ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

Med J SDU / SDÜ Tıp Fak Derg ▶ 2019:26(1):96-103 doi: 10.17343/sdutfd.511364

DOES SHILAJIT HAVE AN EFFECT ON NEW BONE REMODELLING IN THE RAPID MAXILLARY EXPANSION TREATMENT? A BIOCHEMICAL, HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

SHİLAJİT'İN HIZLI MAKSİLLER GENİŞLETME TEDAVİSİNDE YENİ KEMİK ŞEKİLLENMESİ ÜZERİNE ETKİSİ VAR MI? BİYOKİMYASAL, HİSTOPATOLOJİK VE İMMÜNOHİSTOKİMYASAL BİR ÇALIŞMA

Mine GEÇGELEN CESUR[1], Gözde ÖĞRENİM[1], Kanat GÜLLE[2], Fevziye Burcu ŞİRİN[3], Meryem AKPOLAT[4], Gökhan CESUR[5]

[1] Department of Orthodontics, Faculty of Dentistry, Adnan Menderes University, Aydın, Turkey.
[2] Department of Histology and Embryology, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey.
[3] Department of Biochemistry, Faculty of Medicine, Suleyman Demirel University, Isparta, Turkey.
[4] Department of Histology and Embryology, Faculty of Medicine, Bulent Ecevit University, Zonguldak, Turkey.
[5] Department of Physiology, Faculty of Medicine, Adnan Menderes University, Aydın, Turkey.

Cite this article as: Geçgelen Cesur M, Öğrenim G, Gülle K, Şirin FB, Akpolat M, Cesur G. Does shilajit have an effect on new bone remodelling in the rapid maxillary expansion treatment? A biochemical, histopathological and immunohistochemical study. Med J SDU 2019; 26(1): 96-103.

Öz

Amaç
Bu çalışmanın amacı sıçanlarda hızlı maksiller genişletme (RME) sonrası shilajit'in yeni kemik şekillenmesine etkilerini histopatolojik, biyokimyasal ve immünohistokimyasal teknikler kullanarak göstermektir.

Gereç ve Yöntem
Wistar Albino türü sıçanlar (12 haftalık) 3 farklı gruba ayrılmıştır (n = 8): üst çenede genişletme yapılmamış (NE), üst çenede sadece genişletme yapılmış (OE), üst çenede genişletmeye ilave olarak shilajit uygulanmış (Shilajit). Sıçanlara 5 günlük genişletme ve 12 günlük retansiyon periyodu süresince shilajit uygulandı. Hayvanlar sakrifiye edildikten sonra gerekli numuneler alınıp histopatolojik, biyokimyasal ve immünohistokimyasal analizler yapıldı.

Bulgular
Shilajit grubundaki glutatyon peroksidaz, süperoksit dismutaz ve katalaz düzeyleri OE grubundan daha yüksek bulundu ($p < 0.05$). C telopeptid tip I kollajen ve kemik alkalen fosfataz seviyeleri gruplar arasında anlamlı farklılıklar gösterdi ($p < 0.001$). İmmünohistokimyasal bulgular, OE grubunun shilajit grubundan anlamlı olarak daha fazla IL-1 ve TNF-α H skorlarına sahip olduğunu gösterdi ($p < 0.05$). Tüm gruplar kılcal damar yoğunluğu, inflamatuar hücre infiltrasyonu ve yeni kemik oluşumu açısından karşılaştırıldığında gruplar arasında belirgin farklılıklar bulundu ($p < 0.05$).

Sonuç
Shilajit'in sistemik kullanımı, midpalatal suturda yeni kemik şekillenmesini hızlandırarak, RME tedavisinden sonra nüksün önlenmesinde ve retansiyon süresini kısaltmada faydalı olabilir.

Anahtar Kelimeler: Hızlı maksiller genişletme, shilajit, kemik oluşumu

İletişim kurulacak yazar/Corresponding author: minegecgelen@hotmail.com
Müracaat tarihi/Application Date: 10.01.2019 • Kabul tarihi/Accepted Date: 12.02.2019

Abstract

Objective
The goal of this study is to show the effects of shilajit on new bone remodelling after rapid maxillary expansion (RME) in rats utilizing histopathological, biochemical and immunohistochemical techniques.

Material and Method
The rats (12-week-old) were divided into the 3 groups (n=8): no maxillary expansion (NE), only maxillary expansion (OE), maxillary expansion + shilajit (Shilajit). Rats received to shilajit throughout the 5 day expansion and 12 day maintenance time. All rats were sacrificed at the same time. After sacrificing the animals, required tissues were taken and histopathological, biochemical and immunohistochemical analysis were performed.

Results
Glutathione peroxidase, superoxide dismutase and catalase levels within the shilajit (shilajit + expansion) group were measurably higher than the OE group ($p<0.05$). C-telopeptide type I collagen and bone alkaline phosphatase levels illustrated significant differences between the all groups ($p<0.001$). The immunohistochemical evidences showed that OE group had essentially more IL-1 and TNF-α H-scores than the shilajit group ($p<0.05$). When the groups were compared for capillary intensity, inflammatory cell infiltration and new bone formation, significant differences were observed between the groups ($p<0.05$).

Conclusion
Systemic utilize of shilajit may accelerate new bone remodelling within the midpalatal suture, which may be beneficial to prevent of and shorten the retention period after the RME treatment.

Keywords: Rapid maxillary expansion, shilajit, bone formation

Introduction

Rapid maxillary expansion (RME) is an efficient orthodontic treatment which has been routinely used procedure by orthodontists. It has two different phases, namely, active stage and passive stage. In active stage, midpalatal suture broadens by expansion forces to disarticulate the two parts of the maxillary bone. In passive stage, suture tissues undergo bone remodeling, resorption and fiber rearrangement up to a new stability is provided (1,2). The expanded maxillary dental arch tends to return to its pretreatment widths after retention period. The literatures reported that prevelance of relapse can approach as high as 45% (2,3).

Numerous researchers have attempted certain methods to enhance bone remodelling in midpalatal suture, such as bisphosphonates (3), vitamins (4), laser incitement (5-8), stem cells (9), low-intensity pulsed ultrasound (10), sex steroids (11), hyaluronic acid (12) and antioxidants (13-15). Right now, antioxidant treatments have been investigated due to their effect on bone metabolism through hindering osteoclastic activity and encouraging osteoblastic activity (14,15).

Shilajit has been known and used as a therapeutic agent in tradiational medicine. Shilajit contains fatty acids, benzoic acid and vitamins, such as B1 and B12, and different other antioxidant compounds (16,17). The antioxidant properties of shilajit can be caused by both dibenzo-α-pyrones and fulvic acid (18). Shilajit is well researched and regarded as a reliable material (16,19). According to this information, it might be advantageous to use shilajit, organic and cheap substance, so as to stimulate bone regeneration at the expanded midpalatal suture.

Materials and Methods

Animals and Groups
Twenty four, 12-week-old male Wistar albino rats used for this study. The animals were taken from the Animal Laboratory at Adnan Menderes University, School of Medicine. Ethical permission was obtained from the University of Adnan Menderes Local Ethics Committee for Animal Experiments (64583101/2014/064).

The rats were randomly divided into the subsequent three groups (n=8): no maxillary expansion (NE), only maxillary expansion (OE) and maxillary expansion + shilajit (Shilajit). Within the shilajit group, 100 mg/kg/day shilajit was administered systemically via orogastric catheter throughout the expansion and retention phases.

Appliance Placement
Expansion appliances consisting of helical springs fabricated from 0.012-inch stainless steel wires were attached to the maxillary incisors of all the rats. The expansion force was adjusted to 30 g and not reactivated during the 5-day expansion period. Spring was fixed to the maxillary incisors with composite (Figure 1). After the 5 days expansion period, the helical

springs replaced with short lengths of rectangular wire for retention phase for 12 days (20).

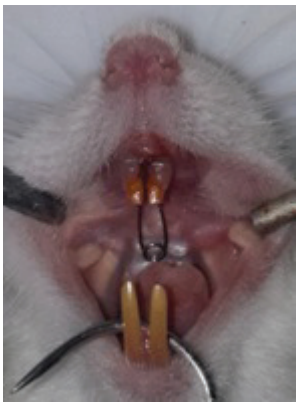

Figure 1. Placement of expansion spring

Histopathological Analysis

At the end of the experiment period, all animals were sacrificed in accordance with animal ethics guidelines. Premaxil¬lae of all animals have dissected. Premaxilla tissues were fixed with neutral formalin. After fixation, samples were decalfied in %10 EDTA melt. approximately 30 days after decalcification, the premaxillae were cut into blocks with one cut passing through the incisor crowns at the alveolar crest and perpendicular to the longitudinal plane, the second cut 4 mm apical to the first cut. All samples were then dehydrated and embedded in hot melting paraffin wax. Paraffin blocks were obtained. All paraffin blocks were sectioned (5 µm thick) and stained with Hematoxylin-Eosine for routine light microscopic analysis. Histopathological analysis was performed by a pair of examiners blind to the identity of the sections. All of groups were compared to establish the new bone formation, inflammatory cell infiltration, and capillary intensity. The intensities were rated as mild (+, score=1), moderate (++, score=2), or strong (+++, score=3) (20).

Immunohistochemical Analysis

Avidin–Biotin Complex (ABC) and the Labeled Streptavidin–Biotin (LSAB) staining methods was used immunohistochemical studies for investigate the TNF-α, IL-1 and IL-6 expression. 4 µm thick sections in premaksilla tissues with poly lysine slides. All sections were taken routine histological processing. With the aim of exposing the receptor areas at intervals the tissue blocked by formaldehyde, citrate buffer (pH 6.0) (Lab Vision, Fremont, USA) was applied to microwave. All slides were allowed in the room tempature to cool down for 20 minutes after antigen retrieval processing. 3% hydrogen peroxide was applied after washing step for 15-20 minutes. Ultra V block was applied for five minutes to stop nonspecific binding (Lab Vision, Fremont, USA). After the blocking stage, the sections were allowed at room temperature for forty five minutes while not being washed and were exposed to anti IL-1 protein (ab9722, abcam, UK), anti IL-6 protein (ab6672, abcam, UK), anti TNF-α protein (NB600-587, novus biological, USA) and primer antibodies that were ready 1/100 proportion. All primary antibodies were incubated at room temperature. Secondary antibody (Thermo Scientific, USA) was exerted for 30 minutes, rinsed with PBS, and exposed to streptavidin peroxidase enzyme (Thermo Scientific, USA) complex for 15 minutes. Lastly, DAB substrat kit (ab64238)) was applied and was allowed for about 5-10 minutes to provide the immune reaction. Counterstaining was done with Mayer's hematoxylin. All slides were mounting with entellan. Then all sections were examined with Axio Scope A1 Imager Microscope (Carl Zeiss, Oberkochen, Germany).

Results from immunohistochemistry were outlined in H-score resulting from the number of cells with activated TNF-α, IL-1 and IL-6 (0%-100% cells) multiplied by the intensity of staining (1=low, 2=medium and 3=high). All slides were analyzed and scored using double-blinded protocol (20).

Biochemical Analysis

Rat serum CTX-I (C-telopeptide type I collagen) and BALP (bone alkaline phosphatase) levels were measured using the rat CTX-I enzyme-linked immunosorbent assay kit and BALP enzyme-linked immunosorbent assay kit (Cusabio Biotech Co, Ltd, Wuhan, China). Malondialdehyde (MDA) was determined by the method of Drapper and Hadley (21). The measurement of Superoxide dismutase (SOD) was based upon the guideline that xanthine reacts with xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT). SOD activity is then measured by the degree of inhibition of this reaction (22). The determination of glutathione peroxidase (GSH-Px) activity was based on the method of Paglia and Valentine (23). Catalase (CAT) activity was measured according to the method of Aebi (24).

Statistical Analysis

The distribution of continuous variables such as BALP, CTX-I, IL-1 H-score were evaluated by Shapiro-Wilk test. Normally distributed continuous variables were shown as mean ± standard deviation (mean±sd), median (min-max) was additionally given for non-normal-

ly distributed continuous variables, while histopathological scores were expressed as both count (n) and median (min-max).

Groups were compared by one-way ANOVA and Kruskal-Wallis test with Bonferroni correction in respect to normally distributed variables, respectively. The Welch's statistics was given for the variables with heterogeneous variances. Bonferroni and Games-Howel tests were used to make pairwise comparisons for the variables with and without homogenous variances, respectively. Histopathological scores of groups were analysed by chi-square tests (linear-by-linear association) and Kruskal-Wallis test with Bonferroni correction as post-hoc. Test statistics and p values were given as a result of all analyses. A $p<0.05$ was accepted as statistically significance. All statistical analyses and calculations were performed by IBM SPSS Statistics 21.0 (Armonk, NY: IBM Corp.).

Results

Biochemical Findings

Serum BALP and CTX-I values were dramatically higher in the shilajit group than the NE group ($p<0.05$). OE group had a significantly greater CTX-I values than the NE group ($p<0.05$). Shilajit group showed an increase in SOD, GSH-Px, and CAT values relative to the OE group ($p<0.05$) MDA values are significantly lower in the NE group than the OE group ($p<0.05$) (Table 1).

Histopathological Findings

The groups were compared based on inflammatory cell infiltration, and significant differences were observed among the groups ($p=0.221$). OE group had significantly more capillary intensity than the NE group ($p=0.049$) (Table 2 and 3).

When the groups were evaluated for new bone formation, considerable differences were found between the groups ($p=0.001$). Increased new bone formation was found in the OE and shilajit groups relative to the NE group (Table 2 and 3; Figure 2).

There were significant differences between the groups in terms of capillary intensity ($p<0.001$). The results showed that there was an increase in capillary intensity in the shilajit and OE groups than the NE group (Table 2 and 3; Figure 2).

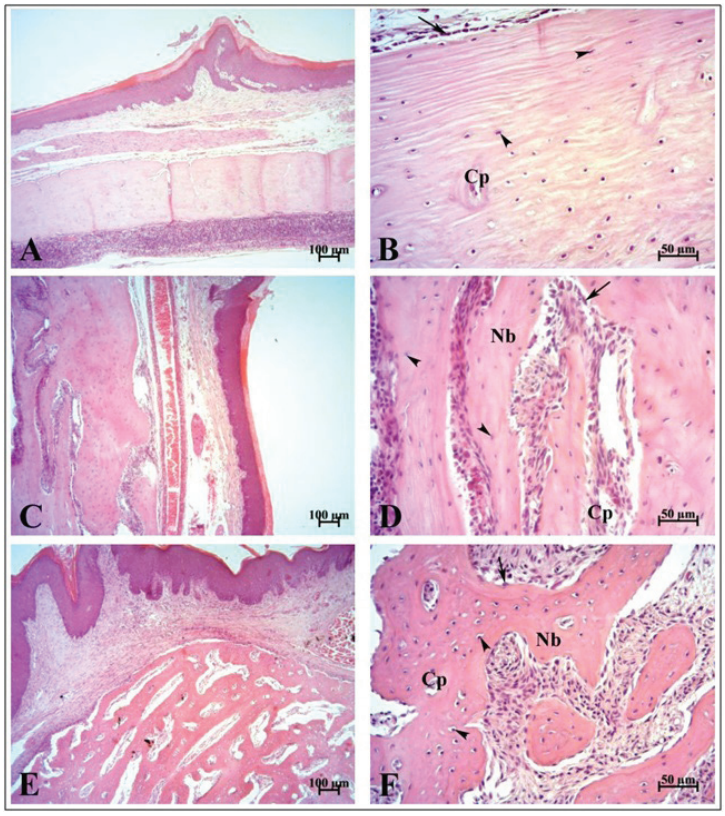

Figure 2. Hematoxylin and eosin staining photomicrographs from the study groups. **A-B:** NE group, **C-D:** OE group, **E-F:** Shilajit group. Increased osteoblastic activity, new bone and capillary formation observed in treatment groups. Nb: New bone, Cp: capillary, arrow: osteoblast, arrowhead: osteocyt. Scale bar; A,C,E,G,I 100 µm, B,D,F,H,J 50 µm.

Immunohistochemical Findings

Results of immunohistochemical examination indicated that IL-1, IL-6 and TNF- α H scores displayed considerable differences between the groups (p<0.001). H-scores of TNF- α, IL-1 and IL-6 were found quite high in the OE group than the other groups (p<0.05) (Table 4; Figure 3).

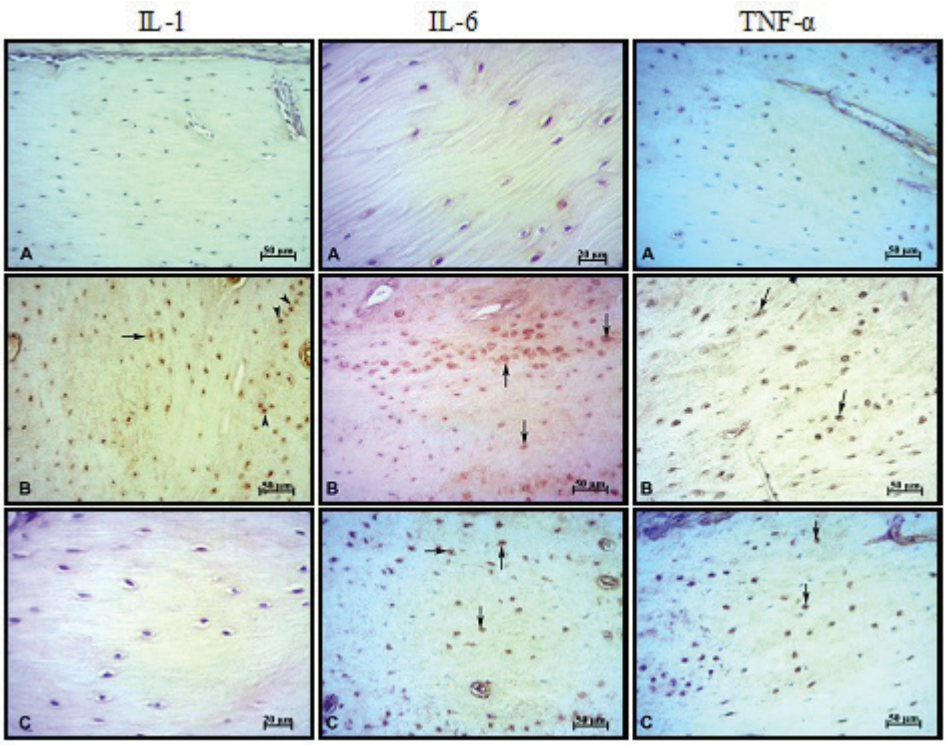

Figure 3. Immunohistochemical staining photomicrographs from the study groups.
A: NE group, **B:** OE group, **C:** Shilajit group. Strongly positive IL-1, IL-6, and TNF-α immunoreactivity were observed in both membranous (arrowheads) and nuclei (arrowheads) in osteocytes at new bone formation sites in OE group. The intensity of staining decreased in shilajit group, especially there was a similarity for NE group and shilajit group in terms of IL-1 (Scale bar=50µm).

Table 1 Effects of Shilajit on the BALP, CTX-I, SOD, GSH-Px, CAT, and MDA levels at the end of the experimental period.

	NE Group	OE Group	Shilajit Group	F, χ^2	p
BALP* **(U/L)**	27.73±3.97* 29.65 (20.8 – 30.8)**,1	40.06±4.55 39.20 (35.6 – 49.3)	55.39±2.99 54.85 (51.1 – 59.5)1	χ^2=20.480	<0.001
CTX-I* (pg/ml)	261.69±36.33[1,2]	359.05±23.19[1]	372.38±49.85[2]	F‡=21.687	<0.001
SOD* (U/g Hb)	2616.20±237.83[1,2]	2281.59±233.70[2,3]	2961.82±277.012[1,3]	F=14.775	<0.001
GSH-Px* (U/g Hb)	31.62±1.93 31.32 (28.64 – 34.49)	26.91±2.94 25.57 (24.31 – 31.33)[1]	34.44±1.50 34.44 (32.54 – 36.35)[1]	χ^2=15.680	<0.001
CAT* (kU/g Hb)	10.56±1.23[1]	9.79±2.08[2]	15.39±3.09[1,2]	F‡=9.407	0.003
MDA* (nmol/g Hb)	182.13±12.52[1]	212.64±9.64[1]	196.29±29.65	F‡=14.268	0.001

*mean±sd;** median (min-max);[1,2,3]p<0.05; ‡Welch statistics

Table 2 Effects of Shilajit on the inflammatory cell infiltration, new bone formation and capillary intensity at the end of the experimental period. Scores indicate the number of subject animals representing that score.

	NE Group	OE Group	Shilajit Group	χ^2	p
Inflammatory cell infiltration				1.500	0.221
Score 1	6	2	3		
Score 2	0	2	3		
Score 3	0	2	0		
New bone formation				11.822	0.001
Score 1	6	3	0		
Score 2	0	2	1		
Score 3	0	1	5		
Capillary intensity				12.201	<0.001
Score 1	6	3	0		
Score 2	0	3	2		
Score 3	0	0	4		

Table 3 Pairwise comparisons of the inflammatory cell infiltration, new bone formation and capillary intensity scores across groups.

	NE Group	OE Group	Shilajit Group	χ^2	p
Inflammatory cell infiltration*	1 (1-1)[1]	2 (1-3)[1]	1.5 (1-2)	6.025	0.049
New bone formation*	1 (1-1)[1]	1.5 (1-3)	3 (2-3)1	12.183	0.002
Capillary intensity*	1 (1-1)[1]	1.5 (1-2)	3 (2-3)1	12.828	0.002

*median (min-max); [1,2]p<0.05

Table 4 Effects of Shilajit on IL-1, IL-6, and TNF-α H score analysis at the end of the experimental period.

	NE Group	OE Group	Shilajit Group	F, χ^2	p
IL-1 H Score*	108.00±3.58[1]	270.00±6.93[1,2]	112.50±4.28[2]	F=1936.394	<0.001
IL-6 H Score*	109.17±1.83 109 (107 - 112)[1]	286.33±6.38 287 (278 - 294)[1]	131.83±6.21 134.5 (120 - 136)	χ^2=15.205	<0.001
TNF-α H Score*	106.50±1.76[1,2]	251.67±9.99[2,3]	124.00±4.73[1,3]	F‡=583.411	<0.001

*mean±sd;** median (min-max);[1,2,3]p<0.05; ‡Welch statistics

Discussion

RME is used to correct transversal discrepancies between the maxilla and mandible. Midpalatal suture remodeling plays an active role in the expansion process, but related molecular mechanisms under expansion force are not fully understood. Orthodontists have used different substances during or after RME to increase bone formation and shorten retention period (11).

Different substances were studied for enhancing bone remodelling during the expanded midpalatal suture in rats to this date. Ozturk et al. (3) investigated the effect of zoledronic acid in the expanded midpalatal suture and showed that significant increases in osteoblast numbers and immunoreactivity in the suture area. Rosa et al. (8) aimed to examine the effectiveness of laser or LED phototherapy after maxillary expansion and reported higher generation of collagen and osteoblastic and fewer osteoclastic activity. Uysal et al. (13) evaluated the local resveratrol injection to the inter-premaxillary suture and stated that bone histomorphometric measurements were higher in the resveratrol treated rats. Cesur et al. (20) used curcumin and melatonin in their rat study model and suggested that systemic application of these agents induce bone remodelling and could abbreviate the retention period. In this study, we studied effects of systemically applied shilajit on bone remodelling in response to expansion of the rat midpalatal suture. According to literature information, our study is the first to research the effects of shilajit use on bone remodelling.

We preferred rat model for experimental maxillary expansion in this study similar to previous studies (1-15). Altan et al. (14) reported that rats and rabbits were well-established animal models for showing the changes in the midpalatal suture under forces.

Various studies have reported a correlation between oxidative stress and bone metabolism. Oxidative stres caused by excessive generation of intracellular reactive oxygen species (ROS) can exert adverse biological effects on bone metabolism. ROS considerably affect the osteoclasts, osteoblasts, osteocytes and play a role in bone resorption. Also, osteoblasts produce antioxidants, such as glutathione peroxidase to protect against ROS (11,15,25).

Shilajit is a multi-component natural occurring mineral substance, recently gained attention because of its antioxidant, immunemodulating, and anti-inflammatory effects (16,18). Wilson et al. (18) reported that intraperitoneal administration of shilajit induced an increase of SOD, CAT, and GSH-Px activities. In the present study, GSH-Px, SOD and CAT levels significantly rised together in the shilajit group relative to the OE group.

Jung et al. (26) reported that shilajit is a potent stimulator of osteoblastic differentiation of mesenchymal stem cells and inhibitor of osteoclastogenesis. Their studies showed that the levels of osteoblastic markers such as ALP activity, the effect of shilajit on osteoblastic differentiation ranges from 35% to 90% of the osteogenic medium stimulatory action. CTX is a biochemical indicator of osteoclastic activity and is used to evaluate the level of bone resorption (27). In the present study, increased CTX-I levels may be related to the acceleration of bone turnover. Elevated BALP levels in shilajit group showed the differentation of mesenchymal stem cells into osteoblasts and increases bone formation.

Pro-inflammatory cytokines (such as IL-1, IL-6 and TNF- α) lead to differentiation of osteoclast precursors and osteoclast activity that then gives rise to bone resorption (28). IL-1, IL-6 and TNF- α H scores were decreased in the shilajit group relative to the OE group in our study. Shilajit decreased the reactive oxygen species production and the level of these cytokines.

The effect of shilajit on the inflammatory cell infiltration, new bone formation and capillary intensity were studied histopathologically. The amount of new bone formation and capillary intensity were increased in the shilajit group relative to the OE group. These results are in agreement with another researchs that used antioxidants and found that increase in bone formation (1,14,15,25). In contrast to previous studies (14,15), there was no significant difference for the inflammatory cell infiltration between the groups in our study. This difference in results may be clarified by the fact that the shilajit could be responsible for preventing the nuclear factor (NF)-kappaB, an osteblastic differentiation marker elicited by ROS, thereby, the reduction of the inflammation.

Our study revealed that application of shilajit during the orthopedically expanded midpalatal suture stimulates bone regeneration, reduces inflammation and improves bone healing. Moreover, it may be beneficial in shortening the retention period and preventing relapse after the RME treatments. Further clinical research is needed to clarify its effects on humans and also ascertain whether it should be used prophylactically.

Funding source, financial disclosures: None.
Conflict of interest: The authors declare no conflicts of interest related to this study.

References

1. Özan F, Çörekçi B, Toptaş O, Halicioğlu K, Irgin C, Yilmaz F, et al. Effect of Royal Jelly on new bone formation in rapid maxillary expansion in rats. Med Oral Patol Oral Cir Bucal 2015;20:651-6.
2. Zhao S, Wang X, Li N, Chen Y, Su Y, Zhang J. Effects of strontium ranelate on bone formation in the mid-palatal suture after rapid maxillary expansion. Drug Des Devel Ther 2015;9:2725-34.
3. Oztürk F, Babacan H, Inan S, Gümüş C. Effects of bisphosphonates on sutural bone formation and relapse: A histologic and immunohistochemical study. Am J Orthod Dentofacial Orthop 2011;140:31-41.
4. Farhadian N, Miresmaeili AR, Zargaran M, Moghimbeigi A, Soheilifar S. Effect of dietary ascorbic acid on osteogenesis of expanding midpalatal suture in rats. J Dent (Tehran) 2015;12:39-48.
5. Da Silva AP, Petri AD, Crippa GE, Stuani AS, Stuani AS, Rosa AL, et al. Effect of low-level laser therapy after rapid maxillary expansion on proliferation and differentiation of osteoblastic cells. Lasers Med Sci 2012;27:777-83.
6. Aras MH, Bozdag Z, Demir T, Oksayan R, Yanık S, Sökücü O. Effects of low-level laser therapy on changes in inflammation and in the activity of osteoblasts in the expanded premaxillary suture in an ovariectomized rat model. Photomed Laser Surg 2015;33,136-44.
7. Amini F, Najaf Abadi MP, Mollaei M. Evaluating the effect of laser irradiation on bone regeneration in midpalatal suture concurrent to rapid palatal expansion in rats. J Orthod Sci 2015;4:65-71.
8. Rosa CB, Habib FA, de Araújo TM, Dos Santos JN, Cangussu MC, Barbosa AF, et al. Laser and LED phototherapy on midpalatal suture after rapid maxilla expansion: Raman and histological analysis. Lasers Med Sci 2017;32:263-74.
9. Ekizer A, Yalvac ME, Uysal T, Sonmez MF, Sahin F. Bone marrow mesenchymal stem cells enhance bone formation in orthodontically expanded maxillae in rats. Angle Orthod 2015;85:394-9.
10. Toy E, Oztürk F, Altindiş S, Kozacioğlu S, Toy H. Effects of low-intensity pulsed ultrasound on bone formation after the expansion of the inter-premaxillary suture in rats: a histologic and immunohistochemical study. Aust Orthod J, 2014;30:176-83.
11. Birlik M, Babacan H, Cevit R, Gürler B. Effect of sex steroids on bone formation in an orthopedically expanded suture in rats: An immunohistochemical and computed tomography study. J Orofac Orthop 2016;77:94-103.
12. Sadikoglu TB, Nalbantgil D, Ulkur F, Ulas N. Effect of hyaluronic acid on bone formation in the expanded interpremaxillary suture in rats. Orthod Craniofac Res 2016;1:154-61.
13. Uysal T, Gorgulu S, Yagci A, Karslioglu Y, Gunhan O, Sagdic D. Effect of resveratrol on bone formation in the expanded inter-premaxillary suture: early bone changes. Orthod Craniofac Res 2011;14:80-7.
14. Altan BA, Kara IM, Nalcaci R, Ozan F, Erdogan SM, Ozkut MM, et al. Systemic propolis stimulates new bone formation at the expanded suture: a histomorphometric study. Angle Orthod 2013;83:286-91.
15. Kara MI, Altan AB, Sezer U, Erdoğan MŞ, Inan S, Ozkut M, et al. Effects of Ginkgo biloba on experimental rapid maxillary expansion model: a histomorphometric study. Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:712-8.
16. Kececi M, Akpolat M, Gulle K, Gencer E, Sahbaz A. Evaluation of preventive effect of shilajit on radiation-induced apoptosis on ovaries. Arch Gynecol Obstet 2016;293:1255-62.
17. Agarwal SP, Khanna R, Karmarkar R, Anwer MK, Khar RK. Shilajit: a review. Phytother Res 2007;21:401-5.
18. Wilson E, Rajamanickam GV, Dubey GP, Klose P, Musial F, Saha FJ, et al. Review on shilajit used in traditional Indian medicine. J Ethnopharmacol 2011;136:1-9.
19. Stohs SJ. Safety and efficacy of shilajit (mumie, moomiyo). Phytother Res 2014;28:475-9.
20. Cesur MG, Gülle K, Şirin FB, Akpolat M, Ögrenim M, Alkan A, et al. Effects of curcumin and melatonin on bone formation in orthopedically expanded suture in rats: A biochemical, histological and immunohistochemical study. Orthod Craniofac Res 2018;21:160-7.
21. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1996;186:421-31.
22. Woolliams JA, Wiener G, Anderson PH, McMurray CH. Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood various breed crosses of sheep. Res Vet Sci 1983;34:69-77.
23. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.
24. Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121-6.
25. Irgin C, Çörekçi B, Ozan F, Halicioğlu K, Toptaş O, Birinci Yildirim A, et al. Does stinging nettle (Urtica dioica) have an effect on bone formation in the expanded inter-premaxillary suture? Arch Oral Biol 2016;69:13-8.
26. Jung CR, Schepetkin IA, Woo SB, Khlebnikov AI, Kwon BS. Osteoblastic differentiation of mesenchymal stem cells by mumie extract. Drug Dev Res 2002;57:122-33.
27. Lee CY, Suzuki JB. CTX biochemical marker of bone metabolism. Is it a reliable predictor of bisphosphonate-associated osteonecrosis of the jaws after surgery? Part II: a prospective clinical study. Implant Dent 2010;19:29-38.
28. Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 1990;85:632-9.