Effects of osthole on bone regeneration in the mid-palatal suture of rats during rapid maxillary expansion

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Abstract: This study aimed at evaluating the effects of osthole on the mid-palatal suture remodeling during rapid maxillary expansion (RME). Rats were randomly separated into three groups: control, expansion only (EO), and expansion plus osthole (OST) groups. An orthodontic appliance was set on rat molars of EO and OST groups and rats in OST group were administrated with osthole (20 mg/kg/day). The suture width and bone volume changes were observed by micro-computed tomography (microCT) and hematoxylin and eosin (HE) staining. Alkaline phosphatase (ALP) and Nel-like molecule-1 (NELL-1) expression were also examined for the assessment of bone formation in the suture. The mid-palatal suture was expanded by the force, but there were no statistically significant differences (P > 0.05) in the width between EO and OST groups. Bone volume of the mid-palatal suture decreased during RME, but increased in the OST group on day 10 compared with EO group. Moreover, ALP and NELL-1 expression of OST group were more than that of the other two groups (P < 0.05). Our data suggest that osthole may be a potential agent to accelerate bone formation of the mid-palatal suture during RME.

Keywords: Suture remodeling, osthole, microCT, alkaline phosphatase, nel-like molecule-1

Introduction

Rapid maxillary expansion (RME) is an effective approach to correct transverse maxillary deficiency [1, 2]. It was originally used for growing adolescents whose suture remains patent, but now the practice can also be for adults with the aid of lateral maxillary and mid-palatal osteotomies [3]. Some studies [4-6] analyzed the long-term effects of RME and proved that a passive retainer was needed for a minimum of three months to prevent the relapse after rapid expansion therapy. This is because, it takes at least three months to complete the process of mineralization in the expanded suture [7]. Inadequate bone formation after expansion may result in an undesired relapse due to the pressure of facial bones and muscles [8]. The effects of RME can be maintained only through establishing a stable structure in the mid-palatal suture. Therefore, to prevent post-treatment relapse, it is extremely important to accelerate bone remodeling in the expanded suture.

Osthole (PubChem CID: 10228), a plant coumarin derivative of Chinese herb medicine, is extracted from many plants, such as Cnidium monnieri (L.) CUSS and Angelica pubescens. Some studies have shown that osthole has vasorelaxation and anti-inflammatory effects [9, 10]. Recent researches [11, 12] have also found that osthole can significantly reverse bone loss and prevent the osteoporosis in ovariectomized rats. However, little is known about the effects of osthole on bone remodeling of other skeletons or sutures. Considering the stimulating effects of osthole on bone formation, we put forward the hypothesis that osthole could promote bone regeneration in the mid-palatal suture during RME. In this experiment, we utilized a well-developed maxillary expansion model of rats to investigate the effects of osthole on bone remodeling of the mid-palatal suture stimulated by expansion force, which will provide additional new insights into preventing the post-expansion relapse and
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shortening the retention period for orthodontists.

Materials and methods

Animals and groups

In this study, fifty-four male Wistar rats (six-week-old, 150±10 g) were randomly divided into the following three groups (n=18 each): control, expansion only (EO), and expansion plus osthole (OST) groups. The rats of OST group were treated by oral administration of 1 ml suspension liquid with 20 mg/kg body weight of osthole dissolved in distilled water every day. Osthole (purity ≥ 98%) was purchased from Dalian Mellon, biological technology co. Ltd (Dalian, China). All procedures were approved by the Animal Welfare Committee of Shandong University, and the study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Rapid maxillary expansion model of rats

A 0.014-inch Australian orthodontic wire (A.J Wilcock Pty Ltd, Australia) was bent into a rectangular form as the orthodontic appliances with two opening loops. After anesthetized, the maxillae in EO and OST groups were performed by inserting the activating appliances between the right and left upper molars. After the end of appliances was inserted to the embrasure between the first and second molars, the light-cured adhesive was attached to all the maxillary molars after etching the teeth with the acid etch (Gluma Etch 35 Gel). The initial expansive force was 0.98 N measured by a strain gauge. All animals were monitored for infection or appliance failure during the experiment. If any complication such as mucosal infection or rapid decrease of body weight was observed, they would be excluded from the study.

MicroCT analysis

Six rats in each group were executed on the 4th, 7th, 10th days. The maxillary bone was exposed by peeling off the nasal and palatal mucosa and fixed in 4% paraformaldehyde buffer at 4°C for 24 h. The whole maxilla was scanned by MicroCT system (Inveon MM CT, SIEMENS, USA) at 80 kV, 500 µA, and 8.5 µm-

Figure 1. MicroCT images of the rat maxilla. A. Suture width was measured on the horizontal surface. B. Bone volume on the coronal plane. ROI was shown in the area in the blue box, and the area in the green box represented the bone volume measured. C. Bone volume on the horizontal plane. D. Suture width in three groups (n=6). *P < 0.05, significant increase versus the control group. E. Bone volume change in three groups (n=6). a: significant decrease versus the control group at P < 0.05, b: significant increase versus EO group at P < 0.05.
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Figure 2. The coronal plane of the mid-palatal suture stained with HE on days 4, 7 and 10. All images are displayed at 400× magnification. The suture consists of central fibrous tissue and secondary cartilage which small-sized immature cells transit to mature chondrocytes. Under the expansive force, the mid-palatal suture was expanded and more bone formation was observed in the cartilaginous area.

Hematoxylin and eosin (HE) staining

The maxilla was surgically removed, trimmed and decalcified in 10% ethylene-diaminetetraacetic acid (EDTA)/phosphate-buffered saline (PBS) solution for three months. Then they were made into tissue sectioning. Serial frontal 5-μm-thick sections of the embedded maxilla were cut at the level of the upper first molar. Then sections were deparaffinized and rehydrated for the following uses. Some sections were stained with HE and then observed under a light microscope. Histological changes and bone remodeling (×400) of the mid-palatal suture were shown in Figure 2.

Alkaline phosphatase (ALP) staining

Some sections were stained using the improved Gomori calcium-cobalt method. Experimental procedures were conducted according to the instructions of ALP kit (JianchenTech., Nanjing, China) and the gray-black granule or block deposit showed positive alkaline phosphatase staining. The results were analyzed by Image-Pro Plus software (IPP 6.0, Media Cybernetics, Inc., USA) to measure the positive area and were taken as an average of five randomly selected fields.

Immunohistochemistry

The other sections were deparaffinized, hydrated, and then washed with PBS. 0.1% (w/v) trypsin was used for antigen retrieval at 37°C for
10 min. Then the activity of endogenous tissue peroxidase would be blocked with 3% H$_2$O$_2$ for 30 min. After pretreatment with normal goat serum for 30 min, the sections were incubated with NELL-1 antibody (1:100 dilution; Biosynthesis Biotechnology, Beijing, China) at 4°C overnight and goat anti-rabbit IgG and StreptAvidin-BiotinComplex at 37°C for 25 min. Diaminobenzidine solution was utilized to visualize localization for 2 min. Next, they were counterstained with hematoxylin. PBS was substituted for the primary antibody as negative controls. The results were analyzed by IPP software to calculate the mean optical density (MOD) of NELL-1 expression. Five fields (×400) randomly selected by a single examiner in a blind study were evaluated with a mean value of five fields.

Figure 3. ALP staining of the suture in three groups on days 4, 7, 10 (n=6). A. The gray-black granule or block deposit represents positive ALP staining at 400× magnification. B. The area of positive ALP staining on days 4, 7, 10. (a: Significant increase versus the control group at $P < 0.05$; b: Significant increase versus EO group at $P < 0.05$).
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Statistical analysis

All the data were presented as the mean ± standard error of mean and analyzed with SPSS software (version 17.0 for Windows; SPSS Inc., Chicago, Illinois, USA). The difference among three groups was analyzed by Kruskal-Wallis one-way analysis of variance, and then we used the LSD method for intergroup differences. A value of $P < 0.05$ was considered statistically significant.

Results

Mucosal infection, dehiscence, or death was not encountered in members of the three
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groups. The body weight in the control group rose steadily. Rats with expansion appliance had difficulty in dieting on the first two days of RME but subsequently recovered.

Suture width change

Morphological changes in three groups were observed from the three dimension images of microCT. The mid-palatal suture of the rats in EO and OST groups became wider ($P < 0.05$) than that in the control group and the tipped molars were observed on day 4, 7, 10. In two-dimensional measurements of the width on the horizontal surface, there was a significant increase in the suture width ($P < 0.05$) in EO and OST groups (Figure 1D). Though mean suture width in EO group was slightly wider than that in OST group on days 7, 10 (0.393±0.017 mm and 0.380±0.015 mm, 0.388±0.018 mm and 0.374±0.027 mm), there was no statistically significant differences ($P > 0.05$). Microscopic observation showed that the suture in the control group was narrow while the suture was expanded and transverse fibers were extended in EO and OST groups.

Bone formation of the mid-palatal suture

The area in the green box represented the bone volume of ROI. The amount of bone formation in the maxilla was measured by comparing bone volume of the three groups on days 4, 7, 10 (Figure 1E). Compared with the control group, bone volume of ROI in the EO and OST groups had a significant decrease ($P < 0.05$). This was because the suture was expanded and bone volume of the same-sized ROI decreased with it. In other words, this also verified that the appliance expanded the suture successfully. Though there was no significant difference between the two groups on day 4, 7 ($P > 0.05$), a significant increase ($P < 0.05$) of bone volume was found in OST group compared with that in EO group on day 10 (0.234±0.008 mm$^3$ and 0.209±0.011 mm$^3$). HE staining could show detailed histological changes of the mid-palatal suture. In the control group, cell components and capillaries of the mid-palatal suture were minimal. After expansion, the fibrous and cartilaginous tissues of the suture widened and new bone was deposited in the EO and OST groups. The chondrocytes proliferated, differentiated, and became hypertrophic. Some cells were extending into the fibrous layer and replaced by osteoblasts. Compared with EO group, more bone deposition and cells occurred within the mid-palatal suture of OST group. The microCT and HE results both indicated that osthole significantly accelerated the progression of bone formation within the expanded mid-palatal suture.

ALP

To further evaluate new bone and cartilage formation, ALP activity was investigated (Figure 3A). In the control group, very weak ALP activity was exhibited along the bone edges of the osteogenic zone. In the EO and OST groups, the ALP activity in the osteogenic zone was higher than that in the control group during the whole experiment (Figure 3B). At each time point, the ALP positive area of OST group was larger than that of EO group ($P < 0.05$).

Immunohistological findings

In the control group, little expression of NELL-1 was seen in the osteoblasts in the osteogenic zone of the mid-palatal suture. In EO and OST groups, marked expression of NELL-1 activity was detected in the osteoblasts in the osteogenic zones of the sutures (Figure 4A). Compared with the control group, the mean optical density (MOD) value of NELL-1 immunoreactivities in the EO and OST groups was significantly high ($P < 0.05$) on days 4, 7, 10 (Figure 4B). Additionally, NELL-1 expression of the OST group was more intense than that of the EO groups ($P < 0.05$) within the whole experiment.

Discussion

Relapse after rapid expansion is one of the challenges clinicians have to cope with. Even after a long retention period, the maxilla after RME still has a strong tendency to return to its previous status. It may be caused by many factors, but many authors hold that one of the major reasons of relapse may be insufficient bone regeneration of the suture [13]. Many experimental approaches have been attempted to accelerate bone formation to shorten the retention period and prevent the post-expansion relapse [14, 15]. But these methods are high in price or there are some difficulties in their clinical practice, which cannot meet the needs of applications in RME patients. Traditional Chinese medicine is well known for lower price and few side effects. Osthole is a natural coumarin and bioactive compound used in traditional Chinese medicine for many years due to its warming the kidney and strengthening.

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Yang [9]. In this study, we demonstrated that the systemic application of osthole could significantly enhance bone regeneration of the mid-palatal suture. As a result, osthole may have a potential therapeutic benefit in promoting bone formation to prevent relapse and decrease the retention period after RME.

Six-week-old rats, which were in a growing phase, were selected as an animal model to investigate bone formation of the suture. Bone formation reduces with age, and it is easier to achieve the desired orthopedic effects of the maxilla before and during pubertal growth [16, 17]. Besides, rabbits or rats are better models to observe bone and suture changes under stress though there are more similarities between monkey and human in RME [18]. Some authors [17, 19] measured the width change and bone formation of the suture by HE staining photomicrographs. In this experiment, we evaluated the suture width and bone volume change through the method of microCT, which is more efficiency in bone microstructure analysis.

In this study, the suture was opened by helical springs and the type of expansion was similar to that used in patients. Furthermore, microCT images showed wide separation of the maxillary bones after expansion. This illustrates that the force applied to the suture is an acute and orthopedic force for growing rats. Widening of the suture by RME can effectively increase the width of dentition and the mid-palatal suture, accompanied by active bone remodeling in the suture. The bone formation-accelerating effects of osthole were also confirmed in our microCT evaluation. A significant increase of bone volume was found in OST group compared with that in EO group on day 10, which indicated that more new bone formation elements were deposited in the expanded suture stimulated by osthole. Of course, the imaging in microCT required bone density to meet a certain standard. Adequate bone deposition needed some time to make new bone formation observed in microCT image, so there was no significant difference between EO and OST groups on day 4, 7. However, detailed histological changes can be revealed by HE staining more sensitively. Compared with EO group, more bone deposition and cells occurred within the mid-palatal suture of OST group on days 4, 7 and 10. These results indicate that osthole can increase bone regeneration of the mid-palatal suture after RME, which are in agreement with the previous experimental results.

ALP, a differentiation marker of osteoblasts, is mainly involved in the mineralization process of the bone matrix. Previous studies [20, 21] have found that osthole can increase ALP activity, maturation and differentiation of osteoblasts in vitro. Our results are consistent with these reports. In this study, we discovered that ALP expression increased in the two groups of expansion and ALP activity in OST group was higher than that of EO group during the whole experiment. The increase of ALP activity reflects high bone matrix mineralization or functional activities of osteoblasts in bone remodeling of the expanded suture. Therefore, the increase of ALP activity of OST group is indirectly supportive of a stimulatory effect of osthole on bone formation of the suture during RME.

Some cytokines also play an important role in bone remodeling of the suture. Nel-like molecule-1 (NELL-1) is a growth factor with osteoinductive properties. Recombinant NELL-1 protein has been observed to induce bone and cartilage formation in numerous in vitro and in vivo models [22-24]. NELL-1 can control cell growth and differentiation, promote differentiation and mineralization of osteoblasts by regulating Runx2 activity and MAPK signaling [25, 26]. These studies all demonstrate that NELL-1 has significant effects on bone regeneration. In our study, weak NELL-1 immunostaining in the control group was detected in some osteoblasts of the suture. For growing rats, these positively stained osteoblasts may actively function during natural growth and remodeling. With expansion forces, the expression of NELL-1 was strongly increased in the suture during RME, which also shows that NELL-1 may play important roles in bone formation in the expanded suture compared with the control group. Furthermore, NELL-1 expression in OST group was both higher than that in EO group. Therefore, the higher expression of NELL-1 in OST group may have been induced by osthole, which means osthole can stimulate the mid-palatal suture remodeling. These experimental results strongly indicate that osthole can promote bone regeneration during RME.

In conclusion, the data collected in the study show that osthole may be a potential agent to promote bone remodeling in the expanded mid-
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Palatal suture, which will provide a better idea for orthodontists to improve the stability of RME and prevent post-expansion relapse. The dose of osthole used in the current study was 20 mg/kg administered oro-gastrically, which was used in many rat models and considered to be relatively safe [27]. Local pharmaceutical delivery may require lower doses and induce fewer side effects, so a local slow-release preparation also needs to be developed for further preclinical trials. Additionally, a longer applied time of osthole will be required to evaluate whether it can shorten the retention period and prevent relapse after RME, so the ultimate validation of osthole as a therapeutic aid for inducing bone formation of the mid-palatal suture after RME needs long-term animal studies and human trials, which is our future research objectives.

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Disclosure of conflict of interest

None.

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