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RANK/RANKL/OPG Expression in Rapid Maxillary Expansion

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The aim of this study was to evaluate osteoclastogenesis signaling in midpalatal suture after rapid maxillary expansion (RME) in rats. Thirty male Wistar rats were randomly assigned to two groups with 15 animals each: control (C) and RME group. RME was performed by inserting a 1.5-mm-thick circular metal ring between the maxillary incisors. The animals were euthanized at 3, 7 and 10 days after RME. qRT-PCR was used to evaluate expression of Tnfsf11 (RANKL), Tnfrsf11a (RANK) and Tnfrsf11b (OPG). Data were submitted to statistical analysis using two-way ANOVA followed by Tukey test (α=0.05). There was an upregulation of RANK and RANKL genes at 7 and 10 days and an upregulation of the OPG gene at 3 and 7 days of healing. Interestingly, an increased in expression of all genes was observed over time in both RME and C groups. The RANKL/OPG ratio showed an increased signaling favoring bone resorption on RME compared to C at 3 and 7 days. Signaling against bone resorption was observed, as well as an upregulation of OPG gene expression in RME group, compared to C group at 10 days. The results of this study concluded that the RANK, RANK-L and OPG system participates in bone remodeling after RME.

Key Words: bone, rapid maxillary expansion, RANK, RANK-L, OPG.

Introduction

Mechanical stimulation, such as orthodontic/orthopedic treatment, may start and promote bone remodeling (1). Rapid maxillary expansion (RME) is usually used in the treatment of maxillary skeletal constriction. This procedure opens the midpalatal suture, and new bone remodeling and formation occur between palatal bones. Clinically, to avoid relapse, retention with a fixed appliance for at least 6 months after RME (2) is recommended. In order to achieve bone formation, as well as biological changes and remodeling activities following RME, a previous inflammatory process is required. Initially, vascular and cellular changes take place, followed by the release of inflammatory mediators, growth factors and neuropeptides in the surrounding tissues. Mechanical stress evokes biochemical responses and structural changes in several cell types *in vivo* and *in vitro* (3). Osteoblasts and osteoclasts are the main cells responsible for the alveolar bone remodeling and the interaction between these two cell types has been investigated (4).

Receptor activator of nuclear factor-kappa B (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) system plays an important role in inducing bone remodeling (3). RANKL and its two receptors RANK and OPG, were shown to play a role in the remodeling process because they are important osteoclastogenesis regulatory molecules (5). RANKL is a downstream regulator of osteoclast formation and activation, by which many hormones and cytokines produce their osteoresorptive effect (3). In bone, RANKL is expressed by osteoblast cell lineage and periodontal ligament (PDL) cells (6) and it exerts its effects after binding to RANK receptor on the cell surface of osteoclast lineage cells as an osteoclast differentiating factor (7). This binding leads to rapid differentiation of hematopoietic osteoclast precursors to mature osteoclasts. OPG is a secreted tumor necrosis factor (TNF) receptor member produced by osteoblastic cells and competes with RANK for RANKL binding. The biologic effects of OPG on bone cells include inhibition of terminal stages of osteoclast differentiation, activation of matrix osteoclasts suppression and accelerating osteoclast apoptosis (3,8). Both RANKL and OPG act as positive and negative regulators of osteoclastogenesis, respectively and are thought to regulate bone remodeling (6). It was found that the ratio RANKL/OPG was significantly higher during orthodontic tooth movement (3). Thus, the signaling and regulation of the expression of RANKL and OPG in PDL may play critical roles in bone remodeling during orthodontic tooth movement (3).

New bone formation is known to occur between the opened palatal bones after RME, although the time-dependent changes in the mid-palatal suture after RME have not been fully evaluated. Even though there are several studies in humans (9) and animals (9,10) regarding the association of RANKL, RANK and OPG levels, there is no information about these biomarkers in mid-palatal suture bone in response to RME. The present study evaluated the expression of RANK, RANKL and OPG genes, and the RANKL/OPG ratio during RME at different times of healing. Therefore, the aim of this study was to evaluate

osteoclastogenesis signaling in midpalatal suture after rapid maxillary expansion (RME) in rats. The study hypothesis was that the osteoclastogenesis process would be stimulated during early periods of healing after RME procedure.

Material and Methods

This animal study was carried out in accordance with the rules of Brazilian Institute for Protection of the Environment and was approved by the Animal Ethics Committee at the Universidade de São Paulo, Ribeirão Preto, Brazil. Thirty male *Wistar albinus* rats (mean weight 280±10 g) were assigned by blind randomization methods to two groups: control (C group, n=15, without RME) and experimental (RME group, n=15). They received a standard diet (C group) or standard chow diet (RME group) and water ad libitum during the entire experimental period. For all procedures, the animals were anesthezied with 40 mg/kg ketamine (Agener®; Agener União, São Paulo, SP, Brazil) and 20 mg/kg xylazine (Syntec®; Syntec, Santana do Parnaíba, SP, Brazil) intramuscularly at 1:2 ratio (1 mL/kg body weight).

Rapid Maxillary Expansion (RME)

The animals were placed on a head-holding device and were submitted to RME. The immediate expansion of midpalatal suture was performed by inserting a stainless steel circular ring (1.5 mm thick and 0.5 mm diameter; Dental Morelli Ltda®, Sorocaba, SP, Brazil), between the maxillary incisors (Fig. 1), as previously described (11). This appliance was kept in position with a light cured adhesive (3M® Unitek, Monrovia, CA, USA). Five animals from each group were euthanized at 3, 7 and 10 days after RME.

RNA Extraction, Reverse Transcription (RT) and Quantitative Polymerase Chain Reaction (q-PCR) Analysis

The palatal mucosa was surgically removed and the newly formed tissue between palatal bones in the suture space was gently collected and the specimens were stored in Eppendorfs at -80 °C. These tissues were sent to gene expressions analysis. The tissue obtained from midpalatal suture of maxilla was used to RNA extraction and complementary DNA (cDNA) synthesis. To produce the cDNA were used 2 µg of total RNA of each sample. The cDNA products were analyzed by q-PCR using TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and primers/probe for Tnfrsf11b (Opg - Rn00563499_m1), Tnfrsf11a (Rank - Rn01426423_m1), Tnfsf11 (Rankl - Rn00589289_m1) and Actb (Beta-actin - Rn00667869_m1; constitutive gene). q-PCR was run on StepOne Plus® machine (Applied Biosystems). Experiments were run in duplicate and water was used as negative control; control groups without RME at 3 days were used as positive control. Amplification conditions were: denaturation at 95 °C (10 min) and 40 cycles at 95 °C (15 s) and 60 °C for 1 min. For mRNA analysis, the relative level of gene expression was calculated in reference to Actb expression using the cycle threshold (Ct) method.

Statistical Analysis

The results were analyzed using the cycle threshold (Ct) values based on the formula: Relative Expression Level (QR)=2-ΔΔCt. Data were submitted to statistical analysis using two-way ANOVA followed by Tukey test (α=0.05).

Results

There was an upregulation of Tnfrsf11a gene, which encodes RANK receptor, in RME group compared to C group at 7 and 10 days of healing ($p<0.05$). The peak of gene expression was observed at 7 days in RME group compared to 3 and 10 days ($p<0.05$) and a similar result was observed in group C (Fig. 2).

Tnfsf11 gene, which encodes soluble factor RANKL, was also upregulated in group RME compared to group C

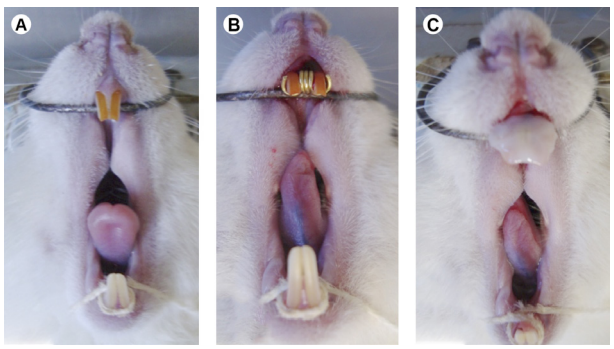

Figure 1. (A-C). RME was performed in midpalatal suture with a circular metal ring (1.5 mm thick and 0.5 mm diameter) placed between the maxillary incisors.

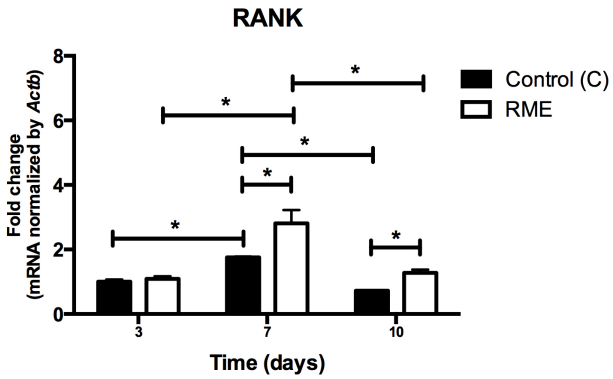

Figure 2. Fold change of mRNA of Tnfrsf11a (RANK receptor) gene expression for RME and C groups over time. $p<0.05$ (*) was considered statistically different.

at 3 and 7 days ($p<0.05$). Tnfsf11 gene expressed a similar pattern of RANK gene in group RME over time ($p<0.05$). There was a peak of Tnfsf11 expression in group RME at 7 days compared to 3 and 10 days. Group C showed an increased expression of RANKL over time ($p<0.05$) (Fig. 3).

There was an upregulation of Tnfrsf11b gene, which encodes OPG, in RME group, compared to C at 7 and 10 days ($p<0.05$). In addition, there was an increased expression of Tnfrsf11b gene in RME group with time ($p<0.05$) (Fig. 4).

The Tnfrsf11a/Tnfrsf11b (RANKL/ OPG) ratio showed a signaling increase favoring bone resorption on RME, compared to C at 3 and 7 days. On the other hand, a signaling against bone resorption was observed in RME group compared to C group at 10 days.

Discussion

Alveolar bone remodeling consists of an interaction of bone resorption by osteoclasts with bone formation by osteoblasts (12). Rapid maxillary expansion (RME) is usually used in the treatment of maxillary skeletal constriction and this procedure can induce a mechanical stress resulting in bone remodeling followed by apposition between the palatal suture borders. Bone resorption involves recruitment of new osteoclasts and activation of existing osteoclasts (13). Osteoclasts are multinucleated cells originating from granulocyte and macrophage colony-forming unit hematopoietic stem cells (5). Recently, RANKL, RANK and OPG were shown to play a key role in bone remodeling (14).

Experimental studies demonstrated that RANKL is expressed by osteoblasts and bone marrow stromal cells (15), whereas its receptor RANK is expressed in preosteoclasts and other cells from this lineage. The interaction between RANKL and RANK stimulates osteoclastic formation, fusion, activation, differentiation and survival (16) by activation of several transcription factors that regulate osteoclastogenesis, thus resulting in bone resorption (17). OPG is a member of the TNF receptor family and a soluble receptor against RANKL, it is produced by osteoblasts and other cells and acts as a decoy receptor that competes with RANKL for binding to its receptor RANK (18). This interaction inhibits osteoclastic proliferation and differentiation and consequently prevents bone resorption (19). Therefore, bone remodeling is controlled by the balance between RANK-RANKL binding and OPG production (20). In this molecular triad, RANKL binds and activates RANK on osteoclast precursors and regulate bone remodeling following orthodontic force application (21,22). Binding of OPG to RANKL inhibits the genesis of clastic cells, thus preventing RANKL linking to receptor activator of NF-kB (21). Bone formation within the suture occurs by intramembranous ossification, but this process after RME is unknown. Time-dependent molecular signaling changes in the midpalatal suture analyzing RANK/RANKL/OPG expression after RME were investigated.

Physiological bone turnover and stable bone mass depend on the balance between OPG and RANKL (20). The present results showed that RANKL/OPG ratio in RME group had an increase at 3 and 7 days compared to C group. On the other hand, this ratio decreased at 10 days. This ratio indicates the activity of osteoclast and it may be the ultimate determinant of bone resorption (23). The increase in the RANKL/OPG ratio during the initial phases may be assigned to the acute inflammation resulting from the RME and the remodeling process. It is known that in the early phases of rapid maxillary expansion there are inflammatory cells inside the sutural tissue. Distraction osteogenesis starts bone formation of an inflammation area around the procedure and the inflammation events bring some growth factors and cytokines by blood flow. Previous

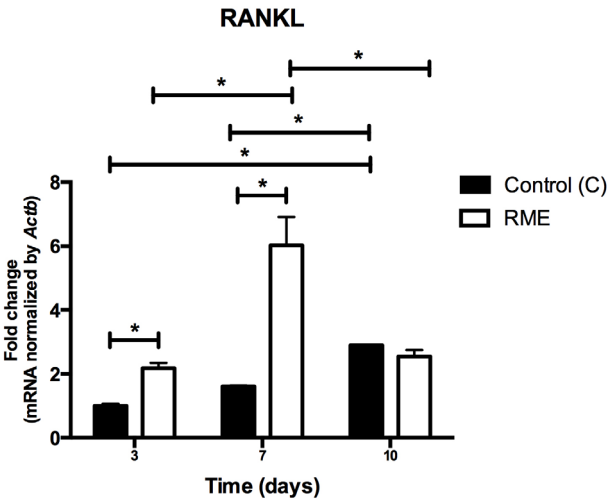

Figure 3. Fold change of mRNA Tnfsf11 (RANKL) gene expression for RME and C groups over time. $p<0.05$ (*) was considered statistically different.

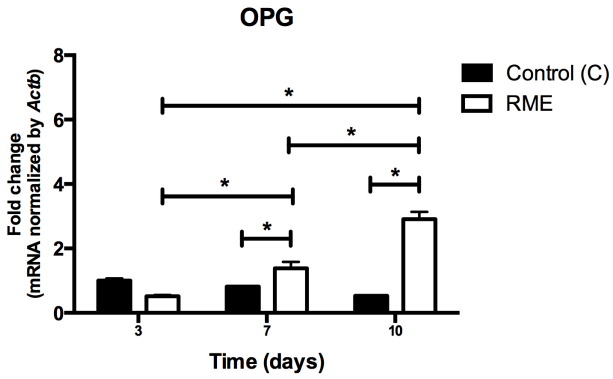

Figure 4. Fold change of mRNA of Tnfrsf11b (OPG) gene expression for RME and C groups over time. $p<0.05$ (*) was considered statistically different.

research (24) suggested that orthodontic tooth movement promotes an inflammation area around the procedure and the inflammation events bring some growth factors and cytokines by blood flow, like Tnfα, MMP1, Interleukin-1, TIMP-1, Col-1 that can contribute to bone healing and then activate bone remodeling genes. Therefore, it is suggested that some inflammatory cytokines may be involved in the regulation of orthodontic tooth movement (3). Although several studies evaluated the proinflammatory cytokines, this study is the first to determine the RANKL and OPG axis, which is a key regulator of osteoclastogenesis after RME in rats.

An increased RANKL/OPG ratio may indicate an increased induction of osteoclast progenitors to mature and active osteoclasts, as well as an extended osteoclast life span (20). It was recently shown *in vivo* that ratio of RANKL levels to OPG in the gingival crevicular fluid was significantly higher in patients with periodontal disease than in healthy subjects (25). Grimaud et al. (26) demonstrated that unbalance in the OPG and RANKL systems may play roles in bone resorption mechanisms. In the present study, RME upregulated the expression of RANKL at 3 and 7 days and it was downregulated at 10 days. Otherwise, OPG expression was upregulated at 7 and 10 days of healing of RME compared to C group.

Several authors (22) showed RANKL expression on periodontal tissues during experimental tooth movement in rats. Previous study (8) showed that human PDL cells might regulate osteoclastogenesis by opposing mechanisms that stimulate resorptive activity by RANKL and inhibition by OPG. In contrast, OPG gene transfer inhibited RANKL-mediated osteoclastogenesis and inhibited experimental tooth movement (3). A recent research (22) also showed that compressive force increased RANKL expression in human PDL cells. These results support the study findings, since it was shown that the RANKL expression was upregulated in rats at tension areas of the suture. This may have stimulated osteoclastogenesis and osteoclastic activity, which subsequently reduced bone mass and promoted bone the remodeling that changed the microarchitecture of bone tissues during the first days of RME.

The results indicated differences between control and RME groups in expression of OPG at 7 and 10 days. RANKL and RANK levels increased at 3 and 7 days in RME group. Increases in RANKL can reflect inflammation and bone resorption process and they may be assumed as potential markers of bone remodeling. Since OPG was strongly increased in the midpalatal suture with RME at 10 days, this marker may play an important role in palatal suture bone formation.

In conclusion, this study demonstrated that in bone recovery process, the cells in the palatal suture expressed RANK, RANKL and OPG under both physiological and mechanical stress conditions, and these factors seem to regulate the osteoclast function and may play important roles in bone formation. Therefore, the hypothesis that oscteoclastogenis process is stimulated during early periods of healing after RME procedure was accepted. The elucidation of RANK/RANKL/OPG system has widely increased the understanding of mechanisms underlying the bone remodeling process. Although this study suggested that several signal pathways are present in bone healing, including RANK, RANKL and OPG genes. Further studies are required to evaluate and confirm these findings using larger periods of study in order to discover the precise signal pathway and possible crosstalks that regulate bone remodeling after RME.

Resumo

O objetivo deste estudo foi avaliar a sinalização osteoclastogenese na sutura palatina após a expansão rápida da maxila (ERM) em ratos. Um total de 30 ratos Wistar machos foram divididos aleatoriamente em dois grupos com 15 animais cada: controle (C) e grupo ERM. ERM foi realizada através da inserção de um anel de metal circular de 1,5 mm de espessura entre os incisivos superiores. Os animais foram sacrificados aos 3, 7 e 10 dias após a RME. qRT-PCR foi utilizado para avaliar a expressão de Tnfsf11 (RANKL), Tnfrsf11a (RANK) e TNFRSF11b (OPG). Os dados foram submetidos à análise de variância de duas vias, seguido pelo teste de Tukey (α=0,05). Houve uma regulação positiva de genes RANK e RANKL aos 7 e 10 dias e uma regulação positiva do gene OPG aos 3 e 7 dias de tratamento. Curiosamente, foi observado um aumento na expressão de todos os genes ao longo do tempo nos grupos ERM e C. O RANKL/OPG mostrou um aumento na sinalização favorecendo a reabsorção óssea no ERM em comparação com o C nos períodos de 3 e 7 dias. Foi observada uma sinalização contra a reabsorção óssea, assim como, uma regulação favorável da expressão do gene OPG no grupo ERM, comparado ao grupo C aos 10 dias. Os resultados deste estudo permitem concluir que o sistema RANK, RANK-L e OPG participa de remodelação óssea após a ERM.

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