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Effect of resveratrol on bone formation in the expanded inter-premaxillary suture: early bone changes

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Structured Abstract

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Objective –The aim of this study was to evaluate the effects of local resveratrol (RSVL) administration on bone formation in response to expansion of the inter-premaxillary suture, in rats.

Material and Methods – Twenty 50- to 60-day-old male Wistar rats were separated into two equal groups. Both groups were subjected to expansion, and 30 cN of force was applied to the maxillary incisors with helical-spring. Twenty-four hours after appliance placement, single-dose 10 μmol/kg RSVL in the dimethylsulfoxide (DMSO) was injected to the inter-premaxillary suture in the experimental group. In the control group, the same amount of DMSO was injected to the suture of rats. Bone formation in the suture was evaluated histomorphometrically. The area of new bone (μm^2), the perimeter around the new bone (μm), Feret's diameter (μm), the percentage of new bone to non-ossified tissue (%), and the number of osteoblast were measured and compared. Mann–Whitney *U*-test was used for statistical evaluation at $p < 0.05$ level.

Results – Statistically significant differences were found between the groups for all histomorphometric parameters. New bone area ($p < 0.001$), bone perimeter ($p < 0.001$), Feret's diameter ($p < 0.001$), percentage of new bone ($p < 0.001$), and the number of osteoblast ($p < 0.001$) were significantly larger in the experimental group when compared with the control. Bone histomorphometric measurements revealed that bone architecture in the RSVL treated rats was improved.

Conclusions – Local application of RSVL during the early stages to orthopedically expanded inter-premaxillary suture area may stimulate bone formation and shorten the retention period, in rats.

Key words: image analysis; maxillary expansion; osteogenesis; rats; resveratrol

Introduction

Rapid maxillary expansion (RME) has become an accepted procedure for the treatment of narrow maxilla or posterior crossbite or to expand arch perimeters to alleviate dental crowding. RME procedure increases the width of posterior dentition rapidly, which is followed by active bone formation in the mid-palatal suture (1, 2). It is well known that even after a retention period, the expanded maxilla has a strong tendency to re-bound to its previous form (3). The extent of this relapse may be as much

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as 90% (4). Reorganization of hard tissue in the suture starts by the end of active treatment phase (5, 6). Haas concluded that ossification of the suture margins is completed in 60–90 days (5).

The relapse phenomenon after RME is complex because the regulation of bone metabolism and stresses generated on mid-palatal and circum-maxillary sutures depend on many factors. Although the reason for the relapse is not fully understood, rate and quality of bone formation in the mid-palatal suture during and after expansion may affect the post-treatment relapse (1). Therefore, it would be potentially beneficial to accelerate bone formation in the mid-palatal suture during and after expansion for preventing relapse of the skeletal base and shortening the retention period (1, 2). Various clinical and experimental investigations have been focused on the acceleration of bone formation and consolidation and thereby aimed to shorten the framing time (1, 2, 7–12).

Resveratrol (RSVL, 3,40,5-trihydroxystilbene) is an edible polyphenolic stilbene phytoestrogen found in the skins of most grape cultivars, peanut roots, mulberries, red wine, and a variety of medical herbs (13). RSVL is an antineoplastic, antioxidant, antiplatelet, and anti-inflammatory agent (14). It prevents osteoclast formation (15) and promotes osteoblast proliferation and induction (16). Recent studies show that RSVL stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells *in vitro* (17). RSVL was proved to act against cyclosporin A inhibition of proliferation and osteoblastic differentiation in mouse bone mesenchymal stem cells (16). Recently, Dai et al. (18) showed that RSVL directly stimulates cell proliferation, osteoblastic differentiation, and osteogenic gene expressions through mechanisms involving the estrogen receptor–dependent mitogen-activated protein kinase pathway in human bone marrow–derived mesenchymal stem cell cultures. The effect of RSVL on bone metabolism in the sutures after RME, however, remains to be unclear.

To stimulate bone formation in mid-palatal/premaxillary suture after expansion, many experimental researches in orthodontic literature has been undertaken. Saito and Shimizu (1) investigated the low-level laser irradiation, and Sawada and Shimizu (2) applied a single dose of transforming growth factor β1 (TGF-β1) for stimulation of expanding suture, in rats. In both studies, stimulation of bone formation in the suture was found significant. In recent studies, Uysal et al. (7) evaluated the effects of dietary boron in rabbits and locally administered pharmacological (8, 9) and mechanical (10–12) stimulants in rats on bone formation in response to expansion of the inter-premaxillary suture and found that all these stimulants could stimulate bone formation during expansion and retention periods.

The goal of combining RSVL administration with RME procedure is to accelerate the bone formation processes in an expanding suture, so that the retention devices could be removed earlier. The aim of this experimental study was to evaluate the effects of RSVL, which is a potent antioxidant phytoestrogen, on bone formation in response to expansion of inter-premaxillary suture, in rats. For the purposes of this study, the null hypothesis assumed that there is no significant difference between the RSVL- and vehicle solution-administered rats on bone formation during expansion of inter-premaxillary suture.

Materials and method

Animals and groups

Twenty 50- to 60-day-old male Wistar rats with a mean weight of 219.94 ± 19.05 g were used. All animals were housed in polycarbonate cages, subjected to a 12-h light–dark cycle at the constant temperature of 23°C and fed a standard pellet diet (Expanded pellets; Stepfield, Witham, Essex, UK) with tap water *ad libitum*. Permission was obtained from the Gulhane Military Medical Academy; Ethics Committee of Experimental Animals after the Research Scientific Committee at the same institution had approved the experimental protocol. The experiments were carried out in the Department of Experimental Animals, Research and Development Center, Gulhane Military Medical Academy.

This study was organized as a parallel group design with one group receiving the experimental protocol and the other receiving the control. The power analysis was performed with G*Power ver.3.0.10. (Franz Faul, Universität Kiel, Germany) software. Based on 1:1 ratio between groups, a sample size of 20 animals would give more than 90% power to detect significant differences with 0.40 effect size and at $\alpha = 0.05$ significance level. Animals were randomly divided into two groups (control and experimental) of ten rats each.

Appliance placement

The animals were anaesthetized with an intramuscular injection of xylasine (Bayer, Istanbul, Turkey) and ketamine (Parke-Davis, Istanbul, Turkey) at 0.5 and 1 ml/kg body weight, respectively. The expansion appliances were helical springs fabricated from 0.014-inch stainless steel wire inserted in holes drilled close to the gingival margins of both upper incisors (8–12) (Fig. 1). The springs were activated to deliver a force of 30 cN and were not reactivated during the 5-day expansion period. After 5 days, the springs were removed and replaced with short lengths of rectangular retaining wire. Tooth separation was maintained for 10 days. The distance between the mesial edges of the upper incisors was measured at the beginning of the experiment and at the end of expansion with a digital caliper (MSI-Viking Gage, SC, USA).

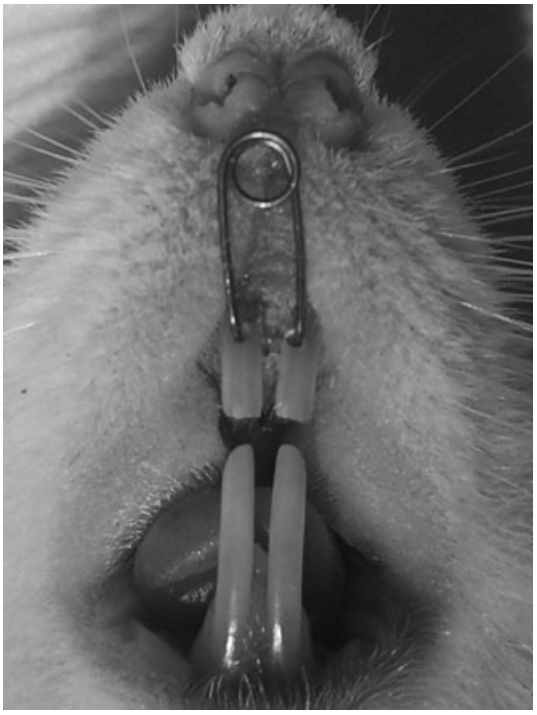

Fig. 1. Appliance *in situ*.

Occlusal radiographs (Dexcowin, ADX 4000; Dexcowin Company, Seoul, Korea) were taken from all animals at three stages to check the sutural opening: at baseline, end of expansion, and at the end of the retention period (Fig. 2).

Administration of solutions

Purified RSVL is only commercially available as the trans-isomer; the most stable and pharmacologically active form of RSVL was purchased from Sigma (500 mg; St Louis, MO, USA) and prepared according to the manufacturer's protocol. Briefly, 100 μmol RSVL was dissolved in dimethylsulfoxide (DMSO) and was prepared stock RSVL solution. It was diluted in serum physiologic as 30 μmol in 100 ml and prepared as 10 μmol/kg.

Animals were randomly separated into two groups of ten rats each. Experimental group was treated with single dose of RSVL (10 μmol/kg). In the control group, the same amount of DMSO was injected to the inter-premaxillary suture. Twenty-four hours after expansion, single-dose 10 μl RSVL or vehicle solutions was injected into the inter-premaxillary suture with a micro-syringe (Hamilton Injection syringe; Hamilton Company, NV, USA).

Specimen preparation

After the retention period of 10 days, the rats were sacrificed with an overdose of ketamine and xylasine and their premaxillae were dissected out and fixed in

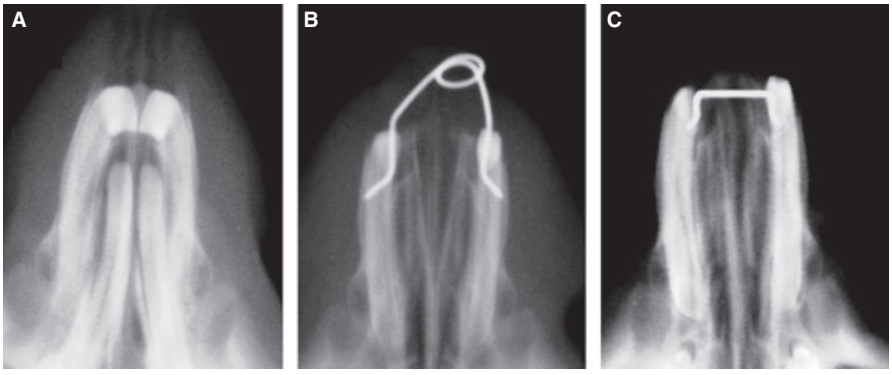

Fig. 2. Occlusal radiographs. (A) baseline, (B) end of expansion, (C) end of the retention period.

10% formalin. After fixation, the retaining wires were removed and the premaxillae were decalcified with 5% formic acid for 4 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 sagittal plane, the second cut 4 mm apical to the first cut. The sections were rinsed, trimmed, and embedded in paraffin. The paraffin blocks were sectioned serially at 5-μm intervals.

Histomorphometric analysis

The histological sections were stained with hematoxylin and eosin. The histomorphometric measurements were performed 200 μm beneath the oral surface of the osseous palate because bone formation in the surface layer was sometimes irregular and unsuitable for quantitative measurement. The sections were viewed under a microscope (Olympus CX41/DP25 Research System; Olympus Corporation, Tokyo, Japan), and the histomorphometric measurements were calculated with an image analysis program. Measurements were performed by two assessors (T.U. and S.G.) who were blinded to the identity of the sections. The final results are averages of these separate evaluations. Two histological sections from each animal were analyzed and representative areas, which were defined beforehand, were captured at ×400 magnification. The image analysis software, Image-J (US National Institutes of Health, Bethesda, MA, USA) was used to compute the histomorphometric measurements (19). The following parameters were measured: new bone area (μm^2), bone perimeter (μm), Feret's diameter (μm), the percentage of new bone to non-ossified tissue (%), and the number of osteoblasts. These basic planimetric measurements provided a description of the amount of new bone. The new bone area is the total cross-sectional area of new bone. The bone perimeter and Feret's diameter are the length of the perimeter around the new bone and the maximum distance between any two points on the perimeter, respectively. Two separate image analysis macro-programs were written by one of the authors (Y.K.) to increase the contrast between the bone and surrounding tissue and display the basic planimetric measurements of the outlined new bone (10). The user was required to draw the outline of the newly formed islands of the bone. At the end of the macro, the results window displayed some basic planimetric measurements of the outlined objects. The second macro enhanced each image and superimposed a grid, consisting of squares with areas of 1000 μm^2, on the image. Intersections of the grid superimposed on new bone were recorded. After recording the new bone, non-ossified areas were recorded in the same manner. At the end of the macro, the program calculated the percentage of new bone (10).

Statistical analysis

All data were analyzed with the statistical package for social sciences, 13.0 (SPSS for Windows; SPSS Inc, Chicago, IL, USA). Descriptive statistics were given as quartiles (25th, 50th – median – and 75th) minimum (Min) and maximum (Max). The group differences were studied by the Mann–Whitney *U*-test. When the *p*-value was <0.05, the statistical test was determined as significant.

Results

All animals survived to the end of the study. Deep mucosal infections, dehiscences, or other adverse effects were not observed in any animals. The expansion appliances were well tolerated and the animals gained weight. As no statistically significant changes in body weight were found during the expansion and retention periods, there was no reason to weight-correct the data.

The histological sections confirmed that the interpremaxillary sutures were expanded in all groups, and there was no statistically significant difference ($p = 0.546$) in the amount of expansion in the groups (Table 1).

Statistical analysis showed statistically significant differences between two groups for all investigated histomorphometric parameters. New bone area

Table 1. **Results and Mann–Whitney *U*-test comparisons of biometric analysis for determination of the amount of expansion (μ)**

Groups	n	25%	50% Median	75%	Minimum	Maximum	*p*-value
Control	10	307.15	330.63	356.53	300.26	386.21	0.546
Resveratrol	10	310.06	334.85	361.37	309.78	380.99	NS

n, sample size; NS, not significant.

($p < 0.001$), bone perimeter ($p < 0.001$), Feret's diameter ($p < 0.001$), newly formed bone percentage ($p < 0.001$), and the number of osteoblast ($p < 0.001$) measurements showed statistically significant differences (Table 2). For all investigated histomorphometric parameters, RSVL group showed more positive results than the control related to the new bone formation and revealed that bone architecture in the treatment group was improved (Figs 3 and 4).

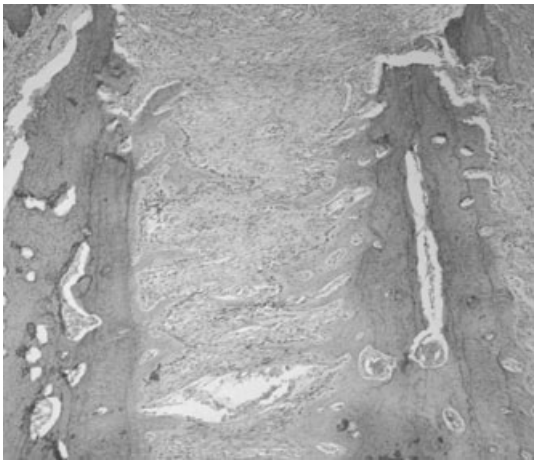

Fig. 3. Photomicrograph of a section in the expansion area of control group showing abundant formation of bone trabeculae. Large connective tissues indicate beginning stages of bone formation (HE 400× magnification).

Discussion

The facial sutures are important mediators of skeletal adaptation to craniofacial growth and biomechanical therapy (20). Expansion of mid-palatal suture is often a key objective in dentofacial orthopedic treatment. Palatal expansion is referred through a multifactorial adaptive response within the mid-palatal suture. Mechanical expansion results in distortion of the sutural structure, inducing a biological chain of events leading to osseous modeling that allows the suture to restore itself to its original architecture (21). Despite the long history of this important clinical procedure, little is known about the cell kinetics of osteogenesis and bone remodeling response associated with it. In the present study, effects of local RSVL administration on bone formation in the inter-premaxillary suture in response to expansion were investigated by using a histomorphometric method and more positive results were determined in RSVL group than the control. Thus, according to formative changes in all histomorphometric parameters in RSVL group, the null hypothesis of this study was rejected.

To accelerate bone healing, experimental studies have been carried out with administration of various antioxidants (22, 23). RSVL has an intrinsic antioxidant capacity (14). *In vivo*, RSVL has been shown to increase plasma antioxidant capacity and decrease lipid peroxidation; however, it is difficult to assess whether these effects are direct or the result of upregulating endogenous antioxidant enzymes (24). Similarly, in stroke-prone rats, RSVL significantly reduced oxidative stress markers and affected focal ischemia most probably by virtue of its antioxidant property (24). Boissy et al. (15)

Table 2. **Descriptive values and Mann–Whitney *U*-test comparisons of histomorphometric measurements**

Parameters	Groups	n	25%	50% Median	75%	Min	Max	*p*-value
Area (μm^2)	Control	10	37.78	58.84	77.67	28.9	89.57	0.000***
	Resveratrol	10	127.35	144.94	164.21	139.79	170.49	
Perimeter (μm)	Control	10	121.73	136.52	152.35	82.47	236.19	0.000***
	Resveratrol	10	274.69	287.52	304.49	238.98	330.87	
Feret's diameter (μm)	Control	10	6.58	16.45	34.86	12.08	17.15	0.000***
	Resveratrol	10	43.74	58.41	75.98	34.45	80.49	
Newly formed bone (%)	Control	10	23.87	35.87	45.25	20.00	55.55	0.000***
	Resveratrol	10	69.45	77.59	87.32	60.00	90.33	
Number of osteoblast (n.Ob)	Control	10	47.00	61.00	69.00	40.00	75.00	0.000***
	Resveratrol	10	90.50	97.00	112.50	85.00	120.00	

n indicates, sample size; Min, minimum; Max, maximum.
***$p < 0.001$.

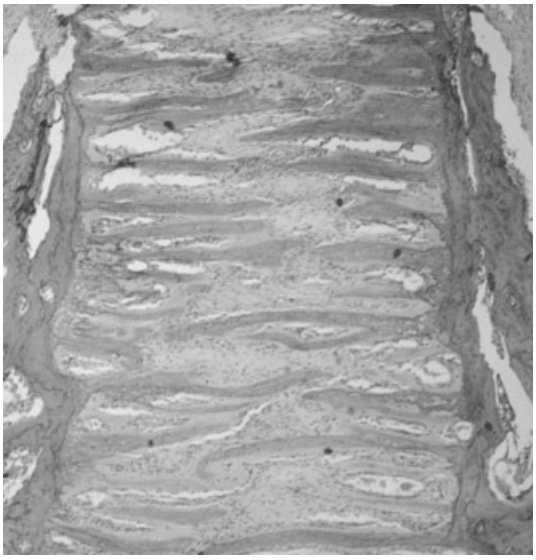

Fig. 4. Photomicrograph of a section in the expansion area of resveratrol-injected group showing larger masses of new bone trabeculae. New bone became attached to old bone at the site of expansion. Large amounts of new bone forming area indicate later stages of bone formation (HE 400× magnification).

stated that RSVL may prevent increased bone degradation by osteoclasts or may stimulate compensative bone formation by osteoblasts. Therefore, to our best notice, this study is the first report to evaluate the effects of RSVL on bone formation in a suture. After application of expansion strain and during retention, more stable and larger callus was formed with stimulation by RSVL than the control.

We administered RSVL in DMSO by intrasutural injection in order to give systematically, thereby maximizing the delivery of the lipophilic protein. In the literature, RSVL was used systemically at a dose of 2.5–100 mg/kg by many investigators (14). RSVL has no known side effects such as mutagenecity, carcinogenecity, cellular toxicity or allergic reactions in clinical and laboratory researches (25). In the current study, locally administered RSVL and its effect on bone formation were evaluated in response to maxillary expansion. To minimize potential, systemically adverse effects, and to support bone formation in definite time interval and area, it is important to apply it locally. Thus, we administered the RSVL to the center of the investigated area to evaluate the pure effects of the product in that region.

While monkey and cat have maxillary sutures similar in most aspects to those of man and have been used in maxillary expansion experiments, the ideal animals to obtain a clear picture of bone and suture changes under stress are the rabbit and the rat (26). Thus, in this study, according to the ethical considerations, the smallest animal model was chosen to test the new material in bone modeling. Results of this study proved that rats are considered a suitable experimental animal model to study the effect of RSVL on bone formation in the sutural area.

Sawada and Shimizu (2) carried out a preliminary experiment to determine the most intense expression of a pharmacological agent, TGF-β1, in response to RME and detected 24 h after expansion was suitable to stimulate bone formation in suture. In the current study, the same period was selected as injection time.

The effects of RSVL on the quantity and the rate of new bone formation during maxillary expansion were examined using a histomorphometric method. The image analysis program evaluated the histological findings objectively and demonstrated that the amount of bone area correlated with newly formed bone. Bone histomorphometry is a reliable technique that is frequently used in quantitative evaluation of bone remodeling (1, 2, 7–12).

Burstone and Shafer (27) reported that the normal premaxillary suture in young rats measured approximately 20–60 μm in thickness and found that expansion of the suture by the rubber wedges over a period of 5 days resulted in an opening of the suture to an average width of 377 ± 10 μm. In the current study, the inter-premaxillary suture was opened by helical springs that were applied buccally, and occlusal radiographs showed wide separation of premaxillary bones after 5 days of expansion and the sutural width measurements were found in range between 312.26 and 386.21 μm. The amount of expansion, determined by image analysis software in all groups, was similar and showed no statistically significant differences ($p = 0.546$).

In the first 14-day period of fracture healing in rats, osteogenic cells differentiate and proliferate (28). After this, ossification begins, along with mineralization of the bone matrix in soft callus filling the fracture gap. RSVL may stimulate compensative bone formation by osteoblasts and directly stimulates cell proliferation and differentiation of osteoblasts (16, 17). In the current study, at the 10th day of retention, histological and histomorphometric assessments showed that local application of RSVL accelerated bone healing.

A 28-day study of the effects of 20 mg/kg oral RSVL in adult rats found no effect on body weight, food or water consumption, hematological or clinical biochemistry variables, or histopathology (29), and no adverse effects were observed at doses of up to 300 mg/kg (30). Besides, Crowell et al. (30) evaluated the potential toxicity of RSVL and found adverse effects in the rats administered 3000 mg/kg body weight per day. According these results, more extensive studies will be required before high doses can be recommended.

Alkaline phosphatase (ALP) is the most widely recognized biochemical marker for osteoblastic activity and this enzyme is believed to play a role in bone mineralization. Mituzani et al. (17) investigated the effects of RSVL on ALP activity and they found that RSVL dose-dependently increased ALP activity and DNA synthesis and accelerated prolyl-hydroxylase activity, indicating that it increased collagen synthesis activity. Also, same researchers established that RSVL inhibited the differentiation from stem cells to osteoclasts. Boissy et al. (15) found that RSVL stimulates vitamin D receptor expression in bone marrow osteoblast precursors. Song et al. (16) indicated that RSVL stimulates proliferation and osteoblastic differentiation of mouse. Besides, Dai et al. (18) reported that RSVL had estrogen-like activity and stimulated the proliferation and osteoblastic differentiation through estrogen receptor–dependent NO/cGMP pathway. RSVL increases the osteogenic response of osteoblasts (31) and bone density in ovariectomized rats (32).

Dioxins and other aryl hydrocarbon receptor (AhR) ligands, such as polycyclic aromatic hydrocarbons, are environmental toxicants generated by the chemical industry (33) or found in cigarette smoke and other sources of air pollution. Additionally, RSVL is a well-known antagonist for the AhR. It has been shown to block aryl hydrocarbon-mediated inhibition of osteoblast differentiation in several tissues including bone (34). Thus, this characteristic can inform as to how the RSVL might work in this model system.

In this study, we determined that RSVL had a marked stimulatory effect on bone formation during the early retention period. It may have a synergistic effect on bone formation in response to mechanical and biological stimuli. RSVL may be useful for a wide range of applications, including the treatment of osteoporosis and the enhancement of bone formation, fractured bone repair or in the reconstruction of bone defects.

Conclusion

Local administration of RSVL during the early stages to orthopedically expanded inter-premaxillary suture areas may stimulate bone formation and shorten the retention period, in rats. Further clinical studies are necessary to validate its effects on humans and also to ascertain whether it should be used prophylactically or continuously until the end of the retention period.

Clinical relevance

This study confirmed that local application of RSVL during the early stages to orthopedically expanded inter-premaxillary suture stimulates bone formation and improves healing. This application may shorten the retention period after maxillary expansion procedures. This principle could potentially be applied during distraction osteogenesis or for the treatment of patients with long bone fractures fixed by external devices and might be particularly helpful in treating fractures with delayed union and non-union.

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