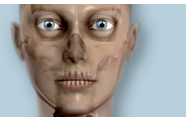
Orthodontics & Craniofacial Research



ORIGINAL ARTICLE

T. J. Franzen
M. Monjo
M. Rubert
V. Vandevska-Radunovic

Expression of bone markers and micro-CT analysis of alveolar bone during orthodontic relapse

Authors' affiliations:
T. J. Franzen, V. Vandevska-Radunovic, Department of Orthodontics, Institute of Clinical Dentistry, University of Oslo, Oslo, Norway
M. Monjo, M. Rubert, Department of Biomaterials, Institute of Clinical Dentistry, University of Oslo, Oslo, Norway
M. Monjo, M. Rubert, Department of Fundamental Biology and Health Sciences, Research Institute on Health Sciences (IUNICS), Universitat de les Illes Balears, Palma de Mallorca, Spain

Correspondence to:
T. J. Franzen
Department of Orthodontics
Institute of Clinical Dentistry
University of Oslo
P.b. 1109 Blindern
0317 N-Oslo
Norway
E-mail: tanyaf@odont.uio.no

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Structured Abstract

Objectives – To investigate biological changes in alveolar bone occurring during orthodontic relapse.

Materials and Methods – Rat maxillary first molars were moved mesially for 10 days. After orthodontic tooth movement (OTM), appliances were removed, and the molars were allowed to relapse for one, three, five, seven, 14 or 21 days. Changes in 3D morphometric parameters of bone located mesial to the first molars were evaluated by micro-CT. Total RNA was isolated from the same bone site, and real-time RT-PCR was used to measure the expression of bone formation and resorption markers.

Results – One day after appliance removal, the molars relapsed to a mean 73% of the achieved OTM and then steadily relapsed to 93% at 21 days. Tissue mineral density and per cent bone volume increased over the experimental period. Inversely, there was a decrease in total porosity. Gene expression of OCN, Coll-I and ALP decreased during OTM, whilst as the molars relapsed showed tended to increase. Gene expression of RANKL and TRAP increased during OTM. Changes in mRNA expression of H^+-ATPase were minor. By 21 days post-appliance removal, the remodelling process in rats appeared to have returned to control levels.

Conclusions – Bone tissue reactions on a molecular level are similar during OTM and orthodontic relapse. These findings validate the importance of immediate retention following active OTM.

Key words: bone remodelling; micro-CT; orthodontic relapse; real-time RT-PCR; tooth movement

Introduction

The biological responses that occur during the bone remodelling sequence induced by orthodontic force application have been well documented (1–3). However, although post-treatment orthodontic relapse remains a clinical problem, relatively few investigations have been performed to explore the biological processes behind this unwanted development.

Orthodontic tooth movement (OTM) occurs in the direction of force and is facilitated by a multifaceted bone and periodontal ligament (PDL) remodelling response (2). It has been reported that during orthodontic relapse, the PDL undergoes remodelling, with bone resorption along the alveolar bone in the direction of relapse, whilst the opposite side of the PDL experiences bone formation, indicating that orthodontic relapse and OTM undergo the same process, with an increase in osteoclast differentiation in areas of compression and a decrease in areas of tension (4–6).

Micro-computed tomography (micro-CT) has been shown to effectively evaluate the mechanical and structural properties of bone during OTM (7,8). It can be used as a substitute for conventional histological analysis of bone structure since high correlations between evaluations of bone structure obtained from conventional two-dimensional (2D) sections and three-dimensional (3D) micro-CT data have been measured (9).

Mechanically induced remodelling of bone and PDL is mediated by the synthesis and release of a diverse amount of molecules, which modulate the differentiation, proliferation and activation of cells of the osteoclast or osteoblast lineage (2,10,11).

In this study, we investigated the changes in a variety of 3D bone morphometric parameters during orthodontic relapse of rat molars and the association of orthodontic relapse with gene expression of osteoblast (runt-related transcription factor 2 (runx2), collagen-I (coll-I), alkaline phosphatase (ALP) and osteocalcin (OCN)), and osteoclast (receptor activator of nuclear factor kappa β ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), and vacuolar-type H^+-ATPase proton pump (H^+-ATPase)) markers. It was hypothesized that bone structure and tissue reactions on a molecular level would be comparable during the processes of orthodontic relapse and OTM.

Materials and methods

Animals and experimental procedure

Forty male six-week-old Wistar rats (HanTac: WH, Taconic, Ry, Denmark), body weight 180–200 g, were used. The animals were housed and treated in the Laboratory Animal Unit at The Norwegian institute of Public Health according to a protocol approved by the Norwegian Animal Research Authority, in accordance with the Animal Welfare Act. Body weight of each rat was evaluated throughout the experimental period, and no significant weight loss was recorded. Three animals were excluded from the study due to appliance loss.

Five animals served as untreated controls (C). The maxillary right first molars of the remaining rats were moved mesially for 10 days by a chrome alloy closed coil spring (0.008 × 0.030, Ormco, CA, USA) ligated to the mesial aspect of the first molar and the eyelet on an incisor band. Approximately 0.5 N of force was applied, with no reactivation during treatment. Force magnitude was calibrated using a Correx dynamometer (Haag-Streit, Bern, Switzerland). Procedures were performed under anaesthesia by intraperitoneal injection of Ketalar 10 mg/ml (Pfizer AS, Lysaker, Norway)/Midazolam 5 mg/ml (Alpharma, Actavis Norway AS, Skøyen, Norway), at a dose of 100 mg/kg body weight/5 mg/kg body weight.

On the tenth day of experimental tooth movement, the appliances were removed, and tooth movement was measured between the distal surface of the first molar and the mesial surface of the second molar using a feeler gauge (Mitutoyo Co., Kawasaki, Japan) with a minimum measurable distance of 0.05 mm. All measurements were taken twice by one operator; no variation was seen in these recordings. The rats were sac-

rificed by guillotine decapitation at each of seven time points (day of appliance removal (A0; n = 5), 1 (R1; n = 4), 3 (R3; n = 5), 5 (R5; n = 4), 7 (R7; n = 4), 14 (R14; n = 5) and 21 (R21; n = 5) days).

Hemi-maxillae were dissected, placed in RNA*later*® solution (Applied Biosystems/Ambion, Austin, TX, USA) at 4°C for 24 h and then stored at −80°C until further use.

Micro-CT imaging

Right-side hemi-maxillae were scanned with a Skyscan 1172 X-ray microtomograph scanner (Skyscan, Kontich, Belgium) (Fig. 1). Images were obtained at 100 kV and 100 μA, with a 0.5-mm aluminium filter and image pixel size 11.96 μm. Scans were reconstructed with a smoothing of 1, beam hardening correction of 40%, ring artefact reduction of 12 and output values from 0 to 0.05 with reconstruction software (NRecon, version 1.6.4.8, Skyscan, Kontich, Belgium). A calibration of the standard unit of X-ray CT density (Hounsfield unit) was performed.

Once the calibration of the micro-CT was completed, the same volume of interest (VOI) was chosen for each sample, and this was the same volume as would later be used for the bone biopsy for gene expression analysis: a cylinder with a diameter of 2.0 mm and a height of 4.2 mm. A 3D morphometric analysis was conducted to determine the architecture of the bone by means of the following:

Tissue mineral density (TMD): (g/cm^3 CaHA) Density measurement restricted to within the volume of calcified tissue, excluding surrounding soft tissue. TMD was calculated using calcium hydroxyapatite phantoms with bone mineral density of 0.75 and 0.25 g/cm^3 CaHA and a threshold for bone tissue selection of 75–255.

Percent bone volume (%BV): The ratio of bone volume to total VOI volume (BV/TV).

Bone surface density (BSD) (mm^{-1}): The ratio of bone surface area to total volume measured in 3D within the VOI (BS/TV).

Total porosity (Po(tot)): The volume of all open plus closed pores as a per cent of the total VOI volume.

This analysis was performed with the CTAn program (version 1.11.1.0), and 3D models were visualized with CTVox (Version 2.1.1.0) (Skyscan, Kontich, Belgium).

Total RNA isolation

Following micro-CT scanning, a sterile 2-mm-diameter biopsy punch (Miltex, York, PA, USA) was employed to remove bone and root tissue of

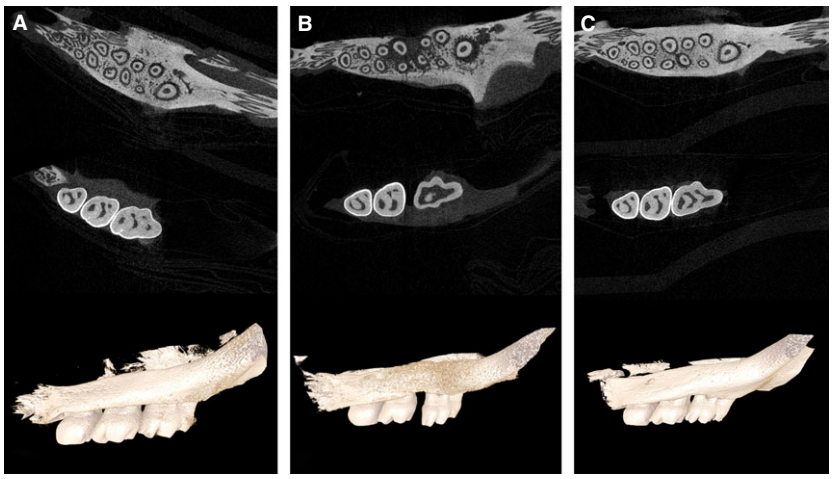

Fig. 1. Micro-CT and 3D reconstructed images of right rat hemi-maxillae. (A) Specimen from the control group with first and second molars in close approximation. (B) Specimen from group A0 exhibiting OTM of the first molar in a mesial direction with resultant spacing between the first and second molars. (C) Specimen from group R14. The first molar has relapsed 100% and resembles the control group sample.

all samples from the mesial aspect of the first molars, parallel to the visible inclination of the first molar mesial root through full thickness of the alveolar bone (Fig. 2). Samples were crushed in liquid N_2, transferred to microcentrifuge tubes containing 1 ml of TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and sonicated in a vibra-cell ultrasonic processor (Sonics & materials, Inc., Newtown, CT, USA). Samples were stored at −80°C. RNA was isolated according to the manufacturer's protocol and quantified at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

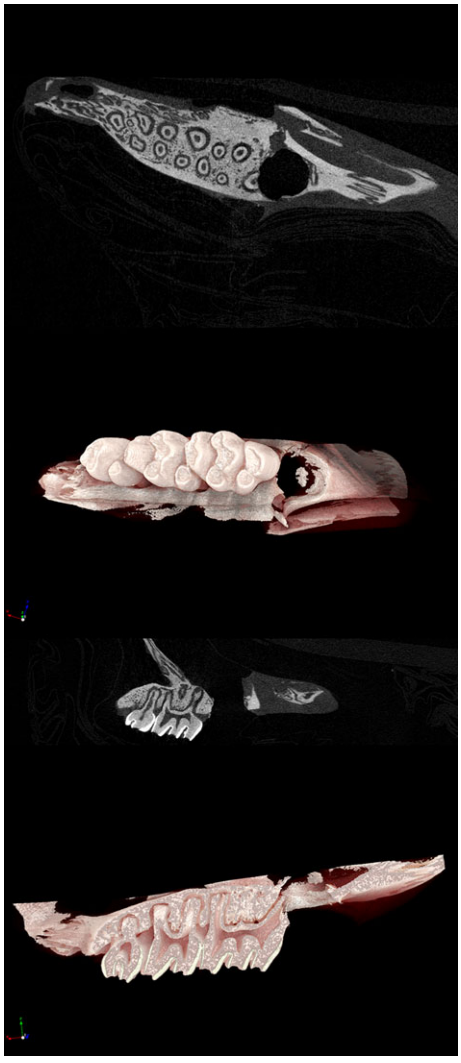

Fig. 2. Micro-CT and 3D reconstructed images of a specimen of right rat maxilla showing a biopsy taken from the mesial aspect of the first molars. The biopsy sample includes bone, PDL and root tissues.

Real-time RT-PCR analysis

One microgram of the total RNA isolated from the tissue was reverse-transcribed to cDNA at 42°C for 60 min using iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) that contained both oligo (dT) and random hexamers. Each cDNA was diluted 1/10, and aliquots were frozen (−20°C) until PCRs were carried out. Real-time RT-PCR was performed in the iCycler (BioRad, Hercules, CA, USA) using SYBR Green detection. Real-time RT-PCR was performed for three housekeeping genes: 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, and seven target genes: bone formation markers runx2, coll-I, ALP, OCN and bone resorption markers H^+-ATPase, TRAP and RANKL (Table 1).

The analysis was performed according to the protocol of Monjo et al. (12). Relative mRNA levels were calculated as the ratio of relative concentration for the target genes relative to that for the mean between the three housekeeping genes (18S rRNA, GAPDH and β-actin), to correct for RNA. Values were expressed as a percentage of control samples, which were set to 100.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Relapse was calculated as a percentage per group after calculating the mean relapse (mm) per group. Comparison between the groups was assessed by analysis of variance (ANOVA). Correlations between different factors were tested by Spearman's rank correlation coefficient (r_s). Results were considered statistically significant at $p < 0.05$. Statistics were performed using Sigmaplot 12 software (Systat Software Inc., San Jose, CA, USA).

Results

Tooth movement and relapse

All appliance-treated first molars exhibited measurable mesial tooth movement following

Table 1. **Primer sequences and specific parameters of the real-time RT-PCR. Sense (S) and antisense (A) primers of target and reference genes, average cycle number (CT, cycle threshold) for the samples, amplicon size (bp) of the resulting PCR products and GenBank accession number for the different sequences are shown**

	Gene		Primer sequence	Average C_T (mean ± SD)	Amplicon size (bp)	GenBank accession number
Bone formation	Osteocalcin	S	5′-CATGAGGACCCTCTCTCTGC-3′	13.7 ± 1.14	135	NM_013414
		A	5′-TTCACCACCTTACTGCCCTC-3′			
	Collagen-I	S	5′-TGCTGCCTTTTCTGTTCCTT-3′	19.1 ± 1.78	179	NM_053304
		A	5′-AAGGTGCTGGGTAGGGAAGT-3′			
	Runx2	S	5′-CAGGGACTGGGTATGGTTTG-3′	22.4 ± 0.97	123	NM_001278483
		A	5′-TGCACTGAAGAGGCTGTTTG-3′			
	ALP	S	5′-AACCCAGACACAAGCATTCC-3′	26.0 ± 1.28	151	NM_007431
		A	5′-GAGAGCGAAGGGTCAGTCAG-3′			
Bone resorption	H^+-ATPase	S	5′-CCGAAACCTCCTGAAGAAAA-3′	22.7 ± 0.87	165	NM_199386
		A	5′-ATAGCCGTGGTGCTGAAGTC-3′			
	TRAP	S	5′-GACGGGAGAGATTGGTGATG-3′	25.8 ± 1.06	110	NM_001270889
		A	5′-CACGAATCTCAGGGTGGAAG-3′			
	RANKL	S	5′-GGCCACAGCGCTTCTCAG-3′	26.7 ± 1.24	141	NM_057149
		A	5′-TGACTTTATGGGAACCCGAT-3′			
Reference	Beta-actin	S	5′-AGCCACCAATCCACACAG- 3′	16.4 ± 0.77	155	NM_031144
		A	5′-CCTCTATGCCAACACAGT-3′			
	18S rRNA	S	5′-GTAACCCGTTGAACCCCATT-3′	8.7 ± 0.71	151	NR_046237
		A	5′-CCATCCAATCGGTAGTAGCG-3′			
	GAPDH	S	5′-ATGATTCTACCCACGGCAAG-3′	18.5 ± 0.71	134	NM_017008
		A	5′-CTGGAAGATGGTGATGGGTT-3′			

10 days of force application, with no apparent spacing developing between the second and third molars. The mean OTM was 0.27 mm ± 0.14. No tooth movement was evident on the untreated contralateral molars.

All treated first molars in the experimental relapse groups underwent orthodontic relapse following appliance removal (Fig. 3A). The molars relapsed rapidly 1 day after the end of active treatment and thereafter demonstrated a steady decrease in relapse rate (μm/d) (Fig. 3B). By 21 days, the first molars had relapsed a mean 93.33% ± 14.91 of their achieved OTM. Statistically significant differences were seen between percentage relapse in the experimental groups and group A0 ($p < 0.001$), no significance was exhibited between relapse groups. The mean total relapse in the experimental relapse groups was 0.18 ± 0.09, which was 69.38% of their initial OTM.

Micro-CT observation

Tissue mineral density, %BV, BSD, and Po(tot) of the VOI were measured to investigate the structural and mechanical properties of alveolar bone (Fig. 4). TMD decreased significantly during OTM and subsequently increased to control levels 3 days after appliance removal (Fig. 4A); however, there was no correlation with percentage relapse or with the other bone parameters. %BV decreased non-significantly during OTM and increased following appliance removal after 7 days ($p < 0.05$) (Fig. 4B). Inversely, Po(tot) decreased in the same time period following appliance removal (Fig. 4C); r_s for %BV vs. Po (tot) = −1, $p < 0.05$. Percentage relapse was positively correlated with %BV ($r_s = 0.62$, $p < 0.05$), whilst Po(tot) was negatively correlated ($r_s = -0.62$, $p < 0.05$). Generally, no significant differences were seen in the mean values of

BSD among the experimental groups (Fig. 4D), nor was there any correlation between BSD and percentage relapse. BSD was positively correlated with %BV ($r_s = 0.74$, $p < 0.05$) and negatively correlated with Po(tot) ($r_s = -0.74$, $p < 0.05$).

Quantitative analysis of mRNA expression of bone activity markers

Bone formation (Fig. 5) and resorption (Fig. 6) were evaluated by measuring levels of mRNA of the relevant markers from all samples taken from the mesial aspect of the first molars. Extraction of total RNA, retrotranscription and amplification of cDNA was confirmed as successful by the high and stable expression levels of the internal control genes that were used for normalization (18S rRNA, β-actin and GAPDH). Taking into account the relationship where higher amounts of mRNA correlate with lower CT values, the highest expression level found in the mesial alveolar bone samples was for OCN and the lowest for RANKL.

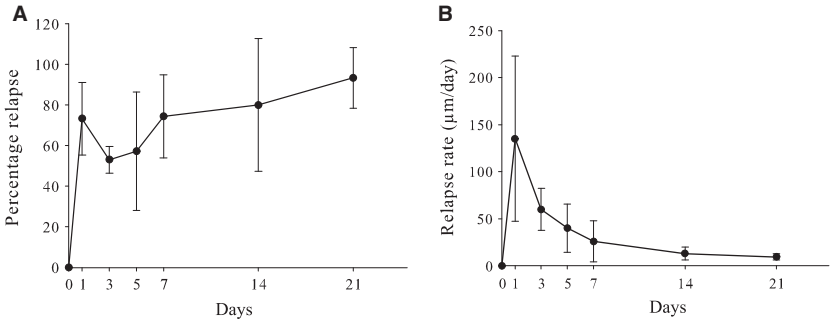

Fig. 3. (A) Percentage relapse and (B) relapse rates of experimental groups of maxillary first molars at 0, 1, 3, 5, 7, 14 and 21 days subsequent to appliance removal ± SD. Day 0 corresponds to the 10 day active (A0) group. SD in some cases is high due to individual differences.

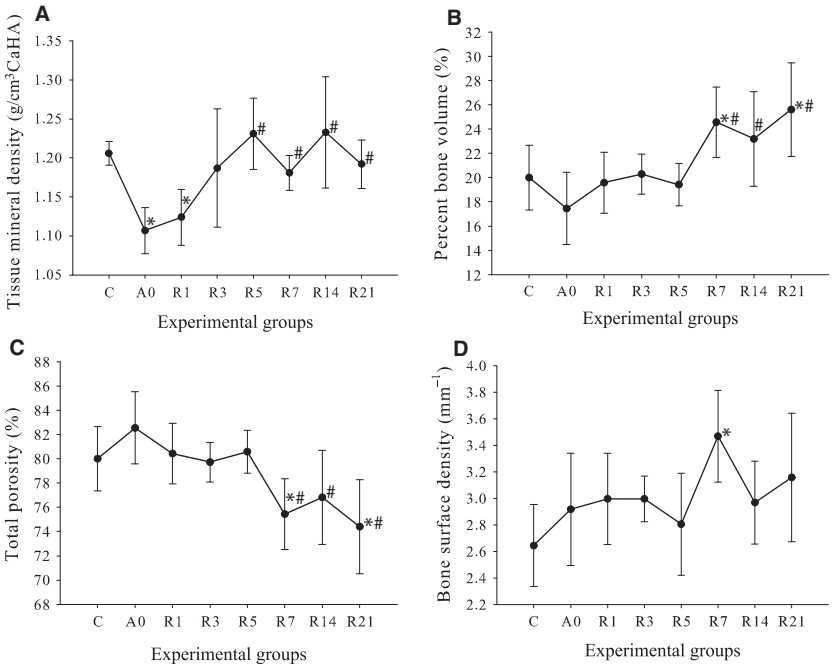

Fig. 4. 3D bone parameters of the VOI taken from bone located on the mesial aspect of the first molars. (A) Tissue mineral density, (B) per cent bone volume, (C) total porosity, (D) bone surface density. Data presented as mean ± SD. $^{*}p < 0.05$ significant differences between control group vs. experimental side bone samples at each experimental time point. $^{\#}p < 0.05$ significant differences between A0 and relapse groups. SD in some cases is high due to individual differences.

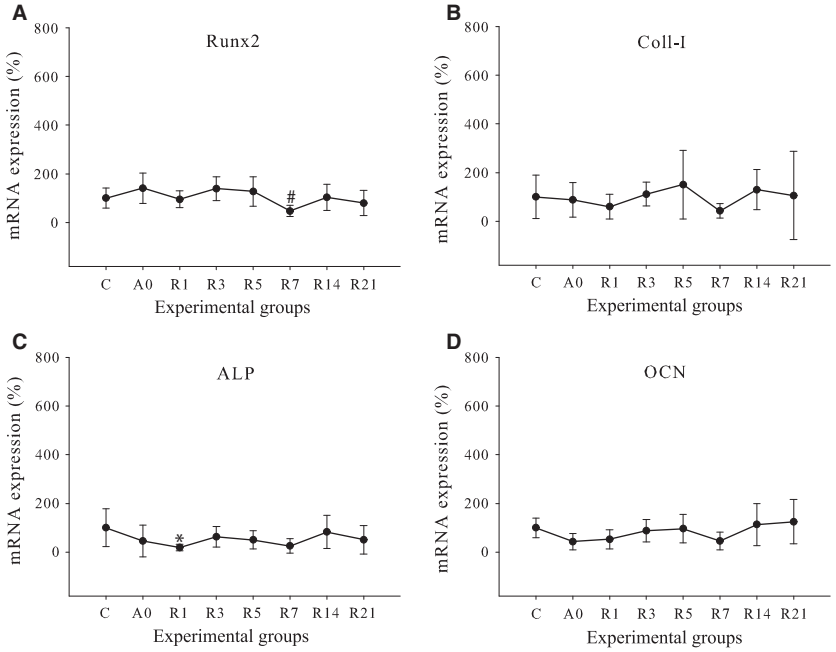

Fig. 5. Relative mRNA levels for the bone formation markers (A) runx2, (B) coll-I, (C) ALP and (D) OCN in the alveolar bone samples taken from the mesial aspect of the maxillary first molars. Ratios of target genes relative to housekeeping genes were expressed as a percentage of control samples, which were set to 100. Data presented as mean ± SD. $*p < 0.05$ significant differences between control group vs. experimental side bone samples at each experimental time point. $^{\#}p < 0.05$ significant differences between A0 and relapse groups. SD in some cases is high due to individual differences.

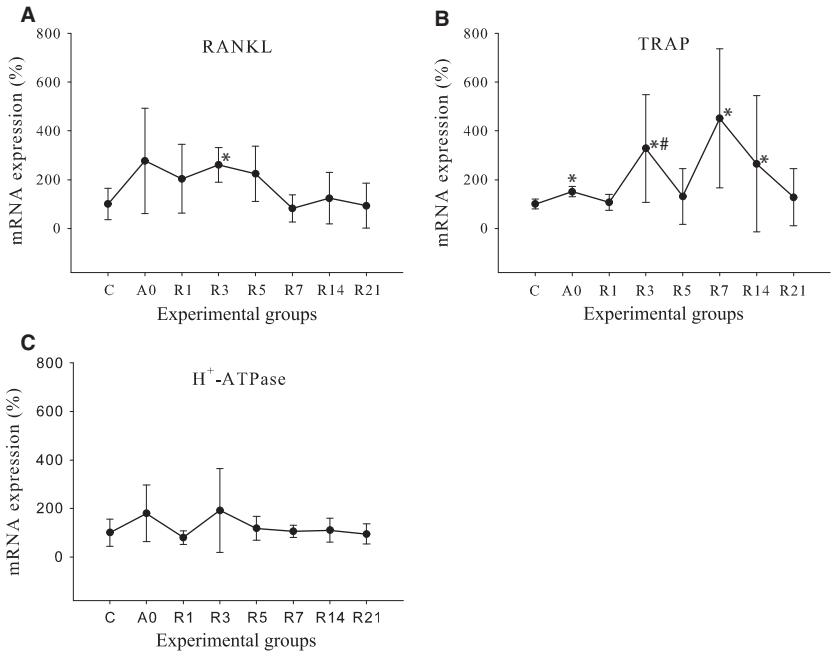

Fig. 6. Relative mRNA levels for the bone resorption markers (A) RANKL, (B) TRAP and (C) H^+-ATPase in the alveolar bone samples taken from the mesial aspect of the maxillary first molars. Ratios of target genes relative to housekeeping genes were expressed as a percentage of control samples, which were set to 100. Data presented as mean ± SD. $*p < 0.05$ significant differences between control group vs. experimental side bone samples at each experimental time point. $^{\#}p < 0.05$ significant differences between A0 and relapse groups. SD in some cases is high due to individual differences.

Although significant differences were seen only at R7 in runx2, mRNA expression of OCN, Coll-I and ALP decreased following OTM and thereafter showed a trend towards higher mRNA levels as the molars relapsed (Fig. 5). However, no correlation was seen between these three

markers and relapse. mRNA expression of runx2 was negatively correlated with relapse, although all osteoblast markers were all positively correlated with each other.

Gene expression of RANKL displayed a non-significant increase in mRNA levels following OTM, and this steadily decreased following removal of the appliances and by 21 days had returned to control levels (Fig. 6A). There was generally an increase in expression of TRAP mRNA after orthodontic force application and until 7 days following appliance removal (Fig. 6B); thereafter, it decreased and had returned to control levels by day 21. The changes in mRNA expression of H^+-ATPase were minor and non-significant (Fig. 6C). Both H^+-ATPase and RANKL were negatively correlated with relapse percentage ($r_s = -0.36$, $p < 0.05$ and $r_s = -0.45$, $p < 0.05$, respectively), whilst TRAP was not significantly associated with the relapse.

Discussion

In the present investigation, alveolar bone tissue from the mesial aspect of the first molars of rats was chosen as the model for studying the biological events occurring during the course of orthodontic relapse. Interindividual variations in all variables were seen in this study; these contribute to the deviation in trends of the variables although they are generally non-significant. The pattern of initial rapid relapse, with a gradual decrease in rate, is similar to that seen in previous relapse investigations in animal models (4–6,13). At 21 days, this distal tooth movement had slowed to a rate of 9.5 μm/d, which is comparable with the rate of 9.9 μm/d in a previous study (4). This low rate of distal movement is marginally greater than the rate of physiological distal drift of adult rat molars (14), and together with the high relapse percentage (93.33%) indicates that a stable situation has been attained and the molars have more or less resumed the process of distal drift.

The biopsy technique was employed to standardize the sample volume and location maximally. The diameter of the biopsy punch was consistent, and all samples were removed parallel to the observed inclination of the first molar mesial root through the full thickness of the alveolar bone. Samples were weighed and no significant differences were seen. The VOI on the micro-CT was cut to replicate the positioning of the biopsies.

As confirmed earlier (4), there is a mesial tipping displacement of the first molars, which reverses following the subsequent relapse after appliance removal. Thus, due to the complex nature of the tipping movement, the VOI and the biopsy sample include areas of both compression and tension. Therefore, due to this and the composition of the tissues in the samples, the results provide a general account of the remodelling process involved in OTM and relapse. Nevertheless, when there is a mesial direction of force, the mesial side of the root is often considered to represent compression and the distal side tension (5,15,16).

Following OTM, the TMD and %BV decreased, whilst Po(tot) increased indicating bone resorption from increased osteoclast activity due to compression of the periodontal tissues. This increased activity corresponds to the previous reports of increased turnover of alveolar bone during OTM in rats (4,16). Bridges et al. (17) also found decreased TMD after 10 days of OTM in rats, and Hsu et al. (18), in an adult sample, observed a reduction in bone density, although in both cases, the samples used encompassed the entire root circumference. Ru et al. (8) using micro-CT to calculate %BV selected samples from the trabecular bone of the mesial root of the maxillary first rat molar from compression and tension areas and found a decrease and increase in %BV, respectively, which is similar to the findings in the larger samples in the present study. After appliance removal, TMD and %BV gradually increased, and Po(tot) decreased as the course of relapse progressed, attaining control levels after 3 days implying decreased osteoclast activity and new bone apposition. This is in agreement with previous studies (4,6), which found that alveolar bone remodelling in the direction of force continued for several days following discontinuation of experimental OTM.

Bone surface density increased during OTM and remained increased above the control levels. Nafei et al. (19) state that BSD is an important explanatory factor of the variations in the compressive elastic properties of bone. This could suggest an increased compressive elastic property of the new bone which is deposited as relapse progresses.

Although non-significant, we saw a decrease in mRNA expression of Coll-I, ALP and OCN, which tended to increase at the start of the relapse period. This trend concurs with other investigations, which have shown decreased promoter expression of coll-I on the compression side of the PDL, and increased coll-I and ALP mRNA expression on the tension sides of orthodontically moved rodent molars (15,20,21) or human OTM samples (22), all signifying increased osteoblastic differentiation and activity in tension areas in response to compressive forces. Although Brooks et al. (23) found increased expression of runx2 in tension areas following force application, in this study, runx2 was not a significant marker of OTM or relapse.

Conversely, on the mesial side during OTM, bone resorption marker expression increased, as seen in previous studies of OTM (6,22–24). During relapse, most markers have returned to control levels by 21 days implying a decrease in osteoclast activity which also corresponds to the observed increased bone apposition, with an increase in TMD and %BV at this site during the relapse process. TRAP expression increased up until 7 days post-force application, and this was similar to the findings of King et al. (6), although they used tissue samples encompassing the entire root area. The significant negative correlation found between mRNA expression of RANKL and H^+-ATPase to percentage relapse indicates that these are perhaps the best biological markers studied here to predict relapse.

It has previously been shown that during relapse, osteoclast differentiation increases with resultant bone resorption in the compression zones in the direction of relapse, whilst there is bone apposition on the former pressure areas (4). In this study, this pattern is confirmed through the changing expression of most of the bone resorption and apposition markers and the increase in TMD and %BV during the relapse process. These results demonstrate molecular evidence of the pattern of bone remodelling occurring during orthodontic relapse.

Conclusions

Orthodontic relapse appears to occur rapidly, particularly during the first week following appliance removal. On the former pressure side (mesial side of first molar), bone resorption continues during the early periods of orthodontic relapse, whilst bone formation appears to start only a few days after removal of orthodontic force.

The results of this investigation, whilst not providing definitive evidence, lend support to the hypothesis that bone structure and tissue reactions on a molecular level are similar during the processes of orthodontic relapse and OTM. Further studies are required to confirm the significance of the pattern of resorption and apposition. These findings validate the importance of immediate retention following active orthodontic tooth movement.

Clinical relevance

The existence of orthodontic relapse requires that we have more knowledge of the biological basis of relapse to aid formulation of therapies that may improve stability following orthodontic treatment. The purpose of this study was to contribute to the paucity of information concerning orthodontic relapse.

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