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Effects of A New Combination Folkloric Medicinal Plant Extract on Bone Formation in Orthopedically Expanded Suture in Rats

Yeni Bir Kombinasyon Folklorik Şifalı Bitki Ekstraktının Sıçanlarda Ortopedik Olarak Genişletilmiş Sütürde Kemik Oluşumu Üzerindeki Etkileri

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Abstract

Aim The aim of this study was to evaluate histological effects of a new combination folkloric medicinal plant extract on bone healing in premaxillary suture expansion in rats.

Material and Method Thirty male Sprague-Dawley rats were used in this study. Rats were evenly divided into three groups (one control and two experimental groups) of ten each. The animals were subjected to premaxillary suture expansion by helix springs. The only expansion group is defined as the control group (Group A). The experimental groups are defined as OstokinPlus-10 (Group B) and OstokinPlus-20 (Group C). In the experimental groups, 10 and 20 ml/kg OstokinPlus herbals were applied systemically after the expansion by use of an orogastric tube during the time of study. The springs were placed and activated to deliver a 30 cN force. After 5 days, the springs were removed and replaced with short lengths of rectangular retaining wire. Tooth separation was maintained for 15 days. After a consolidation period of 15 days, the animals were euthanatized and the maxillary bone containing the midpalatal suture cartilage was surgically removed. The specimens were prepared for histomorphometric assessment of the regenerated bone.

Results The midpalatal suture was successfully distracted following application of the activated helix spring. The distracted premaxillary suture was filled with new bone formation and unorganized fibrous tissues. Newly formed bone percentage and the bone area were found to have significant differences ($p< 0.05$). For investigated parameters, Group B and Group C revealed more positive results than Group A.

Conclusion OstokinPlus herbal had positive effects on bone healing and formation during premaxillary suture expansion.

Keywords Bone regeneration, histomorphometry, OstokinPlus herbal, rapid maxillary expansion

Özet

Amaç *Bu çalışmanın amacı, sıçanlarda premaksiller sütür ekspansiyonunda yeni bir kombinasyon folkrorik tıbbi bitki ekstratının kemik iyileşmesi üzerindeki histolojik etkilerinin değerlendirmektir.*

Gereç ve Yöntem *Bu çalışmada otuz erkek Spraque-Dawley sıçanı kullanılmıştır. Çalışma her biri eşit olmak üzere 10 sıçandan oluşan bir kontrol iki deney grubuna ayrılmıştır. Sıçanlar sarmal yaylar ile premaksiller sütür genişlemesine tabi tutulmuştur. Tek genişleme grubu kontrol grubu olarak (Group A) tanımlanmıştır. Deney grubu OstokinPlus-10 (Group B) ve OstokinPlus-20 (Group C) olarak tanımlanmıştır. Deney gruplarında, 10 ve 20 ml/kg OstokinPlus bitkileri, çalışma süresi boyunca orogastrik tüp kullanılarak genişletildikten sonra sistemik olarak uygulanmıştır. Yaylar yerleştirilmiş ve 30 SN kuvveti sağlayacak şekilde etkinleştirilmiştir. 5 gün sonra, yaylar çıkarılmış ve kısa uzunluklarda dikdörtgen tespit teli ile değiştirilmiştir. Diş ayrımı 15 gün sürdürülmüştür. 15 günlük bir konsolidasyon periyodundan sonra, hayvanlara ötenazi uygulanmış ve midpalatal sütur kıkırdağını içeren maksiller kemik cerrahi olarak çıkarılmıştır. Örnekler, rejenere kemiğin histomorfometrik değerlendirmesi için hazırlanmıştır.*

Bulgular *Aktive heliks yayı uygulamasının ardından midpalatal sütur başarılı bir şekilde ayrılmıştır. Ayrılan premaksiller sütur yeni kemik ve organize olmayan fibröz dokular ile dolmuştur. Yeni oluşan kemik yüzdesi ve kemik alanı arasında önemli farklılıklar bulunmuştur ($p< 0,05$). Araştırılan parametreler için Group B ve Group C, Group A daha iyi sonuçlar ortaya koymuştur.*

Sonuç *OstokinPlus ekstaratı premaksiller sütur genişlemesi esnasında kemik iyileşmesi ve formasyonunu olumlu yönde etkilemiştir.*

Anahtar Kelimeler *Kemik rejenerasyonu, histomorfometri, OstokinPlus bitkisi, hızlı maksiller genişleme,*

INTRODUCTION

Expansion of the median palatal suture with rapid maxillary expansion (RME) is a common procedure in orthodontic practice. This procedure is a well-accepted treatment for narrow maxilla in patients with posterior cross bite or dental crowding. In the RME, first the width of posterior dentition is increased, and then active bone regeneration occurs in the expanded suture area.[1-4]

RME was originally advocated for use in growing adolescents, with the sutures remaining patent, and this procedure has also been successfully used in skeletally mature adults.[5] Even after a retention period, the expanded maxilla has a strong tendency to rebound to its previous form, with up to a 90% relapse.[6] Although the actual causes of relapse are not yet fully understood, regulation of bone metabolism, retention period duration, rate and quality of bone regeneration in the midpalatal suture during and after expansion, soft-tissue adaptation, and age may all affect post-treatment relapse.[1,3,7-9]

The literature contains many studies about bone formation in the expanded suture or distracted callus that has been stimulated by herbals, using low level laser therapy (LLLT) or pharmacological, mechanical, electrical, or electromagnetic methods.[1-3,6,7,10-12] However, no study about the application of combination plant extracts on bone healing during premaxillary suture expansion was observed in the literature. Therefore, we aimed to investigate whether there is enhanced bone healing and regeneration during premaxillary suture expansion with the application of a new combination plant extract. In addition, if relapse after the expansion could be minimalized, it would be major interest for maxillary enlargement.

We used an Ostokin (Crystal Natural Pharmaceuticals, Yunnan, China) herbal product known in the China as OsteoKing. This product has been approved by China's State Food and Drug Administration (Guo yao zhun zi No. Z20025103), and it is a combination plant extract from the herbs astragalus membranaceus (9 gr/100 mL), panax ginseng (15 gr/100mL), carthamus tinctorius (7.5 gr/100 mL), citrus reticulata peel (6 gr /100 mL), pure water (62.2 gr/100 mL) and sorbic-benzoic acid (0.3 gr/100 mL). According to producers, Ostokin can be used for the following diseases: osteoarthiritis, osteonecrosis, osteoporosis, herniated disc and bone fractures. Till now it was reported that ostokin has notable effect in the treatment of bone illnesses such as femoral head necrosis or bone fractures.[13] Additionally, ostokin was found effective on prevention of osteoporosis in rabbits.[14] According to this literature support, Ostokin can be found effective on stability of maxillary expansion and stability. So, the purpose of the present experimental animal study was to evaluate the effect of this new combined folkloric medicinal plant extract when applied systemically for bone regeneration during premaxillary suture expansion in rats.

MATERIAL and METHOD

Sample and ethical statement

Thirty 50- to 60-day-old male Sprague-Dawley rats with a mean weight of 222.76±18.44 g were used in this study. The study was conducted in accordance with the accepted guidelines for the care and use of laboratory animals in research. All of the rats were housed in polycarbonate cages in an experimental animal room (22-24 o C, 55%-70% humidity, 1 atm pressure, with a 12-hour light/dark cycle). The rats were fed a standard laboratory diet, and drinking water was available ad libitum during the time of the study. This study's experimental procedures were approved by the Institutional Review Board and the Animal Use Committee of Bezmialem Vakif University (Animal Ethics Approval No. 2013/107). This study was conducted according to the principles of the Basel Declaration of 2010.

Experimental protocol and study design

The animals were randomly divided into three groups (a control group and two experimental groups) of 10 rats per group. The subjects were subjected to premaxillary suture expansion by helix springs. The expansion only group was

defined as the control group (Group A). The experimental groups were defined as Group B (10 ml/kg herbals) and Group C (20 ml/kg herbals). In the experimental groups, 10 and 20 ml/kg/d Ostokin (Yunnan Crystal Natural Pharmaceutical Co. Ltd 9/F JinTai Blds. No.48 Dong Feng Dong Lu, Kunming, Yunnan, China) herbals were systemically applied after the expansion, while 10 ml/ kg/d saline solution was systemically applied by means of an oro-gastric tube in the control group. It was applied systemically by the same person with a nasogastic tube, once a day at the same time of the day, for 15 days in both the experimental groups and the control group.

Placement of helix springs

The rats were anesthetized by intramuscular injection of 3 mg/kg xylazine hydrochloride (Rompuns, Bayer, Leverkusen, Germany) and 35 mg/kg ketamine hydrochloride (10% Ketasol®, Richter Pharma AG, Wels, Austria). After general anesthesia, a helix spring wire fabricated from a 0.012-inch piece of stainless-steel wire was used to perform the expansion of the midpalatal suture. The springs were placed on a grid and activated using pliers. The spring force, measured with a gauge, was 30 g. To obtain retention, a stainless-steel disc was used to prepare a groove at the level of the gingival papilla on the distal sides of the maxillary incisor teeth. Next, a 0.009-inch stainless-steel ligature wire was used to fix the spring to the maxillary incisors. The helix springs were placed and activated to deliver a force of 30 cN. During the expansion period of 5 days, a distance of minimum 1.5 mm was maintained between the maxillary incisors (Figure 1.). After the expansion period, the springs were removed and replaced with short lengths of rectangular retaining wire. Tooth separation was maintained for a consolidation period of 15 days.

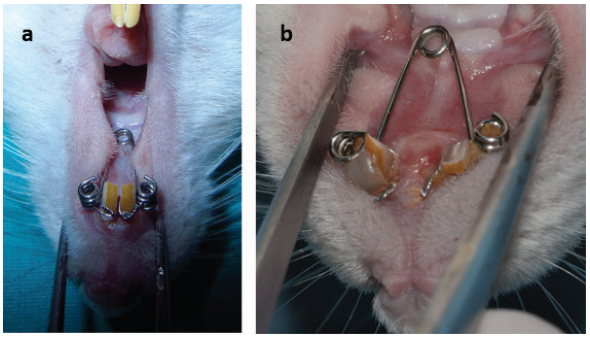

Figure 1. a) The helix spring was placed between the incisors. b) The spring is shown after an expansion period of 5 days.

Specimen preparation

After the consolidation period, the animals were sacrificed with an overdose of 200 mg/kg IV pentobarbital (Pentothal, Abbott, USA). The premaxillae of the animals were dissected out and fixed in 10% neutral formalin. After fixation, the retaining wires were removed. The premaxillae were rinsed with water and decalcified in 10% formic acid solution. After decalcification, the premaxillae were cut into blocks. One cut passed through the incisors at the alveolar crest and perpendicular to the sagittal plane; the second was cut 4 mm apical to the first. The plane passed through the center of the incisor at its gingival portion, and the maxillary incisor acted as the primary guide for orienting the sections. These specimens were dehydrated and embedded in paraffin. The paraffin blocks were sliced into sections 5 μm thick which were then stained with hematoxylin–eosin (HE) for histologic and histomorphometric analysis under light microscopy (Nikon, Tokyo, Japan).

Histologic and histomorphometric assessment

Histologic and histomorphometric analyses were performed by the same histologist, who was also blinded to the identity of the samples. Histomorphometric analysis was performed centered around the premaxillary suture and sections below the surface of the osseous palate facing the oral cavity, because bone regeneration of the surface area was sometimes irregular and unsuitable for quantita-

tive measurement. The presence of inflammatory infiltrate, connective tissue, material resorption, and bone regeneration was assessed. Computer-assisted histomorphometric measurements were performed using an automated image analysis system (ScanScope CS, Aperio®, Vista, California, USA). The images of the histologic sections in all groups were examined with a photomicroscope (Nikon Eclipse i5, Tokyo, Japan) coupled with a video camera on a light microscope (Nikon, DS-Fi1c, Tokyo, Japan), and downloaded to a computer. The number of osteoblasts and osteoclasts were measured with Image J software (US Institute of Health, Bethesta, MA, USA). Two straights were determined on the suture area, one beginning at the incisors and the other 2.5 mm from the beginning straight (Figure 1). Drawings were performed on the images and related areas were calculated in milimeter squares, so differences in the same sections could be compared. Afterwards, the regenerated new bone area (mm2) and the percent of the new bone formation were calculated using the NIS Elements version 4.0 image analysis system (Nikon Instruments Inc., Tokyo, Japan) in the expanded suture area with an original magnification of X40 on the fluorescent images (Figure 2). Eosin staining was used for fluorescent imaging and stained bone tissues can be seen in these images. The same imaging was used in a similar study.[15]

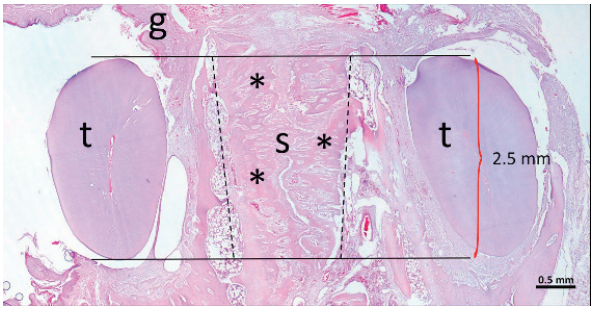

*Figure 2. Two straights were determined on the suture area. g: gingiva, s: suture area, t: tooth, *: new bone area.*

Statistical analysis

The statistical analysis was performed using a commercially available software program (SPSS 20; SPSS Inc., Chicago, IL, USA). To define the normality, the Shapiro–Wilk statistical test was used. For normally distributed data, one-way ANOVA followed post hoc (Tukey's) tests were performed to determine the presence of any significant difference between groups. The difference between groups was considered significant when a value of $p < 0.05$. For each group, the results were reported as median and mean ± standard deviation (n=10).

RESULTS

The expansion of the interpremaxillary suture was well tolerated. No adverse effects such as inflammation, dehiscence, or mucosal trauma were observed in any of the rats; however, three animals were excluded from the study as a result of spring appliance failure, and were replaced by three other rats. The average weight of each group was slightly decreased on Day 1 and had recovered by Day 2. No significant differences in mean body weight among the groups during the course of the experiment were observed. The midpalatal suture was successfully distracted following application of the activated helix spring. After an expansion period of 5 days, a digital caliper measured a distance of approximately minimum 1.5 mm between the maxillary incisors. It was reported that at least 1. 5 mm diastema is acceptable for maxillary expansion in rats.[16]

Figure 4. shows histological images of all the groups. The distracted premaxillary suture was filled with new bone formation and unorganized fibrous tissues. Percentages of newly formed bone and bone area revealed significant differences ($p<0.05$) (Table 1.) (Figure 3. and 4.).

Table 1. Results of the histomorphometric measurements.

Measurements	Control (Group A)	OstokinPlus-10 (Group B)	OstokinPlus-20 (Group C)	Results
Area (mm2) x ±Sd [median]	1.32±0.169* [1.40]	1.51±0.169 [1.57]	1.61±0.147 [1.68]	F= 8.06 **p=0.002**
Newly formed bone (%) x ±Sd [median]	42.68±1.632§ [42.92]	47.33±2.163† [46.96]	53.40±1.529‡ [53.14]	**F= 89.52 p<0.001**
x: Mean average, Sd: Standard deviation p<0.05: area (groups A vs all); percent (Group A vs all, Group B vs all)				

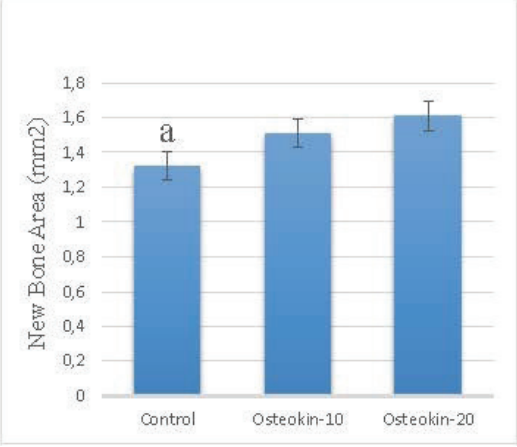

Figure 3. New bone area in all groups. p<0.05) formed bone

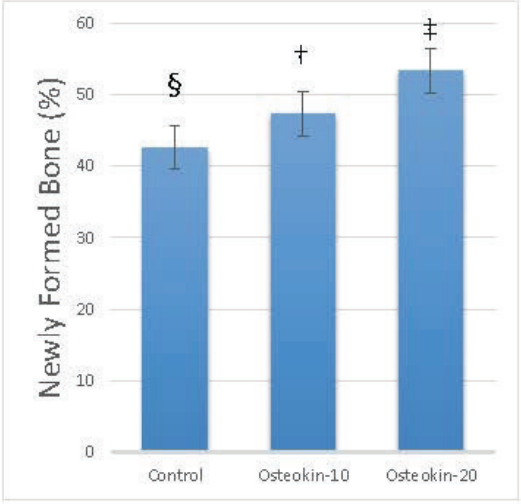

Figure 4. Newly formed bone in all groups (p<0.05)

For the investigated parameters, Group B and Group C revealed more positive results than Group A, with statistically significant differences (p<0.05) (Figure 5. and 6.). Group C also significantly differed from Group B with regard to percentage of newly formed bone (Figure 4.).

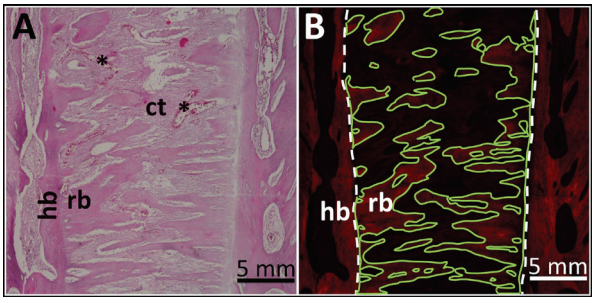

*Figure 5. The regenerated bones shown at original magnification (X40) in the expanded suture area (A: hematoxylin–eosin stain, B: regenerated bone areas in the fluorescent image, hb: host bone, rb: regenerated bone, ct: connective tissue, *: capillary).*

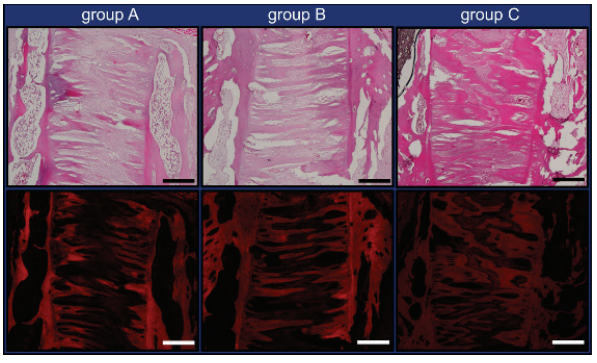

Figure 6. The histological images of all groups are shown as originally magnified (X40) with hematoxylin–eosin stain and fluorescent images (bars are 5 mm long).

DISCUSSION

One of the main causes of posterior cross bite is maxillary atresia. Expansion of the maxilla with RME is one of the most common treatments for maxillary atresia and has been used for more than 30 years.[1,17-19] The changes caused by this treatment are primarily located in the basal bone, increasing the upper jaw arch dimensions through the midpalatal suture, with posterior tooth movement through the alveolar processes.[17]

In this study, rats were used as the experimental animal model because rats have been used in so many similar studies and therefore the results of our study could be compared with those.[2,3,6,7,10-12,17,20-24] Different study protocols have used various parameters for analysis, such as number of osteoblasts[3,10,11] and osteoclasts[3,10], capillaries[3,10], proliferative and mineralizing hypertrophic chondrocytes in the suture cartilage[24], the mineralized area, the fibrosis area, the mineralized area/fibrosis area[7], bone perimeter, Feret's diameter, the newly formed bone percentage[6,11,12], and new bone area.[3,6,7,10-12,23,24] The common feature of most of these studies is the use of the amounts of new bone area as evaluation criteria. We assumed that other parameters, such as Feret's diameter, bone perimeter, and number of osteoblasts, and osteoclasts, were not particularly objective although we used number of osteoblasts and osteoclasts to check cellular activities as similar studies did. Determination of the exact number of osteoblasts and osteoclasts in the expanded area could not be realistic without immunohistochemical staining. The amount of the new bone area is already associated with the osteoblast number, so in this study we focused the new bone area and percentage of newly formed bone. In addition to histologic examinations, other researchers have used different analysis methods in their studies. Da Silva et al.[20] evaluated in vitro osteogenesis parameters and gene expression markers in their cell cultures after treating midpalatal suture expansions with LLLT. Rosa et al.[17] evaluated Raman spectroscopy, and Kobayashi et al.[25] researched alkaline phosphatase activity with histochemical staining.

The procedure for maxilla expansion includes an active phase associated with lateral forces and a passive phase using a retainer. The active phase lasts about 1-2 weeks, depending on the maxillary atresia[17] and expanded maxilla can relapse rapidly if a retainer is not used for a long time1, i.e., a minimum period of 3-5 months after the RME.[17] One of the main causes of relapse is insufficient new bone formation in the midpalatal suture. To avoid relapse, accelerated bone regeneration in the midpalatal suture would be beneficial.[1] The main benefit of accelerating bone formation in the palatal suture during and after expansion is to shorten the time required for bone remodeling in order to shorten retention time and to impede relapse of the skeletal base.[26] Various studies in the literature have investigated increasing bone regeneration in the midpalatal suture by using different mechanical stimulations or various pharmacological agents.[2,3,6,7,17] Saito and Shimizu1, da Silva et al.[20], and Rosa et al.[17] showed that applications of various LLLTs had a positive effect on bone healing in the midpalatal suture. Sawada and Shimizu[2] applied a single dose of Transforming Growth Factor-β1, and Uysal et al.[12] applied[16] a single dose of vitamin C for stimulation of the expanding suture. These studies found significantly stimulated bone regeneration in the midpalatal suture. Jiang et al.[23] showed that the local administration of a glycogen synthase kinase-3β inhibitor could stimulate bone formation in the midpalatal suture. Altan et al.[3] found that systemic use of propolis, a substance made by honeybees, may accelerate rats' new bone formation at the expanded suture. We investigated the effect of the Ostokin herbal product, a combination plant extract, on bone regeneration in the midpalatal suture in rats and found that this herbal agent stimulated bone regeneration during the expansion and consolidation periods. This product contains astragalus membranaceus, panax ginseng, carthamus tinctorius, and citrus reticulate peel.

Astragalus membranaceus is one of the most widely used medicinal herbs in Asian traditional medicine; it has an estrogenic effect that inhibits osteoclast development and

it improves MG-63 cell proliferation so this plant extract modified osteoclast numbers in the current study. This extract may have a synergic effect on improving bone mineral density.[27] In addition, Astragalus membranaceus has been used in traditional Chinese medicine to treat osteoporosis;[28], it has been found effective on rising osteoblastic activity. It improves intestinal calcium absorption, so it has an effect on bone metabolism.[27] In the current study, the number of osteoblasts was raised.

Ginseng has also been used for years in Asian countries. Panax ginseng (PG) is a tonic drug in traditional Chinese medicine. This agent could enhance bone marrow stromal cells and endothelial progenitor cell proliferation, and can increase the number of osteoblasts and bone formation[29] Avsar et al.[30] evaluated the protective effect of PG on bone metabolism in an experimental ovariectomy rat model of osteoporosis and showed that PG has a preventive effect against bone loss.

Carthamus tinctorius (safflower) seeds contain high level of minerals such as calcium, potassium, aluminum, iron, zinc, magnesium, and phosphorous. Traditionally, safflower has been used for purgative and alexipharmic effects in some Asian countries. In China, it has been used as a folk medicine to enhance bone formation.[31] Alam et al.[32] found that safflower seeds have possible roles in the improvement of osteoporosis induced in ovariectomized rats. Citrus reticulate peel comes from the mandarin[33] and has anti-carcinogenic effects.[34,35] No study on bone effects of citrus reticulate was observed in the literature.

Studies associated with the Ostokin or OsteoKing herbal products were also rare in the PubMed database. Hu et al.36 found that OsteoKing could effectively help repair steroid-induced femoral head necrosis in the early stages. In our study, the beneficial effect of bone formation was observed in premaxillary suture expansion.

CONCLUSIONS

The conclusion of the present study is that systemic application of this new combination plant extract can stimulate bone formation and increase bone regeneration in the midpalatal suture in a rat model. In the plant extract group, the number of osteoblasts and osteoclasts were supported this conclusion. This conclusion is only a beginning, and further experimental and clinical studies are needed to evaluate the effects of this herbal extract in humans.

This study showed that systemic application of the new combination plant could be enhanced osteoblastic activity. So, bone formation and improves healing increased in the interpremaxillary suture area during expansion. Eventually, this product could be applied for accelerating bone formation in the palatal suture. Relapse of expanded maxilla could be avoided and also retention period could be not a long time.

Ethics Approval

Ethical approval was obtained from the Institutional Review Board and the Animal Use Committee of Bezmialem Vakif University (Animal Ethics Approval No. 2013/107).

Peer-review

Externally and internally peer-reviewed.

Author Contributions

Concept: S.E., H.Ö., M.B., Design: S.E., H.Ö., H.O.K., Data Collection or Processing: M.E., H.Ö., Analysis or Interpretation: S.E., H.O.K., S.A., Literature Search: S.E., H.Ö., H.O.K., M.B., S.A., Writing: S.E., H.O., M.B., H.O.K., S.A., M.E.

Conflict of Interest

The authors declared no conflict of interest.

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