Accelerating Bone Generation and Bone Mineralization in the Interparietal Sutures of Rats Using an rhBMP-2/ACS Composite after Rapid Expansion

Ren-Fa LAI, Zhi-Ying ZHOU, and Tie CHEN

The Medical Centre of Stomatology, The 1st Affiliated Hospital Jinan University, No. 613 Huangpu Road, Tianhe District, Guangzhou 510630, P. R. China

Abstract: This study aims to investigate the effects of rhBMP-2/ACS composite on bone regeneration and mineralization during expansion of the interparietal suture in rats. Forty 10-week-old Sprague-Dawley rats were divided into four groups (n=10). The first group (intact group) did not receive any intervention. The second group (expansion control group) received an expansion force of 60 g. The remaining two groups received an expansion force of 60 g and were implanted with an atelo-type I absorbable collagen sponge and rhBMP-2/ACS composite positioned on the suture beneath the periosteum. The relapse, relapse ratio, relevant bone remodelling, and calcium and osteocalcin contents were evaluated. Bone regeneration in the interparietal suture was estimated by the histological method. The osteocalcin content was measured by radioimmunoassay, and the calcium content was measured by atomic absorption spectrophotometry. Bone regeneration was more active in the suture after application of the expansion force compared with that of the suture without any intervention. Bone bridges formed in the rhBMP-2/collagen composite group. Both osteocalcin and calcium content were higher in the rhBMP-2/collagen composite group than in the other three groups (P<0.01). The relapse ratio in the rhBMP-2/collagen group was much lower than that in the other two expansion groups (P<0.01). RhBMP-2/ACS composite can promote bone regeneration and bone mineralization in the expanded suture and decrease the relapse ratio. Thus, the rhBMP-2/ACS composite may be therapeutically beneficial to the inhibition of relapse and shortening of the retention period during rapid expansion.

Key words: bone regeneration, interparietal suture, rapid expansion, relapse, rhBMP-2/ACS

Introduction

Rapid palatal expansion (RPE) is the preferred treatment approach for constricted maxillary dental arch. In clinical orthodontics, the relapse of a previously expanded suture is regarded as cumbersome. Currently, relapse is prevented by mechanical retention using various appliances to maintain long-term stability of treatment outcomes [14]. However, a long period of retention is necessary to prevent early relapse of the expanded arch. Although the reason for early relapse is not clear, bone regeneration and mineralization in the mid-palatal suture after expansion may cause posttreatment relapse. Thus, accelerating bone formation and mineralization in the mid-palatal suture after expansion is beneficial in preventing relapse of the arch width and...
reducing the retention period [6].

Sutural expansion with mechanical forces is accomplished by stretching collagenous fibers accompanied by new bone formation with associated mitotic figures. Although force delivery methods have been refined, the mechanism of stress-mediated osteogenesis in the expanded suture remains unclear. The sutural response to direct or indirect force application can be divided into different stages. First, an initial traumatic response is followed by a period of connective tissue repair and wound healing. The expanded suture contains large, thin-walled blood vessels, and the collagen fibers are aligned in the direction of the force. Afterwards, a new bone is deposited perpendicular or parallel to the edges of the suture in tension areas. Finger-like projections of the new nonlamellar bone extend into the suture. Vascular distribution, cellular activities, and fiber orientation reflect the functional repair status [4, 6].

Although many cytokines, growth factors, hormones, and extracellular matrix components are capable of regulating specific aspects of bone regeneration and remodelling during bone growth and repair, bone morphogenetic proteins (BMPs) are among the most potent of the osteoinductive factors [4, 9, 17, 19, 22]. Urist [22] discovered that demineralized bone induces new bone formation when implanted intramuscularly. Subsequent purification of BMPs has opened a new venue for skeletal tissue engineering. Among the BMPs, BMP-2 reportedly has pleiotropic functions ranging from extraskeletal osteogenesis to bone regeneration [17, 19, 23]. Recombinant human bone morphogenetic protein-2 (rhBMP-2) acts primarily as a differentiation factor in bone and cartilage precursor cells. Various preclinical models have revealed that rhBMP-2 induces bone formation and heals bony defects [18, 19].

RhBMP-2 can be applied to enhance bone regeneration and prevent skeletal relapse after rapid expansion of the suture. This study examines the effect of subsequent relapse and expansion forces on the remodelling of sagittal sutures of rats to eliminate the influence of occlusal forces or mastication. This study aims to explore the possibility of pharmaceutically controlling or decreasing skeletal relapse.

**Materials and Methods**

**Animals**

Forty 10-week-old male Sprague-Dawley strain rats weighing 250 ± 10 g were divided into four groups (three expansion groups and one control group) of 10 animals each. This study was carried out in strict accordance with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Jinan University. The rats were fed an ordinary solid diet with water *ad libitum* and were kept in cages at 24°C under alternating 12-h periods of light and dark conditions.

**Mechanical expansion of the sagittal suture**

Two rats died of anaesthetic accidents before reaching the experimental period. These rats were excluded from the experimental groups. Suture expansion was carried out for 21 days using a 0.18-inch-diameter stainless steel round wire (3M Unitek, Monrovia, CA, USA) expansion appliance with 2 helices (Fig. 1). The rats were given general anaesthesia with sodium pentobarbital (Guangzhou Chemical Reagent, Guangzhou, P. R. China). Afterwards, a mid-sagittal incision was made anteroposteriorly through the scalp to expose the sagittal suture. Two holes were symmetrically placed in the parietal bones on opposite sides of the suture with a hole-to-hole distance of 6 mm. The expansion appliance was calibrated to exert an initial expansion force of 60 g and was then
placed into the holes. Finally, the scalp was sutured with an absorbable suture (Fig. 2). Based on the planned experimental protocol, the rats were divided into four
groups: (1) the intact group, which was left untreated; (2) the expansion control group, in which the sagittal suture was expanded but no other material was implanted; (3) the absorbable collagen sponge (ACS) group, in which the sagittal suture was expanded and buffer/ACS was surgically implanted into the sagittal suture; and (4) the rhBMP-2 group, in which the sagittal suture was expanded and rhBMP-2/ACS was surgically implanted into the sagittal suture.

RhBMP-2 or control interventions were administered immediately at the beginning of suture expansion. RhBMP-2 was delivered to the sagittal suture via surgical implantation of rhBMP-2 soak-loaded onto an ACS. Control groups were divided into the following groups based on the procedure applied: 1) buffer/ACS was surgically implanted into the sagittal suture, 2) suture expansion was performed, and 3) no intervention was performed. For the rhBMP-2/ACS group, 100 µl of a 20 mg/ml solution of rhBMP-2 (2 mg total dose of rhBMP-2) was dripped onto a piece of ACS (0.8 × 0.5 × 0.3 cm³) placed in a sterilized plastic tube and then allowed to soak for 30 min prior to use. For the buffer/ACS group, a similar volume of buffer was placed in an identical fashion on an ACS with similar dimensions. A 1-cm cut was made in the center of the periosteum of the parietal bone, and the cranial periosteum was elevated to form a pocket beneath using blunt dissection. Afterwards, rhBMP-2/ACS or buffer/ACS was inserted into the periosteal pocket and positioned on the sagittal suture. The incision was sutured following buffer/ACS insertion. The final control group was not subjected to any intervention [18, 25]. The expansion appliances in the expansion groups were removed at the conclusion of the 21-day expansion period without using any mechanical retaining device. At the conclusion of the 7-day relapse period (day 28), the animals were sacrificed under general anaesthesia by lethal intravenous injection of sodium pentobarbital. All procedures followed the international guidelines for experiments on animals [10, 13, 18, 23, 25].

**Amount of sutural expansion and the relapse ratio**

The distance between the two holes on the parietal bones was measured using Vernier calipers (± 0.02 mm) before appliance insertion, immediately after expansion,
during appliance removal, and at the end of the experiment. The relapse ratio or the rate of decrease in distance was calculated according to the following equation described by Kayou [13]:

\[(A - B)/(A - C) \times 100,\]

where \(A\) is the distance at removal of the expansion appliance (day 21), \(B\) is the distance after the expansion appliance was removed after the 7-day relapse period (day 28), and \(C\) is the distance at the beginning of the experiment (day 0).

**Histological observations**

At the end of each experimental period, the animals were sacrificed under general anaesthesia by lethal intravenous injection of sodium pentobarbital. The heads of animals were dissected. Half of the heads were frozen in liquid nitrogen for biochemical examinations, and the other half were fixed in 4% neutral-buffered formalin for 2 days. The specimens were rinsed with phosphate buffer solution decalcified in 14% ethylenediaminetetraacetic acid (EDTA) for 4 weeks, and washed with the same buffer solution. After dehydration in ethanol, the parietal bones and sagittal suture were carefully removed and embedded in paraffin. The specimens were cut into 4.5-µm-thick frontal sections. For histological examinations, the sections were stained with hematoxylin and eosin and were observed under a light microscope (BX50F-3, Olympus, Tokyo, Japan).

**Biochemistry analysis**

Based on the method described by Aerssens et al. [1], the parietal bone and the sagittal suture were cleaned, and the surrounding tissues were removed. The parietal bone was defatted for 2 days in trichlorethylene (100%), which was renewed twice a day. Pulverization was carried out with a beater mill cooled with liquid nitrogen. Bone powder was stored at −20°C until used in analysis.

Based on the method described by Reddi [8], small amounts (approximately 10 mg) of bone powder were dissolved in 2.5 ml of 1 N HCL. Aliquots of the acid-soluble extract were suitably saluted in 0.5% (v/v) HCL containing 0.1% lanthanum oxide (w/v) for determination of calcium content by atomic absorption spectrophotometry (Z-5000, Hitachi, Tokyo, Japan). The calcium content (expressed as micrograms of calcium per milligram of tissue) represented the total extent of mineralization throughout the experimental period.

**Osteocalcin determination**

For osteocalcin measurement [1], 10 mg of bone powder sample was extracted in 1.0 ml 0.5 M ammonium-EDTA containing protease inhibitors (5 Mm benzamidine, 10 Mm 6-aminocaproic acid, 100 µM p-hydroxymercuribenzoic acid, pH 6.2) at 4°C. The extraction was carried out overnight in microcentrifuge tubes by end-over-end rotation. After 18 h, the solution was centrifuged (12,000 rpm, 30 min), and the non-collagenous supernatant was separated from the collagenous residue. Osteocalcin was measured in the supernatants of the EDTA extracts with radioimmunoassay based on the serum sample method. Dilutions of samples were made in assay buffer that contained 0.025 M Na2EDTA. The presence of EDTA is required because osteocalcin conformation is dependent on the calcium concentration. Antibody subpopulations specific to the alpha-helical form and random coil configuration of osteocalcin were presumably present. Antiserum against bovine osteocalcin produced in rabbits and homogenous bovine osteocalcin were used for standards and the tracer in a sequential saturation analysis. Separation was performed by the double antibody method. The sensitivity of the assay was 0.22 ng of bovine osteocalcin/ml. All values for standards and samples were determined in duplicate.

**Statistical analysis**

All data were first subjected to an F-test to examine the difference in variance between two groups. Fisher’s protected least significant difference test was then used to examine the mean differences between the two groups. The results are presented as the mean ± SD for each group. Data were analyzed by one-way ANOVA. \(P<0.05\) was considered a significant difference.

**Results**

**Histological results**

Microscopic observation of the sagittal suture in the intact groups revealed that the suture was narrow and that cell components and capillaries were minimal. The transverse fibers were arranged, and a layer of osteoid was present on the front of the edge of the parietal bones. In the expansion control group, microscopic observation of the sagittal suture revealed the presence of extended transverse fibers and capillary enlargements in the suture. A new bone was also deposited perpendicular or parallel
to the edges of the suture, and finger-like projections of new nonlamellar bone extended into the suture, which may have been caused by mechanical tension (Fig. 3B). In the buffer/ACS group, microscopic observation of the sagittal suture revealed the ACS completely absorbed, and neither bone nor cartilage was present on the surface of the parietal bone (Fig. 3C). In the rhBMP-2/ACS group, microscopic observation of the sagittal suture also revealed the presence of extended transverse fibres and capillary enlargements in the suture. A new bone was also deposited perpendicular or parallel to the edges of the suture, and finger-like projections of new nonlamellar bone extended into the suture. The carrier collagen was fully absorbed, and a bony augmentation was found. The bony trabeculae were connected directly to the parietal compact bone of the skull; numerous osteoblasts were irregularly packed beneath the bony trabeculae in the transverse fibers. The surface of the augmented bone partially presented an irregular and uneven structure (Fig. 3D).

Fig. 3. Appearance of the sagittal suture in different groups (finger-like projections of new nonlamellar bone are indicated by arrows; capillaries are indicated by asterisks; and osteoblasts indicated by triangles). (A) Intact group, (B) expansion control group, (C) buffer/ACS group, (D) rhBMP-2/ACS group. A, B, and C, hematoxylin and eosin (HE) and ×10 magnification. D, HE and ×4 magnification. Scale bars=200 µm.
Biochemical characteristics

The total amount of calcium and osteocalcin in the intact group was significantly less than that in the other three groups ($P<0.01$). In the rhBMP-2/ACS group, the total amounts of calcium and osteocalcin were significantly higher than those in the expansion control group and buffer/ACS group ($P<0.05$). Although the total amounts of calcium and osteocalcin in the buffer/ACS group were higher than that in the expansion control group, no significant difference was found between these two groups ($P>0.05$) (Fig. 4).

Relapse ratio

The distance of $A–C$ showed no significant difference among the three groups ($P>0.05$). The relapse ratio in the rhBMP-2/ACS group was significantly less than in the expansion control group and buffer/ACS group ($P<0.01$) (Table 1).

Discussion

In clinical orthodontics, RPE is a common treatment strategy. However, expanded arches easily relapse unless retained for a prolonged period. The rate of relapse gradually decreases as the duration of retention increases [8, 12]. Relapse may be caused by unstable oral myofunction, regeneration of sutures connected to other facial bones, tension of palatal connective tissue and alveolar bone remodelling [6, 12, 18]. However, we argue that one of the major causes of early relapse after expansion can be insufficient bone regeneration at the mid-palatal suture. In this study, we demonstrated that a single application of rhBMP-2 by implantation significantly enhanced bone regeneration in the sagittal suture, that the calcium and osteocalcin contents were higher in the rhBMP-2/ACS group, and that the overall relapse ratio after expansion was lower.

Luo et al. [16] reported that many cytokines, including BMPs, transforming growth factor-β (TGF-β), insulin-like growth factors, fibroblast growth factors, and platelet-derived growth factors (PDGFs), are secreted by the cells in the suture when a tension force is applied. These cytokines regulate bone regeneration in a complex manner. The secretion will gradually decrease when the tension force disappears. Among these cytokines, BMP-2 is the most effective.

To enhance bone regeneration in the expanded suture, numerous methods have been applied, including Ga-Al-As laser irradiation and the local application of TGF-β1, recombinant human endothelial cell growth factor, and other cytokines [7, 12, 18], among others. Thus, we deduced that applying BMP-2 to the expanded suture would possibly promote bone regeneration in the suture. BMP-induced bone formation in vivo is clearly a complex

Table 1. Relapse ratios and differences in the four experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Expansion control group</th>
<th>buffer/ACS group</th>
<th>rhBMP-2/ACS group</th>
</tr>
</thead>
<tbody>
<tr>
<td>B–C</td>
<td>1.354 ± 0.268</td>
<td>1.482 ± 0.184</td>
<td>0.073 ± 0.019</td>
</tr>
<tr>
<td>B–A</td>
<td>2.720 ± 0.177</td>
<td>2.696 ± 0.149</td>
<td>2.711 ± 0.142</td>
</tr>
<tr>
<td>R (%)</td>
<td>53.25 ± 8.80</td>
<td>51.98 ± 5.55</td>
<td>2.50 ± 0.71</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD (n=10). *Versus the other two groups $P<0.01$.
multistage process and probably involves the activities of multiple locally produced growth factors and systemically available hormones [9, 18, 19, 23]. Osteoblastic or osteoprogenitor cells generally respond to treatment with BMPs by increasing cell proliferation [23]. BMP-2 has also been shown to induce differentiation of osteoprogenitor cells to osteoblastic cells [3, 5, 15, 17]. BMPs have chemotactic effects on mesenchymal, osteoblastic [5, 17, 24], and endothelial cells [11, 15], suggesting that enhancement of bone formation using rhBMP-2 may be related to increased bone-forming cells and enhanced neovascularization. The implanted rhBMP-2 is unlikely to remain at the site long enough to direct all processes in vivo. A single administration of rhBMP-2 may induce a cascade of multiple BMPs and growth factors that regulate bone formation processes. The expanded suture is a rich source of osteoprogenitor cells during rapid palatal expansion. The primary effect of rhBMP-2 in this setting is to cause cells to differentiate into mature osteoblasts rapidly, thereby resulting into more rapid bone formation, which is consistent with our study. In the rhBMP-2/ACS group, numerous osteoblasts were packed irregularly beneath the bony trabeculae in the transverse fibers. The best carrier for a given BMP may vary depending on the specific clinical indication and skeletal site being treated. Considerations include biodegradability, structural integrity, absence of immunogenicity and rate of release of BMP [18, 21, 25]. Collagenous materials are available in various types and forms, such as solution, sponge, membrane, bead and gel. An acid-soluble type I atelocollagen solution was selected in this study because an equal composite can be obtained by mixing the rhBMP-2 solution and collagen solution at any ratio. The composite can also be prepared in any shape after lyophilization of the mixed solution. Thus, the procedure is extremely simple. The easy handling of this material is important for various clinical applications [2, 5, 20]. One disadvantage of using a collagen sponge to deliver rhBMP-2 is that it requires a second surgery for implantation. Some positive efferent of the secondary operation on bone regeneration were noticed in the surgical control group. For instance, the calcium and osteocalcin contents in the buffer/ACS group were greater than those in the expansion control group. These data indicate that surgical interventions (implantation) at early stages of bone consolidation may induce some moderate positive effects on bone formation. These effects may be due to trauma-induced inflammation that releases certain growth factors and cytokines, such as PDGFs, TGF-β, and interleukins, thereby promoting bone formation [23]. Kayou [13] reported that the relapse of mechanical sutural expansion is controlled or reduced by the injection of bisphosphonate and etidronate using a mechanical retainer. In the current study, a similar result was obtained, but a mechanical retainer was not used. The bony connection formed in the rhBMP-2/ACS group serves as a mechanical anchorage to resist the tension released by the suture fibers when returning to their original forms.

In conclusion, we demonstrated that implantation of rhBMP-2 in an ACS significantly enhanced the bone regeneration of expanded sutures in a rat sagittal suture expansion model. Although this model cannot imitate the complex masticatory systems of the human body and further studies are still required, introduction of rhBMP-2 during RPE in orthodontic treatment is apparently feasible and may be therapeutically beneficial to the inhibition of relapse or shortening of the retention period or both.

Acknowledgments

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