Effects of *Ginkgo biloba* on experimental rapid maxillary expansion model: a histomorphometric study

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**Objective.** We aimed to investigate the effect of systemic *Ginkgo biloba* in rapid maxillary expansion (RME).

**Study Design.** We randomly divided 24 rats into 3 groups: expansion only (EO), expansion plus *Ginkgo biloba* (GB), and no expansion (NE). Expansion appliances were affixed to the maxillary incisors. After a 5-day expansion period, there was a consolidation period of 15 days, following which the rats were killed. Histomorphometric examination was performed to determine the number of osteoclasts, osteoblasts, and capillaries, the number and intensity of inflammatory cells, and new bone formation.

**Results.** New bone formation, number of capillaries, and the ratio of inflammatory cells in maxillary sutures were higher in the GB group than in the other groups. Statistical analysis demonstrated that the GB group had more osteoblasts and osteoclasts than the other groups.

**Conclusions.** GB may hasten new bone regeneration in RME and prevent relapse after RME. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:712-718)

Sustained exogenous forces transmitted as mechanical stresses to craniofacial sutures are a well known controlling stimulus for modulating craniofacial growth in patients suffering from craniofacial anomalies and dentofacial deformities. Sutural mechanical strains, such as those caused during rapid maxillary expansion (RME), trigger a biologic chain of events leading to deposition of new bone in the midpalatal suture.1

RME and surgically assisted RME (SARME) are widely used and well established treatment options in orthodontics and maxillofacial surgery. These approaches are effectively used to treat patients with maxillary collapse, pseudo–class III malocclusions, cleft lip and palate, and rhinologic and respiratory ailments.2

Although these techniques cope with various orthodontic abnormalities, several complications are encountered in RME, such as external root resorption, microfractures at the midpalatal suture, microtrauma of the temporomandibular joint, and postexpansion relapse. Of these, relapse is one of the most frequently encountered complication and an important consideration in RME.3

Relapse is identified as a gradual recurrence of the abnormality for which distraction was initially performed. Relapse after RME is a widely recognized yet poorly described occurrence. Although there is no consensus regarding the main reason of relapse, various risk factors during RME have been proposed. Mode of distraction, tipping of the maxillary segments instead of parallel expansion, overcorrection, scar tissue contraction, duration of retention, stresses accumulated between the articulations of the craniofacial complex, and tension produced in the palatal mucosa have been considered to be possible risk factors.3-5 In addition, individual patient factors, such as age, sex, local and systemic status, regulation of bone metabolism, and supracrestal fibers may also influence relapse. Finally, researchers have emphasized the effect of velocity and the quality of bone formation in the intermaxillary suture in early postexpansion relapse.6

After RME and SARME, bone formation, resorption, and fiber rearrangement continue in the midpalatal su-
ture until the architectural environment achieves equilibrium. Generally, long retention periods are required to prevent posttreatment relapse. Therefore, identifying the mechanism of active bone formation in the intermaxillary suture in response to expansion would aid in understanding the cause of relapse and consequently in developing more effective expansion techniques.

Increasing the growth and growth rate of new bone in the midpalatal suture after expansion may help to inhibit relapse of arch width and decrease the retention period. To this end, researchers have focused on how to hasten new bone generation with the use of various approaches, including laser stimulation and agents such as transforming growth factor, vitamins, lithium chloride, bisphosphonates, and antioxidants. Currently, antioxidant-related studies are being conducted by researchers attracted to the stimulatory effect of antioxidants on bone metabolism through inhibiting osteoclastic activity and stimulating osteoblastic activity.

*Ginkgo biloba* (GB) leaf extract is primarily used as food and in traditional medicine in China, Japan, and Korea. It is widely used as an herbal dietary supplement in the USA. In Europe, GB is clinically approved for the treatment of memory impairment and dementia as well as Alzheimer’s disease. Studies suggest that GB contains pharmacologically active components exhibiting antioxidant, antiplatelet, antihypoxic, antiedemic, hemorheologic, and microcirculatory properties. Furthermore, it can help to increase antioxidant activity, reduce lipid peroxidation injury caused by free radicals, improve athletic ability, and promote recovery.

Free radicals function as intermediates in osteoclast activation, which is vital for bone resorption. These radicals can cause differentiation of osteoclast precursors and osteoclast activity, which results in bone resorption. Owing to its antioxidant properties, GB may play a crucial role in the hastening of bone regeneration and in decreasing of the retention period involved in SARME or RME. We expect the study of the effects of GB on RME to provide additional new insights into strategies for preventing relapse after both these treatment options.

**MATERIALS AND METHODS**

For this study, we obtained 24 12-week-old adult male Wistar albino rats (average weight 200 ± 10 g) from the Animal Laboratory at the Cumhuriyet University Faculty of Medicine, Sivas, Turkey. The rats were housed separately in plastic cages under artificial lighting from fluorescence lamps with a 12:12-hour light: dark cycle. The temperature of the cages was set at 25°C, and food and water were provided ad libitum. This study was approved by the Animal Ethics Committee of the Cumhuriyet University, and the study was carried out in accordance with the guidelines for the use of laboratory animals.

The rats were randomly divided into the following 3 groups (n = 8 each): expansion only (EO), expansion plus GB (GB was given to the rats during the 15-day retention period), and no expansion (NE) (rats in this control group received no procedure or GB). In the GB group, 28 mg GB/kg body weight (Tebokan; Abdi Ibrahim Pharmaceutical Co., Istanbul, Turkey) was systemically administered per day via the orogastric route.

All of the animals were anesthetized by intraperitoneal administration of xylazine (3 mg/kg Rompun; Bayer, Leverkusen, Germany) and ketamine (90 mg/kg Ketalar; Pfizer, New York, NY, USA). Then expansion appliances consisting of helical springs fabricated from 0.012-inch stainless steel wires were affixed to the maxillary incisors of all the animals for expansion of premaxillary sutures (Figure 1). The springs were placed on a grid and activated using pliers. The initial expansion force was measured with a gauge and adjusted to 30 g. A stainless steel disk was used to prepare a groove at the level of the gingival papilla on the distal sides of the incisor teeth for providing retention. Then a 0.009-inch stainless steel ligature wire was used to fix the spring.

The body weight was measured every day throughout the experimental period. The animals were also monitored for infection or appliance failure throughout the treatment period.

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the study. If any complications such as infection, rapid decrease in body weight, or appliance failure were encountered, the animals were excluded from the study.

The consolidation period was initiated after an expansion period of 5 days when \( \geq 1.5 \) mm of distance was provided between maxillary incisors, which was reported to be sufficient to induce the maximum rate of midpalatal suture expansion (Figure 2).\(^{16}\) After a consolidation period of 15 days, all of the animals were killed by injecting 200 mg/kg of sodium pentothal (Pentothal; Abbot, North Chicago, IL, USA). The maxillary bone containing the midpalatal suture cartilage was surgically removed, trimmed, and subjected to further fixation in a 10% neutral buffered formalin solution at room temperature for 24-48 hours.

After fixation, the springs were removed and the specimens were decalcified with aqueous 10% formic acid solution, after which they were dehydrated and embedded in paraffin. The maxillary incisor acted as the primary guide for orienting the sections. The section was cut perpendicular to the sagittal plane and was determined by 2 points, one at the alveolar crest and the other 4 mm apically. This plane passed through the center of the incisor crown at its gingival portion. The paraffin blocks were sliced into 5-\( \mu \)m-thick sections and prepared for hematoxylin and eosin staining before optical microscope examination. Measurement for bone histomorphometry was performed centering on the premaxillary suture and 175-250 \( \mu \)m (sections 35-50) below 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.

Histomorphometric evaluation was performed by 2 examiners blinded to the identity of the sections, and the average of the counts was obtained. Three histologic sections from each animal were analyzed. The study and control groups were compared to establish the number of osteoclasts, osteoblasts, and capillaries as well as the intensity of inflammatory cells and new bone formation. The sections were rated as mild (+: 0-15 cells), moderate (++: 15-30 cells), or strong (+++: >30 cells) for osteoblastic cells. However, new bone formation and inflammatory cell infiltration were qualitative features and evaluated in a subjective manner (mild: +; moderate: ++; or strong: +++).

**Statistical analysis**

All data were analyzed with the use of a commercially available software program (SPSS 14.0; SPSS, Chicago, IL, USA). Differences in the number of osteoclasts and osteoblasts among the 4 groups were evaluated with the use of the Kruskal-Wallis test, and pairwise comparisons were made with the use of the Mann-Whitney \( U \) test. The difference was considered to be statistically significant at a \( P \) value of \(<.05\). The results were presented as the mean ± SD.

**RESULTS**

The animals did not show obvious signs of systemic illness throughout the study period. Deep mucosal infection, dehiscence, or other adverse effects were not encountered in any of the animals in our study. Weight loss did not increase to \(<10\%\) in all groups throughout the study. During the study, owing to appliance failure, 1 rat in the GB group and 1 in the EO group were excluded from the study and substituted with new ones. The midpalatal suture was successfully expanded after application of the activated helical spring.

**Histologic findings**

**Number of osteoblasts.** Histologic findings indicated that the GB group showed a significantly higher number of osteoblasts compared with the other groups (\( P < .05 \)). The NE group had the lowest number of osteoblastic cells (Table I; Figure 3).

**Number of osteoclasts.** All 3 groups were found to have osteoclasts. Tables II and III present the number of osteoclasts present in the groups. The GB group had a significantly higher number of osteoclasts than the other groups (\( P < .05 \)). Furthermore, the EO group had a significantly higher number of osteoclasts than the NE group.

**New bone formation.** When new bone formation was analyzed, considerable differences were found among members of the GB group compared with other groups. The results revealed increased new bone formation in the GB group, which was significantly higher than the corresponding values in the other groups. The EO group showed increased growth that exceeded that of the NE group (Table I; Figure 3).
Number of capillaries. The GB group showed a similar increase in capillaries compared with the other groups. Furthermore, the EO group had a higher number of capillaries than the NE group (Tables I and II; Figure 3).

Intensity of inflammatory cells. There was a significantly higher ratio of inflammatory cells in the GB group than in the EO and NE groups. Furthermore, the EO group had more inflammatory cells than the NE group (Table I).

DISCUSSION
Several potential drawbacks can emerge during the course of RME. Among these, relapse is considered to be most challenging and frequent issue encountered by clinicians during RME. Although the exact reason of
Table II. Effects of Ginkgo biloba on the Number of Osteoclasts and Capillaries at the End of the 12th Day of the Experimental Period (Mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>NE</th>
<th>OE</th>
<th>GB</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>No. of osteoclasts</td>
<td>1.14 ± 0.37</td>
<td>4.14 ± 0.69</td>
<td>7.57 ± 0.53</td>
<td>.000**</td>
</tr>
<tr>
<td>No. of capillaries</td>
<td>3.71 ± 0.75</td>
<td>6.86 ± 0.69</td>
<td>10.86 ± 0.69</td>
<td>.000**</td>
</tr>
</tbody>
</table>

**P < .01 (Kruskal-Wallis variant analysis).

Table III. Pair-Wise Comparisons of the Groups

<table>
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<tr>
<th></th>
<th>NE-OE</th>
<th>NE-GB</th>
<th>OE-GB</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of osteoclasts</td>
<td>.001**</td>
<td>.001**</td>
<td>.001**</td>
</tr>
<tr>
<td>No. of capillaries</td>
<td>.001**</td>
<td>.001**</td>
<td>.001**</td>
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**P < .01 (Mann-Whitney U test).

relapse is not fully understood, recent studies have focused on improving the velocity and the quality of bone formation in the intermaxillary suture. To this end, various studies were designed with the aim of accelerating and increasing new bone formation. Kara et al. investigated the effect of systemic thymoquinone, an antioxidant, in a rat RME study and demonstrated that thymoquinone effectively accelerated new bone formation and prevented relapse and reducing the retention period in RME. Taking these previous results into consideration, the present study aimed to examine the effectiveness of GB to accelerate the formation of new bone and increase bone quality for reducing the retention period and decreasing the risk of relapse.

Various reports have been published on the association between oxidative stress and bone metabolism. Oxidative stress due to excessive production of reactive oxygen species (ROS) and/or impaired antioxidant defense mechanisms can result in adverse biologic effects on bone by inhibiting bone cell differentiation in the preosteoblastic cell line and in the marrow of the stromal cell line. Evidence indicates that ROS considerably affect the generation and survival of osteoclasts, osteoblasts, and osteocytes. ROS play a role in bone resorption, with a direct contribution of osteoclast-generated superoxide to bone degradation. Moreover, osteoblasts produce antioxidants, such as glutathione peroxidase, to protect against ROS. However, the precise mechanism of ROS-mediated destruction of calcified tissue and the role of ROS in bone resorption is as yet unknown.

Therefore, researchers have recently focused on different types of host modulatory agents, such as antioxidants, to cope with the deleterious effect of oxidants and oxidant-related breakdown of hard tissues and for their ability to improve bone remodeling.

Antioxidants are commonly used as nutritional supplements and evidently neutralize harmful free radicals in the body, which can lead to numerous serious or life-threatening diseases, including atherosclerosis, heart disease, cancer, immune dysfunction, metabolic bone diseases, diabetes, and degenerative brain disorders. They also exert favorable effects on bone metabolism, which promotes bone regeneration. Thus, antioxidants are an interesting field of study, particularly because of their effect on new bone formation.

Various studies in literature have emphasized that antioxidant agents may accelerate new bone formation and prevent bone resorption. Ozdemir et al. demonstrated that the oral administration of thymoquinone decreased alveolar bone resorption in a rat periodontitis model owing to its potent antioxidant and antiinflammatory properties. Demirer et al. suggested that boric acid promoted osteoblastic activity and reduced osteoclastic activity and alveolar bone loss by promoting neutralizing agents of ROS.

Ginkgo biloba has been used as folk medicine in China for ~5,000 years. Recently, because of its unique physicochemical characteristics and positive biologic abilities, such as scavenging free radicals, lowering oxidative stress, reducing neural damages, reducing platelets aggregation, antiinflammation, antitumor activities, and antiaging, GB has gained considerable attention in the medical research area. Phytochemical research has demonstrated that standard composition of GB contains 24% flavonoids (ginkgo-flavone glycosides) and 6% terpenoids (including ginkgolides A, B, C, J, and bilobalide). It is also well known for its antioxidant property due to its ability to scavenge free radicals and to neutralize ferryl ion-induced peroxidation.

Bing et al. investigated the effect of GB extract on free radical metabolism in mice livers. They suggested that GB extract helps in increasing antioxidant enzyme activity in liver tissue and reduce lipid peroxidation injury in liver tissue caused by free radicals. Bridi et al. demonstrated the possible role of GB in the treatment of diseases involving free radicals and oxidative
damage. Lucinda et al.\(^1\) examined the effects of GB extract on rat mandibular glucocorticoid-induced osteoporosis and demonstrated that GB recovered the periodontal bone support and increased the mandibular cortical thickness. In the present study, we investigated the antioxidant property of GB on accelerating new bone formation and improving bone quality. We found that the rats administered GB showed increased new bone formation and higher numbers of osteoblastic cells compared with the control rats. The presence of osteoclastic activity was also higher in the GB groups than in the other groups. This elevation of the osteoclast number is most probably related to the acceleration of bone turnover. These results show that systemic administration of 28 mg GB/kg body weight can improve bone formation and may be useful in preventing relapse following RME.

Currently, there is considerable interest in animal research of bone healing, because such research may not be conducted on humans for practical or ethical reasons.\(^2,21,23\) Therefore, animals such as rats,\(^7,9\) rabbits,\(^22\) cats,\(^23\) and monkeys,\(^24\) have been frequently favored for RME models. In the present research, we used rats, considering the cost, size, and maintenance requirements and because rats are more commonly preferred by investigators.

Earlier studies\(^25,26\) revealed that light forces up to 20 g could produce orthodontic tooth movement without the opening/expansion of the premaxillary suture. On the other hand, continuous heavier forces cause necrosis of periodontal ligament and hyalinized areas at alveolar bone and prohibit the tooth movement, leading to sutural expansion by the residual forces without requiring any surgical acceleration. The expansion protocol used in the present study has been found to be efficient in several studies performed on rats and typically preferred in recent research.

Some researchers\(^3,7\) have used calipers for measuring the space between the mesial corners of the upper incisors at the beginning and on the fifth day of the expansion period. Because it has been previously established that a 5-day expansion period is sufficient for midpalatal expansion, and that 1.5 mm between incisors is adequate for validation of the sutural opening/expansion, the distance between the maxillary incisors was not noted but was checked for confirming that a distance of ≥1.5 mm was gained on all rats in our study. Furthermore, during the histomorphometric examination, an expert histologist confirmed that the intended amount of expansion occurred in the midpalatal areas of all rats.

In a study by Sawada and Shimizu,\(^8\) bone formation at the expanded suture was investigated via serial killing at different times, and they considered the 6-12 days after expansion to be the late stage. In our study, to be able to compare the groups more indisputably and to observe a more significant effect, the late stage of the consolidation period was chosen.

Ginkgo is generally a well tolerated drug with scarce and often mild adverse effects, including intracerebral hemorrhage, gastrointestinal disturbances, headaches, dizziness, and allergic skin reactions.\(^27\) In animal experimentation with gingko extracts, no mutagenic or teratogenic effects were found. Oral administration of up to 1,600 mg/kg in rats daily did not produce teratogenic effects. No reproductive and developmental toxicity report was exhibited related to ginkgolide or bilobalide.\(^28\) The dose used in the present study was 28 mg/kg body weight administered orally.

**CONCLUSION**

This study is the first to present data indicating that *Ginkgo biloba* improves new bone formation in RME in rats. This suggests that systemic administration of GB may be useful in the prevention of relapse following RME.

**REFERENCES**

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