Effects of strontium ranelate on sutural bone formation: a histological and immunohistochemical study

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Aim: Rapid maxillary expansion is performed to correct a skeletal transverse deficiency of the maxilla, which is a frequently-encountered orthodontic anomaly. Strontium ranelate (SrR) is a novel agent that has a dual action, involving anti-resorptive and bone-forming effects. The aim of this study was to evaluate the effects of systemically applied SrR on osteoblastic bone formation after maxillary expansion on the mid-palatal suture of rats using histological and immunohistochemical tests.
Materials and methods: A total of 24 Wistar rats were randomly divided into two equal groups. In all animals, five-day inter-premaxillary expansion was applied and maintained for a seven-day retention period, during which 625mg/kg/day SrR diluted with saline solution was administered orally to the experimental group. The rats were sacrificed and the tissues prepared for histological and immunohistochemical examinations after the retention period.
Results: Osteoblastic activity and the width of the blood vessels in the suture area were significantly increased in the SrR group compared with the control group ($p < 0.05$). Ossification was also observed to be active under light microscopy by staining with hematoxylin and eosin in the experimental group. Immunohistochemical labelling performed using osteonectin, osteocalcin, TGF-β and VEGF antibodies revealed significant immunoreactivity in the experimental group ($p < 0.05$).
Conclusion: It may be concluded that SrR contributed to stimulatory osteogenesis in the expansion region. Therefore, a retention period may be shortened and relapse possibly reduced, following the application of SrR after the expansion.
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Introduction

Orthodontists commonly apply forces on facial sutures to overcome transverse maxillary deficiencies by stimulating bone deposition. Rapid maxillary expansion is the primary treatment approach to correct malocclusions caused by a narrow maxillary dental arch and skeletal base deficiency. After expansion, a series of biological events occurs, in which sutures undergo remodelling identified by bone formation, resorption, and fibre rearrangement in the expanded area.[1] This process continues until the architectural bony environment reaches equilibrium.[2] It has been well documented that, even after a retention period, an expanded maxillary dental arch has a strong tendency to rebound to its previous state.[3-5] Relapse rates after retention have been reported between 0% and 45%,[5-7] so long retention periods are usually necessary to avoid potential post-treatment change. Da Silva Filho et al.[8] investigated the expanded suture by computerised tomography after the application of a conventional six-month retention period and concluded that the mid-palatal suture in humans was fully ossified

along its length. The growth rate of new bone and the acceleration of bone formation in the mid-palatal suture after expansion are essential to limit subsequent relapse of the arch width and to shorten the retention period.[9-12] Consequently, research has focused on the maintenance of maxillary expansion. The stimulation of bone formation in the expanded suture has been studied by the application of external factors, such as low-power laser irradiation[9] and low intensity pulsed ultrasound (LIPUS),[10] transforming growth factor-β (TGF-β) 1 injection[11] or with chemical factors, such as antioxidants, bisphosphonates and a vitamin D analog.[12-15]

Strontium ranelate (SrR) is a novel oral anti-osteoporotic agent. In clinical trials, SrR has shown potential in the treatment of osteoporosis and reducing the risk of vertebral and non-vertebral fractures in post-menopausal osteoporotic women.[16,17] Many bone resorption inhibitors have been developed to decrease the risk of osteoporotic fractures, including SrR, which has a dual effect on bone metabolism. Preclinical studies have shown that SrR reduces bone resorption and stimulates bone formation concomitantly in the remodelling areas.[18,19] Bone formation and bone cell replication are stimulated through the amplification of pluripotent and pre-osteoblastic mesenchymal cell effects. SrR has been shown to reduce bone resorption by decreasing osteoclast differentiation and bone resorbing activity.[20]

SrR has been systemically used to accelerate fresh fracture healing,[21] increase bone proliferation,[22] treat osteoporosis,[23] increase osteo-integration[24] and reduce the risk of fracture formation.[25] However, to the best of our knowledge, there has been no study on the use of SrR after maxillary expansion.

Therefore, the objective of the present study was to evaluate the effect of SrR on osteoblastic activity in the mid-palatal suture of rats after expansion, and thereby contribute to the understanding of strategies for preventing relapse after treatment. The null hypothesis states that the administration of SrR has positive effects on bone formation following inter-premaxillary suture expansion in rats.

Materials and methods

Animals and groups

To determine the sample size of this study, G Power software version 3.1.9.2 (Franz Faul, University of Kiel, Germany) was used. With a type I error of 0.05 and a type II error of 0.20, at least 16 animals, each in two groups of eight, would be required for a power greater than 80%. Therefore, 24 male Wistar rats with a mean weight of 200 ± 20 g and mean age of 11–12 weeks were included in the study and divided into two groups of 12 rats: a control and SrR-test groups. Both groups underwent five-day maxillary expansion followed by a seven-day retention period. The control animals received saline solution orally once a day via a syringe during the retention period. The study group was treated with a dose of SrR (625 mg/kg/day) administered orally via a syringe during the seven-day retention period. All rats were caged individually at a constant temperature of 23ºC with a 12 hour light-dark cycle and fed water and standard rat pellets, ad libitum.

Ethical approval for the study was granted by the Animal Research Ethics Committee at İnönü University School of Medicine, Malatya, Turkey (2012/A-65). The study was conducted in accordance with the Declaration of Helsinki, Guiding Principles in the Care and Use of Animals in Research.

Interpremaxillary suture expansion

The animals were anaesthetised by one investigator with a subcutaneous injection of 4 mg/kg of xylazine hydrochloride (Alfazyne; Alfasan International BV, Woerden, Netherlands) and 40 mg/kg ketamine hydrochloride (Alfamine; Alfasan International BV Woerden, Netherlands). Two grooves were prepared in the mesial and distal proximal surfaces of the upper incisors. The legs of a helical spring made of 0.014 inch stainless steel wire (Dentaurum, Germany) were inserted in the grooves to expand the inter-premaxillary suture (Figures 1, 2). Pre-activation of the spring was conducted to apply a force of nearly 50 g without further activation. Following expansion, the springs were deactivated under general anesthesia and left in situ. Using a digital micrometer (Mitituyo, Tokyo, Japan), the amount of expansion was measured between the distal edges of the upper incisors at two time points: prior to expansion (T1) and at the end of the expansion (T2). Each parameter was measured twice and the average measurement was recorded.

Specimen preparation

At the end of retention, all rats were sacrificed using ketamine hydrochloric and xylazine hydrochloric and the rats' maxilla was surgically dissected for

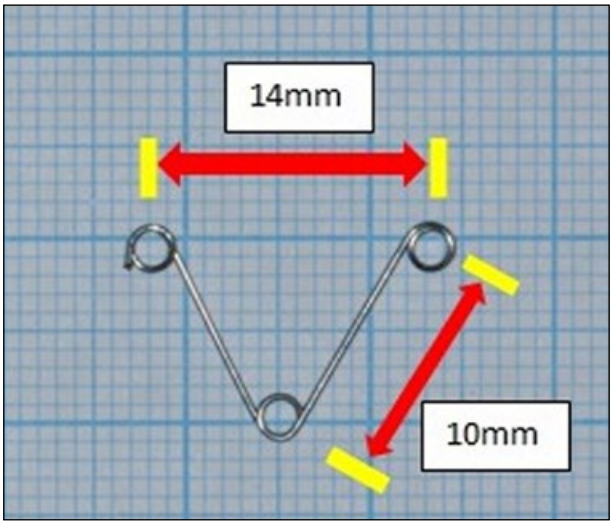

Figure 1. Schematic view of the expansion appliance used.

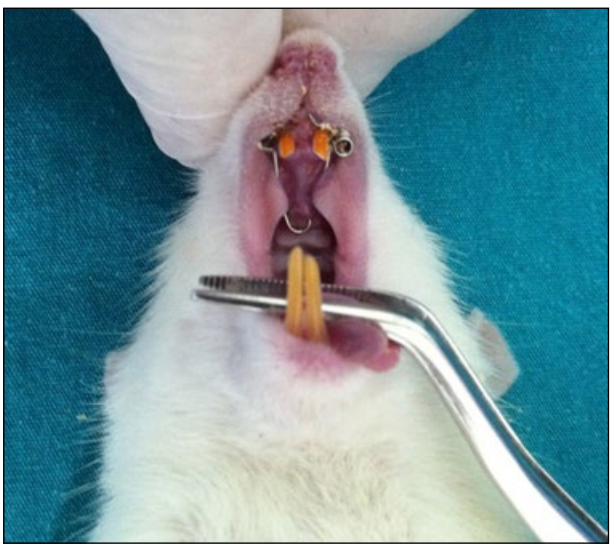

Figure 2. Intra-oral view of the expansion appliance.

assessment. After fixation in 10% neutral buffered formalin solution at room temperature for 24 hours, decalcification of the premaxilla commenced using ethylene diamine tetra-acetic acid (0.1 mol/L, pH 7.1) solution, which was changed on alternate days for three weeks at 14°C. Following decalcification, using the maxillary incisor as the primary guide for orientation, the inter-premaxillary suture areas were excised. The excised tissue was prepared for standard histological examination by paraffin embedding and slices of 5 µm thickness sectioned in the coronal plane and mounted. The prepared sections were stained using haematoxylin and eosin, after which, tissue specimens were randomly selected and histologically evaluated (HE, Harris haematoxylin, 09-182-1, DDK Italia, Italy; Eozin, MOS, 0712012, Turkey). All of the formalin-fixed, paraffin-embedded tissue blocks were evaluated in the Pathology Laboratory of the Medical Faculty, University of Necmettin Erbakan in Konya, Turkey.

Immunohistochemical staining procedure

After overnight incubator storage at 60°C, sections were dewaxed in xylene and rehydrated through a series of ethanol solutions. A solution of distilled water and phosphate-buffered saline (PBS; P4417, Sigma-Aldrich, MO, USA) was applied for 10 minutes to rinse the sections, which were then treated with 0.1% trypsin (Zymed, 00-3008, CA, USA) for 10 minutes and washed with PBS. Sections were delineated with a PAP pen (Super PAP PEN, IM3580, Beckman Coulter, Marseille, France) and incubated with a non-immune serum (Ultra V block, cat. No: TA-125-UD, Lab Vision, CA, USA), followed by incubation with the primary antibodies of monoclonal mouse anti-osteonectin (SPARC, 251503, Abbiotec, CA, USA), monoclonal mouse anti-osteocalcin (OC4-30, Novus Biologicals, Cambridge, UK), monoclonal mouse anti-VEGF (PU483-UP, Biogenex, CA, USA) and polyclonal rabbit anti-TGF-β (ab66043, Abcam plc, Cambridge, UK), for one hour at 4°C in a humidity chamber. The sections were rinsed three times in distilled water and PBS for five minutes, and subsequently incubated with biotinylated secondary antibody followed by streptavidin conjugated to horseradish peroxidase in PBS for 30 minutes each (Lab. Vision Cat:TS-125-HR, Thermo Scientific, MA, USA). After a further three washes with PBS, the sections were incubated with Lab. Volume diaminobenzidine substrate (Lab. Vision CAT: TS-125-HR) for 15 minutes for immuno-staining. The sections were rinsed with distilled water, counterstained with Mayer's haematoxylin (Lillie's modification, ScyTek Laboratories, UT, USA) and rewashed with distilled water before mounting in an aqueous medium.

The sections were examined with a BX 43 bright-field microscope (Olympus, Tokyo, Japan). A precipitate of reddish-brown colour indicated positive findings for the primary antibodies. The staining scores were independently evaluated by an observer (S.K.) blinded to the clinical information. Semi-quantitative grading of the mean values of the immunohistochemical staining intensities was graded as mild (+), moderate (++), or strong (+++).

At 40-times magnification, the number of active osteoblasts was scored as + (1–10), ++ (11–20), or +++ (>20). The width of the vessels was scored as + (narrow), ++ (moderate), or +++ (wide).

Statistical analysis

An analysis of the measurements was conducted using the Statistical Package for the Social Sciences 20.0 (SPSS for Windows, SPSS Inc., Chicago, Ill, USA). The normality of the data was evaluated by the Shapiro-Wilk test. Descriptive statistics were stated as median, minimum, and maximum for ordinal data, and as mean and standard deviation for continuous data. To evaluate the distance measurements in the T1, T2, and T3 periods for group comparisons, the Mann-Whitney U test was applied. To assess the differences in each group over the various periods, the Friedman and Wilcoxon tests were used. Histological and immunohistochemical measurements (T3) were evaluated with the Freidman and Mann-Whitney U tests. A value of $p < 0.05$ was considered as statistically significant.

Results

A temporary reduction in the animal's body weight was seen in the first few days due to the application of the expansion and retention appliance. Thereafter, the spring was well tolerated and no weight loss was observed. The desired sutural opening successfully occurred over the five days of the expansion period.

The mean measurement and standard deviations at T1, T2, and T3 are shown in Table I. There were significant differences between the two groups at the different time periods (T1, T2, T3) and related to the width measurements between the distal corners of the incisors ($p < 0.05$). In addition, there were significant differences in the distances between the T1–T2 and T1–T3 periods ($p < 0.05$). However, there was no significant change between T2 and T3 ($p > 0.05$) for either group.

There was no statistically significant difference in the mean amount of expansion (T1–T2), between the two groups (Table II). Both samples were, therefore, accepted as similar as no difference was observed in the width between the distal corners of the maxillary incisors in the animals in each group (Table II).

The histological and immunohistochemical images of both groups are shown in Figures 3–7. The sagittal suture samples stained with haematoxylin and eosin from the SrR group (five-day expansion, strontium ranelate, and seven-day retention) under the light microscope revealed that 11–20 (++) active osteoblasts were localised along the suture and the blood vessels were evaluated as wide (+++) (Figure 3). In this group, strong (+++) osteocalcin, osteonectin, VEGF and TGF-β immunoreaction was observed in the osteoblasts, and moderate (++) immunoreactivity was observed in the sutural connective tissues (Figures 4 – 7).

The light microscopic examination of the sagittal suture sections stained with haematoxylin and eosin from the control group (five-day expansion and seven-day retention) revealed 1–10 (+) active osteoblasts localised along the suture, and blood vessels were of moderate width (++) (Figure 3). In this group, minimal (+) osteocalcin and moderate (++) osteonectin immunoreactivities were observed in osteoblasts, and minimal (+) immunoreactivity was

Table I. The mean measurement and standard deviations on the caliper measurements of width between the distal corner of the incisor at T1, T2, and T3 (μm).

	T1			T2		T3		Paired *t*-test		
	N	Mean	SD	Mean	SD	Mean	SD	T0-T1	T0-T2	T1-T2
SrR	12	2757.33	78.94	5342.08	128.42	5360.00	203.18	***	***	NS
Control	12	2680,08	87.95	5187.67	70.02	5186.50	77.05	***	***	Ns

N: sample size; NS: non-significant; ***: $p < 0.001$

Table II. Comparison of biometric analysis for determination of the amount of dental expansion (μm).

	SrR			Control				
	N	Mean	SD	N	Mean	SD	*p* value	Significance
Amount of expansion	12	2473.8	93.1	12	2389.75	98.13	0.729	NS

N: sample size; *p* value: independent samples *t*-test; NS: non-significant

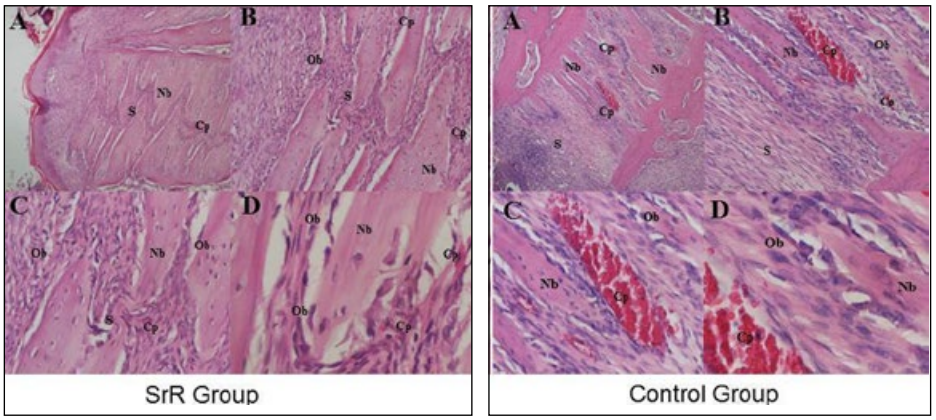

Figure 3. Photomicrographs of histologic sections of sutural areas stained with Hematoxylin and eosin (H&E). Ob: Osteoblastic activity; S: Sutura connective tissue; Cp: Capillary; Nb: New bone (A:5×, B:10×, C:20×, D:40× magnification).

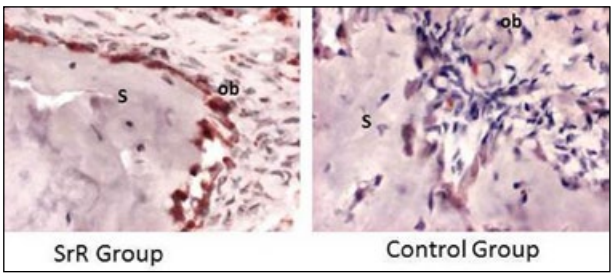

Figure 4. Photomicrographs of immunohistochemical sections from sutural areas stained with anti-osteonectin antibody. Ob: Osteoblastic activity; S: Sutura connective tissue (40× magnification).

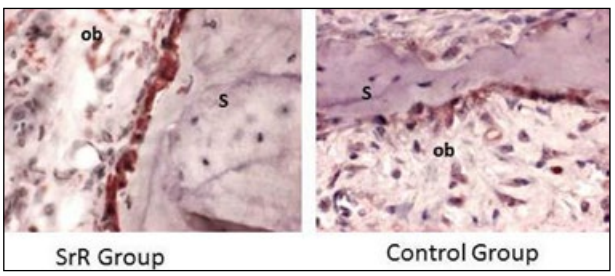

Figure 5. Photomicrographs of immunohistochemical sections from sutural areas stained with anti-osteocalcin antibody. Ob: Osteoblastic activity; S: Sutura connective tissue (40× magnification).

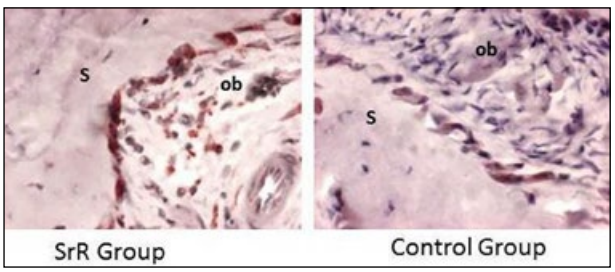

Figure 6. Photomicrographs of immunohistochemical sections from sutural areas stained with anti-TGF-β antibody. Ob: Osteoblastic activity; S: Sutura connective tissue (40× magnification).

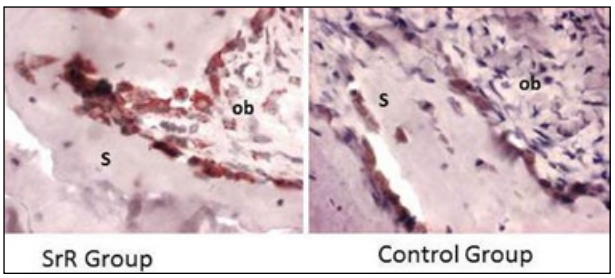

Figure 7. Photomicrographs of immunohistochemical sections from sutural areas stained with anti-VEGF antibody. Ob: Osteoblastic activity; S: Sutura connective tissue (40× magnification).

observed in the suture connective tissues (Figures 4 and 5). In the control group, moderate (++) VEGF and TGF-β immunoreactivity was observed in active osteoblasts and minimal (+) immunoreactivity was observed in the sutural connective tissues (Figures 6 and 7).

When the scores of staining intensity were compared, a statistically significant increase was found in Group 1 compared with Group 2 (Tables III and IV).

Discussion

The results of the present study showed that SrR stimulated new bone formation after the expansion of inter-premaxillary sutures in rats. If the expansion used to treat transverse maxillary discrepancies and the retention period is inadequate, there may be a reduction in the width of the expanded maxillary arch.[7] Previous research has examined the application of several pharmacological agents to increase bone formation. There have also been investigations into the stimulation of bone regeneration in the inter-premaxillary suture after expansion.[26] However, to the best of current knowledge, this is the first experimental study to investigate the effect of SrR on inter-premaxillary sutures after maxillary expansion.

Unlike other available anti-osteoporotic agents, SrR is

Table III. Result of histological evaluations.

	SrR (N=12)					Control (N=12)					Mann-Whitney U test	
	Mean ± SD	SE	Median	Min	Max	Mean ± SD	SE	Median	Min	Max	*p* value	Significance
Number of Ob	2.16 ± 0.71	0.21	2.00	1.00	3.00	1.33 ± 0.49	0.14	1.00	1.00	2.00	0.006	***
Vascularity	2.25 ± 0.45	0.13	2.00	2.00	3.00	1.58 ± 0.66	0.19	1.50	1.00	3.00	0.011	**

SrR: Strontium ranelate; Ob: Osteoblast; Min: Minimum; Max: Maximum; SD: Standard deviation; SE: Standard error; **: $p < 0.01$; ***: $p < 0.001$

Table IV. Result of immunohistochemical evaluations.

	SrR (N=12)				Control (N=12)				P[a]
	Mean ± SD	Median	Min	Max	Mean ± SD	Median	Min	Max	*p* value	significance
Osteonectin-osteoblast	2.41 ± 0.66	2.50	1.00	3.00	1.58 ± 0.66	1.50	1.00	3.00	0.09	*
Osteonectin-fibrous tissue	1.41 ± 0.51	1.00	1.00	2.00	1.08 ± 0.28	1.00	1.00	2.00	0.06	NS
Osteocalcin-osteoblast	2.08 ± 0.90	2.00	1.00	3.00	1.66 ± 0.77	1.50	1.00	3.00	0.24	NS
Osteocalcin-fibrous tissue	1.25 ± 0.45	1.00	1.00	2.00	1.16 ± 0.38	1.00	1.00	2.00	0.62	NS
VGEF-osteoblast	2.83 ± 0.38	3.00	2.00	3.00	2.00 ± 0.85	2.00	1.00	3.00	0.01	**
VGEF-fibrous tissue	1.91 ± 0.79	2.00	1.00	3.00	1.25 ± 0.45	1.00	1.00	2.00	0.02	*
TGF-β-osteoblast	2.41 ± 0.79	3.00	1.00	3.00	1.75 ± 0.75	2.00	1.00	3.00	0.04	*
TGF-β-fibrous tissue	1.25 ± 0.45	1.00	1.00	2.00	1.16 ± 0.38	1.00	1.00	2.00	0.62	NS

SrR: Strontium ranelate; NS: Non-significance; *: $p < 0.05$; Max: Maximum; Min: Minimum; SE: Standard error; SD: Standard deviation; P[a]: Mann-Whitney U test

novel and has been shown to induce bone-formation and anti-resorption effects in vitro and in vivo. Several in vitro studies in models of bone cell resorption and formation have been conducted to clarify the effects of SrR on bone cells, with evidence indicating that SrR is an anti-resorption agent. In mouse calvaria, cultures of SrR have been found to inhibit bone resorption by approximately 30%, as assessed by calcium release.[27] In addition, from measurements of a resorbing pit assay in isolated rat cells, SrR has been shown to decrease osteoclast activity by approximately 30%.[28] These data indicate that SrR decreases the activity of osteoclasts, induces osteoclast apoptosis and decreases osteoclast differentiation, which leads to a decrease in bone resorption in vitro.

In addition, there is evidence that SrR has a positive effect on bone formation in vitro.[17] In isolated rat calvaria cells, SrR was found to stimulate preosteoblastic cell replication and bone collagen synthesis.[29] Therefore, in the present study, the histological and immunohistochemical effects of SrR were investigated on new bone deposition after mid-palatal expansion with an osteoblast cell line and related bone-specific proteins. The present results indicated that SrR increased new bone formation.

In previous animal model experimental studies, the rate of bone mineralisation after the application of force has been evaluated. Although the maxillary sutures of monkeys and cats are considered to be the most similar to those of humans, to achieve a clear representation of bony and sutural influences under force, the rabbit and the rat are the most suitable animals.[30] Therefore, rats were selected as the animal model in the present study.

The normal premaxillary suture has been reported to be approximately 20 µm to 60 µm in thickness, and an average opening of 377 ± 104 µm was achieved by rubber wedges in a five-day expansion period in young rats.[31] In the present study, the inter-premaxillary suture was expanded by a helical spring. Both SrR and control groups were considered to be similar as the measurement of width between the distal corners of the maxillary incisors did not show any statistically significant difference between the two groups.

The capacity of SrR to stimulate bone formation and resorption has been reported in previous animal studies.[32-34] In normal adult mice, SrR (200-1800 mg/kg/day, two years) administration increased vertebral bone formation and decreased bone resorption, which

resulted in increased bone mass.[35] The most affirmative effect of SrR on bone mass in rats was achieved with a dose of 225–900 mg/kg for its anti-resorption and bone forming activity for improved bone strength.[34] SrR also increased the extent of mineralising surfaces.[4] Therefore, the dose of 625mg/kg SR was selected for the present study.

The application of several chemical and pharmacological agents to improve new bone formation in the expanded mid-palatal sutures has been studied previously.[9-11,26,36] Saito and Shimizu[9] evaluated the effects of low-power laser irradiation on bone regeneration during expansion of the mid-palatal suture in rats and suggested that laser therapy has the advantage of inhibiting relapse and shortening the retention period through acceleration of bone regeneration.

Alternatively, researchers applied vitamin E injections at the end of the expansion of the inter-premaxillary suture to evaluate the effect on bone regeneration, and it was determined that vitamin E application stimulated bone formation and shortened the retention period.[36] More recently, in a study by Öztürk et al.,[13] the effects on sutural new bone formation and relapse were evaluated after expansion and the application of injected bisphosphonates in rats. Positive outcomes were observed in the stimulation of bone regeneration and a decrease in the relapse ratio. However, after tooth extraction, an association was found between bisphosphonate therapy and jaw osteonecrosis.[37] As a comparison, it may be accepted that SrR has an important advantage over other available invasive treatment modalities, because of its dual action on bone regeneration and minimal side effects.

Immunohistochemical staining is one of the most widely known and used methods to determine osteoblastic activity and therefore, in the present study, osteonectin, osteocalcin, VEGF and TGF-β were mainly investigated.[11,13,38] Osteonectin is a bone-specific protein with the capacity to specifically bind to hydroxyapatite and collagen.[39] In the current study, there was a statistically significant difference between the groups related to osteonectin activity ($p < 0.05$). Öztürk et al.[13] indicated that, with the application of bisphosphonate in an expanded suture, osteocalcin activity was increased. Since osteocalcin, present in bone and dentin as a non-collagenous protein,[39] plays an active role in regulating the process of ossification, extra-cellular matrix remodelling and maturation, it is not unexpected that the intensity of bone mineralisation increases when osteocalcin activity is higher than normal. After SrR application in the present study, there was a significant increase in osteocalcin activity.

VEGF is a glycoprotein growth factor that has been shown to have major effects on tissue formation, cell migration, and inflammation and wound healing.[40] Öztürk et al.[13] investigated the effects of zoledronic acid (ZA) on the production of VEGF by odontoblast-like cells and reported that ZA accelerated the level of VEGF and that there might be an autocrine effect generated by VEGF on these cells. These findings were consistent with the results of the present study. The results showing significantly increased staining of osteocalcin, TGF-β and VEGF in the mineralised tissue of the tested samples may indicate significant proliferative cellular immunoreactivity following the application of SrR.

In the present study, the number of osteoblasts and the width of vessels in the SrR-applied group were observed to have histologically increased compared with the control group.

Moreover, the data obtained along the suture and the vascular effects in the SrR group are consistent with increased immunoreactivity of osteoblasts in that area compared with the control group. The effect of an increased number of osteoblasts in the SrR group also seems to mirror the effects of agents reported in previous studies.[6,12,13,20,25,41,42]

Öztürk et al.[13] showed an increased immunoreactivity of osteoblasts in the suture area compared with control groups and determined that ZA in the suture contributed to new bone formation ($p < 0.05$). When the results of the indirect immunohistochemical staining using osteonectin, VEGF and TGF-β in the test group of the present study were compared with those of Öztürk et al.,[13] similar findings of osteoblast immunoreactivity were observed. This result suggests that strontium ranelate could be effective in the ossification of the rat sutures.

Several studies have been conducted related to the effect of strontium ranelate on increasing bone formation and reducing destruction. Ozturan et al.[21] investigated the effect of SrR on fracture healing in rats and found that SrR caused a significant increase in bone mineral density (BMD), mechanical strength, callus maturity and the numbers of osteoblasts. Brüel et al.[43] evaluated the effects of systemically-administered SrR on bone

mineral density and bone mineral content in healthy rats and showed that SrR is safe and improves bone resistance by increasing bone mass and improving its architecture while maintaining bone rigidity.

The results of the present study demonstrated that the SrR-applied group had statistically significant increases in osteoblast numbers and immunoreactivity in the suture region, compared with the other groups. This report is the first study to examine the effect of SrR on the inter-premaxillary suture after rapid maxillary expansion. However, the use of SrR in human children for the purpose of reducing relapse of rapid palatal expansion may be ethically questionable.

Conclusions

The present findings suggest that systemically-administered strontium ranelate can stimulate new bone formation. Furthermore, a 625 mg/kg concentration of SrR therapy daily was an effective dose for bone regeneration in rats. Therefore, systemic administration of SrR may be helpful in the prevention of relapse following a maxillary expansion procedure. These results suggest the possibility of pharmaceutically-assisted retention to maintain the outcome of sutural mechanotherapy in clinical orthodontics. It was also shown that oral administration of SrR can be considered to be relatively safe. However, further research is needed to answer the following questions:

1. Does the short-term SrR usage stimulate more bone in maxillary expansion in humans?
2. Does the short-term increased bone production decrease long-term relapse after maxillary suture expansion in rats and humans?
3. Does short-term SrR therapy decrease tooth movement?

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