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Detectability of microscopic findings of fracture healing in the early stages of the healing process at various postmortem intervals and evaluation of wound vitality

Selcuk Cetin[1], Dilek Durak[2], Ulviye Yalcinkaya[3], Elif Cetin[4], Filiz Eren[4], Bulent Eren[1], Vahide Aslihan Durak[2]

[1]Tokat Gaziosmanpasa University, Faculty of Medicine, Department of Forensic Medicine, Tokat, Turkey
[2]Uludag University, Faculty of Medicine, Forensic Medicine Department, Bursa, Turkey
[3]Uludag University, Faculty of Medicine, Pathology Department, Bursa, Turkey
[4]Tokat Gaziosmanpasa University, Faculty of Medicine, Pathology Department, Tokat, Turkey
[5]Council of Forensic Medicine, Bursa Morgue Department, Pathology Division, Bursa, Turkey

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Abstract

In our study, it is aimed how long do the microscopic findings of fracture healing in postmortem interval can be detected and whether the detected findings can be used in differentiation of fractures occur in the period of antemortem or postmortem or not.

In our study, microscopic findings of 10 study groups included manually fractured bone samples that were created in antemortem period in New Zealand White rabbit fibulas. The specimens, in a closed environment under constant temperature and humidity that exposed to 15-day process of putrefaction, microscopic signs of inflammation, fibrin, granulation tissue and periosteal new bone formation in fracture edges were evaluated.

In the control group, none of the evaluated parameters were detected microscopically. In microscopic evaluation of the study groups; fibrin was detected in the all study groups including postmortem 360th hour samples. As a result in our study; in bone fractures, inflammation findings, fibrin, granulation tissue and the presence of periosteal new bone formation, have indicated even in cases where advanced processes of autolysis and putrefaction or soft tissue has disappeared, it revealed significant findings in terms of vitality by histopathological examination.

Keywords: Postmortem interval, forensic pathology, bone; fractures, microscopy

Introduction

The determination of vitality, namely whether an injury occurred during life, and the age of internal injuries, skin wounds, or bone fractures are fundamental issues in forensic medicine [1-4]. In this context, internal injuries and skin wounds are of interest, as well as bony or skeletal injuries, particularly in terms of determining vitality [5,6]. The case of dry bones, in which soft tissues have been removed widely from a corpse, depending on the decomposition process, is different. With dry bones, a fracture can only be identified with certainty as produced antemortem if there are signs of macroscopic or radiological findings of fracture healing. The beginning of the healing process, such as periosteal bone production and callus formation, can be detected both macroscopically and radiologically. However, these processes require long periods. The macroscopic appearance of bone fractures enables a diagnosis of "perimortality" when no new bone is visible and a diagnosis of "antemortem" origin only when woven bone is present. According to previous research, the latter does not occur before 10–14 days [7]. A perimortem fracture is one that appears elastic, such as a green fracture. This morphological characteristic means that the fracture was inflicted when the bone still had elastic properties. This could be in life or just after death. Thus, the term perimortem is not useful in defining the vitality of a fracture.

Although there are similarities between fracture healing and soft tissue healing, fracture healing is more complex and takes much longer than soft tissue healing[8]. Fracture healing begins from the moment of occurrence of the fracture and continues until the fracture ends unite with regular bone formation [9,10]. There are mainly two types of fracture healing: primary (direct) and secondary (indirect). The secondary fracture healing process is characterized by three phases: Inflammation, reparation, and remodeling [11,12]. These stages can be divided into more detailed stages, including hematoma formation, inflammation, angiogenesis, soft and hard

***Coresponding Author:** Bulent Eren, Tokat Gaziosmanpaşa University Faculty of Medicine Department of Forensic Medicine, Kaleardı Mahallesi, 60030 Tokat, Turkey, E-mail: drbulenteren@gmail.com

callus formation, and remodeling phases [13].

When a fracture occurs, cortical bone, periosteal tissue, and surrounding soft tissues are ruptured, destroying blood vessels and consequently causing tissue bleeding [14]. Thus, blood and bone marrow cells, such as immune cells, erythrocytes, and stem cells, ingress and are disrupted from the oxygen and nutrient supply at the injury site. This process leads to local tissue hypoxia and an inflammatory response, which is a result of migration of inflammatory cells, leukocytes, and macrophages into the fracture gap, thereby triggering the formation of granulation tissue[15]. About 48–72 hours after the occurrence of a fracture, an acute hemorrhage at the point of fracture secondary to vessel rupture leads to the production of a fusiform fracture hematoma, which surrounds and joins the ends of the bone [14,16-18]. Subsequently, clotting factors in the damaged area lead to fibrin formation. The latter provides a suitable environment for the formation of fibroblasts and new capillary buds. As time passes, granulation tissue and fibrin are gradually replaced with fibroblasts and new capillary structures develop [8]. In addition to the secretion of prostaglandins from the wound site, the presence of necrotic material plays an important role in the initiation of acute inflammation.

Fibrin is deposited in the hematoma. This is followed 24 to 48 hours later by an inflammatory response, with edema, additional fibrin deposition, and the accumulation of large numbers of polymorphonuclear cells. The next stage begins 48 hours after the injury and is characterized by the appearance of fibroblast and mesenchymal cells, with gradual development of granulation tissue. Approximately two or three weeks after the injury, osteoblasts produce a matrix of collagen and polysaccharide, which becomes impregnated with calcium to produce immature woven bone. Three to four weeks later, a hard bony callus appears, with the bone forming from periosteal and endochondrial ossification. In the last stage, bone remodeling occurs, with woven bone replaced by mature bone.

Autolysis and putrefaction limit the detection of usable microscopic, toxicological and biochemical findings in the postmortem interval. Thus, forensic-histological diagnostics are limited due to. Two to three days after death, the effects of autolysis and putrefaction make it difficult to obtain useful findings from a microscopic examination, and useful information is impossible to obtain after this point (i.e., at longer postmortem intervals) [19].

To our knowledge, other than a pilot study by Cattaneo et al. [7], there are no published studies on the detectability of microscopic findings of fracture healing in decomposed corpses and dry bones. The aims of the present study were two-fold: 1) to determine how long after the postmortem interval microscopic findings of fracture healing can be detected and 2) to determine whether the detected findings can distinguish between fractures that occur in the antemortem period versus those that occur in the postmortem period.

Material and Methods

In the study, 55 female 3-month-old New Zealand White rabbits weighing 2,500–3,000 g from Uludağ University Application and Research Center Experimental Animal Breeding were used. This study was approved by the Board of Ethics in Animal Experiments of Uludağ University.

The New Zealand White rabbits were decapitated three days after the creation of bilateral fibula fractures using a manual method [Fig. 1]. The animals were divided into 10 groups, and microscopic findings in the antemortem period and postmortem periods (0, 12, 24, 48, 72, 120, 168, 216, 288, and 360 hours) were analyzed. The control group consisted of the fractured bone samples created in the postmortem 1st hour. They were evaluated by sampling 10 specimens from each group.

The specimens were then placed in a closed environment under constant temperature and humidity and exposed to a 15-day process of putrefaction. At the end of this period, microscopic signs of inflammation, fibrin, granulation tissue, and periosteal new bone formation at the fracture edges were evaluated. Samples of bone fractures were stained with phosphotungstic acid hematoxylin (PTAH) and hematoxylin & eosin (H&E) for the histopathological examination.

All the preparations were evaluated under a light microscope. In all the samples, microscopic signs of inflammation, fibrin, periosteal new bone formation, granulation tissue, inflammation, fibrin intensity, and autolysis were evaluated. The intensity of inflammation was evaluated according to the number of inflammatory cells observed in the fracture area at a magnification of ×400. The samples were divided into four groups and assigned scores of between 0 and +3, as follows: no inflammatory cells = 0, 1–10 inflammatory cells = +1 [Fig. 2a], 11–20 inflammatory cells = +2 [Fig. 2b], and 21 or more inflammatory cells = +3 [Fig. 2c]. The fibrin density between the ends of the fractures was evaluated according to the ratio of the total fracture surface coverage of fibrin. The samples were assigned scores of 0 to +3 and divided into the following four groups: 0 = no fibrin; +1 = the fracture surface occupied less than 50% of the fibrin sample [Fig. 3a,b], +2 = the fracture surface occupied 50% fibrin of the sample [Fig. 4a,b], and +3 = the fracture surface occupied more than 50% fibrin of the sample [Fig. 5a,b]. These groups are related to inflammation and fibrin formed by semi-quantitative assessment criteria. Granulation tissue and periosteal new bone formation and density were evaluated according to the presence or absence of these findings.

All inflammation, fibrin, granulation tissue, and periosteal new bone formation measurements are given with median (minimum-maximum) values. Regarding the postmortem 0 hour (baseline), the comparison of the measurements obtained in the other time frames was done using Wilcoxon's rank sum test. The SPSS 13.0 (Chicago, IL) program was used for all analyses, and a value of $p < 0.05$ was considered significant.

Results

In all the samples, the macroscopic analysis of the bone fracture edges showed no macroscopic signs of bone healing. In the control group, which included fractured bone samples created in the postmortem 1st hour, none of the parameters evaluated in the study were detected microscopically.

Primary flaccidity of soft tissues and a hemorrhagic appearance

were observed macroscopically in the fracture edges in all the samples in the postmortem 0-hour group. Although there were no signs of putrefaction, rigor mortis was observed in the postmortem 12-hour group. In addition, the fracture edges of all the samples in this group showed a hemorrhagic appearance macroscopically. Rigor mortis was absent in the postmortem 48-hour group. The fracture ends of the samples in this group exhibited a dark red color due to hemolysis of erythrocytes.

The microscopic evaluation of the samples in the postmortem 48-hour group revealed autolytic changes in inflammatory cells. The postmortem groups after 48 hours exhibited greenish and greenish black discoloration due to the putrefaction process. Fly larvae (1–3 mm in length) were seen in the postmortem 120-hour group. The microscopic evaluation of the samples in this group revealed intense autolytic findings. In the postmortem 168-hour group, the samples showed destruction of the reticular structure of fibrin and a reduction in staining of fibrin with H&E and PTAH. In the postmortem 288-hour group, cutaneous structures were detached from the subcutaneous soft tissue layers. In this group, there were no macroscopic signs of hemorrhages in the fracture ends, and osteocytes appeared as pink silhouettes in lacunae. In the postmortem 360-hour group, soft tissues remained in the form of a thin brown layer. There were no signs of macroscopic hemorrhages in the fracture ends, and the medullary cavity was viewed as an empty groove. The microscopic evaluation of the samples in this group revealed only destruction of fibrin deposition in the fracture site, with no inflammatory cells, granulation tissue, or periosteal new bone formation. The microscopic findings in all the groups, including the control group, are presented in Table 1.

The results of the statistical analysis of the data obtained from the microscopic evaluation of the samples revealed significant differences in the detectability and density of inflammation in the postmortem 0-hour samples in the control group ($p = 0.004$) versus these parameters in the samples in the postmortem 120-hour ($p = 0.046$), 168-hour ($p = 0.01$), 288-hour ($p = 0.006$), and 360-hour ($p = 0.004$) groups. All the statistical analysis results are shown in Table 2.

In terms of the detectability and the ratio of total fracture surface coverage of fibrin, there were statistically differences in the postmortem 0-hour control group ($p = 0.004$) versus the postmortem 360-hour ($p = 0.021$) group. Table 3 shows the distribution of the number of samples according to the ratio of total fracture surface coverage of fibrin and the results of the statistical analysis

In terms of granulation tissue, there was a statistically significant difference only between the postmortem 0-hour samples and 360-hour samples ($p = 0.024$). The distribution of the number of samples according to the presence or absence of granulation tissue at the site of fracture and the results of the statistical analysis are presented in Table 4.

There was no statistically significant difference between the study groups and control group in terms of the detectability of the presence or absence of periosteal new bone formation. The distribution of the number of samples according to the presence or absence of periosteal new bone formation and the results of the statistical analysis are shown in Table 5.

Table 1. Microscopic findings in all of the study groups and control group

Groups	Inflammation				Fibrin				Granulation tissue		Periosteal new bone formation	
	0 (n)	+1(n)	+2(n)	+3(n)	0(n)	+1(n)	+2(n)	+3(n)	Yes (n)	No (n)	Yes (n)	No (n)
Group 1	0	6	3	1	0	4	1	5	6	4	4	6
Group 2	2	4	3	1	0	3	5	2	3	7	7	3
Group 3	1	8	1	0	0	2	2	6	6	4	4	6
Group 4	1	8	1	0	0	3	2	5	2	8	5	5
Group 5	2	6	2	0	0	6	3	1	3	7	1	9
Group 6	4	5	1	0	0	5	1	4	5	5	4	6
Group 7	8	2	0	0	1	4	5	0	3	7	5	5
Group 8	3	6	1	0	2	1	6	1	5	5	7	3
Group 9	8	2	0	0	2	2	3	3	1	9	5	5
Group 10	10	0	0	0	2	6	2	0	0	10	0	10
Control group	10	0	0	0	10	0	0	0	0	10	0	10

Table 2. Distribution of number of samples according to the intensity of inflammation and statistical analyze results[a]

Groups (PMI, hour)	Inflammation				Z*	p*
	0 (n)	+1(n)	+2(n)	+3(n)		
Group 1 (0)	0	6	3	1	-	-
Group 2 (12)	2	4	3	1	-0.412[b]	0.680
Group 3 (24)	1	8	1	0	-1.667[b]	0.096
Group 4 (48)	1	8	1	0	-1.667[b]	0.096
Group 5 (72)	2	6	2	0	-1.414[b]	0.157
Group 6 (120)	4	5	1	0	-1.994[b]	0.046
Group 7 (168)	8	2	0	0	-2.565[b]	0.010
Group 8 (216)	3	6	1	0	-1.725[b]	0.084
Group 9 (288)	8	2	0	0	-2.754[b]	0.006
Group 10 (360)	0	0	0	0	-2.877[b]	0.004
Control Group	0	0	0	0	-2.877[b]	0.004

PMI: Postmortem interval.
*Obtained values from comparison of the group 1 with other all groups.
[a] Wilcoxon Signed Ranks Test.
[b] Based on positive ranks.

Table 3. Distribution of number of samples according to the ratio of total fracture surface coverage of fibrin and statistical analyze results[a]

Groups (PMI, hour)	Inflammation				Z*	p*
	0 (n)	+1(n)	+2(n)	+3(n)		
Group 1 (0)	0	4	1	5	-	-
Group 2 (12)	0	3	5	2	-0.632[b]	0.527
Group 3 (24)	0	2	2	6	-0.828[c]	0.408
Group 4 (48)	0	3	2	5	-0.322[c]	0.748
Group 5 (72)	0	6	3	1	-1.730[b]	0.084
Group 6 (120)	0	5	1	4	-1.000[b]	0.317
Group 7 (168)	1	4	4	1	-1.387[b]	0.165
Group 8 (216)	2	1	6	1	-1.249[b]	0.212
Group 9 (288)	2	2	3	3	-0.921[b]	0.387
Group 10 (360)	0	0	0	0	-2.309[b]	0.021
Control Grubu	0	0	0	0	-2.859[b]	0.004

PMI: Postmortem interval.
*Obtained values from comparison of the group 1 with other all groups.
a Wilcoxon Signed Ranks Test.
b Based on positive ranks.
c Based on negative ranks..

Table 4. Distribution of the number of samples according to the presence or absence of granulation tissue at the site of fracture and statistical analyze resultsa [a]

Groups (PMI, hour)	Inflammation		Z*	p*
	0 (n)	+1(n)		
Group 1 (0)	6	4	-	-
Group 2 (12)	3	7	-1.667[b]	0.096
Group 3 (24)	6	4	0.000[c]	1.000
Group 4 (48)	2	8	-1.186[b]	0.236
Group 5 (72)	3	7	-1.089[b]	0.276
Group 6 (120)	5	5	-0.351[b]	0.726
Group 7 (168)	3	7	-1.414[b]	0.157
Group 8 (216)	5	5	-0.412[b]	0.680
Group 9 (288)	1	9	-1.725[b]	0.084
Group 10 (360)	0	10	-2.264[b]	0.024
Control Group	0	10	-2.264[b]	0.024

PMI: Postmortem interval.
* Obtained values from comparison of the 1st study group with other all groups.
a Wilcoxon Signed Ranks Test.
b Based on positive ranks.
c The sum of negative ranks equals the sum of positive ranks.

Table 5. Distribution of the number of samples according to the presence or absence of periosteal new bone formation and statistical analyze results[a]

Groups (PMI, hour)	New bone formation		Z*	p*
	Yes (n)	No (n)		
Group 1 (0)	4	6	-	-
Group 2 (12)	7	3	-1.730[b]	0.084
Group 3 (24)	4	6	-0.966[b]	0.334
Group 4 (48)	5	5	-0.877[b]	0.380
Group 5 (72)	1	9	-0.134[c]	0.257
Group 6 (120)	4	6	-0.816[b]	0.414
Group 7 (168)	5	5	-1.225[b]	0.221
Group 8 (216)	7	3	-1.552[b]	0.121
Group 9 (288)	5	5	-0.557[c]	0.577
Group 10 (360)	0	10	-1.633[b]	0.102
Control Group	0	10	-1.633[b]	0.102

PMI: Postmortem interval.
* Obtained values from comparison of the 1st study group with other all groups.
a Wilcoxon Signed Ranks Test.
b Based on positive ranks.
c Based on negative ranks.

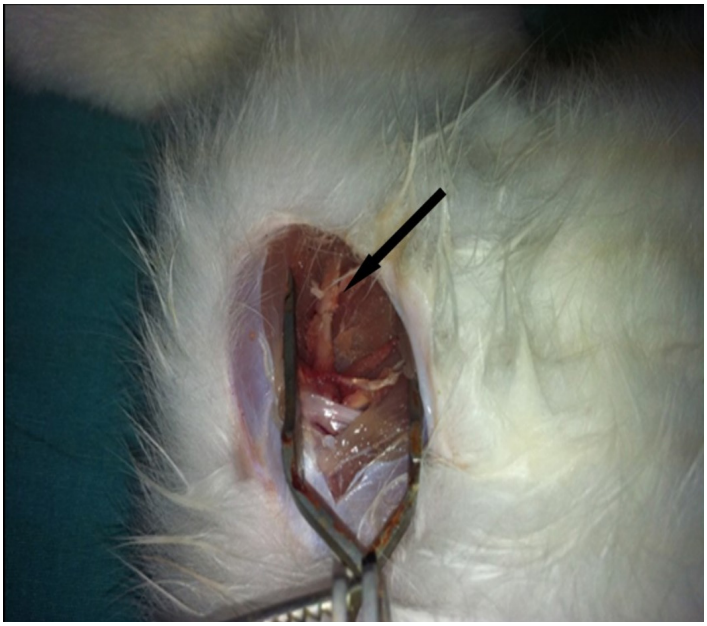

Figure 1. Created fracture in the rabbit fibula (black arrow)

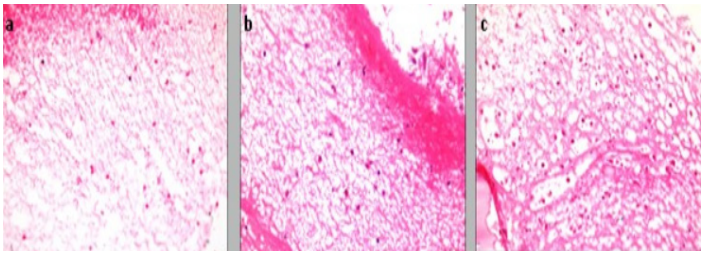

Figure 2 (a,c). Intensity of inflammation evaluated according to number of inflammatory cells observed in the fracture area (H&E, x400). a) +1 inflammation at the site of fracture. b) +2 inflammation at the site of fracture. c) +3 inflammation at the site of fracture

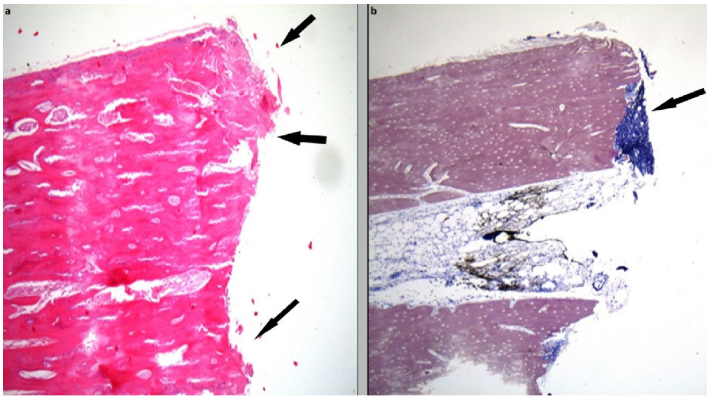

Figure 3 (a,b). Density of fibrin observed between the ends of fractures was evaluated, according to the ratio of total fracture surface coverage of fibrin. a) +1 fibrin in the fracture edge (H&E, x400 PTAH, x40). b) +1 fibrin in the fracture edge (PTAH, x40)

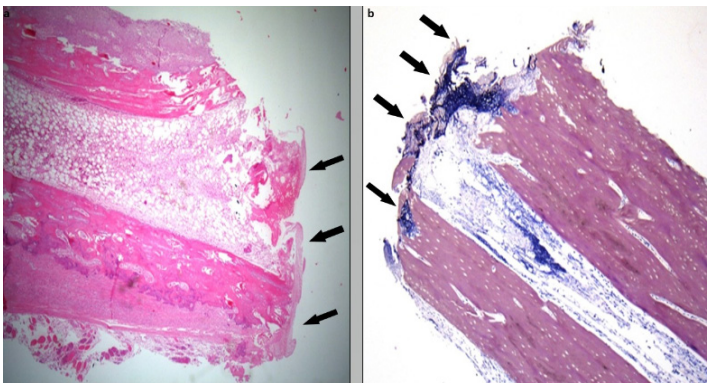

Figure 4 (a,b). Density of fibrin observed between the ends of fractures was evaluated, according to the ratio of total fracture surface coverage of fibrin. a) +2 fibrin in the fracture edge (H&E, x40). b) +2 fibrin in the fracture edge (PTAH, x40)

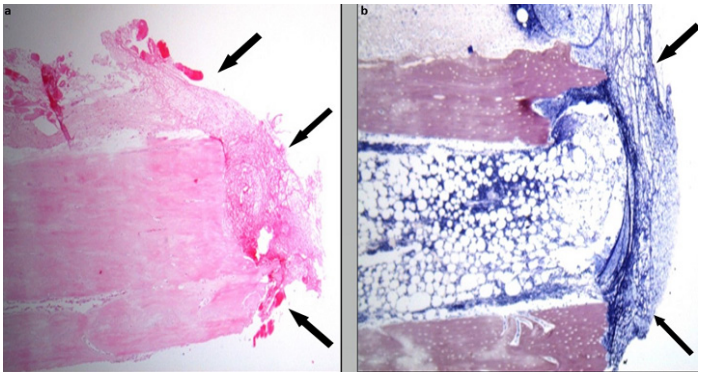

Figure 5 (a,b). Density of fibrin observed between the ends of fractures was evaluated, according to the ratio of total fracture surface coverage of fibrin. a) +3 fibrin in the fracture edge (H&E, x40) b) +32 fibrin in the fracture edge (PTAH, x100)

Discussion

Previous research examined lesional age in soft tissues and vital reactions [20-26]. However, depending on the decomposition of soft tissues disappeared dried bone fractures, and vitality in terms of the availability of early detection of microscopic signs of improvement, and for the evaluation of first and only pilot study conducted in 2007 by Cattaneo et al. [7], was published in 2010. In the present study, although the putrefaction process was artificial, on fracture ends that have no macroscopic evidence they showed us together with red blood cells and fibrin deposition monitored the construction of new bone can be determined by microscopic findings. In common with the study by Cattaneo et al. [7], the present study evaluated the construction of new bone and fibrin deposition, as well as inflammation and the formation of granulation tissue. In contrast to the study by Cattaneo et al. [7], the samples were exposed to a natural putrefaction process, so involvement of entomological factors in the environment has been provided.

According to the results of the present study, in the postmortem interval, inflammatory cells were easily detected until the 5th postmortem day. In accordance with the results of a study by Dokgöz et al. [27], we observed no significant between-group difference until the 120th postmortem hour. This indicates that inflammatory findings maintain the continuity until postmortem 120th hours. However, thanks to the immunohistochemical methods used, the duration of the detection process related to findings of inflammatory cells in postmortem is increased. In relation to this, on a corpse exhumed 391 days after death, findings related to visibility of bronchopneumonia were reported [28]. One reason for the much shorter period in the present study was the low amount of soft tissue extremities of rabbits as compared with that in humans. On the other hand, in the detection of remains belong to cells superiority of immunohistochemical methods to conventional histological techniques allows detection of findings, even in longer postmortem interval cases.

Another parameter evaluated in our study was the presence of fibrin on fracture ends. Fibrin is a fibrillary protein produced by polymerization of fibrinogen. Fibrin is an important component of acute inflammatory exudate, and it is found in areas of tissue damage [29]. As shown by previous studies [7,30], the identification of fibrin alone in the postmortem period cannot be regarded as an indicator of vitality because fibrin may also be present in postmortem clots, especially in cardiac chambers and great vessel lumen. However, the results obtained in a thesis study showed that antemortem and postmortem fibrin fibers and fibrin of different ages exhibited different features in terms of the prevalence and severity of staining when different satins and techniques were used [31].

In the current study, fibrin was observed until the postmortem 360th hour in the fracture edge sections. Fibrin was not observed in any of the samples in the control group, but it was detected in eight of the postmortem 360-hour samples. In these groups, deformed fibrin deposits were also detected on fracture end sections stained with H&E and PTAH. Although there are no clear data about fibrin availability in terms of showing vitality, fibrin detected on dried bones can help to determine the age of a fracture. It can also provide strong evidence about whether a fracture has an intravital origin when evaluated together with other findings. We conclude that the absence of fibrin in all the samples in the control group and the detection of fibrin in all the samples in the study groups increased the contribution to evaluations of bone fractures in terms of vitality. On the other hand, studies on postmortem fibrin are concerned with postmortem clots located in the vessels lumen and heart chambers [31,32]. Therefore, the detection of fibrin located around damaged tissues can provide support for antemortem trauma as a sign of vitality.

Even if they do not show a correlation with the postmortem interval, the presence of periosteal new bone formation, with or without granulation on the fracture ends provides evidence that a fracture is intravital. In previous studies, granulation tissue was detected from the time of the fracture occurrence to 48–72 hours postfracture, and temporary fibrous callus was clearly observed from 3–6 days postfracture [30,33]. Fracture healing differs among individual rabbits. The correlation of these two parameters with the postmortem interval may be explained by the decapitation process, with new bone formation not commencing immediately in some rabbits and the gradual replacement of fibrin granulation tissue.

In the study regarding musculoskeletal system, Alibegovic stated that cartilage could be a new parameter for PMI determination, he mentioned that the structure and anatomical localisation of the cartilage also its mechanical, physical and chemical characteristics as a compartment, provide vitality for chondrocytes for several weeks after the individual's death, and, expressed idea that we can use the decrease of chondrocytes' viability for an scientific PMI detection [34].

Conclusions

In conclusion, based on the results of this study, after bone fractures, the presence of inflammation, fibrin, granulation tissue, and periosteal new bone formation reveal significant findings in terms of vitality by a histopathological examination, even in cases where advanced processes of autolysis and putrefaction or soft tissue have disappeared.

Conflict of interest
The authors declare that there are no conflicts of interest.
Financial Disclosure
All authors declare no financial support.
Ethical approval
The study was approved by Board of Ethics in Animal Experiments of Uludağ University.

Selcuk Cetin ORCID: 0000-0002-3001-2745
Dilek Durak ORCID: 0000-0003-3469-340X
Ulviye Yalcinkaya ORCID: 0000-0002-2097-7842
Elif Cetin ORCID: 0000-0001-5055-5854
Filiz Eren ORCID: 0000-0003-1542-8694
[reviewer] Bulent Eren ORCID: 0000-0002-8296-5484
Vahide Aslihan Durak ORCID: 0000-0003-0836-7862

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