Stimulation of bone formation in the expanding inter-premaxillary suture by vitamin E, in rat

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Objective: The aim of this study was to evaluate the effects of vitamin E (α-tocopherol) administration on bone formation in response to expansion of the inter-premaxillary suture, in rats, histomorphometrically.

Methods: Thirty 50 - 60 day old Wistar rats were separated into five equal groups (one control and four experimental). All groups were subjected to inter-premaxilla expansion with 50-gram of force. Six control animals received saline solution (Group I) and three experimental groups were treated with a single dose of α-tocopherol injected into the inter-premaxillary suture after one day after appliance placement (Group II: 2 mg/kg; Group III: 10 mg/kg; and Group IV: 50 mg/kg). A further group of six animals received three injections of 10 mg/kg α-tocopherol, one each on days 3, 6, and 9 (Group V). Bone formation in the suture was evaluated by bone histomorphometry. Kruskal-Wallis rank and Mann-Whitney U tests were used for statistical evaluation at \( p < 0.05 \) level.

Results: New bone area, bone perimeter, feret’s diameter and newly formed bone measurements were significantly higher in the experimental groups than the control (\( p < 0.001 \)). Bone architecture in α-tocopherol administrated groups was improved, and bone formation during the expansion period was stimulated significantly, in a dose-dependent manner.

Conclusions: The application of α-tocopherol during the early stages to orthopedically expanded inter-premaxillary suture areas may stimulate bone formation and shorten the retention period, in rats. (Korean J Orthod 2009;39(5):337-347)

Key words: Vitamin E, α-tocopherol, Expansion, Histomorphometry, Rat

INTRODUCTION

Widening the mid-palatal suture with rapid palatal expansion (RPE) is an important part of the routine clinical practice in the correction of malocclusions. This method increases the width of the posterior dentition rapidly, which is followed by active bone formation in the suture. 1,4

Orthodontists have studied the force system that induces change of sutural structures for decades. 5 Different orthodontic appliances based on these results have been found clinically successful. Although application methods have been refined, the mechanism and stimulation by external factors of stress-mediated osteo-
genesis in the expanded suture is unclear.

One major problem with the RPE method, however, is the prolonged time required for the new-formed bone in the expanded sutural area to become strong enough to resist the stresses generated on mid-palatal and circum-maxillary sutures. Velocity and quantity of bone formation in the sutural area during and after expansion may affect the post-treatment relapse. 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. Various clinical and experimental investigations have been focused on the acceleration of bone formation and consolidation, and thereby aimed to shorten the framing time.

Oxygen-derived free radicals are formed by a number of phagocytes including monocytes, macrophages, and neutrophils and have been reported to be increased in the normal bone formation process, chronic inflammatory diseases, aging, and osteoporosis. The in vivo and in vitro findings indicate that free radicals generated in the bone environment enhance osteoclast formation and bone resorption. Antioxidant administration has been shown to be beneficial in suppressing the damaging effects of oxygen free radicals in cells during bone formation. Various experimental studies have been carried out to accelerate and shorten bone formation with the administration of antioxidants. GökTürk et al. demonstrated that administration of zymosan - which induces oxygen-free radicals through stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in polymorphonuclear leukocytes - impaired fracture healing in a rat model. Yilmaz et al. have demonstrated the positive effects of ascorbic acid, a well-known antioxidant on fracture healing.

Vitamin E (alpha-tocopherol) is an important lipid-soluble non-enzymatic antioxidant, preventing oxidative attack of membrane lipids and other membrane associated compounds. It has also been shown to be a very effective scavenger of free radicals. It is a strong biological antioxidant and has been shown to suppress the production of certain pro-inflammatory mediators such as interleukin (IL)-1, IL-6, prosta-
Appliance placement

Expansion appliance comprised of helical-springs that were fabricated from 0.014-inch, stainless-steel wires. Springs were placed on a grid and activated on a single arm with pliers. The force was measured with a gauge (50-gram), and the springs were not reactivated during the 5-day expansion period. Appliances were attached to maxillary incisors of all animals under anesthesia (Xylasine + Ketamine combination, 0.5 ml/kg and 1 ml/kg intramuscular, respectively). A hole was drilled in both incisors at the lingual gingival level and springs were inserted into the holes, buccally (Fig 1).

After the expansion period, helical-springs were removed and a piece of rectangular retaining-wire was inserted into the holes between two incisors, for a 10 day retention period. Tooth separation was maintained during the retention phase. Occlusal radiographs (Dexcowin, ADX 4000, Dexcowin Company, Seoul, Korea) were taken at three stages: at baseline, end of expansion and at the end of the retention period (Fig 2).

Administration of solutions

Animals were randomly separated into five groups of six control each. In this histomorphometrical study, six control animals received saline solution (Group I) and three experimental groups were treated with a single dose of α-tocopherol (Ephy addisonal®, Roche, Basel, Switzerland) (Group II: 2 mg/kg; Group III: 10 mg/kg; and Group IV: 50 mg/kg). All amounts of solutions in experimental groups were the same (10 μl), only the concentration of α-tocopherol was changed. Twenty-

![Fig 1. Appliance in situ.](image1)

![Fig 2. Occlusal radiographs. A, Baseline; B, end of expansion; C, end of retention.](image2)
four hours after appliance placement, α-tocopherol or saline solutions were injected into the inter-preamaxillary suture with a micro-syringe (Hamilton Injection syringe, Hamilton Company, NV, USA). A further group of six animals received three injections of 10 mg/kg α-tocopherol, one each on days 3, 6, and 9 (Group V).

Specimen preparation

After the experimental procedure, the rats were sacrificed with an overdose of Ketamine/Xylasine combination and their pre-maxillae were dissected and placed in bottles containing 10% formalin. During decalcification, the solution was changed three-times a day. After fixation, the retaining wires were removed, and the pre-maxillae were decalcified with 5% formic acid for 3 days. The decalcified pre-maxillae were fixed again in the same manner and sectioned. The maxillary incisors acted as the primary guide for orienting the sections. The section was cut perpendicular to the sagittal plane and was determined by two points, one at the alveolar crest and the other 4 mm apically. This plane passed through the centre of the incisor crown at its gingival portion. The sections were rinsed, trimmed and embedded in paraffin. Paraffin blocks were sectioned serially at 5 μm intervals.

Image acquisition

Histological sections were stained with hematoxylin-eosin prior to optical microscope examination (Fig 3). Bone histomorphometric measurement was performed 200 μm under the surface of the osseous palate facing the oral cavity, because bone formation of the surface area was sometimes irregular and not suitable for quantitative measurement. Measurements were based on observations of the sections under a microscope and calculated using an image analysis program. For this purpose a microscope and digital camera system (Olympus CX41/DP25 Research System, Olympus Corp. Japan) were used for histomorphometric evaluation.

Histomorphometric analysis

Measurements were performed double-blinded by two of the contributors and final results were an average of values from these two separate evaluations. Two histological sections were analyzed for each animal. The relevant areas on the slides were predefined and two representative digital images were captured under ×400 magnification. Computer-assisted image-analysis software, Image-J, was used to make measurements, histomorphometrically. For this purpose, two separate image analysis macro was prepared by one of the authors (Y.K.). In the first macro, images were enhanced to increase the contrast between bone and surrounding tissues. The user needed to draw the outline of the newly formed islands of the bone. The program allowed the user to outline more than one bone islands (Fig 4). At the end of this macro, results window appeared showing some basic planimetric measurements of the outlined objects.

With the aid of second macro, the obtained images were opened, enhanced in the same manner, and a grid system consisting of squares with areas of 1,000 μm² each was superimposed. Then, the user was required to click on the intersections of the grid just only over
Fig 4. Histomorphometric measurements of newly formed bone area (μm²). **Left**, Image enhancement and outlining of the area of interest, which is newly formed bone. Histomorphometric measurements of newly formed bone (percentage): **Right**, marking the intersections of the grid just over the newly formed bone and non-osseous connective tissue by mouse-clicking. At the end of this macro, the program gives a percent indicating the ratio of bone to the connective tissue.

**Table 1.** Body weight changes (kg) between groups during expansion and retention periods

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>T1−T0 Mean</th>
<th>Standard deviation</th>
<th>T2−T0 Mean</th>
<th>Standard deviation</th>
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<tr>
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<tr>
<td>Group V</td>
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<td>0.156</td>
<td>0.305</td>
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T₀, baseline weight; T₁, end of expansion (5-day) weight; T₂, end of retention (10-day) weight; NS, not significant.

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 are given as mean, standard deviation, minimum, and maximum. A non-parametric Kruskal-Wallis rank test was used to evaluate the significance of the differences observed in the comparisons of all groups. To identify the significant differences, Mann-Whitney U test was used. When the p-value was less than 0.05, the statistical test was determined as significant.

**RESULTS**

In experimental and control groups, all animals survived to the end of the study. Deep mucosal infection, dehiscence or other adverse effects were not observed in any of the animals. There was no evidence of diarrhea or other gastrointestinal symptoms. Weight loss was observed for two days after appliance placement, for all animals. In the second half of the experimental period the weight gain increased. No statistically significant changes in body weight were observed among
groups during expansion and retention periods (Table 1).

Biometric analysis for the amount of expansion was done by image analysis software at the most anterior part of the pre-maxilla on histological sections. Sutural width measurements from histological sections showed that the inter-premaxillary suture was expanded following application of an activated helical loop. The results indicated that the mean amount of expansion was less in experimental groups (Group II = 330.59 ± 22.73 μm, Group III, 10 mg/kg α-tocopherol; Group IV, 50 mg/kg α-tocopherol; Group V, 10 mg/kg α-tocopherol at days of 3, 6, and 9.

### Table 2. The amount of expansion of each group and biometric analysis

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Table 3. Descriptive values, Kruskal Wallis and Mann-Whitney U results of histomorphometric measurements

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SD, standard deviation; Min, minimum; Max, maximum; NS, not significant; *p < 0.05, †p < 0.01, ‡p < 0.001; Group I, control; Group II, 2 mg/kg α-tocopherol; Group III, 10 mg/kg α-tocopherol; Group IV, 50 mg/kg α-tocopherol; Group V, 10 mg/kg α-tocopherol at days of 3, 6, and 9.
Group III = 317.35 ± 24.79 μm, Group IV = 312.78 ± 22.27 μm and Group V = 318.11 ± 21.14 μm) than the control (338.23 ± 19.79 μm). However, Kruskal-Wallis test showed no statistically significant differences (p = 0.067) (Table 2).

Kruskal-Wallis rank test results showed significant differences among groups for all investigated histomorphometric parameters. New bone area (F = 136.327; p < 0.001), bone perimeter (F = 126.239; p < 0.001), feret’s diameter (F = 29.612; p < 0.001), and newly formed bone percentage (F = 22.256; p < 0.001) measurements showed statistically significant differences (Table 3).

According to Mann-Whitney U analysis, all α-tocopherol administrated groups resulted in increased bone area (p < 0.001). Table 3 shows the bone formation area data after a single injection of α-tocopherol, 2 mg/kg; 10 mg/kg and 50 mg/kg; 24 hours after appliance placement and the course of three injections (10 mg/kg α-tocopherol each) on days 3, 6, and 9 into the rat inter-premaxillary suture. The bone area increased in a dose-dependent manner. According to bone area measurement, the highest value was observed in Group IV (139.117 ± 10.322 μm²) and the lowest value was observed in Group I (66.755 ± 13.449 μm²) and the difference was found to be statistically significant (p < 0.001). The course of three α-tocopherol injections (10 mg/kg) also stimulated bone area (mean: 127.324 ± 10.620 μm²), which did not differ significantly from that of the 10 mg/kg single injection group (mean: 125.687 ± 12.009 μm²).

It was determined that administration of α-tocopherol into the inter-premaxillary suture affected the bone perimeter measurements (p < 0.001). Mann-Whitney U test showed that bone perimeter of group IV was significantly higher than group I; group II; group III and group V (p < 0.001). Only similar bone perimeter changes were observed between three α-tocopherol injections and the 10 mg/kg single injection groups (p = 0.90).

For Feret’s diameter measurement, statistically significant differences were determined among all groups (p < 0.001). Significant difference was also found between Group II and Group IV (p = 0.001).

Table 3 shows newly formed bone percentage data after various injection conditions of α-tocopherol into the rat inter-premaxillary suture. It was determined that the newly formed bone percentage increased in all experimental groups than the control (p < 0.001). For this measurement the highest newly formed bone percentage was observed for Group III (72.055 ± 7.086) and the lowest was for Group I (35.365 ± 12.947); and the difference was found to be statistically significant (p < 0.001). Significant differences were also determined between groups II and IV (p < 0.05).

Thus, according to formative changes in inter-premaxillary sutural area for all histomorphometric parameters, the null hypothesis of this study could not rejected.

**DISCUSSION**

The facial sutures are important mediators of skeletal adaptation to craniofacial growth and biomechanical therapy. Expansion of the mid-palatal suture often is a key objective in dentofacial orthopedic treatment. Although the potential for sutural expansion has been appreciated since the middle of the nineteenth century, Haas introduced the modern clinical concepts of RPE in the last half of the twentieth century. Despite the long history of this important clinical procedure, little was known of the cell kinetics of osteogenesis and the bone remodeling response associated with it. Sutures and the periodontal ligament were widely assumed to have similar mechanisms of osseous adaptation.

Orthopedists have carried out many research series that have shown application of different mechanical stimulations or several pharmacological agents for increasing bone formation. A limited number of published reports have examined the relationship between the fat-soluble antioxidant, α-tocopherol, and bone. Durak et al. reported that prophylactic administration of α-tocopherol may be beneficial in suppressing the damaging effects of oxygen free radicals in cells during fracture healing. Turk et al. concluded that α-tocopherol has a positive effect on both early and late-phase of bone formation. Findings from laboratories indicate that α-tocopherol supplementation protects against bone loss and restores bone strength in osteopenic ani-
Fig 5. Photomicrograph of a section in the expansion area of the control group showing that it appears to be occupied by fibrous tissue, but not the trabecular bone. Large connective tissues indicate beginning stages of bone formation (HE 200 × magnification) (bar = 30 μm).

Fig 6. Photomicrograph of a section in the expansion area of the experimental group (Group IV) 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 indicates later stages of bone formation (HE 200 × magnification) (bar = 30 μm).

mal models, that is, the aged mouse$^{22}$ and the ovariectomized rat.$^{23}$ In these studies,$^{22,23} \alpha$-tocopherol supplementation was shown to have positive effects on bone strength and indicators of bone metabolism. To our knowledge, this study is the first to report a faster formation of bone in a sutural area during expansion, by $\alpha$-tocopherol administration. After application of expansion strain and during retention, more stable and larger callus were formed with stimulation by $\alpha$-tocopherol than the control (Figs 5 and 6). Also, more new bone formation in the expansion region could be observed, leading to a more advanced stage of bone healing.

In orthodontic literature, few researches have been done to stimulate formation in the mid-palatal/inter-premaxillary suture after expansion. Saito and Shimizu$^{1}$ investigated the laser irradiation and Sawada and Shimizu$^{2}$ applied a single dose of transforming growth factor $\beta_1$ (TGF-$\beta_1$) for stimulation of expanding suture, in rats. In both studies significantly stimulated bone formation in the mid-palatal suture was found. In recent studies, Uysal et al.$^{3,4}$ evaluated the effects of dietary boron in rabbits$^3$ and locally administered ED-71 in rats$^4$ on bone formation in response to expansion of mid-palatal suture and found that these agents could stimulate bone formation during expansion and retention periods. In the current study, a pharmacological stimulation, the effects of $\alpha$-tocopherol administration on bone formation in response to expansion of inter-premaxillary suture was investigated in rats and demonstrated an increase of newly formed mineralized bone area in the suture.

Sawada and Shimizu$^{5}$ carried out a preliminary experiment to determine the most intense expression of a pharmacological agent, TGF-$\beta_1$, in response to RPE and detected that 24 hours after expansion was suitable to stimulate bone formation, and this period was selected as the injection time in single injection groups, in the present study.

Various animal models have been described in the literature to investigate the process of bone formation. Swennen et al.$^{24}$ reported that different animals such as dogs, rabbits, sheep, minipig, monkeys, rats and cats were used as an animal model to study the effects of mechanical stimulation on bone. While the maxillary sutures of monkey and cat are similar in most aspects...
to that of man and have been used in maxillary expansion studies, the ideal animals with which to obtain a clear picture of bone and sutural changes under stimulation are rabbits and rats.\textsuperscript{25} In this study, rats were used as the experimental animal model. Results of this study proved that rats are considered a suitable experimental animal model to study the effect of α-tocopherol on bone formation in the sutural area. The primate animal may be the ideal model for bone formation experimentation because of anatomical and wound healing similarities to the human facial skeleton.\textsuperscript{26} Therefore, further clinical studies are needed to confirm the effect of α-tocopherol on bone formation in maxillary expansion, in primate models.

In this study, possible favorable effects of α-tocopherol on the quantity of new-bone formation during maxillary expansion were investigated by using a histomorphometric method. The image analyzer 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, in experimental,\textsuperscript{1-4} in vivo and in vitro conditions.\textsuperscript{27}

The thickness of the normal inter-premaxillary suture in young rats, measured approximately 20 - 60 μm.\textsuperscript{28} Burstone and Shafer\textsuperscript{29} determined that expansion of the suture over a period of 5 days resulted in an opening of the suture to an average width of 377 ± 104 μm. In the current study, the inter-premaxillary suture was opened by helical springs which were applied buccally, and occlusal radiographs showed wide separation of inter-premaxillary bones after 5 days of expansion and the sutural width measurements were found in a range between 287.35 μm and 365.63 μm. The amount of expansion, determined by image analysis software in all groups was similar and showed no statistically significant differences (p = 0.67). In experimental groups, less sutural width measurements was determined that indicates new regenerating bone along the medial margins of bone segments.

With respect to fundamental bone physiology, sutural expansion is similar to surgically mediated DO.\textsuperscript{20} Gradual traction can be applied not only to long bones but also to the maxillofacial area, usually to form new bone.\textsuperscript{29} In most studies of DO for long bones or mandible lengthening, the authors have reported new bone formation by intramembranous ossification.\textsuperscript{29,30} In the present study, it was seen that there was no cartilaginous or fibrocartilaginous tissue in newly formed bone, and the ossification was defined as intramembranous.

When α-tocopherol was injected into the area activated by inter-premaxillary suture expansion during the early stage, bone formation in the suture was markedly stimulated in a dose-dependent manner consistent with the previous report testing TGF-β1. Histomorphometric measurements showed faster bone formation in the highest dose of α-tocopherol administrated group (50 mg/kg).

Additionally, the amount of formed bone after a course of three injections of 10 mg/kg, on days 3, 6, and 9 was almost equal to that induced by a single 10 mg/kg injection of α-tocopherol on day 1, even though the stimulation of bone formation in response to a single injection was dose-dependent. Similar to the present results, Sawada and Shimizu\textsuperscript{2} determined three injections of 200 ng rhu TGF-β1, was equal to that evoked by a single 200-ng injection of rhu TGF-β1.

The mechanisms by which such stimulation could interact with biological systems to accelerate formation have not been yet explained. Garrett et al.\textsuperscript{31} suggested that oxygen free radicals are generated during the formation and activation of osteoclasts, and are associated with osteoclastic bone resorption stimulated by parathyroid hormone, IL-1 and TNF. We suggest that this effect may be partially reversed by α-tocopherol. Additionally, the mechanism of the synergism between α-tocopherol and new bone formation may be the differentiation of mesenchymal cells into osteoblasts and the proliferation of osteoblasts in the expansion zone.

It was determined that α-tocopherol 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. α-tocopherol may be useful for a wide range of applications, including the treatment of osteoporosis and the enhancement of bone formation, as might be helpful during DO, fractured bone repair or in the reconstruction of bone defects.
CONCLUSION

Administration of supplemental vitamin E 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.

REFERENCES