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The effect of edaravone on a rat fracture model complicated with ischemia

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Abstract

Background/Aim: Ischemia is a common issue in fracture healing due to vascular injury, compartment syndrome, and long-term tourniquet use. With ischemia-reperfusion injury, normal tissue repair can become complicated. In our study, we aimed to investigate the effect of edaravone on fracture union in a rat fracture model complicated with ischemia.

Methods: We conducted our study on forty-four rats divided into four study groups and a control group, as follows: Group 1- Fracture, Group 2- Fracture-ischemia, Group 3- Fracture-ischemia-edaravone, Group 4- Fracture-edaravone, and Group 5- Control group. The control group comprised the contralateral intact tibia of the rats in Group 1 that were not complicated with ischemia and were not given edaravone treatment. The effects of edaravone on fracture healing were evaluated histologically, radiologically, and biomechanically.

Results: There were no differences between the groups in terms of biomechanical and radiological indices, except for hardness values in the control subjects (Group 5), which were significantly higher than those of the other groups ($P<0.05$ for all). Similarly, in terms of histological evaluations of fracture healing between groups, group 3 has better improvement fracture healing scores than group 2 ($P=0.042$), and Group 4 has better improvement fracture healing scores than group 1 ($P=0.049$).

Conclusion: These findings suggest that the administration of edaravone, independent of ischemia, had positive results on histopathological fracture healing.

Keywords: Fracture healing, Edaravone, Ischemia-reperfusion injury, Antioxidant agents, Rat fracture model

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Introduction

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, $C_{10}H_{10}N_2O$, MCI-186) is a strong synthetic free oxygen radical (FOR) scavenger that inhibits lipid peroxidation. In addition, edaravone exerts positive effects on cerebral ischemia and stroke models by suppressing hydroxyl and superoxide anion levels [1]. Molecules and mechanisms implicated in ischemia-reperfusion injury include endothelial damage, FORs, polymorph nuclear leukocytes, and complement system activation. FORs are short-lived molecules, containing unstable electrons that negatively interact with cell components, notably cell lipids. Lipid peroxidation is triggered by FORs and includes multiple unsaturated fatty acids that can cause tissue damage. Reactions proceed until terminated by antioxidants or the end of the lipid chain is reached. The basic approach to deactivating FORs is by using antioxidants.

Due to long-term tourniquet use, vascular injury, and compartment syndrome, the circulation of a fracture can be impacted. With reperfusion, blood circulation is not only provided, but also damaged because ischemia is also attempted to be remedied [2]. Reperfusion of ischemic tissue causes tissue damage through a series of cellular reactions. Ischemia-reperfusion injury produces increased toxic metabolites when compared with ischemic damage alone [3].

Numerous studies have examined the effects of FORs and other antioxidant molecules in healing fractures. Although some studies have focused on the antioxidant effects of edaravone on muscles and the skeletal system, no studies have focused on the healing process of edaravone in an ischemia-reperfusion injury fracture model.

In the current study, we assessed edaravone as a FOR scavenger in fracture healing, and evaluated its effects in terms of radiological, biomechanical, and histopathological outputs.

Materials and methods

Following permission from the Tokat Gaziosmanpaşa University Experimental Animals Ethics Committee (2016 HADYEK-01), 44 Wistar-albino rats (aged 10-14 weeks and weighting 250 (30) g) were divided into four experimental groups, with eleven in each group. Rats were kept in separate cages, away from air flow, at a standard temperature 23(2) °C and received periodical lighting for 14 hours daily. Feeds were filled to 50 g per rat every other day, and water containers were renewed to 50 ml per rat every other day.

The groups were designated as follows: Group 1: Fracture only, Group 2: Fracture-ischemia, Group 3: Fracture-ischemia-edaravone, Group 4: Fracture-edaravone, and Group 5: Control group. The control group comprised the intact tibia of the Group 1 (fracture group) rats (Table 1).

Table 1: Methods applied according to working groups

Group Number	Fracture application	Edaravone application	Ischemia application
Group 1	+	-	-
Group 2	+	-	+
Group 3	+	+	+
Group 4	+	+	-
Group 5	-	-	-

For prophylaxis, 20 mg/kg cefazolin sodium (Cezol®, Deva Saba Inc, İstanbul, Turkey) was administered intraperitoneally 30 min before surgery, which was repeated at the 8th postoperative hour. General anesthesia was achieved with 90 mg/kg of intraperitoneal ketamine hydrochloride (HCL) (Ketalar®, Pfizer, Inc., Istanbul, Turkey) and 10 mg/kg of Xylasine (Rompun®, Bayer Healthcare AG, Leverkusen, Germany). The lower right extremities were aseptically shaved and prepared. Then, the femoral artery was dissected from the nerve distal to the inguinal ligament. A microvascular clamp was used as described by Skjeldal et al. [3] to prevent femoral artery blood leakage. After clamping, loss of pulsation and volume in the distal femoral artery were evaluated macroscopically. Whether adequate ischemia occurred in rats with appropriate clamping was also observed macroscopically at the 30th minute of clamping. Adequate ischemia was considered to have occurred if loss of capillary refill, pallor and coldness occurred in the limb. Arterial clamping was applied for 5 hours at 24°C - and the rats were fixed in supine position. During this period, the incision line was closed with a wet sponge to prevent the tissues from drying. No qualitative method was used to evaluate the effects of the ischemia procedure. The process was terminated after 5 hours.

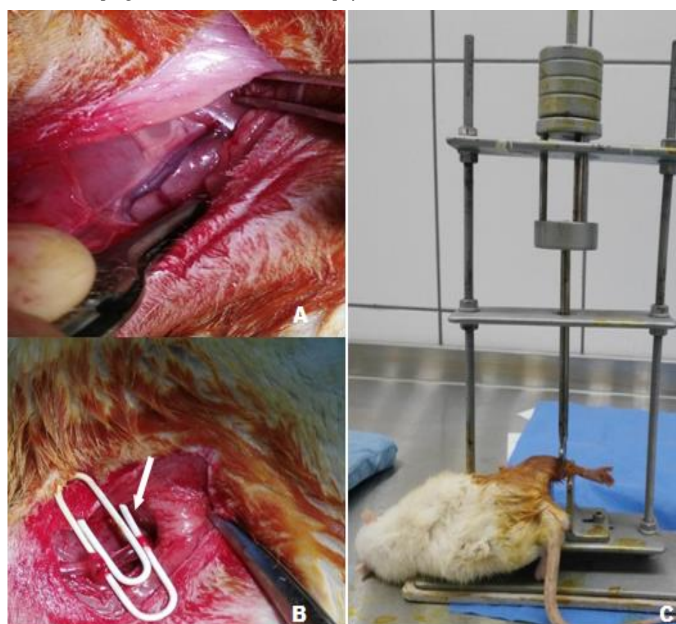
First, intramedullary fixation was applied to all rats, except the control group. The patella was everted laterally by entering through the right knee joint, with a 1 cm medial parapatellar incision. The tibia was cavitated using a 21-gauge needle (Beybi®, Inc., İstanbul, Turkey) over the tuberosity tibia, and a 0.8 mm stainless steel Kirschner wire was inserted into the tibia intramedullary. After bending and cutting the wire and reducing the patella, the incision layers were closed (Figure 1).

Figure 1: Intramedullary fixation technique; A) Medial parapatellar incision, B) Tibia cavitated through a number 21 needle, C) 0.8 mm stainless steel wire inserted intramedullary, D) Bending and cutting of K wire

Then, the closed tibial fracture model was realized in all tibias, except the control group, by exerting equal weight to the midline of tibias. Following the fracture, femoral artery clamping was performed on rats in the ischemia groups (Groups 2 and 3; Figure 2).

Edaravone (Mitsubishi Tanabe Pharma Corporation®, Tokyo, Japan) was provided in a powdered form. The powder was dissolved in 1N sodium hydroxide (NaOH) and titrated to pH 7.4 with 1N HCL. The final concentration was adjusted to 0.3 mg/ml. The intraperitoneal dose was determined as 3 mg/kg/day, which was repeated for seven days. Drug administration was performed under deep anesthesia.

Figure 2: Ischemia and fracture model A: Femoral artery and vein dissection (white arrow), B: Vascular clamping (white arrow), C: Mid-diaphysis fracture creation

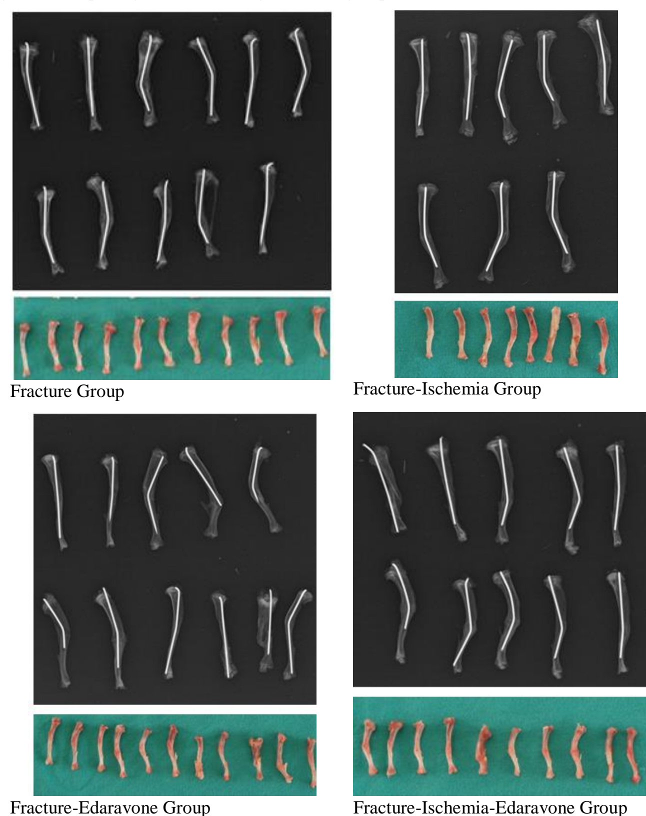


Edaravone was administered to Groups 3 and 4, prior to ischemia, at 3 mg/kg/day over seven days. Hourly observation was performed for 4 hours after intraperitoneal drug administration. Urine and feces were checked for hydration levels for 12 hours. Skin lesion, tumor appearance, and incision controls were performed hourly for the first 4 hours. After 5 hours of ischemia, the procedure was terminated by clamp removal. Femoral artery blood flow was generated. Afterwards, the groin incision was closed. During and after ischemia, rats were kept in a supine position at 24°C. The groin area was closed with a wet sponge to prevent local tissue dehydration. A 4 ml 0.9% Sodium chloride (NaCl) isotonic solution was administered to rats with ischemia for the same reason. Each day, two doses of 200 mg/kg paracetamol were also administered to control pain. During the study, three rats in Group 2 and one rat in Group 1 died; therefore, the study was completed with the following rat numbers per group: Group 1: n = 10; Group 2: n = 8; Group 3: n = 11; and Group 4: n = 11.

Animals were euthanized by cervical dislocation at the end of the 8th week of the experiment. Tibias were collected and cleaned from soft tissue. Radiologic evaluation was performed first, provided with the graphs of the samples by author (MA) unaware of the group number to avoid selection bias. Fracture areas were assessed according to the Lane and Sandhu radiological scoring system [4], which assesses new bone constitution and bone union degree (Figure 3).

After radiological analysis, tibias were stored at -20°C for biomechanical analyses. One day after radiological evaluation, tibias were thawed at room temperature. Three-point bending tests were applied to all specimens using a compression distraction device (Hounsfield H50KM, Surrey, England). Tibia samples were placed into the biomechanical test device after the intramedullary wire was removed. A stable 10 mm/mn pressure was applied to the fracture-healing section to create re-fracture. Breaking force and hardness (N/mm) were recorded separately. All biomechanical tests were performed by one author (SA) unaware of the group number to avoid the selection bias.

Figure 3: Morphological and radiologic view of groups at the end of the 8th week



After the biomechanical evaluation, the tibias were stored in saline until the decalcification process. Morse's Solution (10% sodium citrate, 20% formic acid) was used for decalcification. After decalcification was complete, the tissues were neutralized by sodium sulfate for 12 hours. 5-µm wide paraffin incisions were prepared and stained with hematoxylin-eosin. Samples were assessed by light microscopy. For each sample, five slides were analyzed, and fracture healing was evaluated histologically. A histologist with at least 10 years of experience evaluated the slides. Histological assessments were conducted according to Huddleston et al. [5], in terms of fibrosis, cartilage, immature bone tissue, and mature bone tissue rates.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 19 (IBM, Somers, NY, USA). Descriptive analyses were conducted to provide general group specifications. Continuous variable data were presented as mean and standard deviation. Differences between groups were assessed by one-way analysis of variance. Multi-comparisons were performed between variables using post hoc and Tukey honestly significant difference tests. A P-value <0.05 was considered statistically significant.

Results

In the histopathological analyses performed, a significant difference was observed between the groups (Table 2) (Figure 4).

When groups were evaluated mutually to determine histological differences between groups, the histopathological bone healing in Group 3 was significantly better than Group 2 (P<0.05), and that of Group 4 was significantly better than Group 1 (P<0.05; Table 3).

Table 2: Histological grade distribution by groups

Grade	Groups Group 1	Group 2	Group 3	Group 4	Total	*P*-value
2	1(100)(10)	0(0)(0)	0(0)(0)	0(0)(0)	1(100)(2,6)	0.018
3	1(100)(10)	0(0)(0)	0(0)(0)	0(0)(0)	1(100)(2.6)	
4	0(0)(0)	0(0)(0)	1(100)(10)	0(0)(0)	1(100)(2.6)	
5	3(60)(30)	2(40)(25)	0(0)(0)	0(0)(0)	5(100)(12.8)	
6	2(33.3)(20)	0(0)(0)	4(66.7)(40)	0(0)(0)	6(100)(15.4)	
7	3(18.8)(30)	6(37.5)(75)	3(18.8)(30)	4(25)(36.4)	16(100)(41)	
8	0(0)(0)	0(0)(0)	2(50)(20)	2(50)(18.2)	4(100)(10.3)	
9	0(0)(0)	0(0)(0)	0(0)(0)	4(100)(36.4)	4(100)(10.3)	
10	0(0)(0)	0(0)(0)	0(0)(0)	1(100)(9.1)	1(100)(2.6)	
Total	10(25.6)(100)	8(20.5)(100)	10(25.6)(100)	11(28.2)(100)	39(100)(100)	

Data were presented as n (Row%) (Column%). Chi-square test was used.

Figure 4: Histopathological views of fracture healing according to Huddlestone Classification A) Group 4 (HE, X20) B): Group 3 (HE, X20) C) Group 3 (HE, X20) *: lamellar bone union tissue

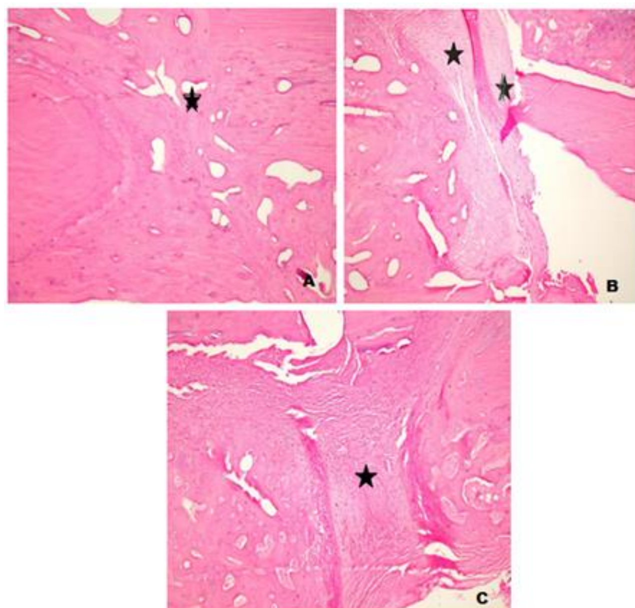


Table 3: Distribution in Comparison Bone Healing Histological Levels of Groups

	Group 1	Group2	Group 3	Group 4
Group 1		0.283	0.193	**0.049**
Group 2			**0.042**	0.057
Group 3				0.072
Group 4				

No side effects were observed after intraperitoneal injection of edaravone according to the above-mentioned criteria.

Four animals died during the experiment. Considering the distribution of dead animals by groups, three animals were in the fracture and ischemia group, one animal was in the isolated fracture group and one animal was in the fracture + edaravone group. Therefore, deaths were not thought to be related to drug administration.

Radiological union was observed in all groups. When bone formation in the fracture site was evaluated according to the Lane and Sandhu radiological scoring system, no significant differences were observed between the groups.

Based on the analysis of biomechanical data, no significant differences were observed between the groups once the control group, comprising rats with a robust tibia, was excluded (Table 4).

Table 4: Correlation between quantitative variables by groups

Group			Breaking force	Hardness (N/mm)	Radiology
Control Group 5	Breaking force	r	1	-0,516	.[a]
		p		0,104	.
		n	11	11	0
	Hardness (N/mm)	r	-0.516	1	.[a]
		p	0.104		.
		n	11	11	0
	Radiology	r	.[a]	.[a]	.[a]
		p	.	.	
		n	0	0	0
Group 1	Breaking force	r	1	0.697*	0.268
		p		**0.025**	0.455
		n	10	10	10
	Hardness (N/mm)	r	0.697*	1	0.296
		p	**0.025**		0.407
		N	10	10	10
	Radiology	r	0.268	0.296	1
		p	0.455	0.407	
		n	10	10	10
Group 2	Breaking force	r	1	0.429	-0.058
		p		0.289	0.891
		n	8	8	8
	Hardness (N/mm)	r	0.429	1	0.217
		p	0.289		0.606
		n	8	8	8
	Radiology	r	-0.058	0.217	1
		p	0.891	0.606	
		n	8	8	8
Group 3	Breaking force	r	1	-0.134	0.112
		p		0.712	0.757
		n	10	10	10
	Hardness (N/mm)	r	-0.134	1	-0.634*
		p	0.712		0.049
		n	10	10	10
	Radiology	r	0.112	-0.634*	1
		p	0.757	**0.049**	
		n	10	10	10
Group 4	Breaking force	r	1	-0.236	0.298
		p		0.486	0.373
		n	11	11	11
	Hardness (N/mm)	r	-0.236	1	-0.336
		p	0.486		0.313
		n	11	11	11
	Radiology	r	0.298	-0.336	1
		p	0.373	0.313	
		n	11	11	11

Discussion

The main finding of our study is that the use of edaravone in the rat fracture model contributes to healing at the histopathological level. This effect occurs both in the presence and absence of ischemia.

Ischemia has potential side effects for fracture healing. After a high energy-fracture, vascular injury, or compartment syndrome, reperfusion generally follows this process. Ischemia negatively affects fracture healing [6-8]. The ischemic period is a well-known part of fracture healing process, after which arterial vasodilation and reperfusion occurs. In our study, edaravone may have affected fracture healing by countering the negative effects of FORs. The fact that this effect was observed in the non-ischemia groups may be due to local ischemia in fracture hematoma at the histopathological level. However, since we did not measure FORs at the fracture hematoma level or in the systemic circulation, it is not possible to reach this conclusion with the results of our study.

Ischemia causes tissue damage, whereas reperfusion causes reperfusion damage, which is a pathophysiological incident [9]. Reperfusion promotes the accumulation of neutrophils in tissues and increases the activity of xanthine oxidase in endothelial cells, thereby accelerating FOR production. Extreme levels of FORs negatively affect fracture metabolism. Cells are exposed to oxidative stress, which results in tissue damage [10, 11]. In the ischemia-reperfusion model of Çetinus et

al. [7], levels of FORs in tibia fractures were higher during the inflammation period of the bone union process.

Several studies have demonstrated that FOR levels increase after ischemia-reperfusion, adversely effecting fracture healing. In our study, histopathological differences, especially between Groups 2 and 3, were observed due to the elimination of the negative effects of FORs. However, the significant differences in histopathological fracture healing between Groups 1-4 without ischemia suggested that the effects of edaravone on fracture healing should be independent of ischemia-reperfusion injury.

Antioxidant molecules help to prevent the negative effects of FORs on bone and fracture metabolism. We used edaravone, which has been used in Japan since 2001 for the treatment of ischemic stroke, as it exhibits FOR scavenging [12-14]. We observed differences at histopathological level, independent of ischemia. Our study differs from similar studies by the fact that biomechanical or radiological evaluation results were similar, but histopathological evaluation results were not.

Edaravone is a molecule that has previously shown efficacy in many animal models. The studies that have been carried out primarily consist of organ damage and ischemic damage models. These animal studies are associated with ischemic stroke [15], myocardial ischemia [16], lung injury [17], and atherosclerosis [18] animal models. To the best of our knowledge, ours is the first animal study to investigate the effects of edaravone on fracture healing.

Edaravone inhibits FORs by inhibiting dependent and independent lipid peroxidation [19]. The side effects of edaravone on the muscles and skeletal system have been previously studied, including the protective effects in osteoarthritis and in skeletal muscles, preventive effects in ischemia-reperfusion damage and muscle atrophy, and positive effects in osteonecrosis [20-23].

Our study suggests that the effects of edaravone may not only be related to ischemia-reperfusion injury but may also have different cytoprotective effects.

Ischemia exerts negative effects on fracture healing in complicated bone fractures following reperfusion. The group exposed to edaravone after reperfusion had better fracture healing in terms of histopathological outcomes. Therefore, edaravone could be beneficial for fractures involving ischemia-reperfusion incidents. However, edaravone may be effective on fracture healing not complicated by ischemia.

Limitations

Our study has several limitations. First limitation was lack of measurements for tissue necrosis scales and FOR levels and the fact that the rats were euthanized at the 8th week of the study period instead of at two different times. The second limitation was lack of standardized ischemia severity assessment. Although we clamped the femoral artery with the same tool for 5 hours in all ischemia applications, differences in the ischemia responses in the tissues may have occurred. In our model of macroscopic interruption of the major arterial circulation, we hope that these differences did not cause any significant changes. Third limitation was an insufficient number of study subjects. It may have been possible to determine significant differences between groups with the inclusion of more rats in the study.

Conclusions

Negative effects of ischemia on fractures complicated by vascular injury and compartment syndrome may be overcome by edaravone. The use of these kinds of molecular scavengers can be extended by conducting larger studies that examine the effects on fracture healing in isolated fractures.

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