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Specific signaling molecule expressions in the interradicular septum in different age groups

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SUMMARY

Introduction. Orthodontic teeth movement is accompanied by the remodeling of alveolar bone, including the interradicular septum. Bone contains three cell types, osteoblasts, osteocytes, and osteoclasts that are in direct contact with all of the cellular elements in the bone marrow. Marrow is the source of both bone-building osteoblasts and bone destroying osteoclasts, and the turnover of bone occurs throughout life. Bone signalling molecules have important functions during osteogenesis, and they are active in the bone remodelling process. Patients involved in orthodontic treatment belong to different age groups: therefore age must be considered as a contributing factor compromising the osteogenetic potential of bone. The aim of the current study was to investigate the specific expression of signalling molecules in the interradicular septum in different age groups.

Materials and methods. The study group included 17 patients to whom the extraction of teeth was recommended as part of further orthodontic treatment. Patients (9 males and 8 females) – were divided into 3 groups 1st group – 12-14 years old); 2nd group – 15-22 years old; 3rd group – 23 years old or older. Expression of BMP 2/4, TGF-α, IL-1, IL-8, OPG, MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, NGFR, NKpB 105, osteocalcin, and osteopontin in interradicular septum tissues was examined. TUNEL staining was also completed. The distribution of these factors was evaluated semi quantitalively.

Results. In the interradicular septum bone structure, the expression levels of osteocalcin, osteoprotegerin, matrix metalloproteinases 8 and 9, and nuclear factor kappa B were determined in all samples. TUNEL staining was also done. Age related decreases in the mean values of signalling factors and the number of apoptotic cells were statistically significant.

Conclusion. Specific to interradicular septum osteoblasts and osteoclasts factors include osteoprotegerin, osteocalcin, matrix metalloproteinase 8, matrix metallproteniase 9, and nuclear factors kappa B. The mean expression levels of these proteins and the mean TUNEL staining statistically significantly decreased with age. This is preliminary study and more patients are necessary for more precise statistical analysis in the future.

Key words: alveolar bone aging, RANK, RANKL, OPG, MMP-8, MMP-9, osteocalcin, apoptosis.

INTRODUCTION

Aging is associated with marked changes in multiple organ systems, including bone. In humans, bone mineral density peaks between 10 and 19 years of age, with continued increases in bone mineral content until 30 to 35 years of age [1].

Bone contains three cell types, osteoblasts, osteocytes, and osteoclasts that are in direct contact with all of the cellular elements in bone marrow. As the marrow is the source of both, bone-building osteoblasts and bone - destroying osteoclasts, turnover of bone occurs throughout life [2].

Aging is thought to be a contributing factor compromising the regenerative potential of bone. An age-related decrease in the number of osteogenic progenitor cells seen in animal models and in humans could be one of the underlying mechanisms [3].

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Typically, the amount of bone removed by the osteoclasts is equal to the amount of bone formed by the osteoblasts, and a constant bone mass is maintained. However, this is not the case during aging. Increased bone resorption and/or decreased bone formation can lead to the bone loss. Based on a number of histomorphometric studies performed using iliac crest biopsies, a decrease in bone formation seems to be the principal pathophysiological mechanism responsible for age-related decreases in bone mass [3].

Although there is no single definitive biological marker that can be used to quantify the rate of aging, the change in the aging rate in mice can be determined through the quantitative measurement of a group of aging-related traits. Lifespan is unquestionably the most important trait reflecting the rate of aging [4].

Additionally, recently developed molecular approaches have rapidly advanced the field through the discovery and extensive characterisation of three new cytokine systems of the TNF (tumor necrosis factor) family. These cytokines have been found to regulate the proliferation, differentiation, fusion, activation, and apoptosis of osteoclasts. RANKL is naturally inhibited by osteoprotegerin and is important in determination of a balance of osteoclast activity [5, 6].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases comprising over 25 enzymes that regulate many biological processes, including development, morphogenesis, and wound healing [7].

MMP-8 (also known as human neutrophil collagenase and, collagenase-2) is produced not only by polymorphonuclear PMN leucocytes but also by certain non-PMN lineage cells, such as gingival fibroblasts, bone and plasma cells. MMP-8 hydrolyses type I collagen is more effective than other MMPs and is the major interstitial collagenase that presents in inflamed human gingival [8]. MMP-9 is known to specifically cleave type 4 collagen, which is a major structural component of the basement membrane [9].

The results of recent studies indicate that activity of MMPs is important for activating osteoclasts to resorb bone. Interstitial collagenase cleavage of type I collagen has been implicated in triggering bone resorption in vitro and in vivo [7].

The cell types within bone and bone marrow interact to promote the differentiation and activity of each cell population. In view of this interdependence, increased apoptosis in all population is likely to trigger changes in other cell populations. For example, controlled apoptosis of osteocytes may be involved in signalling the need for the repair of microdamage. Glucocorticoids affect the apoptosis of both, osteoblasts and osteocytes, which lead to reduced bone formation. Thus, apoptosis plays a role in normal tissue maintenance, abnormal bone turnover, and aging. It is difficult to determine if changes in the pattern of apoptosis in aged bone are the result of aging itself, or if they are secondary, as reaction to hormonal and other changes occurring with age [2].

The aim of the current study was to investigate the specific expression of signalling molecules in the interradicular septum in different age groups.

MATERIALS AND METHODS

Study group

The study group included 17 patients for whom extraction of teeth had been recommended as a part

Table1 Analysis of specific signaling molecules

Age	Sex	IL-1	IL-8	BMP 2/4	TGF-α	OPG	NKpB105	Osteocalcin
12*	F	0/+	0	0	0	+++/++	++/+++	++++
14*	F	0	0	0	0	++/+++	+	++++
15**	F	0	0/+	0	0	++	+++/++	++++
16**	M	0	0	0	0	++/+++	+++	++++
23***	M	0/+	0/+	0	0	+/++	++	++++
25***	F	0	0	0	0	+	0/+	++++
Age	**Sex**	**Osteopontin**	**NGFR**	**MMP-1**	**MMP-2**	**MMP-8**	**MMP-9**	**MMP-13**
12*	F	++++	0	0	0	++	+	0/+
14*	F	++++	0	0	0	++	0/+	0
15**	F	++++	0	0	0	++/+	+	0
16**	M	++++	0	0	0	+	+	0
23***	M	++++	0	0	0	+	++	0
25***	F	++++	0	0	0	+	+/++	0/+

The levels factors were determined semi-quantitatively by counting the number of positive structures in the visual field. 1st group (12 – 14 years old) – *; 2nd group (15 – 22 years old) – **; 3rd group (23 years old and older) – ***.

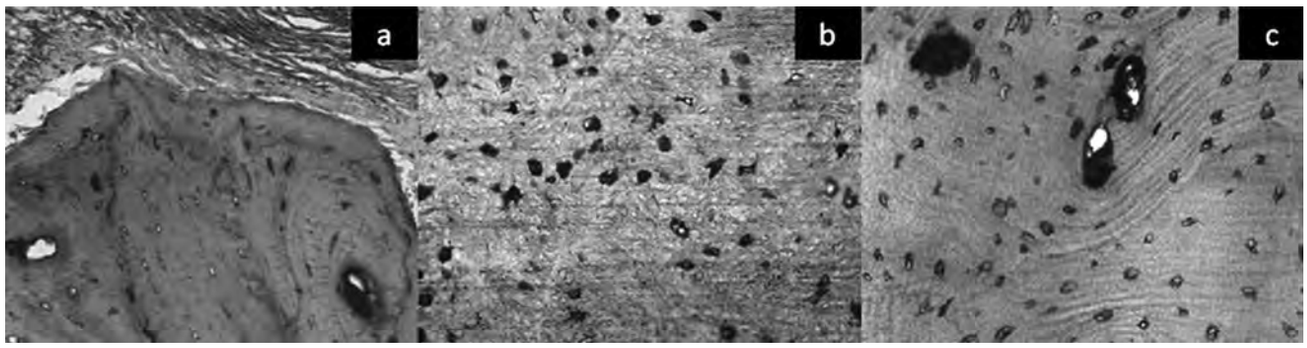

Fig. 1. Microphotograph of osteocalcin expression in bone from the interradicular septum of a 12 (a), 17 (b) and 29-year-old (c) patients, IMH, ×400

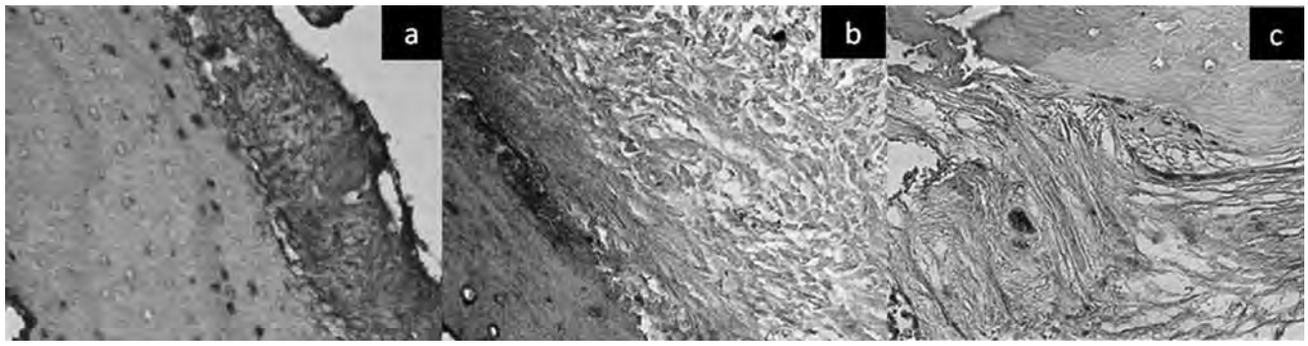

Fig. 2. Microphotograph of OPG expression in bone from the interradicular septum of a 12 (a), 17 (b) and 29-year-old (c) patients, IMH, ×400

of further orthodontic treatment. Exclusion criteria were apical periodontal and periodontal inflammation of teeth. 17 patients (9 males and 8 females), were divided into three groups as follows: 1) 12-14 years old (4 patients), 2) 15-22 years old (5 patients) and 3) 23 years old or older (8 patients). Tooth extractions were performed before orthodontic treatment by four surgeons in the Department of Oral and Maxillofacial Surgery, Riga Stradins University. Tissue samples from the interradicular septum that contains cortical bone were collected after the surgical extraction procedure.

Histological procedure

The tissue samples were fixed in 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). Histological investigation was performed in the Riga Stradins University Institute of Anatomy and Anthropology. Fixed tissue samples were washed in phosphate-buffered saline for 12 hours, embedded in paraffin and cut into 6 to 7 μm thick sections. Levels of the specific signalling molecules were determined by immunohistological analysis of three samples from each study group, with the aim to clarify which signalling molecules present. The following signalling molecules were selected: NGFR (nerve growth factor) [ab3125; Abcam; 1:150, Cambridge UK]-, BMP 2/4-(bone morphogenetic protein) [AV1024011; R/D; 1:100, Germany], TGF-α (transforming growth factor α) [ab27969; Abcam; 1:1000, Cambridge, UK], IL-1 (interleukin 1) [B-7 SC-9983; 1:50, Santa Cruz Biotechnology, Inc. California, US], IL-8 (interleukin 8) [C-19 SC-1269; 1:50, Santa Cruz Biotechnology., Inc., California, US], OPG (osteoprotegerin) [N-20 SC-8468; 1:40, Santa Cruz Biotechnology, Inc., California, US], MMP-1 (matrix metalloproteinase 1) [3-B6 SC-21731; 1:100, Santa Cruz Biotechnology, Inc., California, US], MMP-2 (matrix metalloproteinase 2)

Table 2. Mean expression levels of osteoprotegerin, matrix metalloproteinases -8 and -9, NKpB 105, and osteocalcin; mean TUNEL staining and apoptosis index by age group

Signaling molecule	1 group Mean age 12.5 Mean value (SD)	2 group Mean age 17.2 Mean value (SD)	3 group Mean age 29.6 Mean value (SD)
OPG	2.25 (0.29)	1.6 (0.65)	1.0 (0.59)
MMP-8	2.0 (0)	1.4 (0.65)	1.125 (0.99)
MMP-9	1.25 (0.64)	1.6 (0.89)	1.125 (1.06)
NKpB105	2.125 (0.85)	2.0 (0.79)	1.312 (0.59)
Osteocalcin	4	4	4
TUNEL	2.5 (0.57)	1.8 (0.83)	1.125 (0.99)
AI%	75.6 (10.2)	51.6 (25.3)	53.6 (28.9)

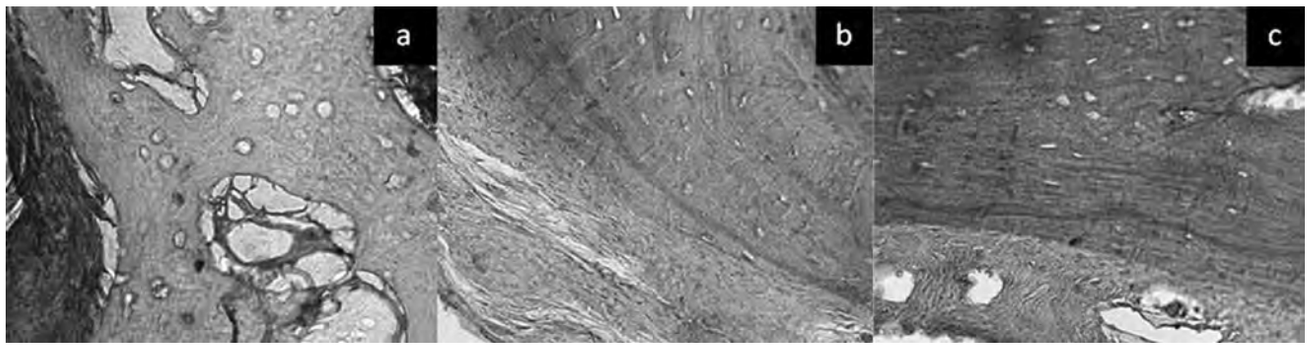

Fig. 3. Microphotograph of MMP-8 expression in bone from the interradicular septum of a 12 (a), 17 (b) and 29-year-old (c) patients, IMH, ×400

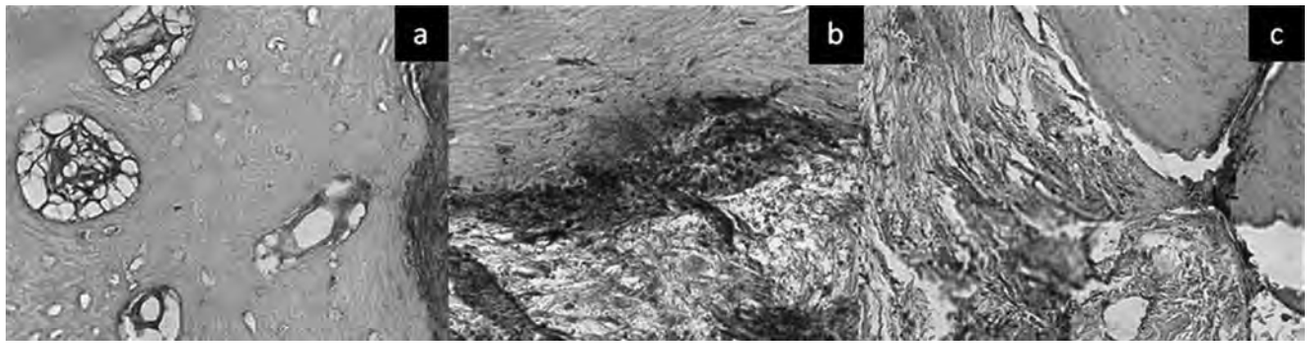

Fig. 4. Microphotograph of MMP-9 expression in bone from the interradicular septum of a 12 (a), 17 (b) and 29-year-old (c) patients, IMH, ×400

[DUB03; R/D; 1:100, Germany], MMP-8 (matrix metalloproteinase 8) [6-19Z: SC-80206, 1:50, Santa Cruz Biotechnology, Inc., California, US], MMP-9 (matrix metalloproteinase 9) [H-129:SC-10737, 1:250, Santa Cruz Biotechnology, Inc., California, US], MMP-13 (matrix metalloproteinase 13) [M-66: SC-81547, 1:100, Santa Cruz Biotechnology, Inc., California, US], NKpB 105 (nuclear factor kappa B protein 105) [p105/p50[ab7971], Abcam; 1:100, Cambridge, UK], osteocalcin [ab13418; Abcam; 1:100, Cambridge, UK], and osteopontin (ab8448; Abcam; 1:100, Cambridge, UK) (see Table 1). After deparaffinisation, the expression levels of osteoprotegerin, osteocalcin, matrix metalloproteinase 8, matrix metalloproteinase 9, and NKpB 105 and the TUNEL, cell apoptosis factor expression, (11684817910; 1:10, Roche, Germany) staining patterns in the tissues were examined using the biotin-streptavidin immunohistochemistry (IMH) [10]. TUNEL kit (11684817910; 1:10, Roche, Germany) was used for apoptotic cell detection [12]. The routine histological method with hematoxyline-eosine staining was used. The expression levels were detected semi-quantitatively by counting the number of positive structures in the visual field ("0/-" – occasional, "+" – few, "++" – moderate, "+++" – numerous, "++++" – abundant positive structures in the visual field) [11]. Counting of positive cells per 4 visual fields and calculation of mean value and standard deviation performed semi-quantative analysis of the slides. Then semi-quantitative results were digitized as "0" – 0; "0/+" – 0.5; "+" – 1; "+/++" – 1.5; "++" – 2; "++/+++" – 2.5; "+++" – 3; "+++/++++" – 3.5; and

Table 3. Relationship between signaling molecules and age (results of the regression analysis)

	Regression coefficient	Age p value	Model p value
OPG	-0.063	0.001	0.0007
MMP-8	-0.227	NS	NS
MMP-9	-0.019	NS	NS
NKpB105	-0.052	0.014	0.0135
TUNEL	-0.057	0.044	0.0437
AI%	-1.63	NS	NS

Table 4. The correlation between age expression of signaling molecules (Spearman's rank correlation coefficient (rho))

	rho	p value
OPG	-0.753	0.0005
MMP-8	-0.3384	NS
MMP-9	-0.1872	NS
NKpB105	-0.5927	0.0122
TUNEL	-0.5765	0.0154
AI%	-0.4475	NS

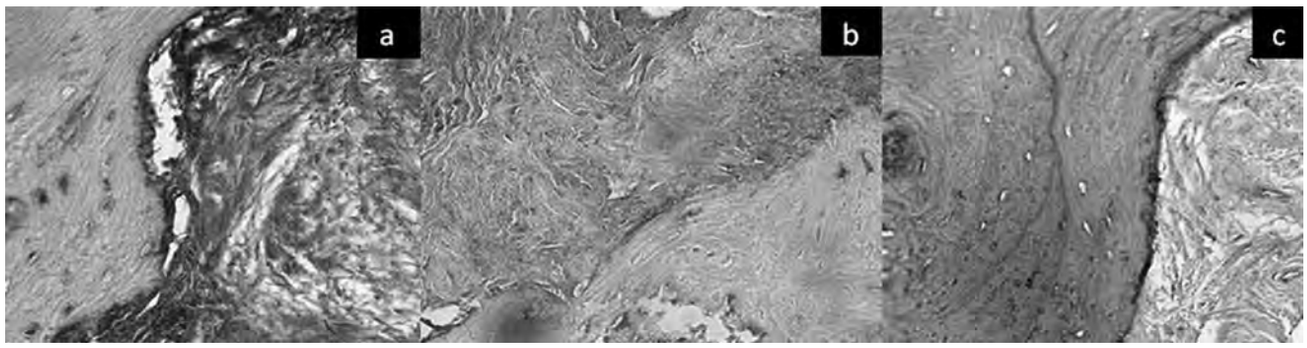

Fig. 5. Microphotograph of NKpbB105 expression in bone from the interradicular septum of a 12 (a), 17 (b) and 29-year-old (c) patients, IMH, ×400

"++++" – 4. The study protocol for work with human materials was approved by the Ethics Committee of Riga Stradins University (2010).

Statistical analysis

Data were analyzed by using descriptive and analytical statistical methods. The mean values and standard deviations (SD) were calculated for all signalling molecules in each group. The statistical significance of the differences among the mean values of the different groups of age was tested by means of one-way ANOVA with the Bonferroni correction. The correlation between age and signalling molecules expression was assessed by using Spearman's correlation coefficient. The association between signalling molecules and age was also assessed by means of ANOVA analysis, where age was included in the model as a continuous variable. A level of significance of 5% was chosen (i.e., p value 0.05).

RESULTS

There were included following groups: 1st group (12-14 years old) – four patients, mean age 12.5 years; 2nd group (15-22 years old) – five patients, mean age 17.2 years; 3rd group (23 years old or older) – eight patients, mean age 29.6 years. No statistically significant difference in patient gender was found. Mean values of expression levels of osteoprotegerin, matrix metalloproteinases 8 and 9, NKpB 105, osteocalcin and mean TUNEL staining are shown in Table 2 and are they were grouped according to patients` age. The following results for the interradicular septum were found: Osteocalcin (see Fig. 1) was found in all groups, and its expression in bone structures was high. Osteoprotegerin (see Fig. 2) was highly expressed in the 1st group, but its expression in the 2nd group was lower than in the 1st group. Significantly lower level of expression was found in the 3rd group. Similar results were found for matrix metalloproteinase-8 (see Fig. 3) that was highly expressed in 1st and 2nd groups and lower in the 3rd group, but matrix metalloproteinase-9's (see Fig. 4) expression in the 2nd group was higher than in the 1st and 3rd groups: the mean expression level was slightly higher in the 1st group than in the 3rd group. Nuclear factor kappa B (NKpB 105) (see Fig. 5) expression in the 1st and 2nd groups was higher than in the 3rd group. The level of apoptosis In the 1st group was higher than in the 2nd group: the level of apoptosis in the 3rd group was significantly lower than in the 1st group (see Table 2). There was statistically significant relationship between age and OPG expression, NKpB expression, and TUNEL staining. The levels of OPG and NKpB 105 expression and level of TUNEL staining decreased with age (see Table 3). Spearman's correlation coefficient also revealed a moderate correlation between age and mean OPG and NKpB 105 expression and TUNEL staining (see Table 4).

DISCUSSION

The role of aging in orthodontics has been investigated from the aspect of tooth movement. Measurements on plaster models and radiographs were done, when a fixed appliance is already worn, but there are few studies that have investigated age-dependant bone remodelling potential using immunohistochemistry. Many studies have been done by using animals. Simonet *et al.* [13] found over-expression of OPG in transgenic mice results in osteopetrosis, and conversely, Mizuno *et al.* [14] found OPG-deficient mice to exhibit severe osteoporosis. However, these studies were done on animals and therefore results cannot be fully extended to humans.

These results are preliminary results of continuing wider study. The main limitation of this preliminary analysis too small sample size, which encounters a higher possibility of exceeding the limit of usually accepted Type II error. However, despite to this limitation, the findings of the study showed statistically significant relationship between age and half of the signalling molecules. Further analysis of the final data probably will provide even more sound proves to the relationship between all studied signalling molecules and age.

Zhang *et al.* [4] stated that, in the period from childhood to adulthood, the bone formation activity is higher than bone resorption activity, which results in a net increase in bone mass. At advanced age, or during particular periods of life, the balance tips toward bone resorption, resulting in a net loss of bone mass. Our study have similar results. OPG and NKpB 105 expression levels were higher in younger patients. This result could be explained by the higher metabolic activity in bone structures and by the occurrence of growth during the pubertal and juvenile periods. With age, all processes become slower, and high levels of signalling molecule expression are no longer needed.

From the biological aspect of aging, this explanation sounds quite logical, but it makes orthodontic treatment more complicated, because as orthodontists we try to stimulate new bone formation during teeth movement, but the response is not equal in patients from different groups of age.

The results showed that matrix- degrading enzymes are also expressed at lower levels in older patients. Ingmann *et al.* [8] measured MMP-8 expression in patients with orthodontic appliances and found that expression increased with bone turnover. However, in their study, patients were not divided into different groups of age. Thus, Ingmann *et al.*'s results revealed only the expression level during mechanical stimulus without separate results for different age groups.

Apoptosis of bone cells was higher in younger patients than in older patients. Similar results were found by Jankovska et al in her study with orthognatic patients. This result could be explained by increased bone growth and the need to reorganize bone structure in younger patients. In older patient samples, the percentage of apoptotic cells was lower, because of bone maturation and less osteocyte activity. In younger patients, apoptosis is needed for new bone formation when growth remodelling occurs.

It remains largely unknown, what kind of molecules can predict possible tooth movement speed when orthodontic force is applied and what molecules are age-specific and are not relevant for the application of orthodontic force.

Research and analysis of signalling molecules that are found in alveolar bone could help to identify the specific substances that depend on human age. Assessment of specific factor expression would provide more knowledge about different patient responses on orthodontic treatment.

CONCLUSION

Interradicular septum specific osteoblasts and osteoclasts factors include osteoprotegerin, osteocalcin, matrix metalloproteinases 8 and 9, and nuclear factor kappa B. The mean expression level of these proteins and the mean TUNEL staining level decreased with age, and these decreases were statistically significant. In younger patients, signal molecule expression is higher because of increased bone metabolic activity. With aging, the bone remodelling process becomes less active, and in adults signalling molecule expression is decreased. This is preliminary study and larger study group is necessary for more precise statistical analysis in future.

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