywords

Splenectomy; Fracture healing; Experimental rat study

1. Introduction

Trauma contributes to morbidity and mortality in patients presented to emergency department (ED). Traumatic individuals, such as from traffic accidents or falling from height, may experience bone fractures and damaged internal organs [1].

Spleen is often injured among the intra-abdominal organs after blunt trauma [2]. Splenectomy is carried out in patients of splenic rupture and bone fracture caused by blunt abdominal trauma, particularly when conventional treatments fail [3]. At present, the spleen trauma is managed through an organized interdisciplinary teamwork. Awareness of potential physiological and immunological disorders following the spleen trauma is emphasized [4]. Spleen function preservation while treating the patients with spleen trauma is especially prioritized. In general, there is a preferred tendency of conservative treatments in medical management [5, 6]. The potential impacts on immunity and fracture healing processes are considered while performing splenectomy on patients with spleen injury and bone fractures. Questions arise regarding effect on immunity and proper healing of fractures. The bones and immune system have the regulatory functions regarding macrophages and osteoclasts which evolve from the same progenitor [7, 8].

Inflammation is the first response towards tissue trauma [6]. Fracture healing also begins with an acute inflammatory response at fracture site and comprises of interrelated complex physiological processes, *i.e.*, inflammatory phase, cartilaginous callus and bony callus formation, and remodelling [9]. The inflammatory response may be short-lived, but its impact can persist in the advanced recovery stages because of the actions of immune cells [9–11].

The objective of fracture healing process is to restore the damaged skeletal structure to its pre-injury cellular and biomechanical state. Numerous local and systemic factors can impact the fracture healing process. Osteoimmunology suggests a significant relation between immune system and skeletal metabolism [12].

Fracture hematoma occurs in the fracture gap immediately after the fracture. It involves the dynamic and tightly choreographed interactions between fibrin matrix, cells, and cytokines for guiding the fracture repair [13]. The inflammatory cells are the first to arrive at fracture site followed by

mesenchymal progenitor cells, endothelial cells, chondrocytes, osteoblasts, and finally osteoclasts [14]. Many cytokines such as tumor necrosis factor (TNF-α), and interleukin (IL)-6 are released through these cells which regulate hemopoietic and immune activities.

Bone fractures are associated with soft tissue injuries and their repair begins with acute inflammation where immune system has active role [3]. Even though the link between immune system and bone development is not completely understood, research work has indicated the effects of immune reaction on fracture healing process [15, 16]. Evidence suggests that immune cells and their secreted factors are important for the physiological response after fracture [17].

The second largest lymphoid organ is spleen which is at the centre of humoral immunity having immunomodulatory role [18].

Fracture healing is a complicated process which includes parameters such as micro-stability, fracture morphology, tissue perfusion, and the immune system [19]. More work is required to understand how spleen affects bone metabolism [20]. Fracture healing mechanisms and the factors affecting them are being researched for the patients underwent splenectomy [21]. There is a hypothesis that the fracture healing repair stages may be negatively impacted by the deficiency of immune cells' functions.

This experimental study evaluates the effect of splenectomy on early and late periods of fracture healing in rats with simultaneous fracture and splenectomy.

2. Methods

2.1 Animals

Each test animal was kept separately in cages with unlimited water and standard pellets. The laboratory conditions were maintained as 20–23 °C, 60–70% humidity, and 12-hour light/dark cycle. Sixty-four healthy male Wistar-albino rats (8 weeks old with average weight of 320 g) were randomly selected and divided into four groups.

2.2 Surgical procedure

Before surgery, an antibiotic (gentamicin, 4 mg/kg) was intraperitoneally administered once to all the rats for preventing infection at the surgical site. Ketamine HCl, 50 mg/kg (Ketalar®, CLB00474, Pfizer, Ortaköy, İstanbul, Türkiye) was intraperitoneally administered as anesthesia. The setting was sterile for all the surgical operations. Closed fracture model was applied to all rats by the same surgeon (MT). The right knee areas of rats were shaved and stained with povidone-iodine (Batticon®, 2441, ADEKA, Samsun, Türkiye).

The right lateral femur was allocated, and 2 cm longitudinal incision was made in the skin along anterior of right knee. The patella was lateralized after medial parapatellar arthrotomy. The femur medulla was entered through intercondylar notch with 1.2 mm drill bit. Nitinol guide wire was inserted after intramedullary reaming through intercondylar notch. A closed fracture was created in the right femur of rats under sterile conditions by employing the Bonnarens and Einhorn's method [22].

A fracture was made and nitinol wire was used in femur medulla to ease reduction after the fracture. Retrograde intramedullary nailing was done over nitinol wire using 16 Gauge intravenous canula (Ayset, Türkiye). Incision was closed following the skin and subcutaneous anatomy with 3/0 coated polyglactin suture (VICRYL, Ethicon, USA). The fixation stability was checked manually and by direct radiography on surgery table with portable X-ray device.

Immediately after fracture creation, 64 rats were randomly assigned to two equal groups. Splenectomy was carried out on one group by the same surgeon. Splenectomy was performed after the depilation of abdominal region followed by 3 cm midline incision. The spleen was examined, dislodged from its ligaments, and extracted from the abdomen. After dissection, ligation with 3/0 silk suture (SILK, Boz Medikal, Türkiye) was made followed by cutting the veins in spleen, punch, and hilus. The spleen was removed from abdomen. The incision was closed following the skin and subcutaneous anatomy with 3/0 coated polyglactin suture (VICRYL, Ethicon, USA).

For evaluating the fracture healing, timing was accepted as three weeks for early period and five weeks for late period [23]. Groups A: splenectomized 3rd week, Groups B: non-splenectomized 3rd week, Groups C: splenectomized 5th week, and Groups D: non-splenectomized 5th week. The experiments had two time points. Groups A and B rats were sacrificed in 3rd week of study to investigate the early phase of fracture healing. Groups C and D rats were sacrificed two weeks later in the 5th week to investigate the late period of fracture healing, and the study was terminated.

Before surgery, an antibiotic (gentamicin, 4 mg/kg) was intraperitoneally administered once to all rats for preventing the infection at surgical site. Rats were anesthetized by intraperitoneally administering 50 mg/kg ketamine HCl (Ketalar®, Pfizer, USA). The setting for all surgical procedures was kept sterile.

There was no complication including the infection during study period. Under general anaesthesia, cervical dislocation was executed in every rat used in the experiments. Afterwards, all fractured femurs were dissected gently from their muscle tissues. Tissue samples were fixed in formalin solution. Soft tissues were removed from the fractured femurs. After radiological evaluations, half of the sixteen rats in each group were sent for biomechanical examinations and other half for histopathological evaluations (Fig. 1).

2.3 X-ray analysis

Rats' femurs were X-rayed (portable X-ray unit, Siemens, Erlangen, Germany) to evaluate the bone union. The femurs were placed in anteroposterior (AP) and lateral planes and exposed to 45 kV voltage for 5 s. Two observers, a radiologist and an orthopaedic surgeon, measured the callus area as per the Radiographic Union Score for Tibial Fracture (RUST) scoring system. RUST scoring evaluated the AP and lateral radiographs of each sample. A score of "1" was assigned to each observable cortex with no callus fracture line, a score of "2" if callus fracture line was present, and score of "3" if callus was present but no visible fracture line. For each sample, the lowest score was "4", indicating that the fracture did not unite,

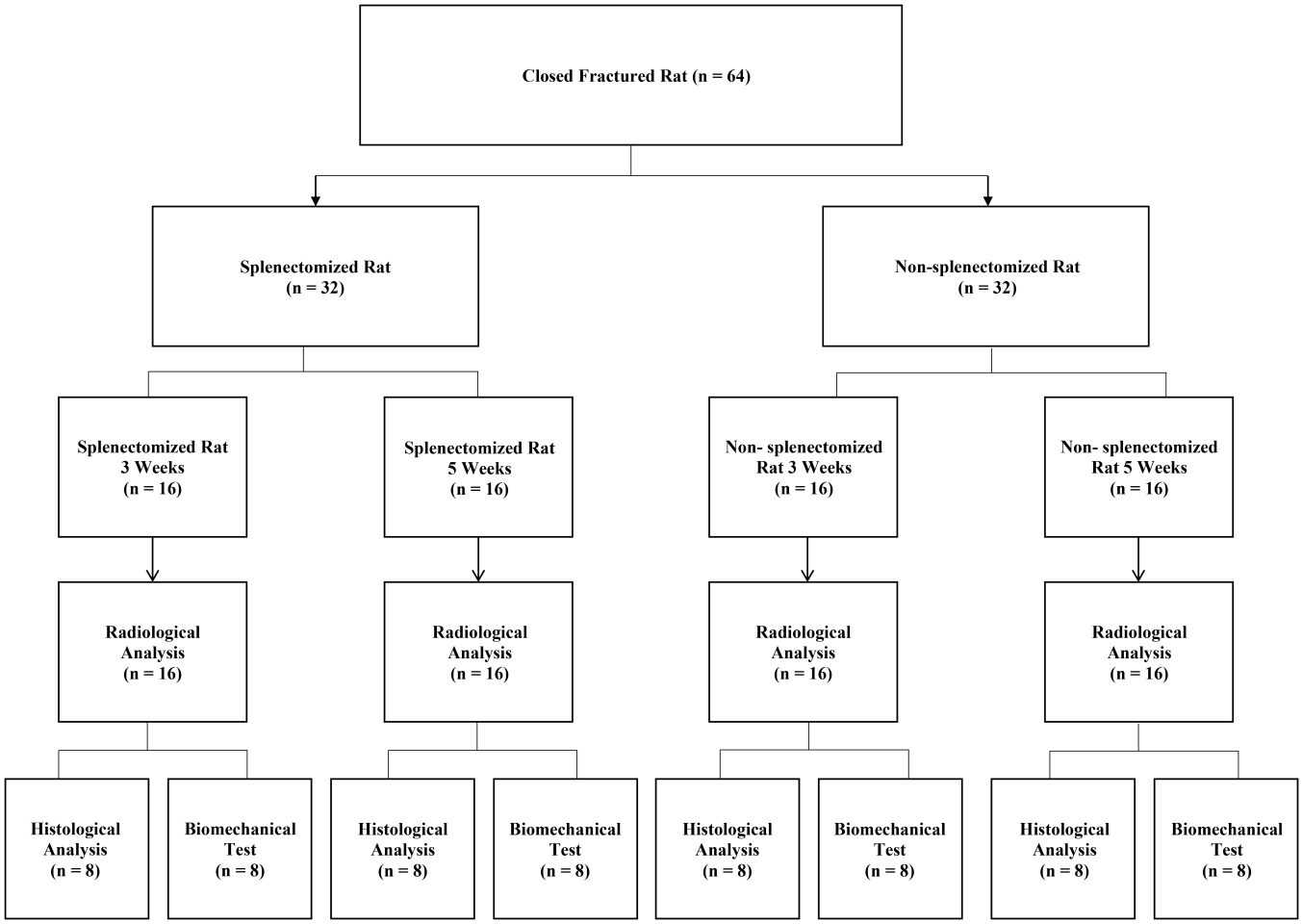

FIGURE 1. The workflow of this study.

while the highest score was "12", indicative of healed fracture. RUST scores determined by the observers were averaged for each sample [24] (Fig. 2).

2.4 Micro-computed tomography (CT)

The samples were placed in micro-CT machine after their formalin fixation for 24–48 hours. The fracture callus was imaged using micro-CT equipment (μCT-80, Scanco Medical, Bassersdorf, Switzerland). Images were collected with 15.6 μm per pixel resolution using micro-CT scanner set to 70 kVp voltage and 114 A current. Serial cross-sectional photographs of callus regions were gathered for the three-dimensional histomorphometric analysis of image acquisition. 500 images were taken while the subject was positioned on turntable which continuously moved in an axial manner over 180°. The region of interest (ROI) encompassed 8.4 mm total range (4.2 mm proximal and distal to the fracture line). The volume of interest was obtained using Scano Evaluation software (NRecon software, Scanco Medical, Brüttisellen, CH, Switzerland) for the quantitative study of bone growth inside the ROI. These quantities were represented in highly mineralized bone (33–60%) and low mineralized callus (17.5–33%) tissue, based on grey threshold. The mineralized callus volume was divided by the tissue density to determine tissue's mineralized density. Bone and callus volumes were also determined using this method (Fig. 3).

2.5 Bone mineral density

Bone mineral density (BMD) (mg HA/cm^3) was measured for each sample. Density calibration data assisted in the BMD estimation through the scans of hydroxyapatite phantom as supplied by the manufacturer [25].

2.6 Histological analysis

Bone tissue samples were fixed in 10% (v/v) formaldehyde for 48 hours. Bone tissues were then taken to decalcification solution containing 8% hydrochloric acid (HCl) and 8% formic acid. The samples were dehydrated by soaking in 75%, 96% and 100% (v/v) ethanol series for 1 hour. For infiltration, tissue samples were kept in an oven at 60 °C (EN 055, Nüve, Ankara, Türkiye) and in molten paraffin wax for 60 min with three changes. Longitudinal sections were cut in 4 μm thickness from bone tissue and stained with Hematoxylin and Eosin (HE). The cell nucleus was stained as purple and cytoplasm as pink with HE staining. Bone tissue samples were examined at $\times$20 magnification under light microscope (BX43 Upright, Olympus, Pennsylvania, USA) equipped with digital camera (DP26 5-megapixel digital camera, Olympus, Pennsylvania, USA). Five sections of each sample were evaluated. In each section, at least five similar areas were examined and scored according to Huo *et al.* [26] histological scoring system. Scoring as per the Histological Scoring Criteria were as fol-

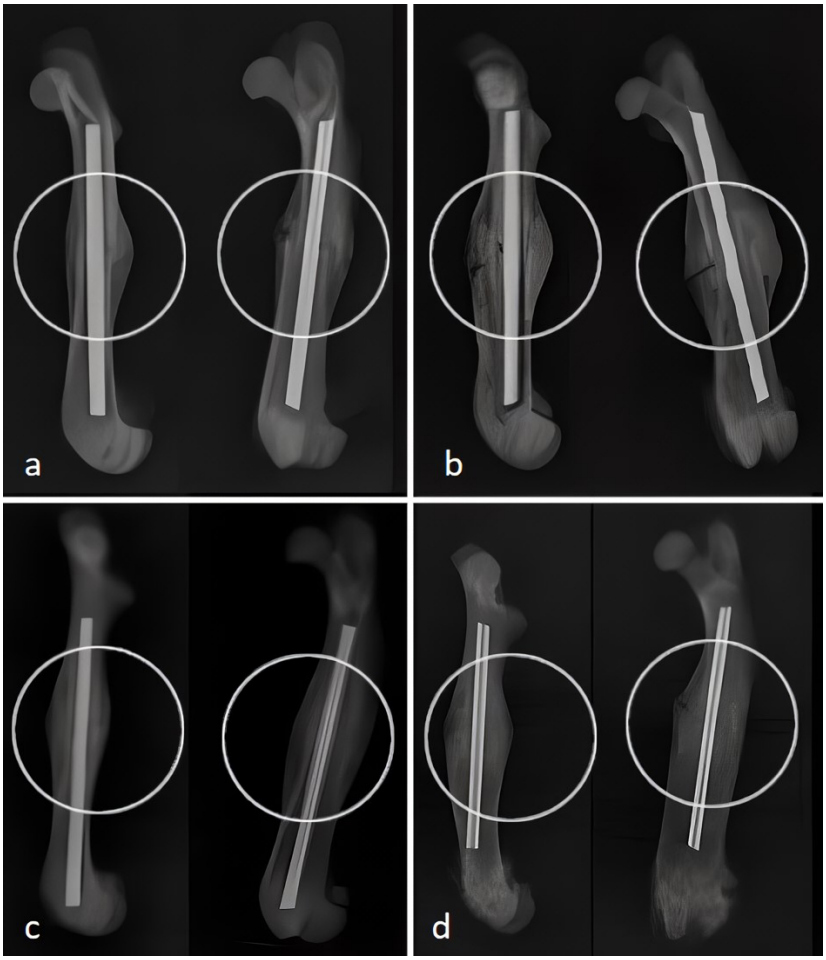

FIGURE 2. Anteroposterior and lateral X-ray examination for fracture lines of rat femurs are marked (in white circles). In the evaluation made with the RUST score, it was determined as 10 in rats with non-splenectomy for 3 weeks (a), 9 in rats with splenectomy for 3 weeks (b), 12 in rats with non-splenectomy for 5 weeks and (c) 11 in splenectomized rats for 5 weeks (d).

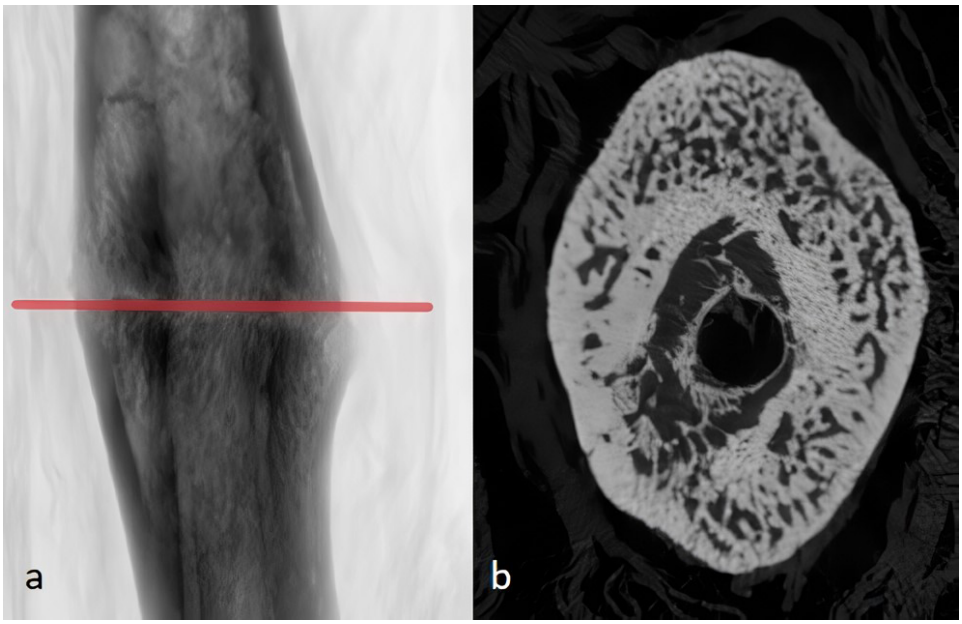

FIGURE 3. Micro-computed tomography sample of splenectomy group at the fifth week; determination of the region of interest (ROI). ROI covered a total range of 8.4 mm (encompassing 4.2 mm proximal and distal to the fracture line (red line) (a), axial section of fracture line (red line). Callus tissue with complete maturation is observed. It is understood that the callus tissue is mineralized (maturation) by giving signals close to the cortical bone in the grey scale (b).

lows: “1” Fibrous tissue, “2” Dense fibrous connective tissue and minor cartilage tissue, “3” Equal amount of fibrous and cartilage tissue, “4” Dense amount of cartilage and small amount of fibrous tissue, “5” Cartilage, “6” Dense amount of cartilage and minor immature bone, “7” Equal amount of cartilage and immature bone, “8” Dense amount of immature bone and minor cartilage, “9” Fracture fused with immature bone, and “10” Fracture fused with mature bone. In summary, system from the lowest number of 1 to the highest number of 10 suggested that the fracture matured and fused as scores increased [26] (Figs. 4,5).

2.7 Biomechanical test

A three-point bending test was conducted for investigating the biomechanical endurance of femoral fracture recovery levels attained after three and five weeks. The biomechanical testing was made by a diagnostic equipment Tensile Strength Tester (Instron 5982, Illinois Tool Works Inc, Norwood, MA, USA) with 100 kN load capacity, 1 kHz sampling frequency, and force measurement precision of 0.5%. The right femoral bones were examined to verify the delivered force meeting the axis perpendicular to fracture line by horizontally placing them on measurement equipment with their fronts pointing upwards. According to Turner and Burr’s advice, 15 mm should be left between bearing and loading bars for each femur [27].

The bones were twisted at a pace of 5 mm/s until a fracture developed. A computer software (Instron Blue Hill 3, Norwood, MA, USA) compatible with the test instrument was used to concurrently capture the force and deflection data. The force maximum (Fmax) curve was identified as the breaking force.

2.8 Statistical analysis

The mean, standard deviation, median, minimum, and maximum values were given for the descriptive statistical data. One-way analysis of variance (ANOVA) was used for the normal distribution data, and Kruskal-Wallis analysis of variance (KWAV) for the non-normal distribution data. The groups having difference in ANOVA were examined with Tukey post hoc test, and those in Kruskal-Wallis Analysis of Variance were examined with Kruskal-Wallis multiple comparison test. Intraclass correlation coefficient (ICC) was employed to examine the inter-observer agreement for Rust scores. Assessments were made using IBM SPSS version 20 programme (Chicago, IL, USA), and statistical significance threshold of $p < 0.05$ was adopted.

3. Results

3.1 Radiological evaluation

Comparison of Groups A and B revealed no statistically significant variation (the early fracture healing period). Groups C and D did not differ significantly (late fracture healing period). Groups C and D had higher RUST scores than Groups A and B (Table 1).

The statistical evaluations of ratios of callus volume to bone volume revealed that the callus/bone volumes of Groups C and D were lower than those of Groups A and B. Moreover, Groups B had lower values as found from Groups A and B evaluations (Table 1).

BMD values in Groups C and D were higher than in Groups A and B. No significant differences were found between Groups A and B or between Groups C and D (Table 1).

3.2 Histological evaluation

Histological evaluation of Groups A rats specimens showed more cartilage and fibrous tissue, and less immature bone tissue. Samples with more immature bone tissue were observed in the early period of fracture healing (Groups B), compared to the splenectomy group having fibrous and cartilage tissues. However, this was not a statistically significant difference.

The histological examination of samples from 5th week of experiment exhibited that both immature and mature bone tissues were dense. There was no statistically significant difference between Huo *et al.* [26] histological scoring of preparations for the late period of fracture healing (Groups C and D). Groups B had higher score than Groups A when compared according to Huo scores. Huo score for Groups D was also higher than Groups C. Significance was found between A–C, A–D and B–D by comparing the Groups of 3rd and 5th weeks, (A–C, $p = 0.004$, A–D, $p \leq 0.001$, and B–D, $p = 0.004$) (Table 2).

3.3 Biomechanical evaluation

In biomechanical results of fracture healing, the values of early fracture healing period of splenectomized rats were lower than the late period (Groups C, D > Groups A, B). When two groups (Groups A and B) of early recovery period were compared, the values in splenectomy group were lower with no statistically significant difference (Table 2).

4. Discussion

The incidence of fractures is rising worldwide because of enhanced population urbanization, increased number of motor vehicles, and aging population. Bone fractures are common in events like traffic accidents, sports, and earthquake, and are costly for the public due to high health care expenditures. A bone fracture is characterized by the mechanical instability of bone due to complete or incomplete breaks in bone continuity. Fracture healing is a complicated process which includes parameters such as micro-stability, fracture morphology, tissue perfusion, and the immune system [19].

In this experimental study, the radiological, biomechanical, and histological effects of femoral fracture healing in rats, with and without simultaneous splenectomy, are investigated in early and late stages of healing process.

In a study made on rabbits, it is noted that hematoma ablation at fracture localization causes impaired fracture healing and hematoma is the trigger for fracture healing [28]. Proinflammatory cytokines released due to bone injury mobilise inflammatory cells to this area and create temporary environment necessary for healing [14]. These cells secrete many cytokines, primarily TNF-α, IL-1 and IL-6, which are the proteins that regulate hematopoietic and immune functions [3, 29]. In this process, the damaged necrotic tissue is removed, and

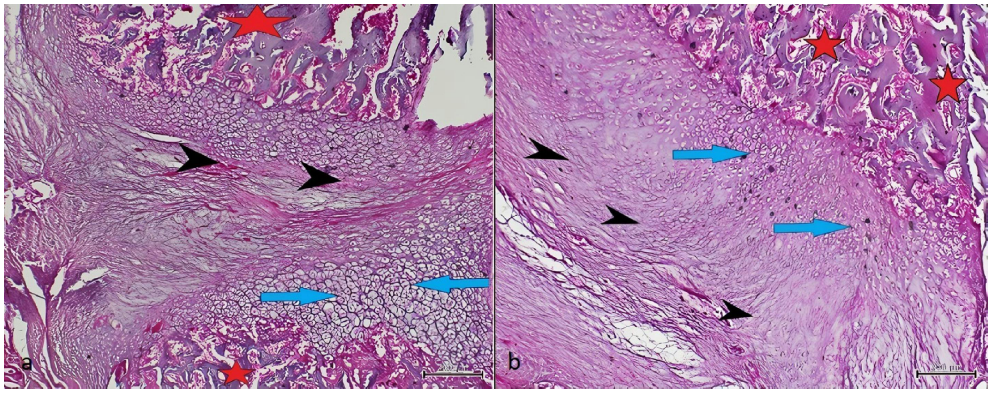

FIGURE 4. Third week histological sections, black arrows indicate fibrous tissue; blue arrows indicate cartilage tissue, and asterisks indicate immature bone tissue. In Groups A, common fibrous tissues and cartilage areas are observed at the fracture line (a). In Groups B, which did not undergo splenectomy, fibrous, cartilage tissue and immature bone areas are observed together (b).

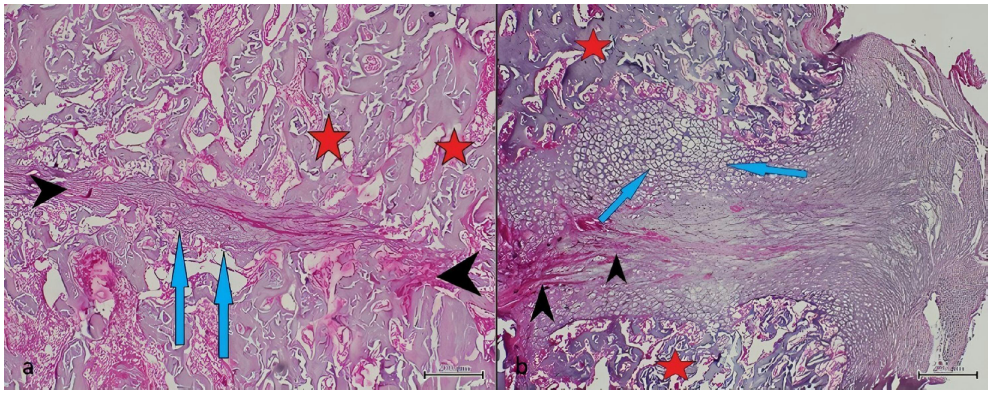

FIGURE 5. Fifth week histological sections, black arrows indicate fibrous tissue; blue arrows indicate cartilage tissue, and asterisks indicate immature bone tissue. Although fibrous tissue is seen in the fracture line of Groups C, cartilage tissue and immature bone areas are also seen (a). In Groups D, which did not undergo splenectomy, the fracture line was observed to merge with cartilage tissue and immature bone (b).

TABLE 1. Radiological evaluating results of the groups.

	Mean ± SD; Median (Min–Max)				*p* value
	Groups A (n = 16)	Groups B (n = 16)	Groups C (n = 16)	Groups D (n = 16)	
RUST Score					
	7.87 ± 0.71	8.94 ± 0.77	11.31 ± 0.60	11.63 ± 0.50	<0.001*
	8 (7–9)	9 (8–10)	11 (10–12)	12 (11–12)	
Post hoc test	A–B: $p = 0.632$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p = 0.001$, B–D: $p \leq 0.001$, C–D: $p = 1.000$				
Callus/Bone Volume					
	1.29 ± 0.09	1.15 ± 0.08	0.72 ± 0.06	0.77 ± 0.03	<0.001**
	1.30 (1.13–1.43)	1.15 (1.02–1.38)	0.73 (0.63–0.81)	0.77 (0.69–0.83)	
Post hoc test	A–B: $p \leq 0.001$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p \leq 0.001$, B–D: $p \leq 0.001$, C–D: $p = 0.239$				
BMD (mg HA/cm^3)					
	0.93 ± 0.02	0.94 ± 0.01	1.06 ± 0.02	1.07 ± 0.02	<0.001**
	0.93 (0.90–0.98)	0.95 (0.92–0.98)	1.07 (0.99–1.10)	1.08 (1.03–1.11)	
Post hoc test	A–B: $p = 0.334$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p \leq 0.001$, B–D: $p \leq 0.001$, C–D: $p = 0.642$				

**Kruskal-Wallis, **ANOVA, SD: Standard Deviation, RUST: Radiographic Union Score for Tibial Fracture. BMD: Bone mineral density, Groups A: splenectomized 3rd week, Groups B: non-splenectomized 3rd week, Groups C: splenectomized 5th week, Groups D: non-splenectomized 5th week.*

TABLE 2. Histology and bending tests of the groups.

	Mean ± SD; Median (Min–Max)				*p* value
	Groups A (n = 16)	Groups B (n = 16)	Groups C (n = 16)	Groups D (n = 16)	
RUST Score					
	7.87 ± 0.71 8 (7–9)	8.94 ± 0.77 9 (8–10)	11.31 ± 0.60 11 (10–12)	11.63 ± 0.50 12 (11–12)	<0.001*
Post hoc test	A–B: $p = 0.632$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p = 0.001$, B–D: $p \leq 0.001$, C–D: $p = 1.000$				
Callus/Bone Volume					
	1.29 ± 0.09 1.30 (1.13–1.43)	1.15 ± 0.08 1.15 (1.02–1.38)	0.72 ± 0.06 0.73 (0.63–0.81)	0.77 ± 0.03 0.77 (0.69–0.83)	<0.001**
Post hoc test	A–B: $p \leq 0.001$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p \leq 0.001$, B–D: $p \leq 0.001$, C–D: $p = 0.239$				
BMD (mg HA/cm^3)					
	0.93 ± 0.02 0.93 (0.90–0.98)	0.94 ± 0.01 0.95 (0.92–0.98)	1.06 ± 0.02 1.07 (0.99–1.10)	1.07 ± 0.02 1.08 (1.03–1.11)	<0.001**
Post hoc test	A–B: $p = 0.334$, A–C: $p \leq 0.001$, A–D: $p \leq 0.001$, B–C: $p \leq 0.001$, B–D: $p \leq 0.001$, C–D: $p = 0.642$				

**Kruskal-Wallis, **ANOVA, SD: Standard Deviation, RUST: Radiographic Union Score for Tibial Fracture. BMD: Bone mineral density, Groups A: splenectomized 3rd week, Groups B: non-splenectomized 3rd week, Groups C: splenectomized 5th week, Groups D: non-splenectomized 5th week.*

vascular endothelial growth factor is secreted which leads to angiogenesis. Cytokines released into the medium, support the transient recruitment of pluripotent mesenchymal stem cells, which later develop into fibroblasts, chondroblasts, or osteoblasts. During bone healing period, chondrogenesis begins to form and collagen-rich fibrocartilaginous network is created with hyaline cartilage sheath surrounding the fracture ends, as well as bone layer adjacent to periosteal layers by the osteoprogenitor cells. Callus is essential for bone remodelling, biomechanical strength and resilience [30]. Callus formation and resolution are coordinated through cellular and molecular factors and follow certain pattern despite their complex configuration [14]. A member of TNF receptor superfamily, receptor activator of nuclear factor kappa beta ligand (RANKL), with macrophage-colony stimulating factor (M-CSF), has role in regulating the osteoclastogenesis [31, 32]. RANKL controls osteoclast formation and regulates bone mass through balanced production of its soluble receptor antagonist osteoprotegerin (OPG) [32, 33]. Literature supports the claim that main bone mass determinant is RANKL/OPG ratio [32]. OPG can bind to RANKL and inhibits osteoclast formation by preventing its interaction with receptor activator of nuclear factor kappa beta (RANK) and influences the bone remodelling [32, 34].

4.1 Radiological evaluation

Holstein *et al.* [35] studied fracture healing in rats with open fractures. It was observed that rapamycin, an immunosuppressive drug, prevented and reduced the callus formation in radiographic examination of two-week fracture healing. However, the difference between this effect and control group disappeared in the 5th week of recovery. The callus diameter was determined by X-ray analysis, measured based on the femur diameter at fracture site. It was noted that rapamycin inhibited the production of vascular endothelial growth factor, which in turn inhibited the angiogenesis and vascularization. However, new bone formation was not completely suppressed despite this inhibitory effect.

Rats with open fractures (osteotomies) were splenectomized in a study, and the impact on fracture was assessed on day 0, 3, 15 and 30. Fibroblast differentiation and chondrocytes were detected at the broken ends after 15 days of fracture, and significant increase in chondrocytes was observed on 30th day along with cartilage ossification. However, fibroblasts differentiation was inhibited, and chondrogenesis was suppressed in splenectomized rats [36].

Macrophages may exhibit phenotypic changes depending on fracture healing phase. It thus has role in other fracture healing phases besides the regeneration phase. The macrophages translocation towards the fracture location has important long-term effect on bone healing [37].

Xiao *et al.* [3] evaluated the splenectomy effect on fracture healing through a mouse study. Osteotomy was performed with an open femoral fracture model. The number of F4/80/cluster of differentiation 11b (CD11b) dual-positive cells were increased which indicated the macrophages increase at injury site after 3 days of splenectomy. This increase was lower in splenectomy rats with time (days 14 and 28) compared to the rats without splenectomy. In conclusion, splenectomy inhibited the immune responses and thus decreased the macrophage flow at fracture site.

Sun *et al.* [38] found that splenectomy reduced the number of T lymphocytes and negatively affected the early phase of fracture healing. There was increase in the interactions between T lymphocytes and RANK-RANKL based on the increase in osteoclastogenesis. Furthermore, splenectomy decreased the IL-1, TNF-α and IFN-y levels. The decrease in cytokines released from active T lymphocytes and monocyte-macrophages because of the splenectomy may affect fracture healing.

A study was made on 40 patients of traumatic extremity fractures aged 15–60 years. Twenty patients underwent emergency splenectomy. Monocytes, lymphocytes, TNF-α

and IL-6 levels were found lower in the peripheral blood of splenectomy patients. It was determined that the numbers of TNF-α and IL-6-positive macrophages and lymphocytes were lower in hematoma tissue. This study also evaluated the X-ray images taken on 1st day, 1st month, and 3rd month after the operation. The bone grey ratio and BMD ratio were reduced in fractured patients with splenectomy [36]. The time frames of three weeks for early period and five weeks for late period were accepted as appropriate times to evaluate the fracture healing [23].

The callus area was evaluated through X-ray analysis by employing RUST scoring system, as assessed by a radiologist and an orthopaedic surgeon. Micro-CT imaging was used to calculate the bone and callus volumes which subsequently determined the Callus/Bone Volume ratio. This method provided more reliable clinical assessment of bone healing. As per the results of our study, callus volumes of rats in Group A were lower than those in Group B. There was no difference between the callus volumes of rats in Groups C and D. These results can be interpreted as the splenectomy rats experience delays in the early stages of fracture healing. The ratios of callus to bone volumes may be lower because of the insufficiency in callus tissue due to decrease in inflammatory cells and cytokines in splenectomy rats.

4.2 Histological evaluation

Sun *et al.* [38] found that T lymphocytes hampered the bone healing, particularly at the early stages of fracture [38]. There is a correlation between decline in serum TNF-α expression and decline in osteoclast count following splenectomy [39]. Movement of monocytic osteoclast progenitor cells stimulate bone resorption-focused process of osteoclastogenesis.

Gültekin *et al.* [40] based on Huo *et al.* [26] histological scoring, found no histopathological difference between splenectomy rats and fracture, and those with only a fracture, at the 4th week of fracture healing [40].

In histological evaluation of this study during early fracture healing period, common fibrous tissues and cartilage regions were detected in the fracture line of splenectomy group. Immature bone regions, fibrous, and cartilage tissues were observed in the group without splenectomy. Immature bone regions, fibrous and cartilage tissues were found in the fracture line of splenectomy group in late phase of fracture healing. Fracture line was combined with cartilage tissue and immature bone in these groups. Although no significant difference was recorded in HUO scores, the absence of fibroblastic differentiation in histological evaluation may be secondary to immune system defect caused by spleen absence.

4.3 Biomechanical evaluation

The biomechanical results of fracture healing in our study depicted that the early fracture healing time values of splenectomized rats were significantly lower than the late period.

In Hao *et al.* [41] rat study, short-term local muscle atrophy and dysfunction secondary to botulinum A toxin caused decreased recovery of femur fractures. In biomechanical tests, the femurs on toxin-administered area displayed lower mechanical functions compared to the control side. Microenvironment surrounding the fracture can be influenced by muscle damage which may impact the healing process.

5. Limitations

One of the drawbacks of this study is the absence of molecular-level analysis for fracture healing. Furthermore, spleen injuries and bone fractures are usually occurring together in patients suffering severe trauma. Studies of injuries involving muscle damage and bone defect, or multi-trauma may provide different results.

6. Conclusions

There were substantial variations between the early and late period scores regarding biomechanical data of fracture repair in splenectomy rats of this study. A significant difference was observed between the splenectomy group in the early period. It is hypothesized that each change affecting the fracture healing process may affect the activities supporting fracture repair.

It is not easy to make a definitive interpretation from this study as the effects of factors such as trauma-related additional factors (such as other organ damage), infection, nutritional disorders, and comorbidity on healing process could not be evaluated. Large scale experimental studies with clinical follow-ups are required for investigating the effects of factors affecting the wound healing and other healing processes.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

NK and MT—designed the study. NK, MT, İG—collected the data. İG, NB, RPK and MS—performed data extraction and statistical analyses. NK, MT, RPK and MS—drafted and revised the manuscript. The final manuscript was reviewed and approved by all writers.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained for the study from the SYLAB animal laboratory (No: 12/Date: 09/22).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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