Histomorphometric analysis of fracture healing using ImageJ software in Sprague–Dawley rat models of fractures with mechanical force to the bone only and to the bone and periosteum

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Abstract. Fractures are major health problems because of their complexity and expensive treatment. Radiological and biomechanical assessments of fracture healing rely on instruments that are not available at all locations. Histological assessment through histomorphometric evaluation can be performed without relying on modern and expensive instruments. This approach is supported by ImageJ software, which can be obtained free of charge. The present study aimed to assess the use of ImageJ software for histomorphometric evaluation of fracture healing. This experimental study included 24 Sprague–Dawley rats. The rats were divided into control (mechanical force to the bone only) and trial groups (mechanical force to the bone and periosteum). Each group was evaluated at 2 and 4 weeks. The parameters were total callus area, total osseous tissue area, cartilage area, and fibrous tissue area. Evaluations were performed by comparing the groups at 2 and 4 weeks and assessing the changes between 2 and 4 weeks in each group. All parameters showed significant differences between the groups at 2 weeks, and all parameters, except total callus area, showed significant differences at 4 weeks. Additionally, the healing process was delayed in the trial group. ImageJ software can be used to perform histomorphometric evaluation of fracture healing.

1. Introduction
Fractures have become major health problems because of their high frequency and complex and expensive treatment [1]. The incidence of fractures has been reported to vary from 9.0 to 22.8/1000 people/year [2], and most fractures occur in the young population [3]. In Indonesia, in 2007, the incidence of fractures was 45.99/1000 people/year, and most fractures occurred in individuals at an active and productive age [4].

Economically, the loss of productivity and the amount of money spent for health services are additional issues. The cost of fracture treatment has been estimated to be around 7,347–19,174 euros per case in Europe and 23.9 billion dollars per year in the USA [5, 6]. Although fractures can be cured, there might be complications, such as delayed union and non-union, which can increase the economic burden, as these complications increase treatment time, treatment cost, and loss of productivity and decrease quality of life [6-9].

There is a need for a strategy or intervention to guard fracture healing and prevent non-union or delayed union. An accurate assessment method is required to assess the effect of an intervention on
fracture healing. The options include radiological, biomechanical, molecular, and histological assessment methods [10]. Recent radiological and biomechanical assessment methods are not widely available in Indonesia because most experimental studies use small animals and small tools/devices. On the other hand, histological methods can be implemented currently. Accurate histological assessment can be achieved by performing histomorphometric evaluations as proposed by Gerstenfeld et al., which can quantitatively assess cellular processes, and the findings can be used as indicators for evaluating fracture healing [10-12].

For histomorphometric evaluation, a tool to conduct quantitative assessments is needed. One such tool that can be used is ImageJ software (National Institutes of Health). ImageJ is an open-source image-processing program that is in the public domain [13]. This program can measure distances and angles, calculate area and image pixels, and perform analysis. The present study aimed to assess the use of ImageJ software for histomorphometric evaluation of fracture healing in rat models.

2. Methods
This experimental study was conducted at the Department of Pathology Faculty of Medicine, Universitas Indonesia between July and September 2013. The study included 28 male Sprague-Dawley rats (weight 250–350 g) aged over 3 months. The rats were maintained from birth, and they did not experience any sickness. The left femur was used in this study. This study protocol has been approved by Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.

The 28 rats were divided into the following 2 groups: control (mechanical force applied to the bone only to achieve fracture) and trial (mechanical force applied to the bone and periosteum). Mechanical force to the bone involved a transverse cut at the diaphyseal part of the femur with a handsaw. Mechanical force to the periosteum involved circular detachment of the periosteum from the bone using a scalpel 5 mm proximal and distal from the fracture site. All fractures were stabilized with intramedullary K-wire fixation.

Histomorphometric evaluations were performed at 2 and 4 weeks after fixation in both groups. Six serial transverse cuts of the callus (thickness, 5 nm) were made at intervals of 300 nm, and the samples were stained with hematoxylin and eosin. The histomorphometric evaluations were performed semi-automatically using a microscope (ICC50 HD; Leica Microsystems, Wetzlar, Germany) at 40× magnification (4× objective; 10× ocular) and ImageJ software. The parameters assessed were total callus area, total osseous tissue area, cartilage area, and fibrous tissue area (Table 1).

| Parameter                        | Unit    | Information                                                                 |
|----------------------------------|---------|-----------------------------------------------------------------------------|
| Total callus area (CAr)          | mm²     | Mean value for measurement of the total callus area inclusive of all tissues outside the original bone cortices |
| Cartilage area (%Cg)             | %       | Mean value for measurement of the total cartilage in the callus expressed as the percentage of total callus volume that is cartilage. |
| Fibrous tissue area (%FT)        | %       | Mean value for measurement of the traced areas of fibrous tissue within the callus. |
| Total osseous tissue area (%TOT) | %       | Mean value for measurement of the total callus area that has osseous tissue. |

A post-hoc statistical evaluation for body weight was performed using the Mann–Whitney test and the independent t-test.
3. Results

The study included 28 rats. However, during the experimental period, 4 rats were excluded. Thus, 24 rats eventually underwent histomorphometric evaluations. The control and trial groups had similar body weight characteristics (Table 2).

| Group                  | Mean/median | p-value |
|------------------------|-------------|---------|
| Body weight            |             |         |
| Control group 2 weeks  | 335 (260–340) | 0.012   |
| Trial group 2 weeks    | 315 ± 18.71 | 0.353   |
| Control group 4 weeks  | 308.33 ± 21.36 | 0.331   |
| Trial group 4 weeks    | 329.17 ± 18.82 | 0.419   |

A post-hoc statistical evaluation for body weight was performed using the Mann–Whitney test and the independent t-test, and a p-value >0.05 was obtained. Thus, the influence of body weight can be ignored in this study.

Histomorphometric evaluations were performed with static and dynamic processes. Static evaluation was performed by comparing the results of each group at 2 and 4 weeks, while dynamic evaluation was performed by comparing the change rate between 2 and 4 weeks in each group. Static evaluation at 2 weeks is presented in Figure 1, and static evaluation at 4 weeks is presented in Figure 2.

![Figure 1](image1.png)

**Figure 1.** Comparison of each histomorphometric parameter at 2 weeks.

The mean values of all parameters showed significant differences between the control and trial groups at 2 weeks (all \( p < 0.05 \)). The total callus area, cartilage area, and total osseous area were significantly smaller in the trial group than in the control group, while the fibrous tissue area was significantly larger in the trial group than in the control group.

![Figure 2](image2.png)

**Figure 2.** Comparison of each histomorphometric parameter at 4 weeks.
The mean total osseous tissue area, cartilage area, and fibrous tissue area showed significant differences between the control and trial groups at 4 weeks (all $p < 0.05$). The total osseous area was significantly smaller in the trial group than in the control group, while the cartilage area and fibrous tissue area were significantly larger in the trial group than in the control group. However, the mean total callus area did not show a significant difference between the groups at 4 weeks ($p > 0.05$).

Dynamic evaluations were performed for the fibrous tissue area, cartilage area, and total osseous area. The findings are presented in Figure 3. Statistical analyses for the dynamic process could not be performed as the data were obtained from 2 different samples.

4. Discussion

In the static histomorphometric evaluations performed at 2 weeks, the total callus area, cartilage area, and total osseous area were smaller in the trial group than in the control group, while the fibrous tissue area was larger in the trial group than in the control group. According to a study by Einhorn et al., in the second week of fracture healing in rats, there are increases in cellularity and cartilage tissue. Additionally, the process of cartilage mineralization and the process of bone formation directly from osteoprogenitor cells that lie beneath the periosteum start at this point [14]. Mechanical force at the periosteal region results in the loss of periosteal blood vessels that supply blood to the outer third of the bone cortex [15]. The area around the fracture shows hypoperfusion and the osteogenic signal decreases. Thus, the cellularity process decreases, cartilage formation decreases, and the fibrous tissue area increases. Mechanical force at the periosteum also decreases the supply of stem cells, as the periosteum acts as a source of stem cells that can differentiate into bone [16], resulting in a decrease in the total osseous tissue area in the callus.

In the static histomorphometric evaluations performed at 4 weeks, the total callus area and total osseous area were smaller in the trial group than in the control group, while the cartilage area and fibrous tissue area were larger in the trial group than in the control group. In the fourth week of fracture healing in rats, bone formation occurs. Cartilage undergoes calcification and forms woven bone, and osteoclasts beneath the periosteum change this bone to lamellar bone. These changes result in a reduction in the cartilage area and an increase in the total osseous tissue area. Mechanical force to the periosteum can cause a delay in the cartilage calcification process, resulting in a small total osseous area and large cartilage area. The study found that the total callus area was smaller in the trial group than in the control group; however, the difference was not statistically significant. This is probably because the remodeling process started in the fourth week, and thus, the total callus area declined.
The study also attempted to assess the dynamic process of fracture healing by comparing histomorphometric changes between 2 and 4 weeks in each group. In the control group, there was an increase in the total osseous area and a decrease in the cartilage and fibrous tissue area. These findings are consistent with the results of a previous study that reported peak osteogenesis during this period, with angiogenesis in the callus, increased mineralization, and formation of woven bone [17]. At the same time, there is also peak intramembranous ossification [14], which results in an increase in the total osseous area within the callus and a decrease in the cartilage and fibrous tissue area. In the trial group, similar trends were observed; however, the areas were smaller when compared to those in the control group. The only difference was that the cartilage area increased in the trial group but decreased in the control group. Mechanical force to the periosteum resulted in disruption of vascularization in the callus, which delayed the calcification process.

An impaired fracture healing model with periosteal stripping has been used by Kokubu et al. [18], Oetgen et al. [19], and Kaspar et al. [20]. In these previous studies, periosteal stripping was performed using an electrocautery device. However, periosteal stripping using an electrocautery device has not only a mechanical effect but also a thermal effect that can cause the death of stem cells around the fracture site. It has been reported that spontaneous regeneration of the periosteum after thermal damage does not occur until the third month [21]. In the present study, periosteal stripping performed with a scalpel that only had a mechanical effect and no thermal effect was associated with disturbances in fracture healing until the fourth week of observation.

5. Conclusion
It is found that ImageJ software could be used to perform histomorphometric evaluation of fracture healing. Our study results indicate that mechanical force to the periosteum involving circular stripping of the periosteum using a scalpel 1 cm around the fracture area inhibits fracture healing until 4 weeks.

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