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Research article

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Investigation of the effects of inflammatory and metabolic factors on fracture union in head trauma and long bone fractures

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Ethics Committee Approval
The work was carried through at the animal experiments laboratory of our institution, with the approval of Namık Kemal University Experimental Animals Local Ethics Commission (date: 20 December 2019, no: T2019-386).

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Fractures are the most common form of trauma in current orthopedic practice. Although studies have shed light on the relationship between the factors affecting the healing process after fracture, this process is still not fully understood. In this study, we aimed to investigate the changes in serum biomediator levels and fracture healing in different trauma patterns, such as head trauma (HT), long bone fracture (LBF), a combination of HT + LBF injury (CI), and in different time points of the healing period.

Methods: Forty Wistar rats were included in the study and divided into five groups. Group 1, the donor group, included rats with HT; Group 2 included rats with LBFs who were administered the serum taken from rats in Group 1; Group 3 included the rats with isolated LBFs; and Group 4 the rats with CI. Group 5 comprised the control rats. An experimental closed HT and fracture model was applied to rats. The rats in Groups 2, 3 and 4 were sacrificed on the 10^{th}, 20^{th}, and 30^{th} days. The biomediator levels in the serum taken after sacrification were studied, while closed femoral fracture models were examined radiologically.

Results: Statistically significant differences were found among the groups regarding radiological scores on the 10^{th}, 20^{th}, and 30^{th} days. On Day 10, Group 2a had significantly higher scores than Group 3a (P=0.03), and Group 3a had lower scores than Group 4a (P=0.01). On Day 20, Group 2b had significantly higher scores than Group 3b (P=0.004) but lower than Group 4b (P=0.03). On Day 30, Group 2c had significantly higher scores than Group 3c but lower than Group 4c (P=0.001). The mean Ca, TGF beta 1, beta-catenin, IL-10, IL-17A, TNF alpha, CRP, Wnt-16, ALP, GH, PTH, IL-1 beta, IL-6, and IL-22 levels were significantly different among the groups (P<0.05). No significant difference was observed in the biomediator levels among the groups at different time points of the healing period.

Conclusion: We concluded that inflammatory cytokines (IL-1 beta, IL-6, IL-17A, IL-17F, IL-23, and TNF alpha) were elevated in the early period in individuals with isolated head trauma and that this effect could be transferred to other individuals by serum transfer. On the other hand, the negative relationship between the IL-10 level, which is a negative modulator in fracture union, and callus thickness was significant. Our study contributes by providing a molecular description of the positive union effect transferred between individuals by serum. We believe our findings will play a significant role in developing new therapeutic agents for fracture healing.

Keywords: biomediator, bone metabolism, fracture healing, interleukin, traumatic brain injury

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Introduction

Fractures are the most common form of trauma in current orthopedic practice, which continues to be a socioeconomic problem. For optimal clinical management and treatment, it is essential to understand the biology behind fracture unions [1].

Fracture healing is a complex but balanced process. Previous studies have shown that healing occurs in four stages: inflammation, soft union formation, hard union formation, and remodeling [2]. The process requires the coordinated involvement of various cell types, such as osteoblasts, mesenchymal stem cells, and osteoclasts [3]. These processes must be active at the right time and intensity for healthy bone fusion. An excessive or random inflammatory process may lead to non-union of the fracture [4].

Although studies have shed light on the relationship between the factors affecting the healing process after fracture, this process is still not fully understood [5]. Similarly, our current knowledge of the healing process of long bone fractures after traumatic brain injury is limited [6]. Understanding the molecular changes in the healing process will accelerate the healing rate [7].

This study aims to examine the changes in biomediator levels and fracture healing in different trauma patterns, such as head trauma (HT), long bone fracture (LBF), a combination of HT + LBF injury (CI), and at different time points of the healing period.

Materials and methods

The work was carried out at the animal experiments laboratory of Tekirdağ Namık Kemal University Faculty of Medicine, with the approval of the Namık Kemal University Experimental Animals Local Ethics Commission (dated 20 December 2019, no: T2019-386) and in compliance with the Helsinki Declaration. The study used 40 male Wistar rats weighing between 360 and 400 g and 3 to 4 months old. Rats were provided with a standard diet and unlimited access to water (ad libitum). Rats were exposed to a 12-h light and 12-h dark cycle at 22°C room temperature. Rats were randomly distributed and assigned to five groups. The donor group (Group 1) included rats with HT (n=8) and the Group 2 rats with LBFs who were administered the serum taken from rats in Group 1 (n=9), while Group 3 included the rats with isolated LBFs (n=9) and Group 4 the rats with CI (n=9). Group 5 comprised the control rats (n=5) (Table 1).

Table 1: Grouping of the rats included in the study

	Group 1	Group 2	Group 3	Group 4	Group 5
Number of rats	8	9	9	9	5
Euthanasia days	1-7	10-20-30	10-20-30	10-20-30	---
Number of femur specimen	-	3-3-3	3-3-3	3-3-3	---

Group 1: donor group, Group 2: serum-administered rats with femoral fractures, Group 3: rats with isolated femur fractures, Group 4: rats with head trauma and femur fracture, Group 5: control group.

Group 1 was divided into subgroups 1a and 1b. Afterward, the three groups in the treatment group were divided into subgroups similarly (titled a, b and c). The trauma models were created on the same day in Groups 1 and 2, while the head and bone fracture models were created simultaneously in Group 4.

Our aim in the study was to form a model like that of humans who experienced closed HT. For this purpose, using an apparatus that was set according to the Marmarou technique, the experimental closed HT model was created (Figure 1) [8]. The force to be applied to the head was determined not to exceed 0.5 joules so that the animals would survive the head injury and maintain their nourishment functions. For this purpose, we used weight and height upper limits of 50 g and 1 m, respectively [9].

All procedures were carried out under general anesthesia, with the inhalation of isoflurane (Forane; Abbott Laboratories, Abbott Park, IL, USA) at 4% for induction and 2% for maintenance. Dexketoprofen 5 mg/kg/day (Arveles; Menarini Turkey, Istanbul, Turkey) was administered to all rats, while one dose of cefazolin sodium 15 mg/kg (Cefozin; Bilim Pharmaceuticals, Istanbul, Turkey) was administered to the animals that were performed fixation.

With an anterior approach over the knee, the patella was lateralized with a longitudinal incision, and the distal femur was exposed. Later, a fracture was created in the femoral shaft. The femoral canal was reamed with a 1.2 mm K-wire, and a 1 mm K-wire crossing the fracture line was inserted into the femoral medulla. The proximal part was bent to prevent the wire from coming back. After the wire was cut, the subcutaneous tissue was closed with 3/0 Vicryl, and the skin was sutured with 3/0 silk sutures (Figure 2). The experimental fracture model described by Bonnarens and Einhorn was applied unilaterally to the left leg of rats following the principles used in numerous studies of experimental fracture healing [10]. For this, a weight of 150 g was dropped on the thigh from a height of 1 m.

Two rats had to be replaced with new rats due to the segmental fractures they developed, which we noticed during our radiological checks. After the procedure, no movement restriction was applied to the animals, and they were placed in cages with four rats in each cage.

Figure 1: Closed head trauma apparatus.

Figure 2: Intramedullary insertion of the K-wire into the femur.

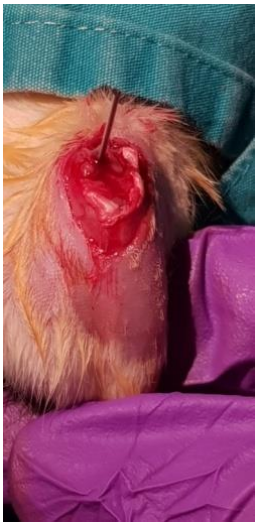
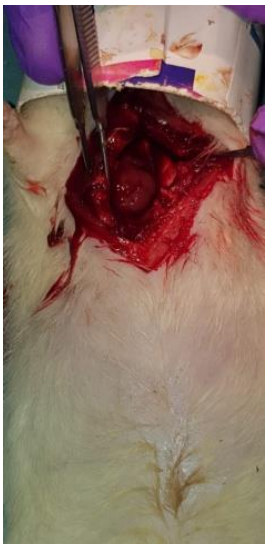


Figure 3: Cardiac blood withdrawal after thoracotomy.



The rats were sacrificed by exsanguination following thoracotomy 24 h later for Group 1a and on the seventh day for Group 1b (Figure 3). To affect the analysis results in the blood samples taken, the blood was drained with a 45-degree slope after the injector was removed to prevent changes in serum content due to hemolysis. The samples were taken to the

centrifuge device and centrifuged at 1000 rpm for 15 min, and the serum portions were separated. Sera (1.5 ccs) from Group 1a was given to Groups 2a, 2b and 2c, and sera from Group 1b (1.5 ccs) were given to Groups 2a, 2b and 2c intraperitoneally and without waiting. On the 10th day, the rats in Groups 2a, 3a, and 4a were sacrificed. Groups 2b, 3b, and 4b were sacrificed on day 20, and 2c, 3c, and 4c were sacrificed on day 30. The biomediator levels were determined by keeping the sera obtained from all groups and healthy control rats at -80°C.

The serum beta-catenin (catalog no: E1153Ra), CRP (catalog no: E0053Ra), TGF beta 1 (catalog no: E1688Ra), Wnt-16 (catalog no: E2542Ra), ALP (catalog no: E0345Ra), GH (catalog no: E0551Ra), PTH (catalog no: E0333Ra), IL-1 beta (catalog no: E0119Ra), IL-6 (catalog no: E0135Ra), IL-10 (catalog no: E0108Ra), IL-17A (catalog no: E0116Ra), IL-17F (catalog no: E0939Ra), IL-22 (catalog no: E1473Ra), and IL-23 (catalog no: E0125Ra), and TNF alpha (catalog no: E0764Ra) levels were tested using commercially available ELISA kit following the manufacturer's instructions (Shanghai Korain Biotech Co. Ltd., Shanghai, China). In addition, serum calcium and phosphorus levels were measured in the C-502 module of the Roche Cobas 8000 analyzer (Roche Diagnostics, Geneva, Switzerland) using commercial kits. All kits' inter-test coefficient of variation (CV) values were 10%. Among the tests, intra-assay CV was <8%.

Following sacrifice, the femurs were disarticulated from the hip joint, and X-rays of the legs were taken (Figures 4–6). In all groups, the ratios defined by Spencer on the lateral radiographs taken on the 10th, 20th, and 30th days were evaluated by an orthopedist blinded to the group information [11].

Figure 4: Radiological appearance of Group 2 on Day 30.

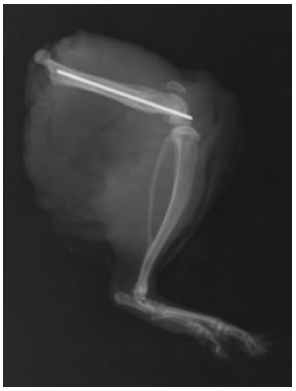


Figure 5: Radiological appearance of Group 3 on Day 30.

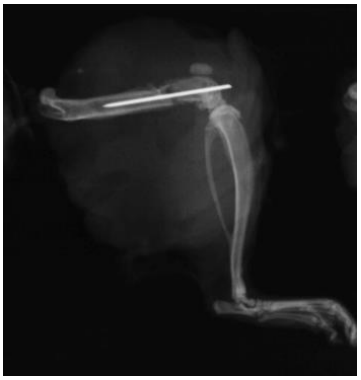
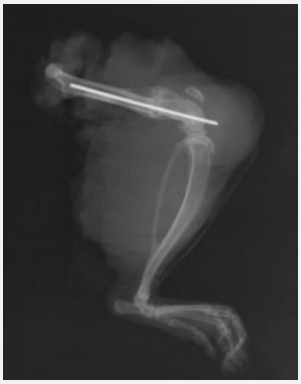


Figure 6: Radiological appearance of Group 4 on Day 30.



Statistical analysis

NCSS 2007 software (NCSS, LLC, Kaysville, UT, USA) was used for statistical analysis. The descriptive statistical methods were defined (mean, standard deviation, median, frequency, ratio, minimum, and maximum), and to evaluate the distribution of the data, normality between the groups was evaluated using the Shapiro-Wilk test. While the differences between 3 or more groups were analyzed with the Kruskal-Wallis test, a non-parametric test, the Mann-Whitney U test was used to compare the two groups. We used the Friedman test to compare three or more time points of the recovery period and the Wilcoxon test to compare two time points. Statistical significance was evaluated at $P<0.01$ and $P<0.05$ levels.

Results

Radiological scores increased significantly in all groups from day 10 to day 30 ($P=0.003$), again showing statistically significant differences between groups. When the 10th-day results were compared, Group 3a had significantly lower scores than Group 2a ($P=0.03$), and group 4a scores were higher than group 3a scores ($P=0.01$). When the results of the 20th day were compared, Group 3b had significantly lower scores than Group 2b ($P=0.004$), while the scores of Group 4b were higher than 2b ($P=0.03$). had scores lower than Group 4c (P = 0.001) (Table 2).

Table 2: Comparison of the radiographic measurements in the groups

		n	Mean (SD)	Min-Max (Median)	*P*-value
Day 10	Group 2	6	1.3 (0.1)	1.2-1.4	0.022*
	Group 3	6	1.1 (0.3)	1.1-1.3	
	Group 4	6	1.4 (0.2)	1.3-1.6	
Day 20	Group 2	6	2.2 (0.2)	2-2.4	0.001†
	Group 3	6	1.8 (0.1)	1.7-1.9	
	Group 4	6	2.4 (0.1)	2.3-2.5	
Day 30	Group 2	6	2.6 (0.2)	2.5-2.7	0.006†
	Group 3	6	2.4 (0.2)	2.3-2.5	
	Group 4	6	2.8 (0.1)	2.6-3	

Kruskal-Wallis test. * $P<0.05$, † $P<0.01$

When serum samples were analyzed, the mean Ca levels in all treatment groups were significantly higher in the control group than in the other groups ($P=0.001$). The CA levels of Groups 2 and 3 were significantly higher than the mean Ca levels of Group 1 ($P=0.001$), while the Ca levels of Group 4 were significantly lower than the mean Ca levels of Groups 2 and 3 ($P=0.001$). No significant difference was observed in Ca levels in the within-group evaluations at all follow-up time points of the treatment groups (Table 3). The mean phosphorus levels among the groups did not differ significantly. No significant differences in terms of phosphorus levels were detected in the intragroup evaluations of all groups at all follow-up time points (Table 3).

The mean beta-catenin levels in all treatment groups, except Group 1, were significantly higher than in the control group ($P=0.001$). The mean beta-catenin level in Group 1 was significantly lower than in Groups 2, 3, and 4 ($P=0.001$). No significant differences in beta-catenin levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean CRP level of the control group was significantly lower than the other groups ($P=0.002$). When the CRP levels of Groups 2, 3, and 5 were evaluated, it was seen that it was significantly lower than the Group ($P=0.001$); when the mean CRP level in Group 4 was considered, it was seen that it was significantly higher than the control group ($P=0.001$). No significant difference was observed in CRP levels in the within-group evaluations of the treatment groups at all follow-up time points (Table 3).

Table 3: Comparison of the serum contents in the groups

		n	Mean (SD)	Min-Max (Median)	*P*-value
Ca	Group 1	7	8.26 (0.38)	7.8-8.9 (8.2)	0.001†
	Group 2	8	9.51 (0.26)	9.1-9.8 (9.6)	
	Group 3	6	9.53 (0.18)	9.28-9.8 (9.55)	
	Group 4	9	8.34 (0.27)	7.9-8.7 (8.4)	
	Control group	2	10.1 (0.42)	9.8-10.4 (10.1)	
P	Group 1	7	3.86 (0.41)	3.01-4.28 (3.87)	0.070
	Group 2	8	3.86 (0.23)	3.51-4.18 (3.87)	
	Group 3	6	4.43 (0.16)	4.12-4.58 (4.46)	
	Group 4	9	4.07 (0.55)	3.26-4.72 (3.93)	
	Control group	2	4.23 (0.27)	4.04-4.42 (4.23)	
Beta-catenin	Group 1	7	3.4 (1.01)	2.4-4.91 (2.89)	0.001†
	Group 2	8	7.7 (0.59)	6.83-8.38 (7.77)	
	Group 3	6	7.24 (0.55)	6.44-7.8 (7.31)	
	Group 4	9	7.44 (0.65)	6.77-8.62 (7.55)	
	Control group	2	2.61 (0.3)	2.4-2.82 (2.61)	
CRP	Group 1	7	94.39 (7.02)	84.75-102.1 (97.2)	0.002†
	Group 2	8	56.72 (16.99)	31.01-78.04 (54.22)	
	Group 3	6	38.95 (17)	23.95-70.31 (34.99)	
	Group 4	9	61.31 (32)	26.62-115.73 (63.3)	
	Control group	2	3.47 (1.25)	2.58-4.35 (3.47)	
TGF beta 1	Group 1	7	263.25 (24.78)	223.79-289.6 (273.79)	0.001†
	Group 2	8	319.33 (32.19)	290.06-367.85 (303.21)	
	Group 3	6	314.58 (25.99)	276.89-344.1 (317.39)	
	Group 4	9	347.78 (45.71)	292.91-406.62 (338.6)	
	Control group	2	218.73 (10.31)	211.44-226.02 (218.73)	
Wnt-16	Group 1	7	540.52 (108.41)	412.83-717.35 (499.64)	0.046*
	Group 2	8	477.53 (32.1)	438.94-539.63 (478.44)	
	Group 3	6	445.24 (63)	371.6-551.3 (439.51)	
	Group 4	9	494.38 (48.79)	431.01-601.12 (489.4)	
	Control group	2	383.61 (14.38)	373.44-393.78 (383.61)	
ALP	Group 1	7	483.84 (9.09)	475.79-502.55 (483.38)	0.002†
	Group 2	8	434.53 (29)	401.06-469.72 (425.22)	
	Group 3	6	401.27 (27.22)	371.43-451.76 (395.72)	
	Group 4	9	466.16 (43.38)	411.81-524.48 (483.1)	
	Control group	2	454.67 (36.05)	429.18-480.17 (454.67)	
GH	Group 1	7	17.41 (1.18)	16.08-18.9 (16.7)	0.002†
	Group 2	8	9.46 (3.7)	5.11-14.45 (8.21)	
	Group 3	6	5.81 (2.91)	3.55-11.34 (4.93)	
	Group 4	9	8.37 (5.04)	4.57-17.37 (7.22)	
	Control group	2	9.53 (0.18)	9.4-9.65 (9.53)	
PTH	Group 1	7	14.17 (3.37)	11.03-18.17 (11.85)	0.006†
	Group 2	8	8.6 (3.18)	5.51-12.48 (6.87)	
	Group 3	6	5.79 (2.58)	4.1-10.87 (4.88)	
	Group 4	9	8.37 (2.91)	4.58-11.93 (7.74)	
	Control group	2	10.43 (0.86)	9.82-11.04 (10.43)	
IL-1 beta	Group 1	7	180.3 (5.35)	174.02-186.34 (181.02)	0.001†
	Group 2	8	164.53 (10.31)	148.8-175.62 (163.42)	
	Group 3	6	140.19 (15.11)	122.26-165.32 (139.56)	
	Group 4	9	192.43 (13.92)	178.32-219.08 (190.02)	
	Control group	2	113.63 (10.72)	106.05-121.21 (113.63)	
IL-6	Group 1	7	81.01 (2.3)	76.84-83.07 (82.21)	0.001†
	Group 2	8	68.79 (3.72)	64.44-73.99 (67.82)	
	Group 3	6	63.45 (3.84)	59.11-69.23 (63.29)	
	Group 4	9	84.04 (8.69)	75.16-95.99 (85.93)	
	Control group	2	46.74 (0.23)	46.58-46.9 (46.74)	
IL-10	Group 1	7	25.69 (2.01)	23.47-28.17 (24.64)	0.001†
	Group 2	8	30.17 (2.26)	30.02-35.96 (33.76)	
	Group 3	6	34.87 (1.71)	33.26-37.23 (34.65)	
	Group 4	9	27.18 (3.42)	21.07-30.95 (27.2)	
	Control group	2	5.65 (0.12)	5.57-5.74 (5.65)	
IL-17A	Group 1	7	220.3 (60.73)	161.3-295.68 (179.88)	0.007†
	Group 2	8	151.7 (14.01)	133.97-167.44 (154.97)	
	Group 3	6	146.41 (5.61)	140.85-154.89 (145.08)	
	Group 4	9	160.37 (14.82)	132.89-174.61 (145.11)	
	Control group	2	141.46 (3.97)	138.65-144.27 (141.46)	
IL-17F	Group 1	7	224.64 (69.4)	152.04-309.8 (174.23)	0.173
	Group 2	8	219.77 (18.07)	222.04-275.34 (243.74)	
	Group 3	6	208.62 (24.98)	198.45-254.2 (233.84)	
	Group 4	9	223.36 (26.53)	206.86-276.59 (230.28)	
	Control group	2	159.88 (13.94)	150.03-169.74 (159.88)	
IL-22	Group 1	7	206.61 (23.7)	175.06-241.08 (199.73)	0.938
	Group 2	8	211.75 (29.2)	170.56-250.12 (219.44)	
	Group 3	6	217.18 (38.8)	173.34-264.89 (212.25)	
	Group 4	9	211.81 (11.63)	198.15-236.61 (209.67)	
	Control group	2	222.23 (19.93)	208.14-236.33 (222.23)	
IL-23	Group 1	7	262 (53.69)	213.37-341.21 (230.99)	0.001†
	Group 2	8	162.34 (12.79)	145.72-183.76 (157.49)	
	Group 3	6	150.58 (6.9)	140.52-160.7 (150.58)	
	Group 4	9	173.3 (26.18)	151.15-226.9 (164.52)	
	Control group	2	154.67 (8.34)	148.77-160.57 (154.67)	
TNF alpha	Group 1	7	139.21 (15)	120.1-166.51 (140.15)	0.001†
	Group 2	8	129.86 (11.07)	115.9-145.1 (129.4)	
	Group 3	6	71.28 (8.24)	60.63-84.32 (69.83)	
	Group 4	9	138.14 (7.15)	129.4-150.31 (137.49)	
	Control group	2	60.92 (0.14)	60.82-61.02 (60.92)	

Kruskal-Wallis test. ALP: alkaline phosphatase, Ca: calcium, CRP: C-reactive protein, GH: growth hormone, IL-1 beta: interleukin 1 beta, IL-6: interleukin-6,IL-10: interleukin 10, IL-17A: interleukin 17A, IL-17F: interleukin 17F, IL-22: interleukin 22, IL-23: interleukin-23, P: phosphate, PTH: parathyroid hormone, TGF beta 1: transforming growth factor beta 1, TNF alpha: tumor necrosis factor alpha. **P*<0.05, †*P*<0.01

When the mean TGF beta 1 levels were evaluated, it was seen that there were significant differences between the groups (*P*=0.001). The TGF beta 1 level in Groups 2, 3, and 4 was significantly higher than Group 1 (*P*=0.001), while the mean TGF beta 1 level in Groups 2 and 4 in the control group was significantly lower than the control group (*P*=0.001). No significant difference was observed in terms of TGF beta 1 level in the within-group evaluations of the treatment groups at all follow-up time points (Table 3).

Significant differences were observed when the mean Wnt-16 levels were compared between the groups (*P*=0.046). The control group was significantly lower when compared to the mean Wnt-16 levels in Groups 1, 2, and 4 (*P*=0.001). No significant difference was observed regarding Wnt-16 levels in the within-group evaluations of the treatment groups at all follow-up time points (Table 3).

Mean ALP levels differed significantly between groups (*P*=0.002). The mean ALP levels in Group 3 were significantly lower than in Group 1 (*P*=0.001), while the mean ALP level in Group 4 was significantly lower compared to Group 3 (*P*=0.001). No significant difference was observed in ALP levels in the within-group evaluations of the treatment groups at all follow-up time points (Table 3). The mean GH levels also significantly differed among the groups (*P*=0.002). The mean GH level in Group 1 was significantly higher than in Groups 2, 3, 4, and 5 (P=0.001), while the mean ALP level in Group 2 was significantly higher than in Group 3 (*P*=0.001). No significant differences in ALP levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

When the groups were compared in terms of mean PTH levels, there were similarly significant differences between the groups (*P*=0.006). The PTH level in Groups 3 and 4 was significantly lower than the mean PTH level in Group 1 (*P*=0.001), while the PTH level in Group 3 was significantly lower than the mean PTH level in Groups 2 and 4 (*P*=0.001). No significant difference was observed in PTH levels in the within-group evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-1 beta levels in all treatment groups were significantly higher than in the control group (*P*=0.001). The mean IL-1 beta level in Group 1 was significantly higher than in Groups 2 and 3 (P=0.001) but lower than in Group 4 (*P*=0.001). The mean IL-1 beta level in Group 2 was significantly higher than in Group 3 (P=0.001) but lower than in Group 4 (*P*=0.001). In addition, the mean IL-1 beta level in Group 3 was significantly lower than in Group 4 (*P*=0.001). No significant differences in IL-1 beta levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-6 levels in all treatment groups were significantly higher than in the control group (*P*=0.001). The mean IL-6 level in Group 1 was significantly higher than in Groups 2 and 3 (*P*=0.001). The mean IL-6 levels in Groups 2 and 3 were significantly lower than in Group 4 (*P*=0.001). No significant differences in IL-6 levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-10 levels in all treatment groups were significantly higher than in the control group (*P*=0.001). The mean IL-10 level in Group 1 was significantly lower than in Groups 2 and 3 (P=0.001), while the mean IL-10 levels in Groups 2 and 3 were significantly higher than in Group 4 (*P*=0.001). No significant differences in IL-10 levels were

observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-17A levels differed significantly among the groups (P=0.007). The mean IL-17A level in Group 1 was significantly higher than in all other groups (P=0.001). No significant differences in IL-17A levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-17F levels among the groups did not differ significantly. No significant differences in IL-17F levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-22 levels among the groups did not differ significantly. No significant differences in IL-22 levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean IL-23 levels showed significant differences among the groups (P=0.001). The mean IL-23 level in Group 1 was significantly higher than in the other treatment groups and the control group (P=0.001). The mean IL-23 level in Group 3 was significantly lower than in Group 4 (P=0.001). No significant differences in IL-23 levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

The mean TNF alpha levels in all treatment groups, except Group 3, were significantly higher than in the control group (P=0.001). The mean TNF alpha levels in Groups 1 and 2 were significantly higher than that in Group 3 (P=0.001), whereas the mean TNF alpha level in Group 3 was significantly lower than that in Group 4 (P=0.001). No significant differences in TNF alpha levels were observed in intragroup evaluations of the treatment groups at all follow-up time points (Table 3).

Discussion

The most important outcome of our study is that inflammatory cytokines in HT cases increased more compared to cases with isolated LBFs in the early period, that there was a negative relationship between anti-inflammatory cytokine levels and fracture callus thickness, and that it presented a perspective on the molecular level to the transfer of the positive effect between cases.

Fracture healing comprises three phases: inflammatory, proliferative, and remodeling. The inflammatory phase includes hematoma formation, releasing inflammatory and growth factors, activating phagocytic cells, and migrating, proliferation, and differentiation of mesenchymal cells. TNF alpha, IL-1 beta, IL-6, and TGF beta can be counted among the active mediators during this period [12]. In the proliferation phase, the hematoma is organized, followed by soft and hard callus formation. When an immature bone is formed, growth factors are very effective during this period. In the remodeling phase, the mineralized callus tissue transforms into a load-bearing, stable lamellar bone tissue [13]. The active mediators in this period are TNF alpha, IL-6, IL-1, growth hormone, and parathormone [14]. Our study's first and seventh days correspond to the inflammation phase, the 10^{th} day to the early callus phase, the 20^{th} day to the mineralization phase, and the 30^{th} day to the ossification phase before remodeling.

HT changes the healing process of the fracture and provides faster healing with abundant callus formation [15]. However, the mechanisms affecting fracture union in such combined traumas have not been fully understood. In the literature, the alkaline blood pH caused by hyperventilation after HT, a central hormonal response, and inhibition of the sympathetic nervous system are listed as possible mechanisms [16]. In the current study, we took the serum samples from Group 1 rats with HT at the 24^{th} hour and on the seventh day and transferred them to Group 2 rats without HT to detect this hormonal effect and see its effectiveness.

In a study performed by Gautschi et al. [17] on human serum samples, the authors observed that the Ca levels in serum samples in patients with HT, LBF, and CI were significantly lower than that of the controls until the seventh day. In our study, despite the serum transfer, the Ca levels in the serum-transferred group of rats were found to be similar to those of rats with LBFs.

In Gautschi et al.'s study [17], the phosphate levels in patients with HT, LBF, and CI were lower than that of the controls until the third day. In our study, phosphate values were not different in inter and intragroup comparisons. It is known that PTH is an effective regulator of Ca and phosphate balance, in addition to its effect in increasing osteoblastic differentiation [18]. In human serum samples taken from patients with HT and CI, PTH levels peaked in the first 24 h and showed higher values than isolated LBFs in Khallaf et al.'s study [19]. In another study, PTH levels were found to peak in the first 6–24 h after HT in samples taken from human cases, while the PTH levels in serum samples with CI were higher at 24 h than in cases with HT and LBFs [17]. In our study, the PTH values of rats with HT were higher than those of other rats because serums were obtained in the early period. PTH values were higher in rats with CI and were given serum than in rats with LBFs was attributed to the effect of HT.

Although GH levels were reported to decrease below normal values in the third week after isolated LBFs, the GH levels peaked in rats with HT and CI in the same period [19]. This suggests that it has more of a stimulating effect on GH. In our study, the fact that the GH levels were higher in the rats with HT than in the group with CI suggests that isolated HT has a more stimulating effect on GH.

CRP, which is a strong acute phase reactant, has been reported to increase in the early inflammatory period of fracture healing, and this increase is even more pronounced after HT [20]. In a study conducted on human serum samples, CRP levels up to the seventh day were higher than the control group in cases with HT, LBFs, and CI, while the cases with isolated fractures had the lowest values [17]. In our study, the highest CRP values were found in the sera of rats with HT in the early period.

Alkaline phosphatase is an indicator of early osteogenic differentiation [21]. It has been reported that ALP levels, which indicate bone regeneration, gradually decrease during the first 4 weeks after LBFs, remaining below the control values [1]. In our study, although ALP levels decreased after LBFs, ALP levels suggestive of increased osteoblastic activity were observed in both the HT and the serum-transferred groups. This was evaluated to indicate that serum could transfer the osteoblastic effect between individuals.

TGF beta 1, a powerful stimulator of osteoblastic bone formation, has been reported to increase up to the sixth week following LBFs [1]. In a study conducted on rat tibia fracture models, the researchers stated that local applications of TGF beta positively affected fracture union [22]. In our study, high TGF beta 1 levels were noticed in both the HT and the serum-transferred groups, consistent with the increased fracture callus tissue in these groups.

It has been reported that Wnt-16 directly proliferates the development of osteoblasts while indirectly suppressing osteoclast formation and differentiation. It is also known that, due to its effects on both osteoblasts and osteoclasts, Wnt-16 does not increase initially but rather later in patients with LBFs [23]. The Wnt-16-beta-catenin signaling pathway is activated after HT [24]. Bao et al. [25] stated that slight activation of the Wnt-16-beta-catenin signaling pathway might positively affect fracture healing in the late phase, while Huang et al. [26] reported that inhibition of the beta-catenin signaling pathway delayed fracture healing in mice. The activation of the Wnt-beta-catenin signal pathway reduces inflammatory markers TNF alpha, IL-6, and IL-1 beta [27]. In our study, we observed that the Wnt-16-beta-catenin signal pathway was activated, especially after HT, and this effect was observed in rats in whom serum was transferred.

Tumor necrosis factor alpha has a dual effect; it can suppress new bone formation by stimulating bone reabsorption and destruction. At low concentrations, it stimulates osteogenic differentiation, while at high concentrations, it inhibits it. Tumor necrosis factor alpha has a dual effect; it can suppress new bone formation by stimulating bone reabsorption and destruction. It stimulates osteogenic differentiation at low concentrations and inhibits it at high concentrations. TNF alpha has the effect of stimulating ossification at an early stage. In the following process, TNF alpha suppresses the non-tissue-specific ALP activity and reduces the mineralization process, thus preventing bone formation. Besides, it promotes the secretion of vascular endothelial growth factor (VEGF), which is secreted by neutrophils and directly affects the formation of blood vessels. Together with IL-1 beta [2], it can promote the mineralization of the bone matrix. Chan et al. stated that TNF alpha injections could have a positive effect in the early period (first 24 h) of fracture healing [28]. The high rates of the union in the serum-transferred group in our study may be related to the early collection of these sera from rats with HT.

Interleukin-1 is an inflammatory mediator that appears in the early post-fracture period and activates healing [29]. However, this positive effect is limited to low doses. Brenner et al. [30] reported that IL-1 beta at high concentrations inhibited osteoblast migration while increasing osteoclast activity, thus delaying fracture healing. Ozeki et al. [31] reported that IL-1 beta increased the proliferation of osteoblast-like cells through the Wnt signaling pathway. In our study, we observed that the Wnt-16 levels and IL-1 beta levels showed similar correlations in the groups.

Interleukin-6 is one of the inflammatory mediators released abundantly by osteogenic cells in the early post-fracture period [32]. Beeton et al. [33] reported higher IL-6 levels in CI cases than in HT cases. It is believed that IL-6 regulates osteoblast and osteoclast activity, increases soft callus formation, and affects osteoclastogenesis [34]. In the current study, the IL-6 levels were high in the early period, while the mean IL-6 level in Group 4 rats was higher than that of Group 2. It has been reported that Interleukin 6 and Tumor necrosis factor-alpha levels, which are pro-inflammatory factors, increase first in the first and second weeks after LBFs, and then gradually decrease [1].

Interleukin-17 is a family of pro-inflammatory cytokines. IL-17A suppresses osteoclast differentiation at high doses, and its effects on bone formation are contradictory [35]. IL-17F induces osteogenic differentiation and positively affects osteoblast maturation [21]. In addition, IL-17F increases in the early phase of fracture healing [21]. Our study shows that both IL-17A and IL-17F are elevated early after HT.

Interleukin-23 is a pro-inflammatory cytokine responsible for bone non-union caused by inflammation [36]. It has been stated that the IL-23 levels in rats with a HT model were above those of the controls until the 14th day, peaking on the third day [37]. Our study showed the greatest increase in IL-23 values in Group 1 rats due to HT.

Interleukin-10 has a strong anti-inflammatory effect. It negatively affects the production of pro-inflammatory cytokines, such as IL-1 and IL-6, and inhibits their release [38]. In addition, the cytokine has been reported to suppress osteoclastic activity [39]. In evaluating the group averages of IL-10 levels in our study, the values were lower in rats that had HT and were transferred to serum. We believe that low IL-10 levels are associated with increased union rates. Although IL-22, a member of the IL-10 cytokine family, showed a similar outcome pattern to IL-10 in the treatment groups, no significant difference was observed between the groups.

Our study had some limitations. First, the number of our subjects was relatively few; by increasing the number of subjects, an evaluation could be made with the data to be obtained in similar periods in all groups. Second, we only had radiological outcomes and molecular parameters. A biomechanical and or histopathological evaluation was not performed. Finally, conventional radiography was employed instead of micro CT for radiological examinations.

The literature suggests that the sera obtained from HT cases in *in vitro* cell culture media positively affects osteoblastic cell proliferation [17]. Sari et al. [40] stated that fracture union could be induced radiologically and histopathologically by *in vivo* serum transfer. The importance of inflammatory and anti-inflammatory cytokine balance in the bone union process has been shown at the molecular level.

Conclusion

Our study demonstrates that, at the macro level, the callus thickness was higher in the serum-transferred group than in the cases with isolated fractures. The study also enabled us to obtain more information about the early inflammatory molecular changes of fracture healing, especially in cases with HT. Based on our findings, we concluded that inflammatory cytokines (IL-17A, IL-1 beta, IL-17F, IL-6, IL-23, and tumor necrosis factor-alpha) were elevated in the early period in HT groups and that this effect could be transferred to Group 2 rats by serum transfer.

It is noteworthy that the Wnt-16, IL-1 beta, and TNF alpha values, which positively affect union, were higher in the serum transfer group than the isolated fracture group and that IL-10 levels, a negative modulator on the union, were negatively correlated with callus thickness. Our findings will contribute to developing new therapeutic agents for fracture healing.

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