The effects of grapeseed extract and low level laser therapy administration on the liver in experimentally fractured mandible

Mehmet Erman Erdemli¹, Hasan Akgul², Bilal Ege³, Zeynep Aksungur⁴, Harika Gozukara Bag⁵, Zeliha Selamoglu⁶

¹Omer Halisdemir University, Faculty of Medicine, Department of Medical Biochemistry, Nigde, Turkey
²Akdeniz University, Faculty of Arts and Science, Department of Biology, Antalya, Turkey
³Adiyaman University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Adiyaman, Turkey
⁴Inonu University, Faculty of Medicine, Department of Medical Biochemistry, Malatya, Turkey
⁵Inonu University, Faculty of Medicine, Department of Biostatistic, Malatya, Turkey
⁶Omer Halisdemir University, Faculty of Arts and Science, Department of Biotechnology, Nigde, Turkey

Abstract
Aim: The present study investigated the changes in the liver tissues of rats with experimentally fractured mandible following the use of Grape Extract (GSE) and Low Level Laser Therapy (LLLT) in healing the fracture in dentistry.

Materials and Methods: 60 adult male Wistar Albino rats were randomly assigned to 5 main groups (Control, Fractured Mandible (FM), FM + GSE, FM + LLLT, FM + LLLT + GSE), and then these groups were divided into two groups of 7 and 21 days (n = 6). A vertical fracture line passing through the molar teeth was formed in the right mandibles of all subjects except for these in the control group, and the fracture was internally fixed with a four-hole microplate and four micro-screws. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) activities were analyzed to determine the changes caused by GSE and LLLT administration in rat liver tissues in fractured mandibles.

Results: It was determined that MDA and SOD levels in FM group and GSH and CAT activity levels in FM + GSE group and MDA levels in FM + LLLT group and GSH and CAT levels in FM + GSE + LLLT group increased statistically significantly to the control group on days 7 and 21.

Conclusion: Biochemical parameters were investigated on the 7th and the 21st days, and it was determined that the oxidative damage caused by mandibular defects could be eliminated substantially in the rat liver especially with the administration of grapeseed that has antioxidant capacity.

Keywords: Low Level Laser Therapy; Mandibular Fracture Recovery; Oxidative Stress; Grapeseed Extract; Liver; Rat.

INTRODUCTION
Destruction of existing anatomical integrity and continuity of the bone that plays a role in protection of organs and systems and metabolic support of the organism with direct and indirect interventions is called fracture. In fractures, only bone is not damaged; surrounding soft tissues suffer damages in different rates as well. The existing fracture heals only through complex cellular and biochemical processes (1). Low Level Laser Therapy (LLLT), determined to have anti-inflammatory, bio-stimulant and wound healing action, is a modern therapy approach. Studies demonstrated that laser therapy facilitates bone cell proliferation, increases new bone formation, and shortens the bone recovery process (2–4).

In dental therapy, the initial month after the surgical operation is quite important since ossification starts in this period and it is the most critical recovery period. Free oxygen radicals have negative impact on fracture recovery and form in excess especially during the early stages of recovery (5). Free oxygen radicals are high reactivity, short half-life molecules that contain at least one unpaired electron in the outer orbit. They easily react with other radicals or non-radical normal cellular components and cause various cell damages such as destruction of DNA, proteins and cellular membrane lips (6). On the other hand, antioxidants are molecules that prevent this damage to the organism and in a healthy organism, the oxidant and antioxidant system stands at a sensitive equilibrium (6).

Liver, which could be affected significantly from the changes that occur in the organism and is tissue with intense metabolic activity, promotes the reduction of free radical damage risk via detoxification and biotransformation events (7-9).

The fact that this equilibrium is disrupted and oxidative stress occurs as a result of fractures and the events that
develop during the initial period after the fracture directed researchers to investigate the effects of molecules with known antioxidant action on this process. Certain antioxidants could be produced in the body, while they could also be taken exogenously when free radicals are produced extensively. Proantocyanodins are a good example for exogenous antioxidants (10). Grapes (Vitis vinifera), extensively cultivated and consumed around the world, are especially a rich source for proantocyanodins (10). The effects of grapeseed extract, which is a more powerful antioxidant when compared to substances with known antioxidant action such as Vitamins E and C in the literature, on fracture recovery have not yet been researched. Furthermore, despite the fact that there are several studies that scrutinized the effect of LLLT on fracture recovery, there is no study that investigated the combined action of LLLT and GSE on bone or fracture recovery.

In the present study, it was aimed to investigate the changes in oxidant / antioxidant system changes in rat liver tissue as a result of LLLT and GSE applications conducted for fracture recovery in mandible fractures, which are frequently encountered in dentistry and especially dental and maxillofacial surgery.

MATERIALS and METHODS

Animals and Experimental Design

In the present study, 60 healthy 4 – 5 months adult male albino Wistar rats (mean body weight 350 ± 50 g) were used. The rats were divided into 10 groups, 6 rats each. Animals were kept in an environment with standardized light cycle conditions (12 hours of daylight / 12 hours of darkness), 22-24 °C temperatures, 55- 70% humidity and free access to food (standard pellet chow diet) and water. The rats were procured from Cumhuriyet University, Experimental Animal Breeding and Research Center, School of Medicine. Ethical rules depicted in “Guide for the Care and Use of Laboratory Animals” (11) were strictly followed while conducting the present study and monitored closely by Cumhuriyet University, School of Medicine Ethics Committee. Identical surgical procedures and postoperative care were applied to all animals with the exception of the control group. Day 0 in all test groups was accepted as the day when the fracture was initiated. Determined main and subgroups were as follows:

1. Control Groups (C7-C21): Control group including healthy rats where no applications were conducted (sacrificed on the 7th and 21st days).

2. Fractured Mandible Groups (FM7-FM21): For standardization purposes, the subjects in this group were administered 1 mL saline solution with orogastric feeding tube after mandibular fracture was induced until they were sacrificed, which was conducted on 7th and 21st days.

3. Fractured Mandible + Grape Seed Extract Groups (FM+GSE7-FM+GSE21): Initial grapeseed extract was administered 1 hour before the operation and continued to be applied in 300 mg/kg/day doses with orogastric feeding tube for a total of 7 and 21 days and the subjects were sacrificed on the 7th and 21st days.

4. Fractured Mandible + Low Level Laser Therapy Groups (FM+LLLT7- FM+LLLT21): Starting from the day surgical operation was conducted, LLLT was applied on 2 different points on the fracture line for 7 and 14 days in 48 hour intervals and 25 seconds in each point (total 50 sec) and in 23 J/cm2 doses in contact with the skin using GaA1As Diode Laser (810 nm, model; Fotona XD-2 diode laser, Fotona, Ljubljana, SLOVENIA) in 0.3 W continuous operation mode and the subjects were sacrificed on the 7th and 21st days.

5. Fractured Mandible + Low Level Laser Therapy + Grape Seed Extract Groups (FM+LLLT+GSE7- FM+LLLT+GSE21): Initial grapeseed extract was administered 1 hour before the operation and continued to be applied in 300 mg/kg/day doses with orogastric feeding tube for a total of 7 and 21 days. Starting from the day surgical operation was conducted, LLLT was applied on 2 different points on the fracture line for 7 and 14 days in 48 hour intervals and 25 seconds in each point (total 50 sec) and in 23 J/cm2 doses in contact with the skin using GaA1As Diode Laser (810 nm, model; Fotona XD-2 diode laser, Fotona, Ljubljana, SLOVENIA) in 0.3 W continuous operation mode and the subjects were sacrificed on the 7th and 21st days.

Surgical Method and Postoperative Care

General anesthesia was performed with 70 mg/kg ketamine and 13 mg/kg xylazin injection to subjects. Right cheek region of the subjects were shaved and cleaned with antibacterial iodine solution and the subjects were prepared for the surgical procedure. Approximately 20 mm long submandibular incision was conducted on the right mandible of the subjects in front-back direction. Subcutaneous soft tissues were retracted starting from foramen mentale region with an obtuse dissection and the external mandible surface was approached by scraping the periosteum.

After the operation region became fully visible, a micro-screw socket was prepared on the mandible to match the second frontal perforation on the micro-plaque at the 1 mm posterior of the linea obliqua externa using a 0.8 mm diameter, 5 mm long drill under saline solution irrigation. Micro-plaque with 4 perforations was loosely adapted to the bone at this reference point with a 1 mm micro-screw. Furthermore, 1.0 mm in diameter and 4.0 mm long micro-screws were adapted loosely at the most posterior, most anterior and the remaining slot, respectively. Following this procedure, reference conduits were formed to create vertical corticotomy lines starting from between the premolar and molar teeth in the mandible and until the lower end of the corpus mandible. Then, these conduits were connected protecting the mandibular medial wall and surrounding soft tissues using a drill. Finally, a full fracture line was induced with a surgical chisel with reference to the corticotomy line. When it was observed that the segments were completely separated, micro-plaque micro-screws were fully screwed and the plaque was rigidly fixed on the bone. Surgical area was washed with
Preparation and Administration of Grapeseed Extract (GSE)

Black grape (Vitis vinifera L.) seeds used in the study were Çalkarası type and harvested in Denizli province in August 2012. Healthy seeds were separated and dried in Gaziantep University, Faculty of Arts and Sciences Biology Department laboratory and crushed into small particles in a mechanic blender. Crushed seeds were placed in Soxhlet Device (Gerhardt EV 14) cartridges in 100 g batches. Six hours of extraction was conducted in the Soxhlet device with 500 mL pure ethyl alcohol (Merck) per cartridge and at 50 – 60°C. Obtained extracts were filtered through Whatman no. 4 filter and condensed in a rotary evaporator (Heildolph Heizbad HB Digit) under high vacuum and at 40°C. Obtained extracts were stored until the tests at +4°C (12). GSE was diluted with 1 mL saline solution to weigh 100 mg and readied for orogastric gavage.

Low Level Laser Therapy (LLLT) Application

In the present study, 810 nm wavelength Fotona XD-2 GaAlAs diode laser (Fotona, Ljubljana, SLOVENIA) with 0.3 W output power was applied at two points on the fracture line in continuous operation mode for 25 seconds on each point (50 sec total) in the dose of 23 J/cm² in contact with the skin. The first dose was given initially after the surgical procedure and repeated at the same hour every 48 hours for standardization.

Sacrifice

Rats were euthanized on the 7th and 21st days, which were the terminal days of the study. Rats in all groups were administered intramuscular 45 mg/kg ketamine hydrochloride and 2.5 mg/kg xylazine to induce general anesthesia and after the subjects were sacrificed, liver tissue samples were stored at -80 °C until the biochemical analysis.

Biochemical Analyses

Liver tissues were homogenized in ice and 0.1 M Tris-HCl buffer (pH 7.5) that includes 1 mM protease inhibitor, phenyl-methyl-sulfonyl fluoride with a homogenizer (IKA Ultra Turrax T25 basic) at 16,000 rpm and at 4 °C for 3 min. MDA, GSH levels and SOD, CAT activities were measured using these homogenates.

Protein assay

Lowry’s method was used to determine liver tissue homogenate sample total protein content (13).
Empirical studies conducted with rats demonstrated that oxidative stress occurs during the first few weeks of the fracture and ROS is one of the factors that affect fracture recovery negatively (5, 19, 20). Studies reported that oxidative stress increases in the liver tissue induced by fracture positively correlated with ROS production. The study compared the statistical significance of SOD activity in all groups 7th and 21st days (P<0.05). GSH level in FM+LLLT+GSE group increased statistically significantly compared to FM+LLLT group in both 7th and 21th days (P<0.05).

Table 1. Liver tissue oxidant–antioxidant parameters of all groups 7th days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/gwt)</th>
<th>GSH (nmol/gwt)</th>
<th>SOD (U/g protein)</th>
<th>CAT (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>282 (269-296)^a</td>
<td>1830 (1780-1860)^a</td>
<td>35 (34-38)^b</td>
<td>32 (31-34)^a</td>
</tr>
<tr>
<td>FM</td>
<td>324 (314-354)^a</td>
<td>1834 (1700-1948)^a</td>
<td>34 (27-41)^b</td>
<td>27 (23-30)^d</td>
</tr>
<tr>
<td>FM+GSE</td>
<td>217 (197-234)^d</td>
<td>2670 (2590-2717)^a</td>
<td>20 (19-23)^c</td>
<td>49 (45-54)^a</td>
</tr>
<tr>
<td>FM+LLLT</td>
<td>358 (298-321)^b</td>
<td>1871 (1780-1900)^c</td>
<td>39 (37-44)^a</td>
<td>26 (22-29)^d</td>
</tr>
<tr>
<td>FM+LLLT+GSE</td>
<td>234 (201-268)^d</td>
<td>2251 (2179-2400)^b</td>
<td>38 (29-45)^sh</td>
<td>36 (32-39)^h</td>
</tr>
</tbody>
</table>

Data are expressed Median (Min-Max) of six animals. gwt; gram wet tissue. Different letters in columns are significant P<0.05.

Table 2. Liver tissue oxidant–antioxidant parameters of all groups 21th days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/gwt)</th>
<th>GSH (nmol/gwt)</th>
<th>SOD (U/g protein)</th>
<th>CAT (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>287 (279-300)^a</td>
<td>1820 (1750-1890)^a</td>
<td>35 (33-39)^b</td>
<td>32 (30-34)^a</td>
</tr>
<tr>
<td>FM</td>
<td>349 (320-408)^b</td>
<td>1846 (1793-1872)^b</td>
<td>46 (39-53)^a</td>
<td>24 (22-29)^a</td>
</tr>
<tr>
<td>FM+GSE</td>
<td>293 (284-308)^c</td>
<td>2325 (2200-2410)^a</td>
<td>36 (30-39)^h</td>
<td>42 (37-47)^g</td>
</tr>
<tr>
<td>FM+LLLT</td>
<td>398 (361-422)^a</td>
<td>1823 (1750-1897)^c</td>
<td>35 (32-42)^b</td>
<td>28 (21-31)^p</td>
</tr>
<tr>
<td>FM+LLLT+GSE</td>
<td>335 (261-361)^h,c</td>
<td>2275 (2200-2400)^a</td>
<td>38 (28-48)^sh</td>
<td>40 (37-45)^a</td>
</tr>
</tbody>
</table>

Data are expressed Median (Min-Max) of six animals. gwt; gram wet tissue. Different letters in columns are significant P<0.05.

SOD activity in MF group increased statistically significantly when compared to the control group on the 7th day (P<0.05). But, it did not change in the 21 days group (P>0.05). SOD activities in FM+LLLT, FM+GSE and FM+LLLT+GSE groups decreased statistically significantly compared to 7th day FM Group. There were statistically significant increases in SOD activities in FM+LLLT and FM+LLLT+GSE groups, while a decrease was observed in FM+GSE group when compared to 21th day MF group (P<0.05). SOD activity in FM+LLLT+GSE group increased statistically significantly compared to 7th day FM+LLLT group (P<0.05). But, it decreased in 21th day group (P>0.05).

CAT activities in FM group statistically significantly decreased when compared to control group on both 7th and 21th days (P<0.05). CAT activities in FM+LLLT, FM+GSE and FM+LLLT+GSE group liver tissues increased statistically significantly when compared to 7th day FM group (P<0.05). There were statistically significantly increases in CAT activities in FM+GSE and FM+LLLT+GSE groups when compared to 21th day FM group (P<0.05). But, CAT activity in FM+LLLT+GSE group did not differ statistically significantly when compared to 21th day FM group (P>0.05) (Table 2). CAT activities in FM+LLLT+GSE group increased statistically significantly compared to FM+LLLT group in both 7th and 21th days (P<0.05).

DISCUSSION

Certain biological events that occur during the first few weeks of the fracture affect the recovery process negatively. Studies reported that oxidative stress is one of the factors that affect fracture recovery negatively (5, 19, 20). Empirical studies conducted with rats demonstrated that ROS induced damage is higher on post-fracture 7th and 14th days and starts to decrease on the 4th week (21,22).

Liver that has a relation with almost all systems of the organism and extremely complex and significant functions is one of the largest organs in our body and has intense metabolic activity which includes detoxification. In this process called biotransformation, free radicals could emerge under physiological conditions. In this situation, sufficient levels of antioxidant intake protect the liver against free radical damage. Sufficient levels of antioxidant intake also help reduce the risk of free radical damage produced in the detoxification process (23,24). In the present study, in order to investigate their effects on rat mandibular fracture recovery, oxidant and antioxidant potentials of low level laser therapy and proantocyanide rich grapeseed extract known for its potent antioxidant properties and their combined use and effects of these applications on rat liver tissue after the experiment was terminated on 7th and 21th days were compared by analyzing biochemical parameters.

Furthermore, the present study is the first study that assesses the changes in rat liver tissue induced by the application of GSE LLLT combination, which is reported to increase antioxidant activity, to ameliorate LLLT application of GSE LLLT combination, which is reported to increase antioxidant activity, to ameliorate LLLT
21 days long recovery process would be sufficient for the immature bone tissue and determined periods were significant to demonstrate the degree that the therapy processes affected the recovery rate. LLLT is among the techniques used to facilitate the recovery process in bone tissue during recent years. Conducted studies analyzed the therapeutic action of LLLT in the first connective tissue and it was determined that in time LLLT reduced inflammation, stimulated fibroblast proliferation and collagen production, increased local micro-vascularization and bone mineralization, and implant osteointegration by stimulating osteoblastic activity, and it was proven that these properties of laser had positive effects on bone recovery (27–29).

Researchers stressed that laser application during the early stage facilitated bone recovery, while its application in late stages did not play an important role in bone regeneration. Furthermore, it is known that laser had effects on reproduction, maturing and bone matrix release of differentiated cells. Thus, it was also mentioned that by triggering vascularization and inflammatory response, it facilitates bone matrix production as well (30). However, in certain studies, although the positive effect of LLLT on hard and soft tissue recovery was demonstrated, it was observed that the estimated effect on recovery was not seen or the laser had negative effects (31–33). In our study, we observed that MDA and CAT levels in the liver of the rats in the groups where mandibular defect + laser were applied both for 7 and 21 days were similar to that of the mandibular defect group (P<0.05). Under certain conditions where free radicals are produced extensively such as inflammation or trauma, endogenous antioxidants could be insufficient. Under such conditions, Halliwell and Gutteridge stated that antioxidant rich nutrients should be consumed to reduce the tissue damage (34). Also in our study, we concluded that intake of an exogenous antioxidant in sufficient dose and duration to supplement the present defense system during the post-fracture recovery period where ROS and oxidative stress increase would be beneficial.

Bagchi et al. stressed that GSE, which has better protective properties than Vitamin E, C and their combination, is a safe antioxidant to add in the daily diet and biologically usable (35). Proanthocyanidins, which are shown to have no adverse effects in previous studies, are food supplements that have been used in America and Europe for many years and because of these properties, they are included in the safe nutrient category by the FDA and recommended GSE dose is 100 – 300 mg per day (36). Numerous studies were conducted on GSE due to its biological and pharmacological properties in recent years. Antibacterial, antifungal, antiviral and anti-carcinogenic effects of GSE on human health were reported (37). It was also mentioned that oligomeric proanthocyanidins included in the extract could have anti-inflammatory and antitumor action, could prevent heart diseases and ageing and acts as a protector against ischemic perfusion damage (37–39). In other study, Khanna et al. investigated the impact of proanthocyanidins on wound healing and created excisional injury on the back of the rats and applied 100 mg/mL topical GSE for 5 days. It was observed that the wound scabbed more rapidly in topical GSE applied group when compared to the control (40).

Although several studies were conducted on GSE in different fields, recent studies that investigated oxidant / antioxidant system changes, which occur due to bone tissue defects and their recovery process, are quite limited, however there are studies that researched directly the effects on bone defects, and in such a study, Park et al. reported that GSE could be beneficial in protection from bone destruction observed in autoimmune inflammatory arthritis (41).

In a study, which scrutinized the effect of GSE on mandible, it was demonstrated that rats fed with standard feed combined with GSE had higher mandibular cortical bone density, cortical and trabecular bone mineral content, in addition to increased mechanical bone properties such as formation, quality and endurance (42, 43). These limited number of studies showed that GSE was quite active on bone tissue. The findings of the present study also demonstrated that GSE was quite effective on mandibular defect induced rat liver as a result of the applied dose and period and displayed protective antioxidant action in liver tissue against the oxidative damage that could occur during bone recovery process. A statistically significant increase was observed in GSH levels and CAT activity in GSE and LLLT + GSE applied groups both on 7th and 21st days when compared to the control group. However, a similar antioxidant potential increase was observed in LLLT + GSE applied groups both on 7th and 21st days only compared to the LLLT applied groups. SOD activity values during fracture recovery period in GSE 7 and GSE 21 groups were similar. The fact that GSE application would prevent Superoxide production and could remove the oxidative damage was observed via the reductions in SOD activity and MDA levels. As a result, we consider that GSE had a good level action against oxidants in early and late periods of fracture recovery. Literature review would demonstrate that radical production had increased after 7th and 14th days and until 22nd day during the fracture recovery process. In fact, reductions in SOD activity during 7 and 21 days long GSE applications in the processes of mandibular fraction generation process and the recovery process of this defect were observed in our study as well. Findings of this study are consistent with the results of previous studies.

Previous studies showed that proanthocyanidins are quite effective against tissue damage and improved wound healing and angiogenesis (44–46). In the present study, it was observed that generally it was possible to remove oxidative damage in all test groups compared to mandibular fracture groups. It was also observed that combined laser and GSE applications were more effective on removal of possible oxidative damage that could develop in liver tissue as a result of mandibular fracture and antioxidant effect was more present when compared to laser monotherapies during fracture recovery. Preventive potential of combined GSE applications in laser therapies in removal of possible
oxidative damage that could occur in liver tissue was also remarkable. Although there are several studies in the literature that addressed GSE activity, no previous study that investigated the effect of mandibular fracture defects and their recovery process on the liver tissue was found. Furthermore, although several studies that investigated the action of LLLT on fracture recovery using different doses, since sufficient information is not available on its combined use with GSE and the biochemical changes it causes especially in liver tissue in this process, the findings of the present study are considered significant for the effects that mandibular fracture defects could cause in the oxidant / antioxidant system in liver tissue. As stressed previously, the most important characteristics of GSE are its wide dose range and lack of toxicity and maintenance of a certain serum level for 7–10 days after the last oral dose (35, 47).

Studies conducted on acute and chronic toxicity of proanthocyanids are also available. It was reported that generally no toxic and mutagenic effects of proanthocyanids were observed even in high doses (1400 – 1500 mg/kg/day) (48). In a study conducted by Yamakoshi et al. on acute and sub-chronic oral toxicity of GSE in rats, it was reported that toxicity and mutagenicity were not observed even in 2 and 4 g/kg doses administered for 14 days (49). Also, the effect of long-term GSE use was scrutinized. Ray et al. supplemented 100 mg/kg GSE for 1 year in the diet of male Bc3F1 mice or 500 mg/kg GSE for 6 months in the diet of female mice and reported that no adverse effects were observed in subjects’ vital organs at the end of the study (50). In addition to all these studies, it was reported that LD50 value of GSE administered to male and female albino rats using gastric intubation method was more than 5000 mg/kg (36).

CONCLUSION

In our study, it was determined that mandibular fracture defect was a factor that increased lipid peroxidation in liver tissue and induced oxidative damage. It was identified that 7 and 21 day long LLLT and GSE applications had different effect on liver tissue oxidant / antioxidant systems in both different time periods. In applications had different effect on liver tissue oxidant / antioxidant systems in both different time periods. In this study, determination of nitric oxide synthase distribution during fracture healing was carried out in different tissue samples in animals. Although there are several studies in the literature that addressed GSE activity, no previous study that investigated the action of LLLT on fracture recovery using different doses, since sufficient information is not available on its combined use with GSE and the biochemical changes it causes especially in liver tissue in this process, the findings of the present study are considered significant for the effects that mandibular fracture defects could cause in the oxidant / antioxidant system in liver tissue. As stressed previously, the most important characteristics of GSE are its wide dose range and lack of toxicity and maintenance of a certain serum level for 7–10 days after the last oral dose (35, 47).

REFERENCES


