Annals of Medical Research
DOI: 10.5455/annalsmedres.2020.03.193 2020;27(3):700-10

Original Article

Histomorphometric analysis of the effects of grape seed extract (*vitis vinifera*) and low-level laser therapy (LLLT) on fracture healing

Bilal Ege[1], Mutan Hamdi Aras[2], Hasan Akgul[3], Muhammed Isa Kara[4], Isil Aydemir[5], Sevinc Inan[6], Seyithan Taysi[7], Sevil Altundag Kahraman[8]

[1]Adiyaman University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Adiyaman, Turkey
[2]Private Dentist, Department of Oral Maxillofacial Surgery, Gaziantep, Turkey
[3]Akdeniz University, Faculty of Science, Department of Biology, Antalya, Turkey
[4]Private Dentist, Department of Oral Maxillofacial Surgery, Sivas, Turkey
[5]Nigde Omer Halisdemir University, Faculty of Medicine, Department of Histology and Embryology, Nigde, Turkey
[6]Izmir Ekonomi University, Faculty of Medicine, Department of Histology and Embryology, Izmir, Turkey
[7]Gaziantep University, Faculty of Medicine, Department of Medical Biochemistry, Gaziantep, Turkey
[8]Gazi University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Ankara, Turkey

Abstract

Aim: In this study, we aim to investigate the effects of supplementary Grape Seed Extract (GSE) and Low-Level Laser Therapy (LLLT) on fracture healing, oxidant and anti-oxidant system in experimental mandible fractures.

Materials and Methods: 48 Wistar Albino rats (adult male, n=48) were used in our study. For all the subjects, a vertical fracture line through molar teeth in right mandibles was created and internally fixed using a four-hole microplate and four micro screws. Firstly, these subjects were randomly divided into 4 main groups (Control, GSE, LLLT, GSE+LLLLT) of 12 animals each, and then these were individually separated into two sub-groups of 7th and 21st days. The number of groups (n=6) was 8 in total. GSE of 300 mg/kg/day were provided to the subjects before they were sacrificed. LLLT of 23 J/cm^2 was administered to two different points along with the fracture line at intervals of 48 hours for 7 days in the 7th day sub-groups and for 14 days in the 21st day sub-groups. After the procedure, while biochemical values such as TAS, TOS and OSI are measured; histopathologically it was examined in terms of capillary number and width, inflammatory cell, fibroblast count, collagen fibers, osteoblast count, ossification and mature bone formations.

Results: For biochemical analyses, there was statistically significant difference only in TAS values on either the 7th or 21st day for the groups. Histological analyses showed that mandibular fracture healing were significantly better in the GSE and GSE+LLLT groups compared to the control group. The group of only LLLT had limited recovery while the combination of GSE+LLLT was the best for ossification.

Conclusions: It is concluded that GSE may be one of the potential methods to accelerate fracture healings in mandibular fractures common in oral and maxillofacial surgery clinic and thus help patients recover in shorter time, and however LLLT can have positive effects on the process of ossification and recovery only when combined with the extract, which may result in better outcomes in clinical use.

Keywords: Low-level laser therapy; fracture healing; oxidative stress; free radicals; grape seed extract

INTRODUCTION

Fracture is a phenomenon in which bone integrity and durability are implicitly or explicitly broken down and not only the bone but also the peripheral tissues are damaged at different levels. Many people are currently exposed to maxillofacial traumas for a variety of reasons and thus different factors may cause fractures to occur in bone tissue. In some cases these fractures may primarily account for morbidity and mortality. Therefore fracture healing has now remained a significant problem in dental practice.

Received: 03.03.2020 **Accepted:** 05.03.2020 **Available online:** 06.03.2020
Corresponding Author: Bilal Ege, Adiyaman University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Adiyaman, Turkey **E-mail:** miregein@gmail.com

For fracture healing the main goal is bone tissue regeneration without a scar, which has no dysfunction and deformity. However, a number of factors negatively influence the fracture healing process, including sub- and malnutrition, age, smoking, drug use and alcohol consumption, systemic diseases, hormones, and growth factors as well as severity of trauma, level of damage in bone and soft tissue, and local blood flow reduction. Accordingly, several therapies are used including different chemical stimulants, autogenous bone grafts, biomaterials, bone morphogenetic proteins, hyperbaric oxygen, electromagnetic field, and laser and ultrasound techniques, in order to stimulate cellular activity for acceleration of fracture healing and hence help patients recover in a shorter time. It is also necessary to take dietary supplements to stimulate fracture healing besides these supportive methods. Research has showed that the supplementation of minerals including calcium and magnesium, which is crucial in bone metabolism, and such vitamins as Vitamin D and Vitamin K can accelerate fracture healing (1,2). Nevertheless, around 5 to 10% of fracture cases still have such problems as bone nonunion or delayed union despite use of new treatments and surgical techniques at present times (3). Thus, studies are still conducted to uncover the aspects of fracture healing and more clearly describe the positive or negative factors contributing to this process and continue to be relevant. For oral surgical interventions and also implantology, the first month of operation is very important due to either inception of ossification or the fact that this is the most critical recovery period.

Known as having anti-inflammatuar, biostimulant and wound healing effects on soft and solid tissue healing, low-level laser therapy (LLLT) is another current treatment approach used to accelerate osseous healing at present. Studies showed that LLLT can accelerate proliferation of osteocytes and promote new bone growing and thus shorten the duration of osseous healing (4,5).

In addition, use of different antioxidant agents has in recent years drawn much attention to reduce adverse effects on tissues of free radicals which occur following tissue damage and accelerate tissue healing. Free oxygen radicals are high-energy and unstable moles with multiple unpaired electrons in outer atomic orbitals (6). Free radicals lead to tissue damage by attacking on several biological materials including protein, lipid, and DNA because these are much reactive and tend to reach steady state. Several moles counteract these radicals and prevent potential damage in body, called antioxidants, and the oxidant and antioxidant system has a delicate balance. The researchers have tended to investigate the effects of the agents well-known with its antioxidant property on healing due to the fact that oxidative stress may appear in this process because this equilibrium is broken down following the developments in early period after fracture formation. In this context, grape seed extract (GSE) is one of the most common antioxidant agents and anti-inflammatory, anti-carcinogenic, antiviral, anti-diabetic, anti-aging, cardioprotective, and neuroprotective in effect (7). The anti-oxidant capacity of GSE is rooted in phenolic compounds and proanthocyanidin, particularly resveratrol. Proanthocyanidins are so effective to protect the damaged tissues by oxidative stress. The free radical scavenging influence of GSE is stronger than Vitamin C, Vitamin E, and other common antioxidants (8). Thus, many researchers have studied grape seed in terms of its pharmacological effects.

Out of common antioxidants, Vitamin C, Vitamin E, allopurinol, and N-acetylcysteine have been studied to analyze their effects on fracture healing in experimental animal models. However, the effect of grape seed extract has not been investigated, which is a stronger antioxidant compared to well-known agents with their antioxidant activity including Vitamin E and Vitamin C. Furthermore, there is also no evidence on the effect of the LLLT combined with GSE on osseous or fracture healing despite many studies focusing on its effectiveness on osseous healing. For all these purposes, we aim to investigate the effects of GSE and LLLT on fracture healing and oxidant/antioxidant system in mandibular fractures which have great part in the practice of dentistry and specifically oral, dental and maxillofacial surgery.

MATERIAL and METHODS

Animals and Experimental Design

Our study was approved by Cumhuriyet University Animal Experiments Local Ethics Committee (B.30.2.CUM.0.01.00.00-50/81-399), and then we used 48 adult male rats (Wistar Albino, average weight: 350±50g) in the Experimental Animals Production and Research Laboratory at Cumhuriyet University. The rats were retained in standardized experimental cages during all the trial period with free access to pellet food and water in animal room (22-24o C, %55-70 humidity, 12:12 light:dark).

Experimental Groups

The animals were randomly separated into four groups in total: three study groups (GSE, LLLT, and GSE+LLLT) and one control group. Then, all the groups were individually divided into two sub-groups: 7th and 21st groups. The total number of groups were 8 (n=6) (Table 1). The main groups and sub-groups specified are as below:

Control group (C7-C21): For standardization, physiological saline solution (1cc) were orogastrically administered to the control group and these rats were sacrificed on the 7th and 21st day.

GSE-Extract group (E7-E21): The first extract administration was carried out one hour before the operation and GSE of 300 mg/kg/day were orogastrically administered to the extract group for 7 and 21 days in total, and then the rats were sacrificed on the 7th and 21st day.

LLLT group (L7-L21): For this group, laser therapy (dosage: 23 J/cm2; output power: 0.3 W) was performed with skin contact to two different points along with the fracture line for 50s (25s each) at intervals of 48h for 7d and 14d

commencing with the day of operation. In this procedure, GaA1As Diode laser (810 nm; Fotona XD-2 diode laser, Fotona, Ljubljana, Slovenia) was used in continuous mode. The rats were sacrificed on the 7th and 21st day.

GSE-Extract+LLLT group (EL7-EL21): Both extract and lazer therapy were used in combination for these group animals in the same procedure as mentioned above, and then the rats were sacrificed on the 7th and 21st day.

Table 1. Distribution of the groups

Groups	Control (C)	Extract (E)	LLLT (L)	Extract + LLLT (EL)
Day 7	6	6	6	6
Day 21	6	6	6	6
Total	12	12	12	12

Surgical Method and Postoperative Care

Ketamine (70 mg/kg) and Xylazine (13 mg/kg) were intraperitonally administered to the rats to achieve general anesthesia. For each animal, the right bucca was shaved and wiped using anti-bacterial iode solution for surgical operation. Then, an anteroposterior sub-mandibular incision (about 20mm in length) was made in the right mandibles. Subcutaneous tissues were bluntly dissected from foramen mentale to reach the outer surface of mandible following after periosteum was peeled. In this region, masseteric muscle was dispensed to make apparent its attachment point to linea oblique externa (Figure 1). Microscrew insert was prepared approximately 1mm posterior to linea obliqa externa and under serum irrigation using titanium drill bit (diameter: 0.8mm, length: 5mm). Afterwards, four-hole microplate was lousely adapted to bone at the reference point using a microscrew (diameter: 1mm). Following its fixation, the other three microscrew inserts were also completed and then the microscrews (d: 1.0mm, l: 4.0mm) were respectively adapted to these wells without much pinning. After that, several guiding channels were prepared in a vertical corticotomy line between premolar and molar teeth vertically down to the lower edge of corpus mandibular (Figure 2). We combined all these channels using a dental bur without harming medial wall and peripheral soft tissues, making a complete fracture line on the bone with a surgical chisel (Figure 3). The segments were exactly separated from each other and then microplate was rigidly fixed to the bone with the pinned microscrews. We washed to clean the operative field with normal saline and checked to ensure that there was no foreign body or tissue residue, and then massetic muscle filaments were sewed up with 5.0 catgut and 4.0 propylene suture (Figure 3).

To all the subjects, Carprofen (Rimadyl® flakon) was administered for pain control immediately after the operation once a day and Seftriakson sodyum (Novosef® i.m.) for infection prophylaxis once a day for 5 day. The same surgical procedure and post-operative care was followed for each animal. In all the control and study groups, the day when fracture was made in the rats was regarded as the zeroth day. The animals were fed only with soft diet (cake) and water in the first 7 days for the fractures on their jaws and from ending of first week with normal diet. Afterwards standard pellet feed was used. The animals were individually weighed to check during the trial period.

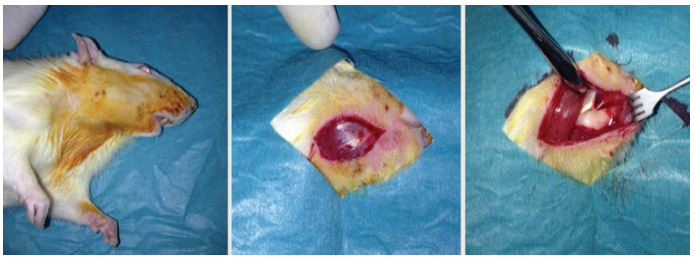

Figure 1. Surgical operation site, anteroposterior submandibular incision and dissection

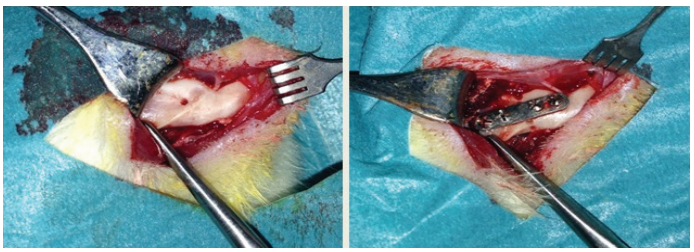

Figure 2. The appearance of guide hole and vertical corticotomy line

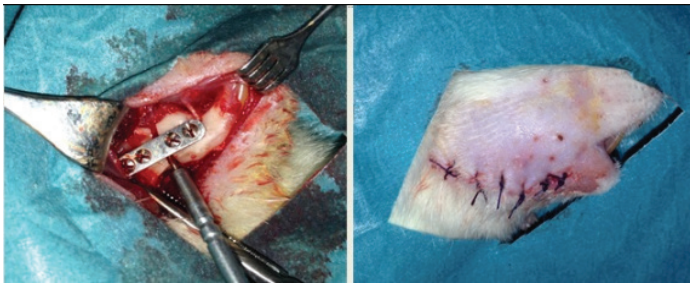

Figure 3. The fixation after full fracture line and surgical wound closure

Extraction of Grape Seed Extract

In the study, Çalkarası-cultivar grape seeds (Vitis vinifera L.) were dried in Laboratuary of Biology Department, Science Faculty, Gaziantep University using the selected healthy seeds and chunked in a mechanical disintegrator. The seed particles were placed in 100 g onto the cartridges of Soxhlet device (Gerhardt EV 14) and extracted at 50-60 °C for 6 h using 500 mL pure ethyl alcohol (Merck) for each cartridge. The obtained extracts were intensified at 40°C under high vacuum on Rotary Evaporator (Heildolph Heizbad HB Digit) following after being filtered with Whatman No. 4 and then preserved at +4 °C for the assay. GSE was rarefied to 100 mg per 1 ml normal saline in order to prepare for orogastric gavage (Figure 4a).

Low-Level Laser Therapy

In our study, 23 J/cm2 was administered to two points on fracture line for 50 s (25 s each) with skin contact using

GaA1As Diode laser (810 nm, 0.3 W, continuous mode, Fotona XD-2 diode laser, Fotona, Ljubljana, Slovenia). In laser therapy, the doses were administered at intervals of every 48 hours for 7 days in the 7th day sub-groups and for 14 days in the 21st day sub-groups (Figure 4b). The first dose was administered immediately after the operation.

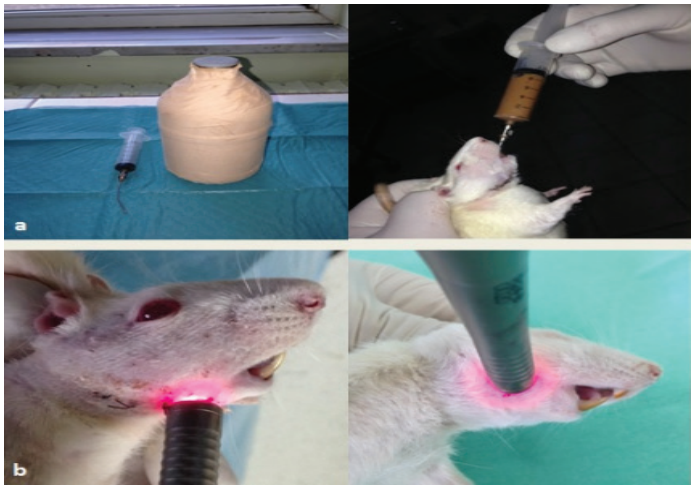

Figure 4. a. Application of grape seed extract with orogastric gavage; b. Application of low level laser therapy

Sacrification and Biochemical Analysis

All group animals were sacrificed on 7th or 21st day by drawing blood intracardiacally. Subsequently the tubes were centrifuged at 10 min x 4000 g and then the serum samples were kept at -80°C for the lab assay to measure total oxidant (TOS) and total antioxidant (TAS) levels as well as their oxidative stress indices (OSI).

Histological and Histomorphometric Examinations and Sample Preparation

Following the sacrification, soft tissues in each rat were dissected to exscind the whole mandibula and bisected to detach microplate and microscrews on the side of fracture. The obtained samples were retained into formalin solution (10%) for 48 h. These were supervised and decalcified into EDTA (ethylenediaminetetraacetic acid) solution (100 ml buffered in 0.1 M phosphate pH: 7.1) at + 4°C alternately every two days for 8 week. The specimens were washed with distilled water and crystallized with xylene following dehydratation using alcohol series with progressive degrees and then embedded into paraffin to produce paraffin blocks. For histological analysis, the serial sections (5 µm in width) removed from the blocks were deparaffinized in an oven at 60˚ overnight and in xylene for 1 h and then rehydrated to dye using hematoksilen-eozin (Surgipath, 01562E, 01602E, Peterborough, UK) and Mason Trikrom (HT15, Sigma).

Histopathological and Histomorphometric Evaluation

For all groups the sections were examined in terms of bone fracture area, fibrosis and ligament, cartilage tissue as well as new and mature bone formations. In groups mandibular fracture healing was evaluated on the 7th and 21st days using the scale proposed by Huo et al. based on the composition of histological order, fibrous tissue, cartilage, new bone growing, and mature bone formation (Table 2) (9). Furthermore, ligament elements resulted from healing in fracture areas in all groups were analyzed and scored as follow: the capillary number and width, the number of inflammatory cells, the number of active fibroblast cells, and the number of collagen filaments and osteoblasts were scored in 400 x magnification, as presented in Table 2.

Statistical Analysis

For normal distribution of continuous variables Shapiro wilk test was used. To compare more than two independent groups, one-way ANOVA and LSD multi-comparison tests were utilized for normally distributed variables, and Kruskal Wallis test and Dunn multi-comparison tests for non-normally distributed variables. In one-way ANOVA variance homogeneity was evaluated using Levene test. For statistical analyses SPSS Windows version 22.0 software package was used. Significance level was statistically defined as $p< 0.05$.

Table 2. Scoring system and evaluation criteria used in histological evaluation of samples

	Scoring system				
Grade 1	Fibrous tissue				
Grade 2	Mainly fibrous tissue, a small amount of cartilage				
Grade 3	Equally fibrous and cartilage tissue				
Grade 4	Mainly cartilage tissue, a small amount of fibrous				
Grade 5	Cartilage tissue				
Grade 6	Mainly cartilage tissue, a small amount of immature bone				
Grade 7	Equal cartilage and immature bone tissue				
Grade 8	Predominantly immature bone, a small amounts of cartilage tissue				
Grade 9	Fracture healing with immature bone				
Grade 10	Fracture healing with mature bone				
	Evaluation criteria				
Per unit area at x400 magnifications	Capillary number and width	Inflammatory cell	Fibroblast count	Collagen fibers	Osteoblast count
	1: little	1: little	1: 1-30	1: normal	1: 1-10
	2: middle	2: middle	2: 30-60	2: middle	2: 10-20
	3:increased	3:increased	3: > 60	3:increased	3: >20

RESULTS

Observational Findings

In our study there was no animal killed or excluded from any reasons during the experiment and within post-operative period. No significant complication was observed in any rats including loosening of the used microscrews, any type of infection in surgical area, and wound dehiscence. Based on our clinical evaluation, all the rats typically well-tolerated the surgical operation although weight loss was found in all groups.

Biochemical Findings

There was no significant difference between the 7th-day groups for TOS and OSI values (p>0.05), and significant difference for TAS values (p=0.042*). Statistical significant difference was found between the TAS values of the E7 and L7 groups (p=0.014) or the E7 and EL7 groups (p=0.037) (Table 3).

Similarly, significant difference was observed between the 21st-day groups for TAS values (p=0.005*), and there was no significant difference for TOS and OSI values (p>0.05). Statistically significant difference was found between the TAS values of the C21 and L21 groups (p=0.027) or the C21 and EL21 groups (p=0.001) or the E21 and EL21 groups (p=0.036) (Table 4).

Histological, Histomorphometric Analysis Findings

7th-Day Groups

Based on our statistical analysis, there was no significant difference between the 7th-day groups (C7, E7, L7, and EL7) in terms of the capillary number, the number of inflammatory cells and the number of fibroblasts and the values of collagen filaments (p>0.05), whereas statistically significant difference was found between the numbers of fibroblasts and the values of collagen filaments of the 7th-day groups (Table 5) (p<0.05).

Table 3. Average, standard deviation and statistical differences of TAS, TOS and OSI values of C7, E7, L7 and EL7 groups

	Mean ± Standard deviation				
	C7 (n=6)	E7 (n=6)	L7 (n=6)	EL7 (n=6)	p
TAS	1.03 ± 0.14	1.09 ± 0.21 §.¶	0.85 ± 0.13 ‡	0.89 ± 0.10 ‡	0.042*
TOS	53.61 ± 20.37	62.03 ± 17.31	35.70 ± 15.73	41.05 ± 21.38	0.097
OSI	5.06 ± 1.29	6.09 ± 1.32	4.07 ± 1.14	4.42 ± 1.70	0.092

* p<0.05 (Intergroup evaluation for TAS value), † C7 group compared to the other 7th day groups, p<0.05, ‡ E7 group compared to the other 7th day groups, p<0.05, § L7 group compared to the other 7th day groups, p<0.05, ¶ EL7 group compared to the other 7th day groups, p<0.05

Table 4. Average, standard deviation and statistical differences of TAS, TOS and OSI values of C21, E21, L21 and EL21 groups

	Mean ± Standard deviation				
	C21 (n=6)	E21 (n=6)	L21 (n=6)	EL21 (n=6)	p
TAS	0.76 ± 0.05§.¶	0.89 ± 0.07¶	0.92 ± 0.18†	1.05 ± 0.12†.‡	0.005*
TOS	24.92 ± 9.18	27.23 ± 14.66	30.80 ± 20.29	45.22 ± 18.19	0.160
OSI	3.25 ± 1.04	2.95 ± 1.27	3.13 ± 1.45	4.21 ± 1.31	0.352

* p<0.05 (Intergroup evaluation for TAS value), † C21 group compared to the other 21st day groups, p<0.05, ‡ E21 group compared to the other 21st day groups, p<0.05, § L21 group compared to the other 21st day groups, p<0.05, ¶ EL21 group compared to the other 21st day groups, p<0.05

21st-day Groups

Based on our statistical analysis there was no significant difference between the 21st-day groups (C21, E21, L21, and EL21) in terms of the capillary number, the collagen values while significant differences were found between the numbers of inflammatuar cells, fibroblasts and osteoblasts, and the new bone growing values (Table 6) (p<0.05). At the end of 21st-day the best result in the values of osteoblasts and new bone growing was observed in Group EL21 for which extract and laser were used in combination.

Histological Evaluations

The sections from the sample rats with mandibular fracture were dyed using hematoksilen-eozin ve Masson trikrom techniques to evaluate histologically.

Table 5. Average, standard deviation and statistical differences of histopathological scores of C7, E7, L7 and EL7 groups

Variables	Mean ± Standard deviation				
	C7 (n=6)	E7 (n=6)	L7 (n=6)	EL7 (n=6)	p
Capillary number and width	2.50 ± 0.54	2.33 ± 0.51	1.50 ± 0.54	2.50 ± 0.54	0.107
Inflammatory cell	1.33 ± 0.51	2.33 ± 0.51	1.50 ± 0.54	2.33 ± 0.51	0.074
Fibroblast count	1.33 ± 0.51	2.16 ± 0.40	1.16 ± 0.51	2.50 ± 0.54	0.097
Collagen fibers	2.66 ± 0.51	2.00 ± 0.63	2.16 ± 0.75	2.50 ± 0.54	0.256
Osteoblast count	1.00 ± 0	2.16±0.40†	1.33 ± 0.51	2.50±0.54†.§	0.001*
Ossification	1.66 ± 0.51	6.00±0.63†	4.50 ± 0.54	7.50±0.54†.§	0.000*

* p<0.05, † C7 group compared to the other 7th day groups, p<0.05, ‡ E7 group compared to the other 7th day groups, p<0.05, § L7 group compared to the other 7th day groups, p<0.05, ¶ EL7 group compared to the other 7th day groups, p<0.05

Table 6. Average, standard deviation and statistical differences of histopathological scores of C21, E21, L21 and EL21 groups

Variables	Mean ± Standard deviation				
	C21 (n=6)	E21 (n=6)	L21 (n=6)	EL21 (n=6)	p
Capillary number and width	2.33 ± 0.51	2.16 ± 0.75	1.16 ± 0.51	2.83 ± 0.40	0.055
Inflammatory cell	1.50 ± 0.54	2.16 ± 0.40	1.16 ± 0.51	2.66 ± 0.51†	0.009*
Fibroblast count	1.50 ± 0.54	2.50 ± 0.54	2.16 ± 0.75	2.66 ± 0.51†	0.031*
Collagen fibers	2.50 ± 0.54	2.16 ± 0.40	2.16 ± 0.75	2.50 ± 0.54	0.573
Osteoblast count	1.50 ± 0.54	2.33 ± 0.51	1.50 ± 0.54	2.83±0.40†.§	0.003*
Ossification	1.66 ± 0.51	6.33±0.51†	5.00 ± 0.63	7.66±0.81†.§	0.000*

* * p<0.05, † C21 group compared to the other 21st day groups, p<0.05, ‡ E21 group compared to the other 21st day groups, p<0.05, § L21 group compared to the other 21st day groups, p<0.05, ¶ EL21 group compared to the other 21st day groups, p<0.05

Control Group (C)

A scar tissue was significantly observed in fracture area. Compared to the 21st day, the disrupted continuity of fibroblasts and collagen filaments which were mainly established in the same direction were monitored as well as a few of capillaries on the 7th day. In the healing process fibrous tissues were already intense and small quantity of cartilage was formed on the 21st day. Further a few of oval, basophilic cytoplasmic osteoblasts and of polynuclear acidophilic cytoplasmic osteoclasts were seen. It was observed that osteocytes in solid tissue outside the fracture area were typical within bone matrix (Figure 5a,b).

GSE-Extract Group (E)

When examining the dyed sections with hematoxilen-eozin and masson trikrom of both the 7th-day and the 21st day groups, which were treated with extract following the fracture, a significant healing was observed and cartilage and immature bone formation started compared to control and laser-therapy groups. Furthermore, it was seen that the fibroblasts and the collagen synthesis increased and inflammation and blood capillaries moderately escalated, and new ossification was irregularly shaped. In osseous area basophilic cytoplasmic oval active osteoblasts were monitored and it was also observed that new ossification becomes significant (Figure 5a,b).

LLLT Group (L)

In this group the sections of the 7th and 21st day healed as relative to control group but not exactly to extract and extract+laser groups. A significant scar tissue was observed in fracture area and however increase were limited in the number of osteoblasts and the capillary number and the quantity of collage ligament. Furthermore, the healing process began with the formation of cartilage and a few fibrous tissue and healing on the 21st day was better than that on the 7th day (Figure 5a,b).

GSE-Extract+ LLLT Group (EL)

The best healing after the fracture was observed in this group in which extract and laser were used in combination. The group animals had a few cartilage and also higher immature bone formation. There was no exact fracture closure and basophilic cytoplasmic oval active osteoblasts were seen in newly grown bone tissue. In addition it was observed that several parameters significantly increased including inflammation, capillary number and width, fibroblasts and collagen synthesis. In comparison of the sections of 7th and 21st day, we found the increased number of filaments and osteoblasts and higher osteogenesis on the 21st day (Figure 5a,b).

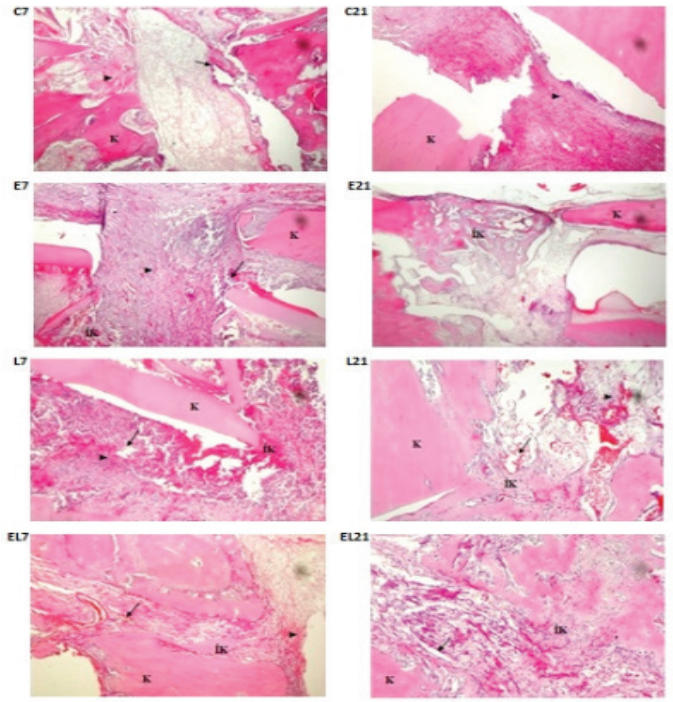

Figure 5a. Analysis of histological findings of the groups, Hematoxylin-Eosin. (C: Control, E: Extract, L: Laser and EL: Extract+ Laser groups; arrow: capillary; arrowhead: inflammatory area; IK: Bone formation; K: Bone)

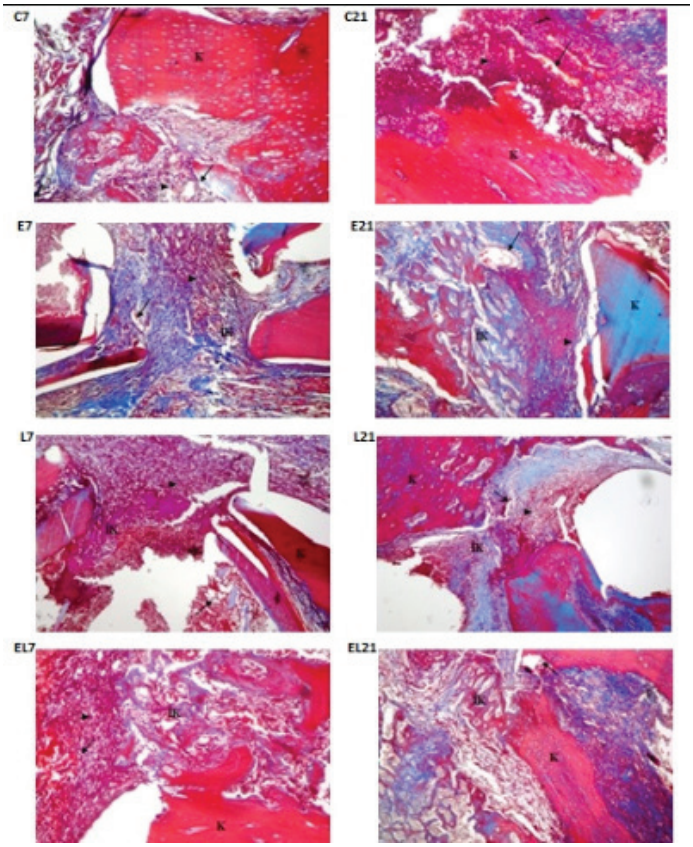

Figure 5b. Analysis of histological findings of the groups, Masson Trichrome. (C: Control, E: Extract, L: Laser and EL: Extract+ Laser groups; arrow: capillary; arrowhead: inflammatory area; IK: Bone formation; K: Bone)

DISCUSSION

Fractures are healed in the closest way to the original shape and function without remaining scar tissue different from other tissues thanks to its ability of remodelation (10,11). However this is a process including the best mechanism among cells, growth factors and extracellular matrix and a proper bone union may occur only if these complicated cellular and biochemical phases are complete (10). A potential lagging at any phase or negative influence from any reason or cause lead to delays in osseous healing.

In literature it has been reported that oxidative stress occurs during fracture healing and free oxygen radicals (FOR) is one of the adverse factors against this process (12). Following fracture occurrence and subsequent transient ischemia, inflammatory cells and osteoclasts in circulation are vital in formation of free radicals (13). Deficiency of endogen antioxidant defense systems result in oxidative damage (14). Göktürk et al. studied the effects of FOR on fracture healing and showed that the increased radical production disrupt the process based on their analyses made on 22nd day following the fracture in rats (15). With this, earlier stages are essential for exact bone union in fractures.

As known, antioxidants are useful to eliminate the negative influences of free oxygen radicals on fracture healing. For this purpose, we sit well with the assessment of healing process on the 7th and 21st day in order to analyze the effects of LLLT, which is currently used in dentistry in recent years due to miscellaneous therapeutic properties, and GSE known with its being strong oxidant and richness in proanthocyanidins on FOR and possible tissue damage in the early period following the fracture. We consider that the specified times are significant to demonstrate if this twenty-one-day healing process will be efficacious and what extent the treatment procedures of choice will affect the healing rate. Recently, a number of studies have been conducted on GSE because of either its biological or pharmacological characteristics. It is known that GSE has antibacterial, antifungal, antiviral, and anticarcinogenic effects on human health (16). Furthermore, it has been also stated that oligomeric proanthocyanidins included in the extract can exhibit anti-inflammatory, and antitumoral activity, inhibit heart diseases and aging and be protective against ischemic perfusion damage (16). Khanna et al. investigated the effects of proanthocyanidins on wound healing, made excisional wound on dorsal part of the rats, administered topical GSE of 100 mg/ml for 5 d and observed that the wound more rapidly closed in the study group compared to the control group (17).

The recent studies on bone tissue are very limited despite many reports including use of GSE in different areas. Among these, Park et al. highlight that GSE may be beneficial in prevention of osteonecrosis in autoimmune inflammatory arthritis (18). In a study, Woo et al. proposed an arthritis model induced by monosodium iodoacetate on knee joints and concluded that GSE (100 mg/kg and 300 mg/kg) reduces osteophyte formation as well as loss

of chondrocyte and proteoglycans in rats three times a week for 4 w following its injection and thus is protective against joint injury and this may become a promising therapy for osteoarthritis (19). In another study, Yahara et al. studied the effect on their tibia diaphysis of GSE mixed with standard pellet food in rats fed free from calcium and consequently determined that GSE enhances the mechanic properties of bone tissue including its formation, mineral content and durability (20). In similar studies, it has been showed that the formation, durability and mechanical properties of mandibular condyl had higher performance in rats for which GSE supplement was added to pellet (21,22). For rats in developmental period, researchers studied what effect GSE has on mandibular and observed that cortical bone intensity and mineral content of cortical and trabecular bone improved with the raised mechanical properties such as formation, quality, and durability and other mechanical properties in mandibula of the rats fed with GSE in combination with standard pellet (23,24).

GSE with a wider dosage range seems to be an effective and safe therapeutic agent since it shows no toxic effect even at higher doses and maintains a certain saline level for 7 to 10 days following the last dose given orally (25). In an acute and subchronic oral toxicity study of Yamakoshi et al., it was reported that GSE had no oral toxicity and mutagenity even at higher doses (i.e. 2 or 4 g/kg) for 14 days (26). Furthermore, the effect of use of GSE has long been studied. Ray et al. supplemented GSE of 100 mg/kg/day to male rats (B6C3F1) for 1 year or of 500 mg/kg/day to female rats for 6 months and reported no side-effect in their vitals (27). In addition to similar evidence proanthocyanides and GSE are also a nutritional supplement used in the U.S. and Europe for many years and due to these features are usually in the list of safe foods approved by Food and Drug Administration (FDA) and it is stated that the prescribed daily dosage of GSE ranges from 100 to 300 mg (28). In the light of all these information we orogastrically administered GSE of 300 mg/kg/gün within safe dose range suggested in literature till the day when the rats were sacrificed (25).

Balcı et al. analyzed the effects of GSE on alveolar bone loss and histopathological variations in rats with periodontitis infected with diabetes and concluded that GSE administration may reduce periodontal inflammation and alveolar bone loss and raise osteoblastic activity in experimental diabetic rats with periodontitis (29).

In a study researchers investigated the effects of GSE on fracture healing and biomechanics of healed bone and suggested that osseous healing is more significant in the groups supplemented with GSE, based on histopathological examination of the samples in bone callus tissue. In the groups administered by GSE, radiological healing scores and callus volumes were observed to be statistically significantly higher. For biomechanical forces, it was determined that GSE promoted bone fracture not only in fracture groups but also positive control group. Consequently, the study showed that GSE as a strong antioxidant either had positive effects on osseous healing or enhanced mechanic strength of healed bone (30).

Our literature review indicated that GSE has much effect on bone tissue. These studied the effects of GSE on osseous healing while recent studies has focused on the effects of resveratrol (Rsvl) known as active compound in the extract. On this topic the first in vitro assay was conducted by Mizutani et al. (31) In that study, the authors reported that Rsvl in vitro stimulated differentiation and proliferation of osteoblastic MC3T3-E1 cells. Alkaline phosphatase is widely known as a biochemical marker for osteoblastic activity and it is believed that this enzyme plays role in bone mineralization. Likewise researchers showed that Rsvl increased the activity of prolyl hydroxylase and alkaline phosphatase in MC3T3-E1 cells and inhibited the production of prostaglandin-E2, an endogen supporter for osteoclast formation. The same authors suggested that Rsvl inhibits differentiation from stem cells to osteoclasts and has stimulating effect on osteogenesis.

Researchers stated that Rsvl may have relative advantages compared to current pharmacological therapies which influence by stimulating bone formation through osteoblastic activity or inhibiting bone resorption through osteoclastic activity. Boissy et al. (32) studied the effect of Rsvl on osteocytes and myelom. In in vitro assay the authors found that this natural compound raised osteoblast differentiation and inhibited osteoclast differentiation. Mobasheri and Shakibaei (33) reviewed the results of in vitro studies and concluded that Rsvl inhibited osteoklastogenesis, stimulated osteoblast formation and hence increased bone mass.

A study of Casarin et al. (34) is the first in vivo study discussing the effects of Rsvl on bone damages. In that study, researchers made a critical-size defect (diameter: 5mm) on the calvarium of the samples and also embedded titanium implant (length: 4mm, diameter: 2.2mm) into their tibia. Rsvl of 10mg/kg was administered via gavage for 30 days. In the evaluation of study the authors showed that the quantity of non-healed defects significantly decreased in Rsvl group compared to the control. These results indicated that the chronic use of this substance can be a useful therapeutic agent in osseous healing processes and in the rehabilitation of edentulous patients with help of dental implants.

Uysal et al. (35) performed an experimental study in rats for which rapid maxillary expansion was made and locally administered Rsvl to midpalatal suture and then examined its effects on bone formation in this part. For trial purpose, only one dose of Rsvl (10μmol/kg) was injected to the suture and ossification was histomorphometrically evaluated. The measurements of the area and percentage of new bone formation, the number of osteoblasts and Feret's diameter (the highest length in a range) were found to be higher in study group compared to the control group. Based on this principle the authors concluded that Rsvl can be used for treatment of bone fractures and bone surgery such as distraction osteogenesis.

In another in vitro study, Singh et al. (36) highlighted that Rsvl may be a promising therapeutic and protective agent in smoking-related alveol bone losses and periodontal diseases. Özcan et al. (37) examined the effects of Rsvl on extraction sockets in rats for which tooth extraction was made with the administration of Siklosporin A and observed that use of Siklosporin A had adverse effects on the socket healing and Rsvl administration could stimulate this process.

For clinical trials, Ornstrup et al. (38) designed a randomized, double-blind, placebo controlled study, randomly distributed middle-aged 74 obese male with metabolic syndrome into three groups, administered to each group the Rsvl of 500 mg and 75 mg and Placebo solution respectively twice a day for 16 weeks and consequently highlighted that high-dose Rsvl administration positively influenced the bone by stimulating mineralization and formation and longer studies were required to enhance these results.

Despite many studies focusing on the efficacy of GSE in literature, there is no evidence on how GSE influence fracture healing. In addition several researchers studied the efficiency of LLLT on either fracture or osseous healing using different doses and however there is limited evidence on what effect its combination with GSE has, and therefore our results in the present study are essential for oxidant/antioxidant system and fracture healing. After all, we found that ossification significantly occurred in study groups administered particularly by extract in mandibular fracture healing at a higher level on both 7th and 21st days, compared to the control groups. Moreover, we suggest that GSE may be a therapeutic agent for its miscellaneous influential potential in either fracture or defect healing.

In recent years, type of GaA1As laser has commonly been used since it offers more effective and positive results in practical applications because of its higher penetration (39). In our study, we also selected to use this device. Biostimulation effect of LLLT is varying on many factors including the wavelength of the laser. It is known that diode laser (810 nm) can penetrate up to 10 mm2 and stimulate in deeper tissues. With this, we identified 810 nm as wavelength so that the laser to be extraorally used could influence bone tissue.

Another key factor in use of LLLT is dose adjustment. In general laser has no effect at low levels while at higher levels it may have inhibitor effect and accordingly determination of wider therapeutic dose range is crucial in laser therapies (40). The therapeutic dose range of LLLT is larger and however no protocol could be established because this therapy has no standard dose and wavelength effective on bone tissue (41). Nevertheless, some of the recent studies have involved the laser therapy of 23 J/cm2 and achieved better outcomes. Khadra et al. provided GaA1As laser (830 nm) at a rate of 23 J/cm2/day for 6 days on the defects made on parietal bone in rats and determined that there was significant increase in revascularization and new bone growing in bone and connective tissues for the laser group (5). In another study of the same author, implant wells were readied in tibia of rabbits to construct the link of titanium implants with bone and again 23 J/cm2 was daily administered for 10 days combined with GaA1As laser at the equal wavelength. Based on the eighth-week results, it was observed that implant- bone contact surface was larger and calcium/phosphorus ratio increased (42). In this study we judged on dosage of 23 J/cm2 and GaA1As diode laser (810 nm) therapy, based on lack of animal study examining the effect of LLLT on mandibular fracture healing in literature and the positive effect from the study of Khadra et al.

In literature it has been reported that laser therapy has effect on the proliferation, maturity and bone matrix construction of differentiated cells. Many authors state that this can stimulate early response to inflammation with vascularization to accelerate bone matrix construction (43). However, several studies suggest that LLLT has lack of expected outcome on osseous healing or adverse effect due to the laser despite the fact that other researchers show its positive role in solid and soft tissue healing (44, 45). In our study it was demonstrated that new bone formation and the quantity of osteoblasts statistically significantly increased in the groups (E7, EL7, E21, and EL21) provided with extract on the 7th and 21st days in post-operative period, compared to the control group, and the best results were achieved from the groups (EL7, EL21) for which extract and laser therapy were used in combination. For the dosage and period extract had higher efficacy when combined with laser and however LLLT itself showed no statistically significant effect on ossification. Based on this result, we consider that LLLT can be used as a supportive treatment and nonetheless further studies should be conducted to achieve clear outcomes in this issue.

In our study biochemical analyses indicated that there were significant difference between the groups related to oxidant and antioxidant systems only for TAS values on both the 7th and 21st days, and the TAS values of 7th day had significant difference only in the extract group while laser therapy negatively influenced TAS. In contrast these results, the laser and extract+laser groups were the best for TAS values among the 21st-day groups rather than the extract group and laser had positive effect on TAS in long term.

CONCLUSION

Consequently, grape seed extract of 300 mg/kg/day can be administered in order to accelerate the fracture healing in mandibular fractures common in Oral and Maxillofacial surgical clinic and thus for patients recover in shorter time. Besides, we conclude that LLLT of 23 J/cm2 (0.3 W, 810 nm GaAlAs) may have positive effects on ossification and healing process when combined with the extract, that's the combination of GSE and LLLT can provide better outcomes in clinical use. In literature there is no evidence on the role of the combined use of GSE and LLLT in fracture healing, and therefore we consider that our findings in

the present study make a significant contribution to the literature. Despite all, further considerations are required to elucidate the exact cause of ossification pattern.

Competing interests: The authors declare that they have no competing interest.

Financial Disclosure: This study was supported by Scientific Research Projects Commission of Gaziantep University with the project numbered DHF.13.03.

Ethical approval: The necessary ethical approval was obtained from Animal Experiments Local Ethics Committee of Cumhuriyet University (Decision number: B.30.2.CUM.0.01.00.00-50/81, 399).

Bilal Ege ORCID: 0000-0002-1279-0893
Mutan Hamdi Aras ORCID: 0000-0002-8907-7340
Hasan Akgül ORCID: 0000-0001-8514-9776
Muhammed İsa Kara ORCID: 0000-0001-7644-3295
Isıl Aydemir ORCID: 0000-0002-4143-7319
Sevinc Inan ORCID: 0000-0003-1971-9720
Seyithan Taysı ORCID: 0000-0003-1251-3148
Sevil Altundag Kahraman ORCID: 0000-0003-3573-1068

REFERENCES

1. Higgins TF, Dodds SD, Wolfe SW. A biomechanical analysis of fixation of intra-articular distal radial fractures with calcium-phosphate bone cement. J Bone Joint Surg Am 2002;84:1579-86.
2. Doetsch AM, Faber J, Lynnerup N, et al. The effect of calcium and vitamin D3 supplementation on the healing of the proximal humerus fracture: a randomized placebo-controlled study. Calcif Tissue Int 2004;75:183-8.
3. Rozen N, Lewinson D, Bick T, et al. Role of bone regeneration and turnover modulators in control of fracture. Crit Rev Eukaryot Gene Expr 2007;17:197-213.
4. Liu X, Lyon R, Meier HT, et al. Effect of lower-level laser therapy on rabbit tibial fracture. Photomed Laser Surg 2007;25:487-94.
5. Khadra M, Kasem N, Haanaes HR, et al. Enhancement of bone formation in rat calvarial bone defects using low-level laser therapy. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:693-700.
6. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47-95.
7. Georgiev A, Ananga V, Tsolova V. Recent Advances and Uses of Grape Flavonoids as Nutraceuticals. Nutrients 2014;6:391-415.
8. Bagchi D, Swaroop A, Preuss HG, et al. Free radical scavenging, antioxidant and cancer chemoprevention by grape seed proanthocyanidin: an overview. Mutat Res 2014;768:69-73.
9. Huo MH, Troiano NW, Pelker RR, et al. The influence of ibuprofen on fracture repair: biomechanical, biochemical, histologic, and histomorphometric parameters in rats. J Orthop Res 1991;9:383-90.
10. Schindeler A, McDonald MM, Bokko P, et al. Bone remodeling during fracture repair: The cellular picture. Semin Cell Dev Biol 2008;19:459-66.
11. Komatsu DE, Warden SJ. The control of fracture healing and its therapeutic targeting: improving upon nature. J Cell Biochem 2010;109:302-11.
12. Duygulu F, Yakan B, Karaoglu S, et al. The effect of zymosan and the protective effect of various antioxidants on fracture healing in rats. Arch Orthop Trauma Surg 2007;127:493-501.
13. Yeler H, Tahtabas F, Candan F. Investigation of oxidative stress during fracture healing in the rats. Cell Biochem Funct. 2005;23:137-9.
14. Prasad G, Dhillon MS, Khullar M, et al. Evaluation of oxidative stress after fractures. A preliminary study. Acta Orthop Belg 2003;69:546-51.
15. Göktürk E, Turgut A, Bayçu C, et al. Oxygen-free radicals impair fracture healing in rats. Acta Orthop Scand 1995;66:473-5.
16. Cos P, De Bruyne T, Hermans N, et al. Proanthocyanidins in health care: current and new trends. Curr Med Chem 2004;11:1345-59.
17. Khanna S, Venojarvi M, Roy S, et al. Dermal wound healing properties of redox-active grape seed proanthocyanidins. Free Radic Biol Med 2002;33:1089-96.
18. Park JS, Park MK, Oh HJ, et al. Grape-seed proanthocyanidin extract as suppressors of bone destruction in inflammatory autoimmune arthritis. PLoS One 2012;7:e51377.
19. Woo YJ, Joo YB, Jung YO, et al. Grape seed proanthocyanidin extract ameliorates monosodium iodoacetate-induced osteoarthritis. Exp Mol Med 2011;43:561-70.
20. Yahara N, Tofani I, Maki K, et al. Mechanical assessment of effects of grape seed proanthocyanidins extract on tibial bone diaphysis in rats. J Musculoskelet Neuronal Interact 2005;5:162-9.
21. Ishikawa M, Maki K, Tofani I, et al. Grape seed proanthocyanidins extract promotes bone formation in rat's mandibular condyle. Eur J Oral Sci 2005;113:47-52.
22. Kojima K, Maki K, Tofani I, et al. Effects of grape seed proanthocyanidins extract on rat mandibular condyle. J Musculoskelet Neuronal Interact 2004;4:301-7.
23. Gunjima M, Tofani I, Kojima Y, et al. Mechanical evaluation of effect of grape seed proanthocyanidins extract on debilitated mandibles in rats. Dent Mater J 2004;23:67-74.
24. Kamitani Y, Maki K, Tofani I, et al. Effects of grape seed proanthocyanidins extract on mandibles in developing rats. Oral Dis 2004;10:27-31.
25. Bagchi D, Bagchi M, Stohs SJ, et al. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 2000;148:187-97.
26. Yamakoshi J, Saito M, Kataoka S, et al. Safety evaluation of proanthocyanidin-rich extract from grape seeds. Food Chem Toxicol 2002;40:599-607.
27. Ray S, Bagchi D, Lim PM, et al. Acute and long-term safety evaluation of a novel IH636 grape seed proanthocyanidin extract. Res Commun Mol Pathol

Pharmacol 2001;109:165-97.
28. Kar P, Laight D, Shaw KM, et al. Flavonoid-rich grapeseed extracts: a new approach in high cardiovascular risk patients? Int J Clin Pract 2006;60:1484-92.
29. Toker H, Balci Yuce H, Lektemur Alpan A, et al. Morphometric and histopathological evaluation of the effect of grape seed proanthocyanidin on alveolar bone loss in experimental diabetes and periodontitis. J Periodontal Res 2018;53:478-86.
30. Gurger M, Yilmaz E, Yilmaz S, et al. Grape seed extract supplement increases bone callus formation and mechanical strength: an animal study. J Orthop Surg Res 2019;14:206.
31. Mizutani K, Ikeda K, Kawai Y, et al. Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 1998;253:859-63.
32. Boissy P, Andersen TL, Abdallah BM, et al. Resveratrol inhibits myeloma cell growth, prevents osteoclast formation, and promotes osteoblast differentiation. Cancer Res 2005;65:9943-52.
33. Mobasheri A, Shakibaei M. Osteogenic effects of resveratrol in vitro: potential for the prevention and treatment of osteoporosis. Ann NY Acad Sci 2013;1290:59-66.
34. Casarin RC, Casati MZ, Pimentel SP, et al. Resveratrol improves bone repair by modulation of bone morphogenetic proteins and osteopontin gene expression in rats. Int J Oral Maxillofac Surg 2014;43:900-6.
35. Uysal T, Gorgulu S, Yagci A, et al. Effect of resveratrol on bone formation in the expanded inter-pre-maxillary suture: early bone changes. Orthod Craniofac Res 2011;14:80-7.
36. Singh SU, Casper RF, Fritz PC, et al. Inhibition of dioxin effects on bone formation in vitro by a newly described aryl hydrocarbon receptor antagonist, resveratrol. J Endocrinol 2000;167:183-93.
37. Ozcan-Kucuk A, Alan H, Gul M, et al. Evaluating the Effect of Resveratrol on the Healing of Extraction Sockets in Cyclosporine A-Treated Rats. J Oral Maxillofac Surg 2018;76:1404-13.
38. Ornstrup MJ, Harslof T, Kjaer TN, et al. Resveratrol increases bone mineral density and bone alkaline phosphatase in obese men: a randomized placebo-controlled trial. J Clin Endocrinol Metab 2014;99:4720-9.
39. Kawasaki K, Shimizu N. Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg Med 2000;26:282-91.
40. Sommer AP, Pinheiro AL, Mester AR, et al. Biostimulatory windows in low-intensity laser activation: lasers, scanners, and NASA's light-emitting diode array system. J Clin Laser Med Surg 2001;19:29-33.
41. Parker S. Low-level laser use in dentistry. Br Dent J 2007;202:131-8.
42. Khadra M, Ronold HJ, Lyngstadaas SP, et al. Low-level laser therapy stimulates bone-implant interaction: an experimental study in rabbits. Clin Oral Implants Res 2004;15:325-32.
43. Salate AC, Barbosa G, Gaspar P, et al. Effect of In-Ga-Al-P diode laser irradiation on angiogenesis in partial ruptures of Achilles tendon in rats. Photomed Laser Surg 2005;23:470-5.
44. Bayat M, Javadieh F, Dadpay M. Effect of He-Ne laser radiation on healing of osteochondral defect in rabbit: a histological study. J Rehabil Res Dev 2009;46:1135-42.
45. Oliveira P, Ribeiro DA, Pipi EF, et al. Low level laser therapy does not modulate the outcomes of a highly bioactive glass-ceramic (Biosilicate) on bone consolidation in rats. J Mater Sci Mater Med 2010;21:1379-84..