Evaluation of the Effects of Topical Ellagic Acid and Graft Application on Bone Regeneration: an Experimental Study

Mehmet Gül[1], Serkan Dundar[2], Akın Yigin[3], Gökhan Artaş[4], Abdulsamet Tanik[5], Muhammet Bahattin Bingül[6], Osman Habek[6]

[1]Department of Periodontology, Faculty of Dentistry, Harran University, Sanliurfa, Turkey.
[2]Department of Periodontology, Faculty of Dentistry, Firat University, Elazig, Turkey.
[3]Department of Genetic, Faculty of Veterinary, Harran University, Sanliurfa, Turkey.
[4]Department of Pathology, Faculty of Medicine, Firat University, Elazig, Turkey.
[5]Department of Periodontology, Faculty of Dentistry, Adıyaman University, Adıyaman, Turkey.
[6]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Harran University, Sanliurfa, Turkey.

Corresponding Author:
Mehmet Gül
Hamidiye Mah. Harran Üniversitesi
Yenişehir Kampüsü, Haliliye, Şanlıurfa
Turkey
Phone: 05359255812
Fax: +90 414 318 31 90
E-mail: m.gul3838@gmail.com

ABSTRACT

Objectives: The aim of this experimental animal study is to investigate the effect of bone graft and topical ellagic acid application on bone regeneration in rats with critical-sized calvarial bone defects.
Material and Methods: A total of 24 male Wistar rats were divided into three groups, and 7 mm critical-sized calvarial bone defects were created surgically in them. In the first group, the created defect was left empty, and this acted as a control group. In the second group, only a bone graft was placed in the created defect. In the third group, in addition to placing a bone graft in the created defect, 0.325 mg/kg ellagic acid (EA) was applied topically to the defect.
Results: As a result of semiquantitative scoring, osteoblast counts were 2 (SD 0.82) in the control group, 2.71 (SD 0.76) in the graft group, and 1.14 (SD 0.69) in the EA + graft group. The number of osteocytes was 2.29 (SD 0.76) in the control group, 2.71 (SD 1.11) in the graft group, and 1.43 (SD 0.54) in the EA + graft group. When inflammations were evaluated, values of 1.71 (SD 0.75), 1.14 (SD 0.69), and 3 (SD 0.82) were obtained in the control, graft, and EA + graft groups, respectively.
Conclusions: Topical ellagic and graft applications show different effects at different doses under topical and systemic conditions. The dose amount of ellagic acid applied, especially in topical applications, has critical importance in bone healing.

Keywords: bone; ellagic acid; fracture healing; graft.

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INTRODUCTION

Ellagic acid (EA) is found in many fruits, such as strawberries, pomegranates, grapes, mangoes, almonds, and walnuts. In addition, EA is known as a polyphenolic component naturally found in hazelnut and plant extracts [1]. EA is known to improve liver functions due to its antioxidant and antihepatotoxic properties when toxic and pathological conditions occur [2]. It has been suggested in previous studies that EA inhibits the proliferation of cancer cells and induces cell apoptosis [3]. EA has been reported to reduce human adipocyte differentiation by causing changes in chromatin remodelling [4]. Researchers have found EA to have a recovery effect against inflammation in the rat brain hippocampus induced by arsenic [5].

When bone damage occurs, inflammation and the process of bone healing begins. After inflammation, the activity of osteoclasts increases, and bone resorption occurs. As a result of the suppression of inflammation, osteoclast activity decreases, and thus, new bone formation occurs [6]. In a previous study, it was suggested that adding factors such as EA to the bone graft results in an increase in its anti-inflammatory properties. The pomegranate plant contains polyphenolic compounds, such as EA, gallotannins, and anthocyanin [7]. It was reported that polyphenolic compounds are the most important bioactive substances in the protection of bone health [8]. EA was reported to prevent the formation of free radicals and have anti-inflammatory, antioxidant, antiapoptotic, antimutagenic, and antiviral properties [6].

Phenolic compounds have been recognized as the most important bioactive compounds responsible for their effects on bone health. EA has antioxidant, anti-inflammatory, radical scavenging, chemopreventive, antiapoptotic, antimutagenic, antiviral, and anti-fibrosis activities [9-13]. The hydroxyl group in its structure has been reported to increase antioxidant activity in lipid peroxidation and protect cells from oxidative damage [14]. It was suggested in a study conducted on female rats that EA has positive effects on the healing process and prevents bone loss [15]. Devareddy et al. [16] suggested that blueberries containing high levels of EA prevented bone loss in rats. They reported that this might be due to the components of blueberries that have free radical scavenging activities [17]. It was determined that polyphenols such as chlorogenic acid and caffeic acid reduce oxidative stress biomarkers and prevent bone loss [18].

In the guided bone repair process, the principle of integration of the biomaterial with the new bone is adopted. A long-term and permanent osteoconductive bone structure is aimed in the defect region [19]. Due to this, guided bone application has limited effect on large bone defects [20,21].

The aim of this experimental animal study is to investigate the effects of bone grafting and topical ellagic acid application on bone regeneration in rats with defects in the head region.

MATERIAL AND METHODS

Study design and animals

This study was conducted at Harran University Experimental Research Center, Sanliurfa, Turkey between the 1st of June, 2020, and the 20th of July, 2020, after obtaining approval from Harran University Animal Experiments Local Ethics Committee (Protocol No. 2019/001/03). Rats were taken from Fırat University, Experimental Research Center (Elazig, Turkey) in accordance with the Helsinki declaration of experiments. Rats were kept in cages in pairs. *Ad libitum* diet and water feeding were carried out. Humidity was kept at 55% and room temperature was kept constant at 22 ± 2 °C. In addition, attention was paid to the 12-hour day and night cycle.

In this study, a total of 24 male Wistar albino rats weighing 250 to 300 g were used. The subjects were randomly divided into three groups. In the control group, a 7 mm critical bone defect was created in the calvarium of the rats (Figure 1A). In the graft group, a 7 mm critical bone defect was created in the calvarium of the rats and a bone graft (β-tricalcium phosphate [β-TCP] - BMT CALSIS Health Technologies Co.; Ankara, Turkey) was placed in the created defect (Figure 1B). In the EA + graft group, a 7 mm critical bone defect was created in the calvarium of the rats. Bone graft and 0.325 mg/kg EA (Fluorochem Ltd.; Derbyshire, UK) were applied topically to the defect area (Figure 1C). The Rats were sacrificed on the 28th day with an overdose of anesthetic agent method. The samples were stored in buffered formaldehyde solution for 48 hours.

Afterward, the extracted specimens were fixed in 10% neutral formalin for 24 hours. The hard bone tissue was decalcified in an ethylenediaminetetraacetic acid (EDTA) solution and turned into soft tissue. The materials were dehydrated by ethanol, cleared with xylitol, and embedded in paraffin. The samples were stained with hematoxylin and eosin and examined under a light microscope. All images of histological samples were taken with a digital camera connected

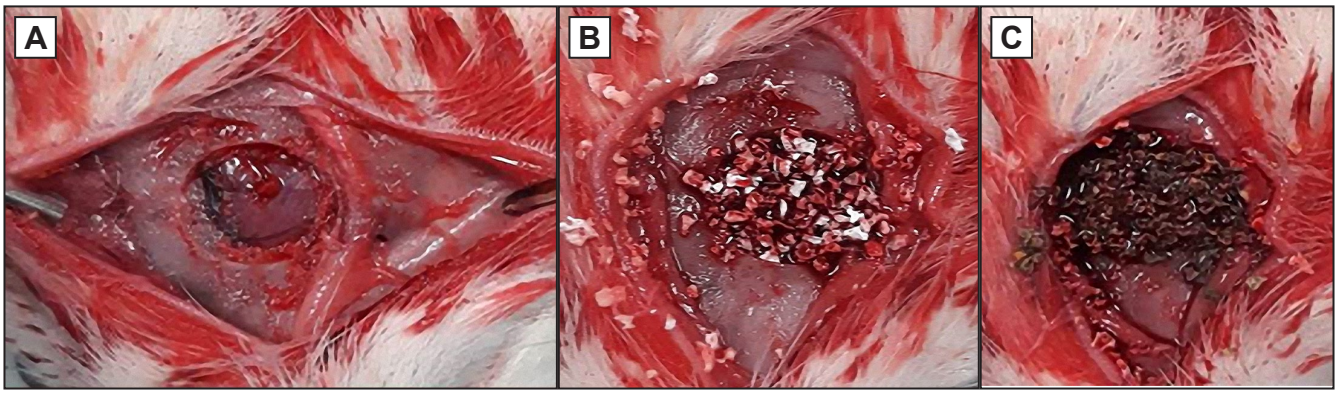

Figure 1. In rats with head defect: A = control group; B = graft group; C = ellagic acid + graft group.

to a light microscope, and the images were transferred to a computer at the original magnification. The Olympus Microscope Digital Camera model DP71 (Olympus Co.; Shinjuku, Tokyo, Japan) software imaging system was used for histological analysis (Figure 2).

Surgical method

The Rats were fasted for 12 hours before the experiment and were anesthetized using 90 mg/kg ketamine hydrochloride (10% Ketasol® - Richter Pharma AG; Wels, Austria) and 3 mg/kg xylazine hydrochloride (2% XylazinBio® - Bioveta; Ankara, Turkey). The bite reflex of the rats were observed, and the procedure was started at the appropriate anesthesia depth. The operation area was shaved, and asepsis was achieved by staining with 10% povidone-iodine (Betakon® - Aroma; Tekirdağ, Turkey). An incision was made in the coronal midline of the scalp. A circular defect in the midline bone was created using a 7 mm trephine bur (SC Medikal Ürünleri Sanayi ve Ticaret A.S.; Izmir, Turkey). The postoperative skin incision of the experimental group was closed with 4/0 silk sutures (Jinhuan Medical Products Co., Ltd.; Shanghai, China). For prophylaxis, a single dose of 50 mg/kg antibiotic (Betamox® LA, Active; İstanbul, Turkey) was injected immediately after the operation.

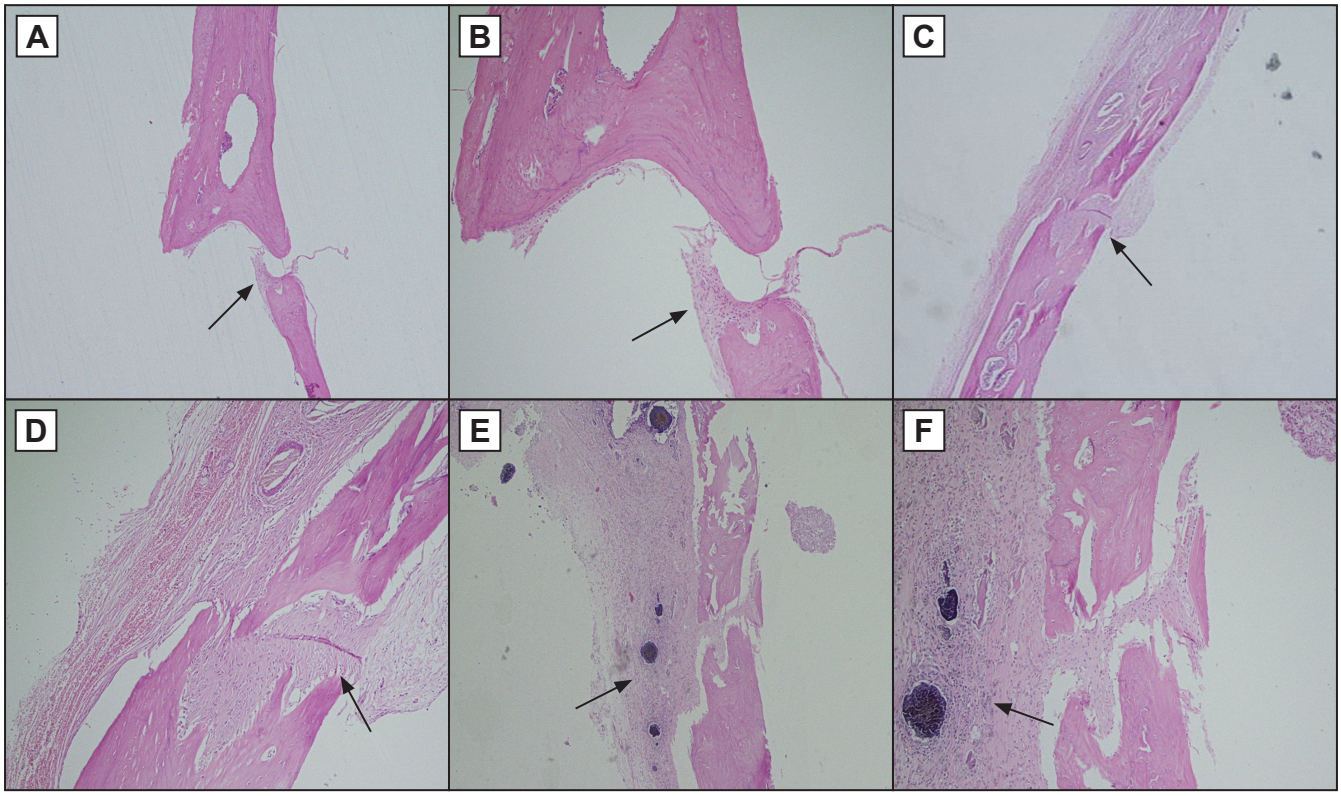

Figure 2. Histological sections (hematoxylin-eosin stain): A = control group (original magnification x40); B = control group (original magnification x100); C = graft group (original magnification x40); D = graft group (original magnification x100); E = graft + ellagic acid group (original magnification x40); F = graft + ellagic acid group (original magnification x100).

Semi-quantitative scoring of histopathological parameters

A semiquantitative scoring was determined by examining osteoblasts, osteocytes, inflammation, new bone formation, and osteoclast cells in the bone tissue. Histological sections were obtained after routine histological follow-up, 15 different areas were scanned for each slide, and the average value of 10 randomly selected cells were obtained. As a result of these averages, 10 average points were obtained for each animal group, and this data was statistically analyzed. While averaging decimals were converted to integers before statistical analysis. Similar semiquantitative methods have been used in previous histological studies of bone tissue [22,23].

Enzyme-linked immunosorbent assay

In this study, enzyme-linked immunosorbent assay (ELISA) kits were used: osteonectin (MyBioSource Inc.; San Diego, California, USA) and osteopontin (Shanghai Korain Biotech Co., Ltd; Shanghai, China). The manufacturer's instructions were followed during use. The specimens were precoated with 96-well anti-osteonectin and anti-osteopontin antibody prior to analysis. The test samples and biotin-conjugated detection antibodies were added to the wells and washed. 3,3',5,5'-tetramethylbenzidine substrates were used to help visualize the enzymatic reaction. The optical density of the yolk was measured using the Epoch® Micro-Volume Spectrophotometer System (BioTek Instruments Inc., Winooski, Vermont, USA) to determine osteonectin and osteopontin in a 96-well plate at 450 nm.

Statistical analysis

Statistical analysis of the data in present study was performed using SPSS (IBM® Ver; 15.0 Windows, USA) statistical program. Parametric data were expressed as mean and standard deviation (M [SD]). The normality of the obtained data was evaluated with the Kolmogorov Smirnov test. Kruskal Wallis test was used for comparisons between groups of more than two data that did not show normal distribution. Bonferroni-corrected Mann-Whitney U test was used to compare paired groups in subgroups of more than two groups. Statistical significance level was defined at $P = 0.05$.

RESULTS

As seen in Table 1, according to the results of semiquantitative scoring, as a result of the analyses performed, a statistically significant decrease in osteoblast cells, osteocyte cells, and new bone formation was observed in the EA + graft group compared with the graft group. Inflammation was found to be significantly higher in the EA + graft group ($P < 0.05$).

As seen in Table 2, after the biochemical analyses of the samples taken from the defect area, although higher osteonectin and osteopontin values were obtained in the graft and control groups compared to the EA group, the differences were not statistically significant ($P > 0.05$). As seen in Supplemental Table 3, after the analyses of the blood serum samples, a statistically significant difference was found only in the osteopontin value between the graft group and the EA + graft group ($P < 0.05$, $P = 0.014$) (Figure 3).

As seen in Table 4, in the biochemical analyses of the blood serum samples, alanine aminotransferase, aspartate aminotransferase, calcium, creatinine, parathormone, and magnesium were evaluated, and a statistically significant difference was obtained between the EA + graft group and the graft group only

Table 1. Changes in histological values in cranial bone defect between test and control groups

Parameter	Group			P-value[d]	Comparison		
	Control	Graft	EA + graft		P[a]	P[b]	P[c]
	Mean (SD)	Mean (SD)	Mean (SD)				
Osteoblast cells	2 (0.82)	2.71 (0.76)	1.14 (0.69)	0.009*	0.133	0.065	0.004*
Osteocyte cells	2.29 (0.76)	2.71 (1.11)	1.43 (0.54)	0.04*	0.421	0.04*	0.027*
Inflammation	1.71 (0.75)	1.14 (0.69)	3 (0.82)	0.004	0.184	0.017*	0.003*
New bone formation	1.86 (0.69)	2.86 (0.69)	0.71 (0.75)	0.001*	0.024*	0.019*	0.002*
Osteoclast cells	1 (0.58)	2.57 (0.78)	2.43 (0.54)	0.002*	0.003	0.002*	0.827

[a]Comparison between control and graft groups.
[b]Comparison of control and EA + graft groups.
[c]Comparison between graft and EA + graft groups.
[d]Kruskal Wallis test; [a,b,c]Mann Whitney U test.
* = polysemy; EA = ellagic acid.

Table 2. Biochemical measurement values in bone defect between test and control groups

Parameter	Group			P-value[d]	Comparison		
	Control	Graft	EA + graft		P[a]	P[b]	P[c]
	Mean (SD)	Mean (SD)	Mean (SD)				
Osteonectin	338.73 (97.42)	431.51 (137.7)	278.47 (101.78)	0.081	NS	NS	NS
Osteopontin	195.49 (53.36)	261.06 (105.06)	158.61 (24.07)	0.149	NS	NS	NS

[a]Comparison between control and graft groups.
[b]Comparison of control and EA + graft groups.
[c]Comparison between graft and EA + graft groups.
[d]Kruskal Wallis test; [a,b,c]Mann Whitney U test.
NS = not significant; EA = ellagic acid.

Table 3. Biochemical measurement values in serum of rats between test and control groups

Parameter	Group			P-value[d]	Comparison		
	Control	Graft	EA + graft		P[a]	P[b]	P[c]
	Mean (SD)	Mean (SD)	Mean (SD)				
Osteonectin	173.46 (38.69)	212.49 (62.63)	245.98 (123.22)	0.498	NS	NS	NS
Osteopontin	110.53 (17.83)	141.89 (29.88)	169.49 (47.42)	0.014*	0.025*	0.013*	0.18

[a]Comparison between control and graft groups.
[b]Comparison of control and EA + graft groups.
[c]Comparison between graft and EA + graft groups.
[d]Kruskal Wallis test; [a,b,c]Mann Whitney U test.
* = polysemy; NS = not significant; EA = ellagic acid.

Table 4. Changes in blood biochemical values between control and test groups

Parameter	Group			P-value[d]	Comparison		
	Control	Graft	EA + graft		P[a]	P[b]	P[c]
	Mean (SD)	Mean (SD)	Mean (SD)				
Alanine aminotransferase	66.14 (27.8)	51.86 (13.45)	57 (19.01)	0.711	NS	NS	NS
Aspartate aminotransferase	436.29 (464.72)	133.71 (37.08)	261.57 (339.09)	0.196	NS	NS	NS
Calcium	1.24 (0.35)	1.77 (0.72)	1.63 (0.59)	0.278	NS	NS	NS
Creatine	0.39 (0.09)	0.31 (0.08)	0.43 (0.24)	0.215	NS	NS	NS
Parathormone	11.01 (4.08)	6.51 (0.88)	13.81 (5.99)	0.014**	0.055	0.482	0.004*
Magnesium	0.68 (0.29)	0.45 (0.25)	0.91 (0.33)	0.044**	0.143	0.196	0.02**

[a]Comparison between control and graft groups.
[b]Comparison of control and EA + graft groups.
[c]Comparison between graft and EA + graft groups.
[d]Kruskal Wallis test; [a,b,c]Mann Whitney U test.
* = polysemy; ** = significance; NS = not significant; EA = ellagic acid.

in terms of their parathormone and magnesium values (Figure 4) ($P < 0.05$, $P = 0.014$ and $P = 0.044$).

DISCUSSION

After a defect in the bone, inflammation begins, followed by regeneration, osteogenesis, blood clots, and the formation of fibrous tissue [24]. Macrophages, neutrophils, cytokines, growth factors (TNF-α, fibroblast growth factor, etc.), and polymorphonuclear cells are known as mesenchymal stimulating cells. After the inflammatory event occurs, the formation of the extracellular matrix and bone tissue, known as the regeneration stage, begins. Events occurring in this phase include the production of bone morphogenetic protein, osteocalcin (OCN), and collagen. As a result of the accumulation of collagen, hydroxyapatite (HA) crystals, a matrix (a structure responsible for growth) are formed. The remodeling phase continues with the participation of OCN, cytokines and type I collagen [25-27].

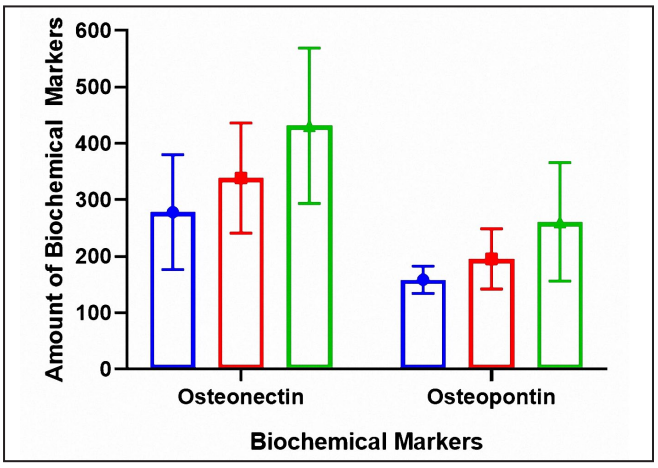

Figure 3. The changes between the biochemical marker values in the bone defect between the test and control groups.
Blue column = ellagic acid + graft group; red column = control group; green column = graft group.

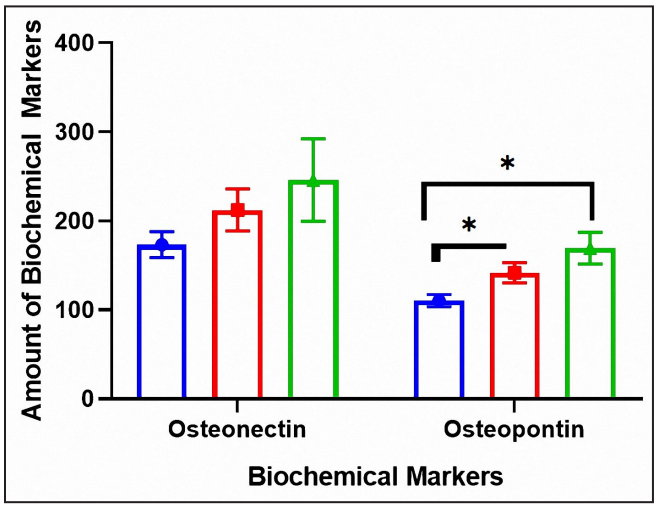

Figure 4. The changes between the biochemical marker values in blood serum between the test and control groups.
Blue column = control group; red column = graft group; green column = ellagic acid + graft group.
* = groups with statistically significant differences between.

Lin et al. [28] showed that EA inhibited osteoclastogenesis and bone resorption under *in vitro* conditions by suppressing RANKL-induced NF-κB and MAPK signaling pathways, and it counteracted bone loss that was induced *in vivo*. It was reported that EA could be a potential compound to treat osteoclast-related bone diseases such as osteoporosis [28].

It has been reported that free oxygen radicals are produced in the inflammatory phase by phagocytic cells on the bone surface. In the study of Wardhana et al. [29], an increase in osteoclast formation and bone resorption was observed, which is thought to prevent free radicals from remineralizating in the bone defect and tooth eruption.

In the study of Wardhana et al. [29], bone defects were treated with HA-EA. As a result of the study, an effect on the inflammatory phase in bone regeneration was observed. It has been reported that HA-EA application reduces bone resorption and keeps osteoclasts under control. It has been reported that EA reduces the production of proinflammatory cytokines involved in osteoclast differentiation and prevents bone resorption. In another study, it was reported that EA decreased the transcription of NF-kB and inhibited cytokine production, resulting in increased osteoclast apoptosis [30].

In this study, 0.325 mg/kg topical EA was mixed with graft material after histological examinations in rats with head defects, and no positive effect was observed in the applied area. After the biochemical analyses, no positive effect of EA application on the bone tissue taken from the region was found. However, in the analyses performed on the serum taken from the rats, a significant positive difference was obtained in the graft group and the EA + graft group.

Studies have reported that osteopontin protein has a very important role in bone formation and resorption mechanisms and is present in high concentrations in bone formation. It has also been reported that while osteopontin has an important role in the bone mechanism, it is also found in non-mineralized tissues and has different functions [31-34]. Studies have interpreted the high level of osteopontin protein in preosteoblastic cells as having an important role in the bone mechanism [31,35]. In the studies conducted by Ram et al. [36], it was emphasized that osteonectin protein has an important role in the collagen matrix mineralization process. It was also reported that osteonectin protein has a high level in the wound healing process and plays an important role in the bone formation mechanism in implant stabilization.

In this study, the highest values of osteopontin and osteonectin in the samples taken from the defect area were obtained in the graft group, but no significant difference was found between the groups. However, higher values were obtained in blood serum values in the osteopontin, graft and EA + graft groups compared to the control group. A statistically significant difference was also found between these groups.

CONCLUSIONS

In conclusion, our data showed that topical ellagic acid and graft application affected significantly at different doses under topical and systemic conditions. The dose amount of ellagic acid applied, especially in topical applications, has critical importance in bone

healing. There were no positive effects on healing in the 0.325 mg/kg defect area we applied. More positive results were obtained in the grafted area than in the ellagic acid + grafted area. This study has limitations. More studies at different doses are needed to determine the relationship between topical ellagic acid and bone healing.

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No conflict of interest was declared by the authors.

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This work was conducted at the Harran University Experimental Research Center. Ethical approval was obtained from the Harran University Animal Experiments Local Ethics Committee (Approved no: 2019/001/03).

Biochemical analyses were carried out by Ismail Koyuncu, an associate professor at the Department of Biochemistry at Harran University.

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