Turk Neurosurg, 2024

Original Investigation

DOI: 10.5137/1019-5149.JTN.44200-23.2

Received: 20.04.2023
Accepted: 01.09.2023

Published Online: 13.02.2024

Effects of Focal Cerebellar Injury on Fracture Healing and Oxidative Stress in Rat Model: An Experimental Animal Study

Fatih DOGAR[1], Kaan GURBUZ[2], Duran TOPAK[1], Aysun OKCESIZ[3], Ayse EKEN[3], Emine KILINC[4], Mustafa ARIK[5], Okkes BILAL[1], Mustafa Abdullah OZDEMIR[1], Mikail TELEK[1]

[1]Kahramanmaras Sutcu Imam University, Faculty of Medicine, Department of Orthopaedics and Traumatology, Kahramanmaras, Türkiye
[2]Kayseri City Education & Research Hospital, Clinical of Orthopedics and Traumatology, Kayseri, Türkiye
[3]Erciyes University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Kayseri, Türkiye
[4]Kahramanmaras Sutcu Imam University, Faculty of Medicine, Department of Pathology, Kahramanmaras, Türkiye
[5]Aksaray Ortakoy State Hospital, Clinical of Orthopedics and Traumatology, Aksaray, Türkiye

Corresponding author: Fatih DOGAR ✉ drfatihdogar@hotmail.com

ABSTRACT

AIM: To examine the effect of cerebellar damage on the process of fracture healing.

MATERIAL and METHODS: A total of forty-two male rats were selected at random and subsequently allocated into three distinct groups. The participants were divided into two subgroups within each group, with the intention of sacrificing them during the third and sixth weeks. Group 1 had isolated femoral fracture, Group 2 had femoral fracture after craniotomy, and Group 3 had femoral fracture accompanying cerebellar injury after craniotomy. Left femoral fractures in rats in all groups were treated using an intramedullary Kirschner wire. Radiological, histological, and biochemical evaluations were conducted at 3 and 6 weeks to assess the processes of fracture healing. To determine the effects of fracture healing and cerebellar injury on oxidant–antioxidant systems, catalase (CAT), malondialdehyde, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were measured.

RESULTS: Between the time frame of 3 to 6 weeks, Group 3 had higher radiography scores, alkaline phosphatase levels, callus/diaphyse ratio, callus improvement, and bone mineral density in comparison to the other groups. The activity of SOD was found to be statistically negligible in all groups, suggesting that SOD does not have a substantial impact on fracture healing in cerebellar injury. However, notable increases in the activity of GPx and CAT enzymes were observed, showing their considerable involvement in the process of fracture healing.

CONCLUSION: Cerebellar injury reduces the oxidative stress in the fracture area and contributes positively to fracture healing both radiologically, biochemically and histopathologically.

KEYWORDS: Cerebellar injury, Fracture healing, Antioxidant, Bone, Rats

ABBREVIATIONS: TBI: Traumatic brain injury, **BMD:** Bone mineral density, **ROI:** Region of interest, **ALP:** Alkaline phosphatase, **S-bone ALP:** Bone-specific ALP, **SOD:** Superoxide dismutase, **GPx:** Glutathione peroxidase, **CAT:** Catalase, **MDA:** Malondialdehyde, **ICC:** Intraclass Correlation Coefficient

Fatih DOGAR : 0000-0003-3848-1017
Kaan GURBUZ : 0000-0002-0144-5517
Duran TOPAK : 0000-0002-1442-3392
Aysun OKCESIZ : 0000-0001-9130-2853
Ayşe EKEN : 0000-0003-4830-5770
Emine KILINC : 0000-0002-1032-651X
Mustafa ARIK : 0000-0001-5596-9175
Okkes BILAL : 0000-0002-7949-5434
Mustafa Abdullah OZDEMIR : 0000-0002-8281-3528
Mikail TELEK : 0000-0002-9163-0205

INTRODUCTION

Every year, roughly 1.2 million individuals die and fifty million more are injured in automotive incidents worldwide (7). Traumatic brain injury (TBI) is divided into primary and secondary types and is most commonly observed after traffic accidents. Primary damage is the mechanical damage that develops at the time of the event, caused directly by the traumatic factor on the nervous system, and cannot be treated. This type of damage is divided into two subgroups as focal (contusion, laceration, bleeding) and diffuse (diffuse axonal injury). Secondary damage develops sometime after the moment of trauma, and all available treatment methods are aimed at secondary damage. Ischemia and increased intracranial pressure are the most common clinical presentations of secondary injury and the main treatment goals (2).

Although the cerebellum comprises around 10% of the brain's volume, it includes more than 50% of the brain's neurons (5). The cerebellum has been deemed a motor structure due to the fact that cerebellar injury causes problems in motor control and posture and the bulk of cerebellar outputs are routed to motor system components. The cerebellum does not originate motor instructions; instead, it changes the motor commands of the descending pathways to make motions more adaptable and precise (25).

It has been proven in clinical experience and in the literature that TBI accelerates fracture healing and heals with an exaggerated volume of callus tissue, regardless of the fracture treatment. However, to date, almost all studies have focused on the mediators, hormones, and cytokines involved in this event, and there are few studies on the role of a damaged coordination center in the brain.

This research attempted to determine if the cerebellum, which is responsible for central nervous system movement coordination, contributed to this phenomenon.

MATERIAL and METHODS

Between September 2020 and March 2021, this study was implemented at the Experimental Research and Application Centre of Sutcu Imam University (Project ID: 2020/4-13M). 42 male rats were utilized in this investigation, and they were kept in individual cages with a constant temperature of 23°C, a 14/10 h light-dark cycle, and other conventional conditions. A commercial pellet meal and tap water were given to each animal. The Medicine School of Sutcu Imam University University's Experimental Animals Breeding and Research Center served as the site for all procedures. Animal care was carried out with the prior approval of the Sutcu Imam University Animal Experimental Ethics Committee on April 2020 with approval number 2020/04-02 and was in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and the Helsinki Declaration of Animal Rights.

The G power software was used to calculate the sample size using the parameters of 0.07, 0.15, and 0.82 for the effect size. The periosteal thickness at young ages and the neuroprotective impact of progesterone hormone were taken into consideration while dividing the 42 male rats (age, 8-10 months; weight, 300-320 g) into six groups (n=7 per group).

There were two control groups and four study groups, as follows:

Group 1–third and sixth week: The control groups had simply a femoral fracture done, and were sacrificed third and sixth weeks later.

Group 2–third and sixth week: The research groups with only a craniotomy (no cerebellar damage) and a femoral fracture that were sacrificed three and six weeks later.

Group 3–third and sixth week: The groups that underwent craniotomy with cerebellar injury and femoral fracture were sacrificed three and six weeks later.

Prior to the fracture of the femur, a blood sample of 1.2 mL was collected for the purpose of analyzing alkaline phosphatase (ALP). Anteroposterior and lateral images of the femur were acquired, thereby facilitating the evaluation of the overall femoral bone mineral density (BMD). Additionally, a 3 cc blood sample was collected from the individuals for antioxidant analysis prior to their euthanasia. Following the sacrifice of the rats, the whole left femurs were removed and for histological analysis, the extracted femurs were kept in 10% formaldehyde solution.

Surgical Procedure

The surgical procedures were conducted under controlled conditions with the use of a heating pad set to a temperature of 37°C. Prior to the procedures, sedatives were administered, specifically xylazine (Rompun 2% solution, 50 mL vial, Bayer-Turk Ilac Ltd., Istanbul, Türkiye) at a dosage of 6-8 mg/kg and ketamine (Ketalar 50 mg/mL 10 mL vial, Pfizer Ilaclar Ltd.) at a dosage of 60-80 mg/kg. A single dosage of antibiotic prophylaxis was supplied before to all surgical operation.

Craniotomy and Cerebellar Damage

The scalp was opened longitudinally, and craniotomy was performed utilizing a 1×1 mm Kirschner (Kr) wire with the help of a high-speed drill on the posterior right side of the lambda, the junction of the sutures of the parietal and occipital bones, which corresponds to the cerebellar localization in accordance with the atlas of Paxinos and Watson Rat Brain Stereotaxic Coordinates (5th edition). By controlling bleeding with bipolar cautery, cerebellar damage was implemented with the help of a sterile micropipette to create a 5-mm-depth penetration in the cerebellum (Figure 1).

Femoral Fracture

A knee midline incision was widened proximally for all rats' arthrotomy. Accessing the knee joint required a median parapatellar incision after crossing the skin-subcutaneous tissue. After exposing the mid-distal left femurs of all rats, this area was osteotomized utilizing an electric knife and retrogradely intramedullary rimiered utilizing a micro-drill. 1.5-mm Kr wires mended it. After fixation, the proximal and distal Kr wires in the femur were carefully maintained. After

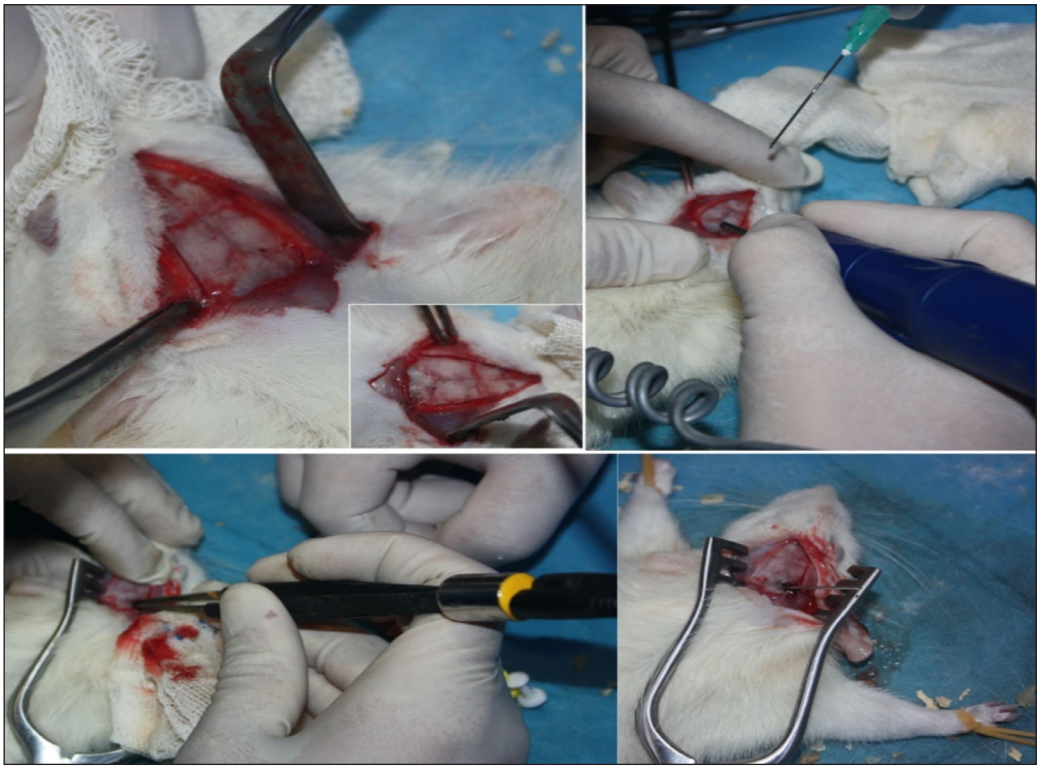

Figure 1: Surgical procedure of craniotomy at the junction points of the sutures of the parietal and occipital bones and cerebellum damage with sterile micropipette.

fixing, 30 mL of 0.09% NaCl washed the broken line and incision. A 3/0 Vicryl needle (Johnson & Johnson, Brussels, Belgium) closed the patellar tendon and epidermis. The wound covering ended fixation.

Radiological Evaluation

At the third and sixth weeks after sacrifice, Warden et al. (31) altered a five-point evaluation method to measure fracture repair in each group: 0 points, no healing; 1 location, callus formed, fracture gap unbridged; 2 points, callus formation visible with bridging of the fracture gap and fracture line; 3 points, callus formation apparent with bridging faint; 4 points, fracture union. At three and six weeks postoperatively, normal radiography assessed the callus/diaphysis proportion and new callus volumes at the femoral fracture area.

Histopathological Evaluation

For the assessment of fracture healing histologically, a 10-point scale was used: One point for fibrosis, 2 points for predominantly with small amount of cartilage, 3 points equal mixture of fibrous and cartilagenous tissue, 4 points predominantly cartilage tissue with little amount of fibrosis, 5 points primarily cartilage, 6 points predominantly cartilage with minimal immature bone tissue, 7 points proportionally equal amounts of cartilage and immature bone tissue, 8 points for embryonic bone tissue that has restricted cartilage, 9 points for recovery with immature bone tissue, and 10 points for mature bone tissue.

BMD Evaluation

Dual-energy X-ray absorptiometry (Hologic QDR 4500 Elite Acclaim Series, Hologic, Massachusetts, USA) measured femur BMD. The 14.5 × 10.8 mm Region of Interest (ROI) provided excellent quality area, bone mineral content, and density data. This ROI covered the osteotomy sites in the afflicted limb (left) and the limb that was not impacted (right).

Evaluation of Oxidative Stress

Preparation of Sample

Plasma was extracted from blood samples by centrifuging them at 4400 rpm for 5 minutes. Until the oxidative stress parameters were measured, the plasma specimens were kept at -80 degrees Celsius. A spectrophotometer (UV-1800, Shimadzu Co., Kyoto, Japan) was used to measure the levels of malondialdehyde (MDA) and anti-oxidant enzyme activity in the samples, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). The protein content was measured by Lowry's technique (17).

CAT Enzyme Activity

Aebi's method was utilized to examine the CAT activity of the samples at 25°C. The rate of H_2O_2 dissolution was determined for 30 seconds using a spectrophotometer at 240 nm. Activity was measured in units per gram of protein (1).

SOD Enzyme Activity

Utilizing the methodology suggested by Fitzgerald et al. (8), SOD activity was examined. 25µL of samples were combined with 850µL of a substrate solution comprising 50 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid and (pH 10.2), 0.05 mmol/L xanthine sodium, and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride. A

spectrophotometer was utilized to measurement the mixture's absorbance at 505 nm for 3 minutes after 125μL of xanthine oxidase (80 U/L) had been added. SOD activity is measured in units per gram of protein.

MDA Levels

By detecting MDA levels in samples, lipid peroxidation was calculated in accordance with the technique described by Ohkawa et al. (23). Then, 100μL of plasma and 50μL of 8.1% sodium dodecyl sulfate (SDS) were combined, vortexed, and incubated for 10 minutes at room temperature. Additionally, 375μL of 0.6% thiobarbituric acid and 375μL of 20% acetic acid (pH 3.5) were added. These mixtures were then heated in a water bath for 60 minutes. For chilling, the specimens were brought to room temperature. The mixture was then centrifuged at 1000 rpm for five minutes after 1.25 mL of butanol:pyridine (15:1) was added. The organic mauve layer was assessed at 532 nm. In specifically, 1,1,3,3-tetraethoxypropane was used as a standard. The provided data was expressed in units of nmol/mg protein.

GPx Enzyme Activity

Utilizing the approach described by Pleban et al. (24), the GPx activity was identified. In a nutshell, we created a 50 mmol/L Tris buffer (pH 7.6) with 1000 U of glutathione reductase, 1 mmol/L Na_2EDTA, 2 mmol/L reduced glutathione, 0.2 mmol/L NADPH, and 4 mmol/L sodium azide. After that, 980μL of the reaction solution and 20μL of sample were mixed, and the combination was incubated at 37°C for 5 minutes. A spectrophotometer was used to measure the decrease in absorption at 340 nm for three minutes after introducing 8.8 mmol/L of H_2O_2 to the reaction. The unit of measure for GPx activity was U/mg protein.

Biochemical Evaluation

The measurement of ALP activity levels has been suggested as a potential biomarker for assessing the advancement and speed of bone healing subsequent to fractures (21). The blood alkaline phosphatase (ALP) levels of rats were tested concurrently using the ARCHITECT systems developed by Abbott Laboratories, located in Abbott Park, Illinois, United States (30).

Intraclass Correlation Coefficient Evaluation

A two-sided variable effects model and intraclass correlation coefficients (ICC) assessed radiological and histological observers' consistency and dissimilarities. ICC values 0.5 indicated poor dependability, 0.5-0.75 moderate reliability, 0.75-0.9 reasonable reliability, and >0.90 faultless reliability (15).

One orthopedic and one radiology specialist with more than 10 years of experience took radiographic measures of all participants twice a month to establish intra- and inter-observer reliability.

ICCs and a two-sided varied effects model implied unanimity in intra- and inter-observer measurements. Its intra- and inter-observer reliabilities were excellent (ICC, 0.975–0.996) and satisfactory (ICC, 0.860–0.995).

Histological proportions of all subjects were repeated again with a one-month interval by two pathologists with at least five years of expertise to measure interobserver reliability.

Intra- and inter-observer consistency of measurement was assessed using a two-sided mixed effects model using ICCs. Inter- and intra-observer pathologists had great reliability (ICC, 0.945–0.999).

Statistical Analysis

IBM SPSS version 23 was used for data input and analysis. The Kolmogorov–Smirnov test assessed factor normalcy across all six groups. Mean, standard deviation, percentage, frequency, and lowest to maximum values are shown. The Mann–Whitney U test analyzed asymmetrical data throughout six categories. Pearson's chi-square test was used for independent and dependent variable univariate analysis. Multiple comparisons using ANOVA and Tukey's test. 0.05 is significant.

RESULTS

Table I shows a difference of statistical significance (p=0.0017) at three and six weeks between the study groups that had craniotomy only (Group 2) and study groups with cerebellar damage (Group 3). The third to sixth weeks study group had the best fracture union, and both the control and study groups' radiological scores at this time were greatest (p<0.05) (Figure 2A–F).

The callus tissue volume, callus/diaphysis proportion, and serum alkaline phosphatase (ALP) levels were assessed in this investigation (Table II) since they are frequently used indicators of fracture unity during radiological and biochemical follow-up.

All three groups had a significant increase in callus production and the callus/diaphysis ratio at the sixth week relative to the third week (p<0.05), with Groups 3-6 weeks seeing the largest increase. Even though the difference between Groups 2 and 3 and the control group at the end of the sixth week was not statistically significant (p>0.05), the rise in callus volume was. Additionally, at the end of the third week, Group 3 and the control group, in addition to Groups 2 and 3, showed substantially distinct increases in callus volume (p<0.05; Table II).

Preoperatively, at weeks 3 and 6, and across all groups, there were statistically significant variations in the blood ALP levels (p<0.05). Although there was not a significant difference between Groups 2 and 3 (p>0.05) between the third-week rise in serum ALP levels between Groups 2 and 3 compared to the control group. By the sixth week, Groups 2 and 3 had considerably higher serum ALP levels than the control group, and Group 3 had significantly higher serum ALP levels than Group 2 (Table II, p<0.05). At six weeks, there was no discernible difference between groups 2 and 3, as shown by the greater histopathological assessment scores for those two (Figure 2G–I).

At three and six weeks, BMD in Groups 2 and 3 was higher than in the corresponding control groups (p<0.05). Between the

third-week and sixth-week groups, there was no discernible change. The BMD was greatest in group 3-6th weeks (respective BMD values (mean SD): group 1-3rdw: 0.1350.003, group 1-6thw: 0.1380.003, group 2-3rdw: 0.1420.002, group 2-6th w: 0.1520.004, group 3-3rd w: 0.1720.002, group 3-6th w: 0.2020.006).

Table I: Radiological Scores* for all Groups

Groups (n=7 for each)	1 Point		2 Points		3 Points		4 Points		p for overall difference
	n	%	n	%	n	%	n	%	
Group 1-3rdw: Control group sacrificed at third week	0	0	5	71.4	2	28.6	0	0	<0.05
Group 1- 6thw: Control group sacrificed at sixth week	0	0	4	57.1	3	42.9	0	0	
Group 2-3rdw: Only Craniotomy performed study group sacrificed at third week	0	0	2	28.6	4	57.1	1	14.3	
Group 2- 6thw: Only Craniotomy performed study group sacrificed at sixth week	0	0	1	14.2	3	42.9	3	42.9	
Group 3-3rdw: Craniotomy with cerebellum damage performed study group sacrificed at third week	0	0	0	0	2	28.6	5	71.4	
Group 3- 6thw: Craniotomy with cerebellum damage performed study group sacrificed at sixth week	0	0	0	0	1	14.3	6	85.7	

** Intra- and inter-observer reliability were evaluated based on radiographic measurements from all subjects repeated twice with an interval of one month by one orthopedic and one radiologic specialists with 10 years of professional experience. A two-way mixed effects model and intraclass correlation coefficients (ICC) were used to evaluate agreement and differences between intra- and inter-observer measurements. Intra- and inter observer reliability were determined to be excellent (ICC:0.975–0.996)and good (ICC: 0.860–0.995), respectively.* ***w:*** *week.*

Table II: Comparison of Callus/Diaphysis Ratio, Callus Formation, and Serum Alkaline Phosphatase Level Between the Sacrification Weeks

Groups (n=14 for each)	Callus/diaphysis ratio		Callus Formation (mm)		Alkaline phosphatase level (U/mL)		
	3rd week	6th week	3rd week	6th week	Preoperative	3rd week	6th week
	Mean ± SD						
Control groups: Group 1-3rdw and Group 1- 6thw	3.25 ± 0.23	4.13 ± 0.45	4.85 ± 0.51	5.55 ± 0.55	270 ± 19.43	307 ± 31.35	321 ± 38.47
Only craniotomy groups: Group 2-3rdw and Group 2- 6thw	3.43 ± 0.32	4.63 ± 0.57	5.08 ± 0.73	5.72 ± 0.91	292 ± 16.45	328 ± 27.67	341 ± 2.84
Craniotomy with cerebellum damage groups: Group 3-3rdw and Group 3- 6thw	3.92 ± 0.14	5.16 ± 0.21	5.68 ± 0.69	6.07 ± 0.02	303 ± 20.56	359 ± 11.48	397 ± 9.37
Significance of time-dependent intergroup callus/diaphyseal change (p<0.05)	F=48.433 **p<0.001**		F=53.657 **p<0.001**		F=108.933 **p<0.001**		
One-way analysis of variance in repeated analysis	*		*		**		

****** Th post hoc test Tukey showed a significant difference between the groups.*

******* In the post hoc test Tukey, there was a significant difference between preoperative Alkaline phosphatase and 3rd and 6th week values. The difference between the only craniotomy groups (Group 2-3rdw and Group 2- 6thw) and craniotomy with cerebellum damage groups (Group 3-3rdw and Group 3- 6thw) was significant.* ***w:*** *week.*

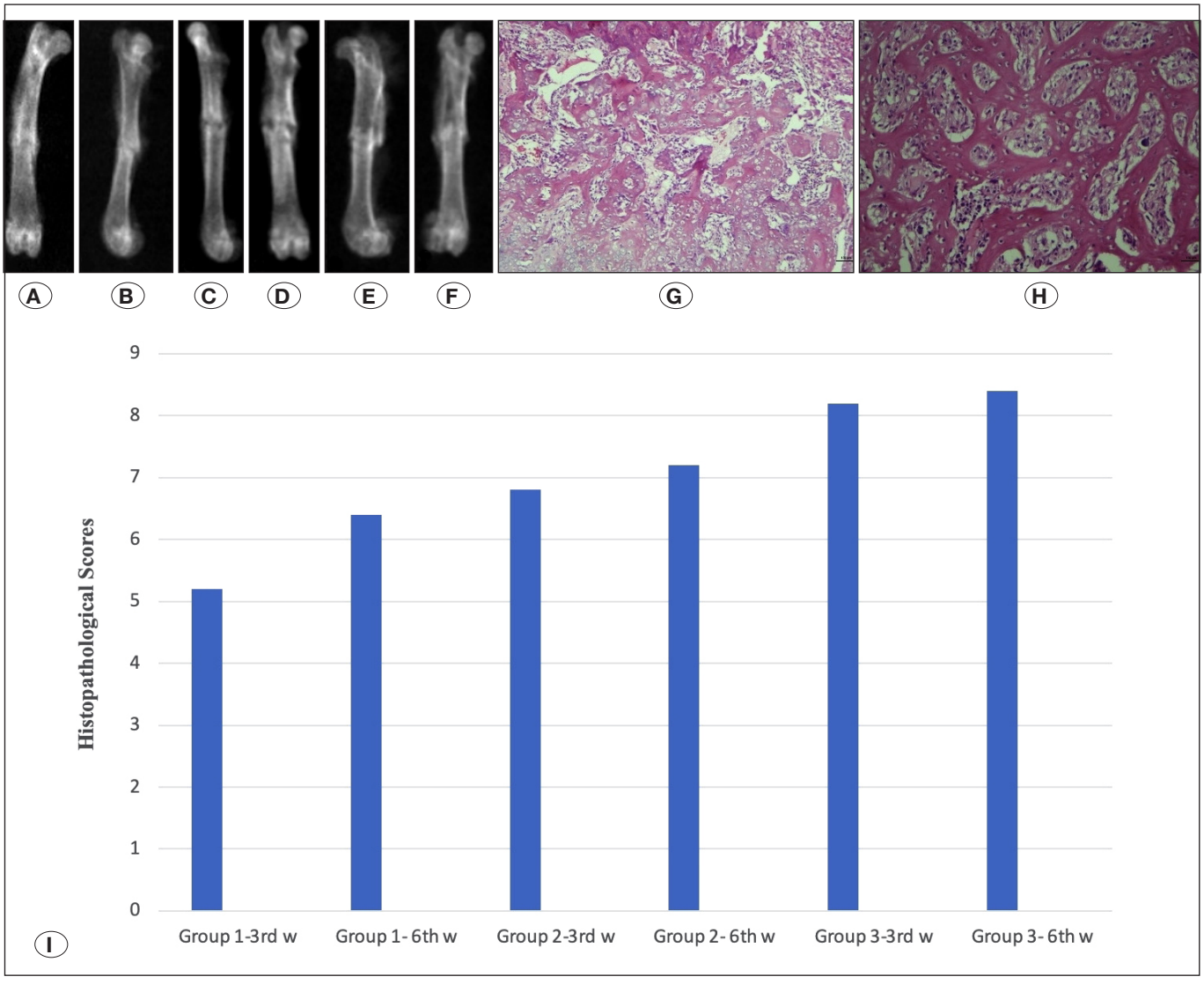

Figure 2A-I: Radiological sample views from each group, and histopathological scores graph and samples from bone healing site. **A)** Group 1-3rdw: Control group sacrificed at 3rd week, **B)** Group 1- 6thw: Control group sacrificed at 6th week, **C)** Group 2-3rdw: Only Craniotomy performed study group sacrificed at 3rd week, **D)** Group 2- 6thw: Only Craniotomy performed study group sacrificed at 6th week, **E)** Group 3-3rdw: Craniotomy with cerebellum damage performed study group sacrificed at 3rd week, **F)** Group 3- 6thw: Craniotomy with cerebellum damage performed study group sacrificed at 6th week, **G)** Dominant proliferation of cartilage tissue at the fracture healing zone (100xH/E) in Group 3- 6thw, **H)** Immature bone-rich proliferation at the fracture healing zone (200XH/E) in Group 3- 6thw, **I)** Histopathological scores of each group.

The metrics for oxidative stress assessed in plasma samples are shown in Figure 3A–D as CAT, SOD, MDA, and GPx values. SOD activity in each group did not differ significantly when oxidative stress indicators were compared, proving that SOD is not involved in cerebellar damage when it occurs in peripheral blood (Table III). SOD also lacked statistical significance, according to subsequent statistical analysis using Tukey multiple comparisons. However, GPx performs a statistically significant and functional role in antioxidant systems (Table III).

DISCUSSION

In this study, it has been shown that radiological, biochemical, and histopathological parameters showing fracture healing are better and higher in fractures developing after standardized TBI and isolated injury to the cerebellum responsible for the movement and coordination center compared to fractures developed after isolated TBI. Although most studies to date have focused on the mediators, hormones, and cytokines involved in this event, there has been no study showing the effects of an isolated damaged coordination center, the cerebellum, outside the brain on the union of the fracture. This paper is the first to demonstrate the impact of cerebellar

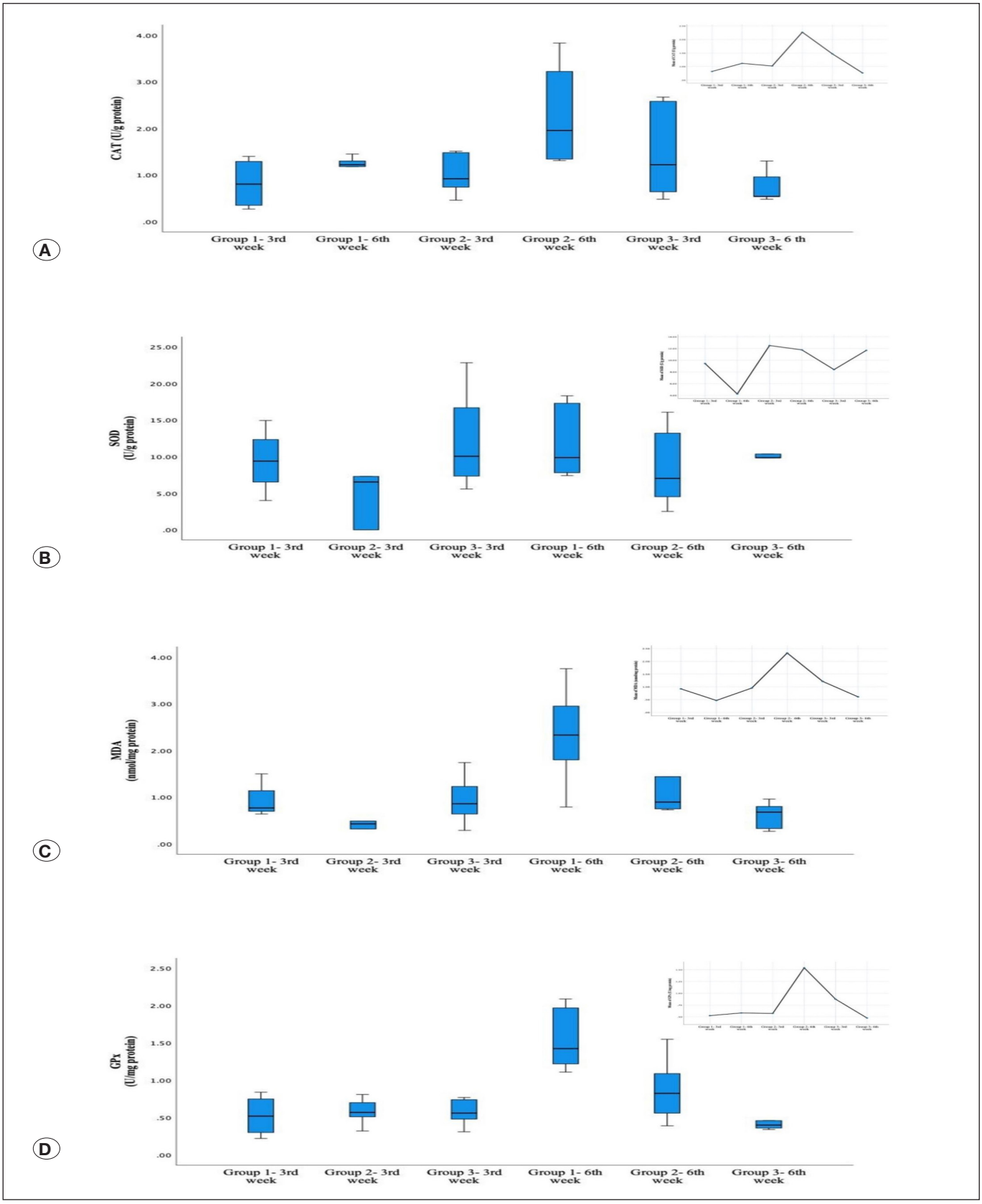

Figure 3A-D: Distribution of each oxidative stress parameters in all groups. (**A:** catalase, **B:** superoxide dismutase, **C:** malondialdehyde, **D:** glutathione peroxidase).

Table III: Comparisons of Oxidative Stress Parameter in Each Group, and Besides Tukey Multiple Comparisons of Groups Regarding the Oxidative Stress Parameters

Dependent Variable	Group 1- 3rdw	Group 2- 3rdw	Group 3- 3rdw	Group 1- 6thw	Group 2- 6thw	Group 3- 6thw	p-value
CAT, Mean ± SD (U/g protein)	0.82 ± 0.55	1.11 ± 0.39	1.02 ± 0.46	2.26 ± 11.76	1.46 ± 0.97	0.76 ± 0.35	**0.021**
SOD, Mean ± SD (U/g protein)	9.43 ± 4.47	4.23 ± 3.87	12.50 ± 7.15	11.76 ± 4.8	8.39 ± 5.21	11.67 ± 5.38	0.157
MDA, Mean ± SD (nmol/mg protein)	0.92 ± 0.39	0.46 ± 0.38	0.95 ± 0.55	2.32 ± 1.05	1.21 ± 0.7	0.60 ± 0.29	**0.001**
GPx, Mean ± SD (U/mg protein)	0.52 ± 0.27	0.58 ± 0.18	0.57 ± 0.19	1.54 ± 0.4	0.87 ± 0.43	0.47 ± 0.19	**<0.001**

	Group versus Group		Mean Difference	Standard Error	p-value
CAT (U/g protein)	Group 1- 6thw	Group 3-6thw	-1.50433	0.44277	**0.025**
SOD (U/g protein)	None	None	-	-	-
MDA (nmol/mg protein)	Group 1- 3rdw	Group 1- 6thw	-1.40500	0.42276	**0.029**
	Group 2- 3rdw	Group 1- 6thw	-1.85900	0.39658	**0.001**
	Group 3- 3rdw	Group 1- 6thw	-1.37300	0.39658	**0.021**
	Group 3- 6thw	Group 1- 6thw	-1.71700	0.39658	**0.003**
GPx (U/mg protein)	Group 1- 3rdw	Group 1- 6thw	-1.01500	0.20037	**<0.001**
	Group 2- 3rdw	Group 1- 6thw	-0.95800	0.18796	**<0.001**
	Group 3- 3rdw	Group 1- 6thw	-0.96800	0.18796	**<0.001**
	Group 2- 6thw	Group 1- 6thw	-0.66667	0.17922	**0.012**
	Group 3-6thw	Group 1- 6thw	-1.06600	0.18796	**<0.001**

SD: *Standard deviation,* ***CAT:*** *Catalase,* ***SOD:*** *Superoxide dismutase,* ***MDA:*** *Malondialdehyde,* ***GPx:*** *Glutathione peroxidase.* ***w:*** *week.*

damage on fracture healing using an experimental fracture model.

The biological procedure of proliferation that occurs to repair a fracture is called fracture healing (37). Some biochemical parameters have received considerable attention as potential mediators between the brain and skeleton, and metabolic and inflammatory processes may also play an important role (10,29,33,35). While the influence of TBI on fracture healing remains an intriguing topic, there is growing clinical evidence that patients with polytrauma, TBI, and long bone fractures demonstrate too much callus formation (9,20,30).

Spencer reported in a clinical trial of 53 cases carried out by TBI that fracture recovery was superior in patients with concomitant TBI compared to patients without radiological brain injury, and that callus volume increased by 73% (28).

In one experimental study by Arik et al., it was found that the callus volume was higher in TBI groups than controls (3). In our study, callus volume and radiological scores were higher in our study groups than controls. Moreover, callus volume and radiological scores were highest in the sixth week group with cerebellar damage. In another study conducted by Giannoudis et al., the callus/diaphysis ratio was higher in the head trauma group than in patients in the non-head trauma group (9).

In another experimental study, fracture healing was demonstrated by micro-computed tomography (CT) from the second week, characterized by excessive callus formation and progressive bone bridging, in mice with external fixation following femoral osteotomy with TBI (30). Arik et al. also showed that callus: diaphysis ratio wa higher in brain injury groups than controls (3). In our study, the callus: diaphysis ratio was higher in our study groups compared to the controls, and this ratio was noted in the sixth week group with the highest cerebellar damage.

Among biochemical markers, ALP and bone-specific ALP (S-bone ALP) activity have been shown to be indications of the course and pace of bone healing after fractures (21,22). Another research discovered a link between an increase in ALP and an increase in bone-specific ALP levels (21). Furthermore, increases in ALP levels on the seventh and fourteenth days following injury mirrored alterations in bone-specific ALP levels on the same day. Similarly, callus capacity is linked to ALP and bone-specific ALP levels decreasing, remaining

unchanged, or increasing. During the first two weeks, absence of change or a minor rise in ALP and bone-specific ALP levels suggested fracture fixation, fast bone healing, and little or negligible callus development. A large rise in ALP activity within the first two weeks suggested that fracture fixation was poor, bone regeneration was delayed, and callus production was high. In another investigation, patients with severe head injury and fracture had significantly higher mean ALP and bone isoenzyme levels at the end of the second week (peaking at the third week post-injury) when compared to patients with isolated fractures or head trauma and healthy participants (34). Our findings show that serum ALP levels are critical for monitoring fracture repair. Furthermore, our study groups' serum ALP levels were greater than the controls'. At week six, the cerebellar damage group had the highest serum ALP level. This finding suggests that there is a link between the groups and radiological scores, callus production, and callus/diaphysis ratios.

Oxidative stress is defined as a shift in the balance between oxidants, which are a normal byproduct of the aerobic process, and antioxidant defense, which attempts to restrict them through enzymatic or non-enzymatic ways, in favor of oxidants (27). The mechanics of physiological fracture healing include the stages of embryonic skeletal development. Under inflammatory and ischemic circumstances, reactive oxygen species (ROS) are created during the first phase of bone regeneration (36). These harmful radicals damage nuclear acids and proteins, induce cell injury, and cause lipid peroxidation, leading to the generation of MDA, a tissue oxidative stress marker (12). ROS affects bone homeostasis by limiting the growth of anabolic cells including osteoblasts, osteocytes, and chondrocytes while increasing the activity of catabolic osteoclasts. This is unquestionably detrimental to fracture healing (4,14,18,19,32).

TBI causes biochemical, molecular, and physiological changes, such as disruption of the blood–brain barrier, inflammation, excitotoxicity, necrosis, apoptosis, mitochondrial dysfunction, and production of oxidative stress (13). The effectiveness of the antioxidant response involving GPx, CAT, and SOD enzymes influences the severity of oxidative stress to some extent. Rapid increases in CAT and GPx enzyme activity are accompanied by an unexpected decrease in SOD activity (6) in rats suffering from traumatic brain injury. Plasma MDA levels, a marker for oxidative stress, and antioxidant enzymes (CAT, GPx) acting to remove oxidative products were statistically significant when compared between groups in this study, whereas SOD activity was not statistically significant. This indicates that SOD plays no function in the repair of peripheral blood fractures in cases of cerebellar injury. In their experimental study, Locher et al. discovered that the combined trauma group (fracture/TBI) had greater bone volume, mineral density, and bridging at the fracture site than the fracture group (16). Similarly, in another study, the combined trauma group (Fracture/TBI) had a lower histopathologically delayed healing rate than the fracture group (26). The results of our study's histopathological assesment of BMD of fracture healing were comparable to those found in the literature, and the highest values were found in the group with cerebellar injury at six weeks.

The first limitation of the current study can be considered as the small sample size in the groups, but the numbers in the groups are suitable according to the power analysis calculation. Second, we measured callus size only with radiography (two-dimensional) and not with the more detailed micro-CT (three-dimensional). Finally, post-fracture biomechanical evaluations could not be performed in this study.

CONCLUSION

In cerebellar injury in addition to TBI, promptly fracture healing (biochemical and radiological) and abundant callus formation (histopathological) were observed in comparison to isolated fractures or fractures associated with brain damage. Only SOD activity was statistically insignificant among the oxidative stress indicators in our study, and in the case of cerebellar damage, SOD played no role in fracture healing in the peripheral blood; however, rapid increases in GPx and CAT enzyme activities were found and performed a significant role in fracture healing.

Funding

The authors are indebted to the Research Foundation of Kahramanmaras Sutcu Imam Univesity (Project ID: 2020/4-13M) for its financial support in this work.

ACKNOWLEDGEMENT

The authors are indebted to the Research Foundation of Sutcu Imam University (Project ID: 2020/4-13M) for its financial support in this work, special thanks to Perihan Gurbuz, Assoc. Prof. PhD. (Department of Pharmacognosy, Faculty of Pharmacy, Erciyes University) for her support and Atilla Yoldaş, Prof. M.D. (Department of Anatomy, Faculty of Medicine, Kahramanmaras Sutcu Imam University) for his tireless assistance at cerebellum damage technique standardization, and thanks are also due to Mr. Onur Öztürk for their tireless assistance at photographs taken, and transportation.

AUTHORSHIP CONTRIBUTION

Study conception and design: FD, KG, DT, OB
Data collection: FD, OB, MAO
Analysis and interpretation of results: FD, KG, AE, AO, EK
Draft manuscript preparation: FD, MA, MT
Critical revision of the article: FD, KG, OB
Other (study supervision, fundings, materials, etc...): FD, DT, MAO, MA
All authors (FD, KG, DT, AO, AE, EK, MA, OB, MAO, MT) reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Aebi H: Catalase in vitro. Methods Enzymol 105:121-126, 1984
2. Algattas H, Huang JH: Traumatic Brain Injury pathophysiology and treatments: Early, intermediate, and late phases post-injury. Int J Mol Sci 15:309-341, 2013
3. Arik M, Ekinci Y, Gurbuz K, Batin S: The effects of focal brain damage on fracture healing: An experimental rat study. Eklem Hastalik Cerrahisi 30:267-274, 2019
4. Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS, Rhee EJ, Han JH, Song KH, Cha BY, Lee KW, Kang MI: Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. Calcif Tissue Int 87:226-235, 2010
5. Bloedel JR, Bracha V: Duality of cerebellar motor and cognitive functions. Int Rev Neurobiol 41:613-634, 1997
6. DeKosky ST, Taffe KM, Abrahamson EE, Dixon CE, Kochanek PM, Ikonomovic MD: Time course analysis of hippocampal nerve growth factor and antioxidant enzyme activity following lateral controlled cortical impact brain injury in the rat. J Neurotrauma 21:491-500, 2004
7. Faul M, Coronado V: Epidemiology of traumatic brain injury. Handbook Clin Neurol 127:3-13, 2015
8. Fitzgerald SP, Campbell JJ, Lamont JV: The establishment of reference ranges for selenium. The selenoenzyme glutathione peroxidase and the metalloenzyme superoxide dismutase in blood fractions. The fifth international symposium on selenium biology and medicine. In: Tennessee, 1992:20-23
9. Giannoudis PV, Mushtaq S, Harwood P, Kambhampati S, Dimoutsos M, Stavrou Z, Pape HC: Accelerated bone healing and excessive callus formation in patients with femoral fracture and head injury. Injury 37:18-24, 2006
10. Hannouche D, Petite H, Sedel L: Current trends in the enhancement of fracture healing. J Bone Joint Surg (Br) 83:157-164, 2001
11. Huo MH, Troiano NW, Pelker RR, Gundberg CM, Friedlaender GE: The influence of ibuprofen on fracture repair: Biomechanical, biochemical, histologic, and histomorphometric parameters in rats. J Orthop Res 9:383-390, 1991
12. Ilyas A, Odatsu T, Shah A, Monte F, Kim HK, Kramer P, Aswath PB, Varanasi VG: Amorphous silica: A new antioxidant role for rapid critical-sized bone defect healing. Adv Healthc Mater 5: 2199-2213, 2016
13. Khatri N, Thakur M, Pareek V, Kumar S, Sharma S, Datusalia AK: Oxidative stress: Major threat in traumatic brain injury. CNS Neurol Disord Drug Targets 17:689-695, 2018
14. Kubo Y, Wruck CJ, Fragoulis A, Drescher W, Pape HC, Lichte P, Fischer H, Tohidnezhad M, Hildebrand F, Pufe T, Jahr H: Role of Nrf2 in fracture healing: Clinical aspects of oxidative stress. Calcif Tissue Int 105:341-352, 2019
15. Lee KM, Lee J, Chung CY, Ahn S, Sung KH, Kim TW, Lee HJ, Park MS: Pitfalls and important issues in testing reliability using intraclass correlation coefficients in orthopaedic research. Clin Orthop Surg 4:149-155, 2012
16. Locher RJ, Lünnemann T, Garbe A, Schaser K, Schmidt-Bleek, K, Duda G, Tsitsilonis S:Traumatic brain injury and bone healing: Radiographic and biomechanical analyses of bone formation and stability in a combined murine trauma model. J Musculoskelet Neuronal Interact 15:309-315, 2015
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin's phenol reagent. J Biol Chem 193: 265-275, 1951
18. Manolagas SC, Parfitt AM: What old means to bone. Trends Endocrinol Metab 21:369-374, 2010
19. Morita K, Miyamoto T, Fujita N, Kubota Y, Ito K, Takubo K, Miyamoto K, Ninomiya K, Suzuki T, Iwasaki R, Yagi M, Takaishi H, Toyama Y, Suda T: Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. J Exp Med 204:1613-1623, 2007
20. Morley J, Marsh S, Drakoulakis E, Pape HC, Giannoudis PV: Does traumatic brain injury result in accelerated fracture healing? Injury 36(3):363-368, 2005
21. Muljacić A, Poljak-Guberina R, Zivković O, Bilić V, Guberina M, Crvenković D: Course and rate of post-fracture bone healing in correlation with bone-specific alkaline phosphatase and bone callus formation. Coll Antropol 37:1275-1283, 2013
22. Nakagawa H, Kamimura M, Takahara K, Hashidate H, Kawaguchi A, Uchiyama S, Miyasaka T: Changes in total alkaline phosphatase level after hip fracture: Comparison between femoral neck and trochanter fractures. J Orthop Sci 11:135-139, 2006
23. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animals and tissues by thiobarbituric acid reaction. Anal Biochem 95:351-358, 1979
24. Pleban PA, Munyani A, Beachum J: Determination of selenium concentration and glutathione peroxidase activity in plasma and erythrocytes. Clin Chem 28:311-316, 1982
25. Rapoport M, van Reekum R, Mayberg H: The role of the cerebellum in cognition and behavior: A selective review. J Neuropsychiatry Clin Neurosci 12:193-198, 2000
26. Seemann R, Graef F, Garbe A, Keller J, Huang F, Duda G, Schmidt-Bleek K, Schaser KD, Tsitsilonis S: Leptin-deficiency eradicates the positive effect of traumatic brain injury on bone healing: Histological analyses in a combined trauma mouse model. J Musculoskelet Neuronal Interact 18:32-41, 2018
27. Sies H: Oxidative stress: Oxidants and antioxidants. Exp Physiol 82:291-295, 1997
28. Spencer RF: The effect of head injury on fracture healing. A quantitative assessment. J Bone Joint Surg 69:525-528, 1987
29. Trentz OA, Handschin AE, Bestmann L, Hoerstrup SP, Trentz OL, Platz A: Influence of brain injury on early posttraumatic bone metabolism. Crit Care Med 33:399-406, 2005
30. Tsitsilonis S, Seemann R, Misch M, Wichlas F, Haas NP, Schmidt-Bleek K, Kleber C, Schaser KD: The effect of traumatic brain injury on bone healing: An experimental study in a novel in vivo animal model. Injury 46:661-665, 2015
31. Warden SJ, Komatsu DE, Rydberg J, Bond JL, Hassett SM: Recombinant human parathyroid hormone (PTH 1-34) and low-intensity pulsed ultrasound have contrasting additive effects during fracture healing. Bone 44:485-494, 2009

32. Wauquier F, Leotoing L, Coxam V, Guicheux J, Wittrant Y: Oxidative stress in bone remodelling and disease. Trends Mol Med 15:468-477, 2009
33. Wildburger R, Zarkovic N, Leb G, Borovic S, Zarkovic K, Tatzber F: Posttraumatic changes in insulin-like growth factor type 1 and growth hormone in patients with bone fractures and traumatic brain injury. Wien Klin Wochenschr 113:119-126, 2001
34. Wildburger R, Zarkovic N, Petek W, Egger G, Leopold U, Schweighofer F: Hypertrophic callus formation and craniocerebral trauma: Early diagnosis and behavior of basic fibroblast growth factor. Unfallchirurg 99:17-23, 1996
35. Wildburger R, Zarkovic N, Tonkovic G, Skoric T, Frech S, Hartleb M, Loncaric I, Zarkovic K: Posttraumatic hormonal disturbances: Prolactin as a link between head injury and enhanced osteogenesis. J Endocrinol Invest 21:78-86, 1998
36. Yeler H, Tahtabas F, Candan F: Investigation of oxidative stress during fracture healing in the rats. Cell Biochem Funct 23:137-139, 2005
37. Yurteri A, Yildirim A, Celik ZE, Vatansev H, Durmaz MS: The effect of quercetin on bone healing in an experimental rat model. Jt Dis Relat Surg 34:365-373, 2023