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ORIGINAL ARTICLE

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Orthopedic protraction of the maxilla may affect cranial base synchondroses indicated by increased expressions of growth factors

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Structured Abstract

Objectives – To examine the biological adaptation of cranial base synchondroses (CBS) when the maxilla was forward positioned by orthopedic force.

Setting and Sample Population – The Department of Orthodontics at Shanghai Jiao Tong University. 50 Sprague–Dawley rats, 4 weeks of age, were divided into experimental (n = 30) and control groups (n = 20).

Material and Methods – An orthopedic appliance was fitted to the cranio-maxillary complex to advance the maxilla forward. The animals in the experimental group, together with the counterparts in the control group, were sacrificed at days 1, 3, 5, 7, and 14, respectively. The whole cranial base housing both the spheno-ethmoid (SES) and spheno-occipital synchondroses (SOS) was removed for tissue processing and immunotest of Sox9, Core-binding factor α 1 (Cbfa1), and vascular endothelial growth factor (VEGF), three carefully selected growth factors that are markers of chondrogenesis in different stages and its transition to endochondral ossification. Semiquantitative analysis was also conducted by using a computerizing imaging system.

Results – The temporal tendency of the changes in the expressions of the three growth factors featured an increase from Day 3 and onwards for Cbfa1 and VEGF, and a following decline after Day 5 for Sox9. In both SES and SOS, the expressions of the three growth factors were significantly stronger in the experimental groups than that in groups ($p < 0.05$).

Conclusions – Protractive orthopedic force imposed on the maxilla provokes an enhancement of chondrogenic process in CBS.

Key words: chondrogenesis; cranial synchondroses; orthopedics

Introduction

A growing number of studies have been devoted to the cranial base and its association with the oral and maxillofacial complex. Growth and development of the cranial vault closely coordinate with that of the facial skeleton and, more importantly, influence the positions of the maxilla and the mandible (1, 2). Dysmorphology of the cranial base structures results in deformities in the maxillofacial region (3, 4). Severe skeletal class III malocclusion characterized by mid-face retrusion is partially caused by defective growth of the bony tissue of the cranium (5). It is widely accepted that reverse-pull headgear (RPHG) is effective in correcting class III malocclusion by enhancing growth and advancing the position of the maxilla (6). However, it remains unclear whether or not this orthopedic modality can affect the deformative structures in the cranial base.

It is well established that the cartilaginous tissues between the cranial base bones, namely, spheno-ethmoid (SES) and spheno-occipital (SOS) synchondroses, play a critical role in growth and development of the cranium both in embryonic, neonatal, and postnatal stages (2). The biological phenotype of the cranial base synchondroses (CBS) remains a disputable topic in the literature. Some researchers liken CBS with the epiphyseal cartilage in the lone bone growth plate, regarding CBS as an independent growth center of the cranium (7, 8). Others assert that the growth pattern of CBS can be influenced by external mechanical forces, a biological nature similar to that of condylar cartilage that is adaptive to mandibular advancement (9).

The transition from cartilage to bone is reported to take place via endochondral ossification. It begins with chondrogenesis where chondrocytes differentiate, mature, and hypertrophy to their terminal phenotype, and concludes with osteogenesis where hypertrophic cartilage degrades and vasculature invades, followed by osteoblastic differentiation and the subsequent bone formation (10). The stage-specific transcriptional factors are reported to govern and regulate the biological process of endochondral ossification (11). Sox9, one of the Sry-related family of HMG box DNA-binding proteins, is a master transcription factor that controls the genetic program of differentiation of mesenchymal cells into chondrocytes (12). It has been reported that when the Sox9 gene is inactivated after mesenchymal condensations, most cells are arrested as condensed mesenchymal cells and will not undergo overt differentiation into chondrocytes (13). Core-binding factor α 1 (Cbfa1), among others, is a pivotal transcription factor involved in chondrocyte maturation and osteoblast differentiation (14). Cbfa1 regulates the postnatal growth of mandibular condyle by coupling the process of chondrocytes maturation and degradation during endochondral bone formation (15). This is further evidenced by mutant experiments where the Cbfa1-deficient mice show chondrocyte maturational disturbance and the absence of hypertrophic chondrocytes (16, 17). It is also reported that Cbfa1 claims its role in the final stage of endochondral ossification firstly by up-regulating all the major osteoblast-specific genes and then by enhancing extracellular matrix mineralization (17). This is further convinced by the studies where osteoclastogenesis is blocked in Cbfa1-mutant mice and resumed when using transgene to restore its expression (18). In the transition toward osteogenesis, the production of VEGF is regarded as an important event where neovascularization is allowed to occur to bring in the osteogenitor cells, leading to the onset of bone deposition (19).

To obtain a better understanding of the linkage between maxillary orthopedic protraction and its possible effect on the cranial base, this study was designed to create an animal model where the maxilla of Sprague–Dawley (SD) rat was advanced by RPHG and to elucidate whether or not the CBS were adaptive to the maxillary repositioning by examining the expressions of the regulatory factors of Sox9, Cbfa1, and VEGF in both SES and SOS.

Materials and methods

Animal model with orthopedic appliances

Sprague–Dawley rats were used for the model (Shanghai Ninth People's Hospital Research Ethical

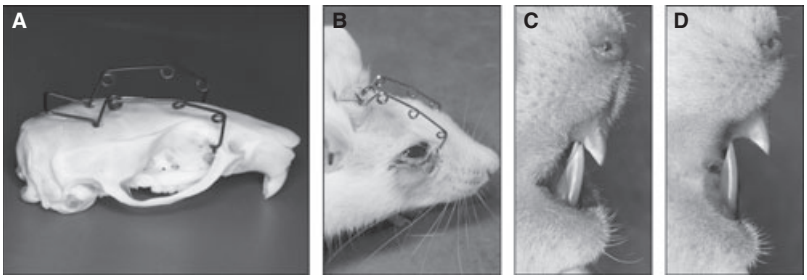

Fig. 1. The design and placement of reverse-pull headgear (RPHG). The four arms of RPHG are inserted into the surgically prepared defects in the parietal bone of the skull and the temporal–zygomatic bone fissures (A). When fitted and activated (B), RPHG advances the maxilla forward, leading to a markedly increased overjet in the experimental animal, while overjet remains normal in the control animal (C, D).

Committee Approval NO. 0097239224). The orthopedic appliance RPHG was made of the 0.018-inch Australian wires with the coils bent in to generate the pushing force of 80 g. The frontal arms of the appliance were inserted into the fissures between the temporal and the zygomatic bones, and the rear arms into the two defects surgically opened on the parietal bone of the skull. The orthopedic force direction was, therefore, almost parallel to the cranial base (Fig. 1A). When fitted (Fig. 1B), the appliance was activated to protract the whole maxilla forward indicated by an increased overjet of 3 mm (Fig. 1D).

Animal grouping and tissue preparations

Fifty SD rats with 4 weeks of age were divided into the experimental (n = 30) and the control (n = 20) groups. The experimental animals were placed with the RPHG orthopedic appliances and were further divided into five subgroups (n = 6) according to the experimental durations. The control group was also further divided into the matched five subgroups (n = 4). The animals in the subgroups were sacrificed at days 1, 3, 5, 7, and 14, respectively. Immediately after death, the parietal bone was surgically opened and the cranial base bone was carefully removed with intact synchondroses (Fig. 2A). The specimens were then decalcified in 12.5% ethylenediamine tetraacetic acid (EDTA, pH = 7.0) at 4°C for 3 weeks and were finally embedded in paraffin. Serial sections of 6-μm thick were cut through CBS at the coronal plane using a rotary microtome (Leica RM 2035; Leica Corp., Nussloch, Germany) and were floated onto glass slides for histological and immunohistochemical analysis.

Immunohistochemical examinations

The primary antibodies for the three growth factors were following: The Sox9 antibody was raised against a recombinant protein (sc-17341; Santa Cruz Biotechnology, Inc., CA, USA) corresponding to an internal region of human origin. Goat polyclonal PEBP2&A antibody IgG (sc-8566, Santa Cruz) that was raised against a peptide mapping at the C-terminus of PEBP2&A was used for immunolocalization of Cbfa1. Goat polyclonal antibody IgG (sc-152, Santa Cruz) that was raised

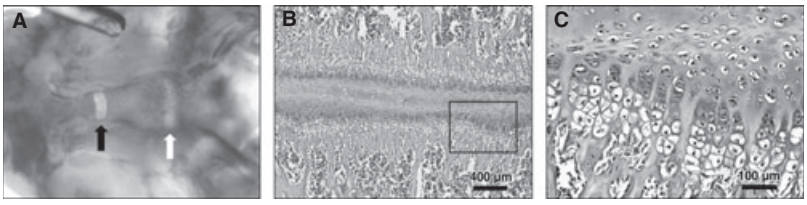

Fig. 2. Tissue sample of cranial base showing anatomical location of spheno-ethmoid (black arrow) and spheno-occipital synchondroses (white arrow) (A), and their histological structures featuring bipolar epiphyseal cartilage (B). Higher magnification of the marked frame in (B) reveals a typical cartilaginous phenotype highlighted by resting, proliferative, hypertrophic, and erosive chondrocytes (C).

against a peptide mapping at the N-terminus of VEGF-A was used for the detection of vascular endothelial growth factor (VEGF). The secondary antibodies were biotin-conjugated donkey–anti-goat IgG (sc-2023, Santa Cruz).

Immunohistochemistry was carried out with a three-step avidin–biotin complex method (19). Briefly, after the sections were dewaxed and rehydrated, they were treated in 3% peroxide for 10 min. Then the samples were covered with 0.05% trypsin for 15 min at 37°C to retrieve the antigen. Nonspecific bindings were blocked with 3% albumin bovine serum before incubating the samples overnight with the primary antibody at 4°C. Free antibodies were removed by washing in phosphate buffer solution. Sections were then incubated with secondary antibody for 30 min at 37°C, followed again by washing. Then the slides were dipped in 3, 3-diaminobenzidine (DAB) for 3 min to identify the binding sites. To ascertain the specificity of the antibodies, negative controls were performed, in which the primary antibody was replaced by nonimmune serum.

Quantitative and statistical analyses

The expressions of the three growth factors were assessed quantitatively by measuring the area of the positively reacted immunostaining signals. A true-color computer-assisted image analyzing system with a digital camera (Pixera Penguin 600CL CCD; Pixera Corporation, Santa Clara, CA, USA) and ImageJ software (Version 1.36b; NIH, Bethesda, ML, USA) was used for quantitative analysis. Measurements were carried out within a fixed frame of 480×360 μm^2 set in the central part of SES and SOS. All of the tissue slides from fifty rats went through the measurement. Statistical analysis was processed with SPSS for Windows (Version 11.0; SPSS Inc. Chicago, Illinois, USA) for *t* test.

Results

Histological examination revealed that cranial synchondroses presented bipolar epiphyseal cartilage phenotype (Fig. 2B), featuring column-arranged chondrocytes (Fig. 2C).

Table 1. **Expressions of three growth factors in spheno-ethmoid (SES) and spheno-occipital synchondroses (SOS), respectively, over experimental time points (μm^2)**

		Sox9			Core-binding factor α 1 (Cbfa1)			vascular endothelial growth factor (VEGF)		
Day		Exp	Con	*p*	Exp	Con	*p*	Exp	Con	*p*
1	SES	17255 ± 4281	16682 ± 8067		26682 ± 2523	26006 ± 6957		36694 ± 9804	35237 ± 15242	
	SOS	12496 ± 2923	11882 ± 3569		24908 ± 2564	23528 ± 6929		26595 ± 13406	23719 ± 6751	
	p									
3	SES	24632 ± 7670	19991 ± 6518		31963 ± 3511	30737 ± 6217		42756 ± 11690	37217 ± 15728	
	SOS	15378 ± 2328	14394 ± 3290		27522 ± 2145	24646 ± 5731		33573 ± 8439	26461 ± 2704	
	p									
5	SES	57550 ± 9461	36631 ± 4482	**	36001 ± 940	32931 ± 1670		47432 ± 18643	37523 ± 1973	
	SOS	29620 ± 4618	21098 ± 2588		31192 ± 6230	26613 ± 914		41598 ± 20672	32937 ± 1316	
	p	**	**							
7	SES	40095 ± 4560	24285 ± 5805	**	41993 ± 5995	35059 ± 3493		57207 ± 17243	40687 ± 8461	**
	SOS	20039 ± 3605	14320 ± 2630		34490 ± 5841	30201 ± 1653		43218 ± 9519	37726 ± 1956	
	p	**	**							
14	SES	29799 ± 10779	14810 ± 2500	**	60520 ± 16765	41752 ± 3353	**	69742 ± 18882	46851 ± 19181	**
	SOS	17394 ± 2512	14265 ± 2377		38687 ± 9434	33781 ± 2378		51059 ± 13409	38392 ± 16045	**
	p	**			**			**		

**$p < 0.05$.

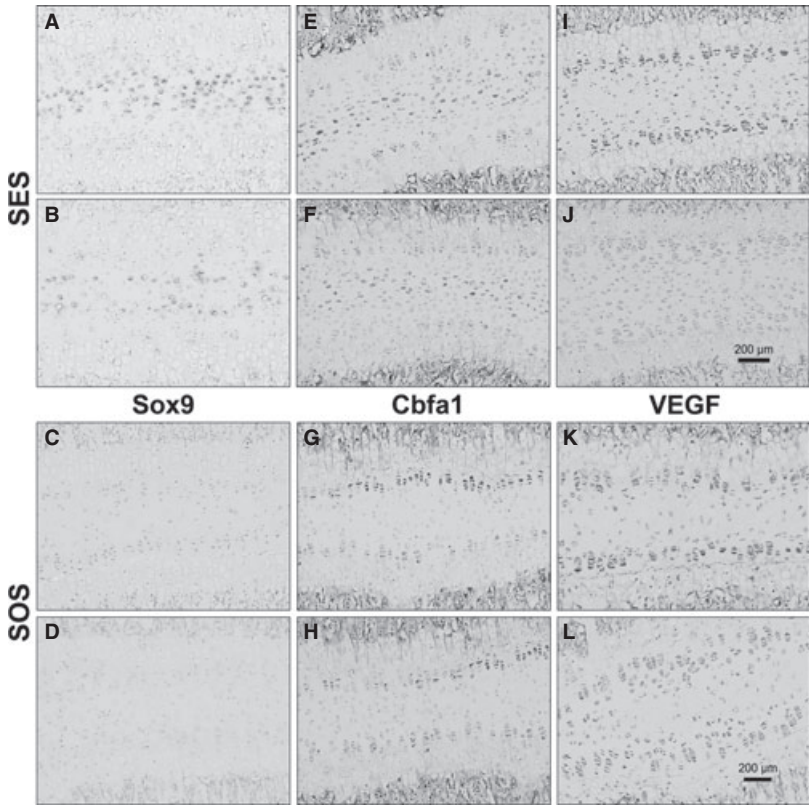

Fig. 3. Photomicrographs of immunoreaction of the three growth factors at Day 7. In spheno-ethmoid, the localization of positive staining for Sox9 in the resting and proliferative chondrocytes (A, B), Core-binding factor α 1 in the hypertrophic chondrocytes (E, F), and VEGF in the erosive cartilage (I, J) indicate that they are stage-specific markers for the biological pathway from chondrogenesis to osteogenesis. The immunoreactions in the experimental subgroups (A, E, I) remain stronger than those in the control subgroups (B, F, J). In spheno-occipital synchondroses, distinctive localization of Sox9 (C, D), Core-binding factor α 1 (G, H), and vascular endothelial growth factor (K, L) in different cartilage zones suggests their respective regulatory roles governing the process of endochondral ossification. Growth factors in the experimental subgroups (C, G, K) are markedly stronger than those in the control subgroups (D, H, L).

The quantitative assessment of the expressions of the three growth factors in both SES and SOS is shown in the Table 1.

The spatial localization of the three growth factors expressed in the cranial synchondroses cartilage was distinctive. Immunoreaction of Sox9 was seen mainly in the resting and the proliferative chondrocytes (Fig. 3A–D); the expression of Cbfa1 was witnessed in the hypertrophic cells (Fig. 3E–H); and the erosive cartilage close to bone tissue was the area where VEGF was positively stained (Fig. 3I–L).

The temporal tendency of the expressions of the three growth factors is shown in Fig. 4 and is described in details as below:

Day 1: The expressions of the three factors in both SES and SOS were at a lower level, which did not show any significant differences between the experimental and control groups. Day 3: The expressions of the three factors in the experimental group exceeded those in the control group. Positive staining of the three factors in SES was higher than that in SOS. Day 5: The immunoreaction of Sox9 reached its peak level, with that in the experimental group being significantly higher than in the control group and that in SES being significantly higher than in SOS. Day 7: Strong positive staining for Cbfa1 and VEGF was seen in both SES and SOS, with the expressions of these two factors in the experimental group being higher than in the control. The level of Sox9 expression declined at this time point. Day 14: The further increased expressions of Cbfa1 and VEGF were evident in both synchondroses, but again, with that in SES and in the experimental group being significantly higher than that in SOS and the control group. Sox9 expression, on the other hand, continued to decline compared with that in the previous time points.

Discussion

Reverse-pull headgear is a commonly used orthopedic approach to correct skeletal class III malocclusion by protracting the deficient maxilla forward. While enhanced growth and forward positioning of the maxilla by RPHG are widely recognized (20), its effect on the cranial base structures remains unknown. As an integral part

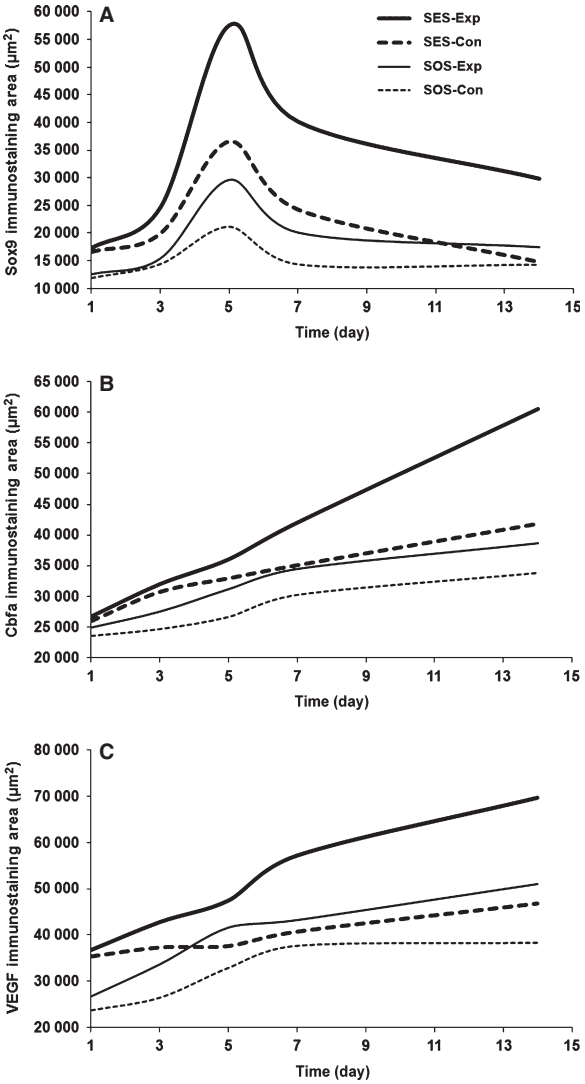

Fig. 4. Statistical diagram showing the temporal patterns of the expressions of the three growth factors in spheno-ethmoid and spheno-occipital synchondroses. The expression of Sox9 (A) increases from Day 1 and reaches a peak level at Day 5 followed by a decline. The expressions of Core-binding factor α 1 (B) and vascular endothelial growth factor (C) keep elevated over the time points. While the three growth factors in the experimental groups (Exp) of both SES and spheno-occipital synchondroses express themselves stronger than those in the control groups (Con), the temporal patterns between them remain identical.

of the oral and maxillofacial complex, the growth increment and the physical contour of the cranium affect markedly those of the maxillofacial region (21). This notion is well reflected by the fact that in severe class III malocclusion, the angulation between the anterior and the posterior cranial floor becomes decreased (22, 23) and the length of the anterior cranial base is also shortened (24). This points to a need for growth modification not only in the maxilla but also in the anterior cranium when RPHG is applied in an attempt to minimize the skeletal discrepancy resulted from class III growth pattern. In this study, a unique animal model was created where the orthopedic appliance was designed specifically for SD rats to mimic the clinical scenario of RPHG mechanism (Fig. 1). This was unlike most of the previous *in vitro* studies where the cranial synchondroses were removed from the cranial base and organ/tissue cultured for direct force loading (4).

Cartilage has been of much interest because of its association with endochondral ossification. The histological architectures and biological features, especially their adaptive responses to external interventions, remain distinctive from cartilages in different regions. The condylar cartilage in the temporomandibular joint (TMJ) features zone-like packing of the chondrocytes in different maturational phenotypes. Chondrogenesis and consequent osteogenesis can be re-activated in condylar cartilage, even after growth cessation, if only the microenvironment in TMJ is changed, for example, the repositioning of the mandible (19). The metaphyseal and epiphyseal cartilages in the growth plate of long bone, on the other hand, consist of the chondrocytes that are column-like arranged. Active chondrogenesis and endochondral bone formation in epiphyseal cartilage occur only during growth puberty, and afterward, the subsequent articular cartilage remains unchanged without adaptation to the external stimuli (10).

The cranial synchondroses cartilage, the target of this study, has a unique histological morphology characterized by symmetrically bipolar packed chondrocytes that are column-like positioned (Fig. 2B). To elucidate whether or not the cranial synchondroses are adaptive to the environmental influences, we carefully selected three transcription factors regulating the biological episodes of differentiation and maturation of the chondrocytes during chondrogenesis and osteogenesis process. In this study, the SD rats were employed with RPHG orthopedic force for maxillary protraction for the periods of 1, 3, 5, 7, and 14 days, respectively. Immunohistochemical

examinations were conducted for cranial synchondroses specimens to identify the changes in the expressions of Sox9, Cbfa1, and VEGF, the three reliable markers indicating the different stages of chondrocytes differentiation, maturation, hypertrophy, and the subsequent bone formation. Compared with the control group, the expressions of the three factors in the experimental group were much more enhanced at most of the five time points (Table 1). The meaningfulness of the higher expressions of these factors has been well documented. The increased expression of Sox9 not only enhances the differentiation of the resting chondrocytes into active ones that will proliferate (12) but also activates the synthesis of collagen II protein that forms the matrix framework for cartilage (25); the emergence of Cbfa1, on the other hand, allows the chondrocytes to progressively mature and terminally hypertrophy, leading to degrading of the chondrocytes and the surrounding matrices (15), followed by the invasion of the new blood vessels evidenced by high expression of VEGF in the hypertrophic and erosive cartilage (11). The final event of the above cascade is highlighted by deposition of the new bone replacing the disintegrated cartilage (19).

Another interesting finding in this study was the differential expressions of the three factors in quantity between SES and SOS. Significantly higher expressions of the three factors in SES than in SOS were found in the experimental group (Fig. 4). This phenomenon was also evidenced even in the control group where the immunostaining for the growth factors in SES remained positive while that in SOS was almost negative, especially so in the later time points (Fig. 4). It has been widely recognized that the major fusion of SES completes in adolescence/juvenile and that of SOS takes place much later, even until the time for second or third molar eruptions (26, 27). Therefore, the differential expressions of the growth factors between the two synchondroses could be explained by the fact that the animals at 4 weeks of age in this study fell into the late stage of pubertal growth (28), where chondrogenesis and osteogenesis in SES were still active and therefore stronger than that in SOS, where the chondrocytes are yet to initiate their phenotypic differentiation. Many studies have emphasized an important association between mid-face deficiency and anterior cranial base anomaly in which SES is situated. Mori-Akiyama et al. (29) have demonstrated a consequent maxillary retrusion as a result of cartilaginous defects in SES triggered by gene mutation of Sox9; some studies have also reported the phenomenon where mid-face retrusion in class III malocclusion is accompanied by a shortened anterior cranial base (30). This points to the necessity for the correction of anterior cranial morphology if a satisfactory correction of class III skeletal deformity is aimed to achieve. The more enhanced biological response in SES than in SOS cartilage found in this study adaptive to the maxillary protraction may add to the rationale for the use of RPHG, which corrects class III malocclusion not only by forward positioning of the maxilla but also by affecting the growth pattern deep in the anterior cranial structures.

As a preliminary attempt, this work only aimed to identify the temporal relation between the change of cranial synchondroses and a certain level of external force. The degree of synchondroses adaptation and its correlation with different magnitudes of force should be explored as a sequel of this study.

Conclusions

The higher expressions of the three growth factors in orthopedic force-loading group suggest an enhanced chondrogenesis taking place in cranial synchondroses in response to maxilla protraction; the higher expressions of the three growth factors in SES than in SOS indicate that the growth patten in anterior cranial base is more adaptive to maxilla forward positioning.

Clinical relevance

To shed light on the conflicting views whether or not the cranial base growth pattern could be affected by orthopedic force, this study created an

in vivo animal model where the maxilla of the Sprague-Dawley rat was advanced by orthopedic appliance. The increased expressions of the transcriptional factors marking the sequential events in endochondral ossification indicate an adaptive response of CBS to the external stretching force. The importance of this study is to suggest that the orthopedic intervention in clinic may not only correct mid-facial deficiency of class III malocclusion but also intervene the accompanied anterior cranial base deformity.

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