

# Expression of $\alpha$ -smooth muscle actin in the periodontal ligament during post-emergent tooth eruption

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## Abstract

**Objective:** This study was performed to explore the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the periodontal ligament (PDL) of young and adult rats during post-emergent tooth eruption in opposed and unopposed teeth at two time points: 3 and 15 days after antagonist loss.

**Methods:** Four-week-old ( $n = 20$ ) and 22-week-old ( $n = 20$ ) male Wistar rats were used. The right maxillary molar crowns were cut down. PDL samples were isolated from the first mandibular molars at two time points: 3 and 15 days after cut-down of the right maxillary molars. Quantitative reverse-transcription polymerase chain reaction and immunohistochemical staining were performed to detect differences in  $\alpha$ -SMA expression in the PDL tissues of unopposed versus opposed molars.

**Results:**  $\alpha$ -SMA was upregulated in the PDL of the unopposed molars in the 3-day group of young rats. The region around the root apex of the unopposed molars in this group exhibited strong immunostaining for  $\alpha$ -SMA. The expression level and immunoreactivity of  $\alpha$ -SMA did not differ in both time points in young controls and among all the adult groups.

**Conclusion:**  $\alpha$ -SMA-positive myofibroblasts are implicated in post-emergent tooth eruption of unopposed molars of young animals.

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## Keywords

Tooth eruption,  $\alpha$ -smooth muscle actin, unopposed tooth, periodontal ligament, myofibroblast, immunostaining, rats

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## Introduction

Tooth eruption is divided in two discernible phases: the pre-emergent phase, when the tooth is still within the alveolar bone, and the post-emergent phase, which starts when the tooth penetrates the gingiva. During the post-emergent phase, teeth exhibit various degrees of overeruption after loss of their antagonists. Bone remodeling and periodontal ligament (PDL) rearrangement are among the biological mechanisms that control this phase of eruption.<sup>1</sup>

Several studies have shown that overeruption occurs after loss of antagonist teeth.<sup>2-4</sup> However, in a cross-sectional study based on the records of 53 adults who had molars without antagonists for  $\geq 10$  years, only 24% of the documented unopposed molars showed moderate to severe overeruption ( $\geq 2$  mm).<sup>2</sup> In a longitudinal study of adult patients with unopposed maxillary molars who were examined for  $>10$  years, both opposed and unopposed molars showed vertical displacement throughout the 10-year period.<sup>3</sup> The displacement was greater in the unopposed teeth, although the difference in the average amount of overeruption was not clinically pronounced. In contrast, another study of children and adolescents who had undergone extractions of upper second molars during orthodontic treatment, the unopposed lower second permanent molars showed clinically significant overeruption 10 years later.<sup>4</sup> Thus, it seems that overeruption of unopposed teeth takes place to a

greater degree during the growth period than in adulthood.

Experimental animal models using molars without antagonists have confirmed these clinical observations. These models were based on elimination of the occlusal contacts, either by extracting<sup>5</sup> or grinding<sup>6</sup> the antagonist tooth, and showed that teeth without antagonists overerupt more than opposed molars. This was more obvious in younger rats, as shown in humans.<sup>7</sup>

Upon occlusion, mechanical stimulation is distributed to the PDL through the teeth and then throughout the alveolar bone. Lack of occlusal forces is known to influence the structure and remodeling of the PDL. Loss of normal occlusal function due to loss of teeth leads to atrophic changes in the PDL,<sup>6</sup> such as narrowing of the periodontal space,<sup>8</sup> vascular constriction,<sup>9</sup> and deformation of the mechanoreceptor structure of the PDL.<sup>10</sup>

Myofibroblasts have been identified in the PDL.<sup>11</sup> These cells are mechanically active and may alter the microenvironment of the extracellular matrix and the overall mechanics of the PDL. Myofibroblasts are responsible for wound healing following injury and for development of fibrosis.<sup>12</sup> Alpha-smooth muscle actin ( $\alpha$ -SMA) is expressed during the phenotypic transition of fibroblasts into myofibroblasts and plays a role in the contractile activity of myofibroblasts. Therefore, the expression of  $\alpha$ -SMA distinguishes myofibroblasts from fibroblasts. In mature tissues,  $\alpha$ -SMA is localized to pericytes of blood vessels and

myoepithelial cells of salivary glands,<sup>13</sup> which are required to generate a force capacity that supports contractile function.

During the pre-emergent phase of eruption,  $\alpha$ -SMA-positive cells are localized to the alveolar bone crypt next to the dental follicle cells after the bell stage of tooth formation, suggesting that these cells differentiate into osteoblasts and participate to alveolar bone formation. During the post-emergent phase and throughout root formation,  $\alpha$ -SMA-positive cells are observed in the PDL, mainly in the apical part of the root and around Hertwig's epithelial root sheath.<sup>14</sup>  $\alpha$ -SMA-positive cells are also present in the PDL during orthodontic tooth movement, indicating that enhanced mechanical stress promotes myofibroblast differentiation.<sup>15</sup>

Upon occlusal force alteration, the over-eruption of unopposed teeth differs between young and adult rats because of a lack of antagonists, with less overeruption occurring among adults. We hypothesized that (a) the cellular and molecular basis of post-emergent tooth eruption is based on the contractile ability of the myofibroblasts, (b) myofibroblasts are more numerous in the PDL of young than adult rats, (c) many more myofibroblasts are present in the PDL of molars without antagonists than in the PDL of opposed molars, and (d) the presence of myofibroblasts decreases with time after antagonist loss.

Therefore, the aim of this study was to compare the expression of  $\alpha$ -SMA in the PDL during post-emergent tooth eruption in opposed and unopposed teeth of young and adult rats at two time points.

## Materials and methods

The experimental protocol was approved by the General Direction of Health, Domain of Animal Experiments, Canton of Geneva, Switzerland.

Forty male Wistar rats were used in this study; 20 were young (4 weeks old) and 20 were adult (22 weeks old). During the experimental period, the animals were fed a soft diet and water ad libitum. The day/night rhythm was ensured by automatic dimmed lighting (08:00 to 20:00). Body weight was measured every 2 days as an indicator of the general physical condition of the rats.

## Interventions

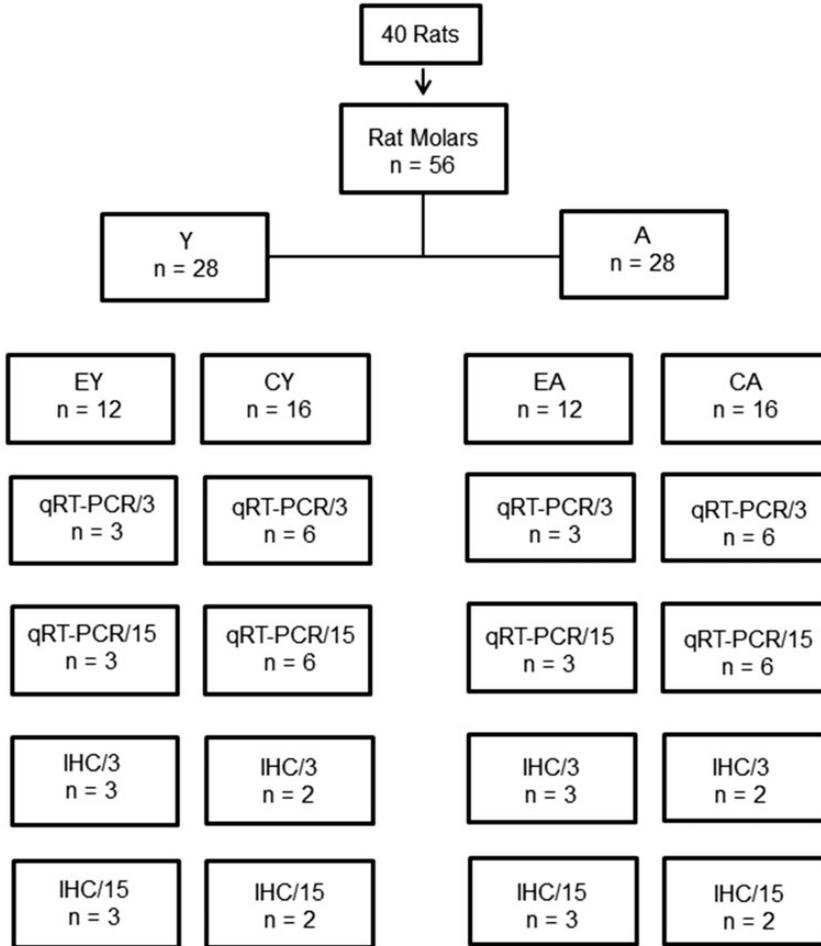
The right maxillary molar crowns of 12 young and 12 adult rats were cut under anesthesia after intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The right unopposed mandibular molars were the experimental teeth and were considered to be hypofunctional. Sixteen age-matched rats were used as controls. In total, 56 mandibular molars were examined. The left and right mandibular molars of the control animals were studied separately, and the mean of the two sides was used for the statistical analysis.

The total experimental period was 15 days. During this period, 10 young rats (6 experimental and 4 control) and 10 adult rats (6 experimental and 4 control) were killed 3 days after the cutting of the right maxillary molars. The remaining 20 young rats (6 experimental and 4 control) and adult rats (6 experimental and 4 control) were killed 15 days later (Figure 1).

The three factors tested in this study were:

- a. The presence of unopposed molars versus controls
- b. The age of the animals (4 vs. 22 weeks)
- c. Time period without antagonists (3 vs. 15 days)

After the experimental period, the mandibular molars that were obtained were divided into the following eight groups: Experimental Young 3 days (EY3),



**Figure 1.** Experimental design. Fifty-six rat molars extracted from 40 rats were divided into 2 main groups [young (Y), n = 28 and adult (A), n = 28] based on the age of the animals (4 vs. 22 weeks). Each group was further divided in two subgroups depending on the presence of unopposed molars [experimental (E) and control (C)]. In the control subgroups of each age group, the right and left molars were considered as one pool of teeth with antagonists. The experimental periods were 3 days (3) and 15 days (15). The right maxillary molar crowns of 24 animals (12 Y and 12 A) were cut down, leading to unopposed molars on the right side (EY and EA, respectively). In the remaining 16 animals (8 Y and 8 A), the mandibular molars were opposed by their antagonists (CY and CA, respectively). Periodontal ligament samples were used for gene expression analysis and quantitative reverse-transcription polymerase chain reaction, in which three samples for each experimental group (EY and EA) and six samples for each control group (CY and CA) were used at each time point. Immunohistochemistry (IHC) was performed using three samples for each experimental group (EY and EA) and two samples for each control group (CY and CA) at each time point.

Control Young 3 days (CY3), Experimental Young 15 days (EY15), Control Young 15 days (CY15), Experimental Adult 3 days (EA3), Control Adult 3 days (CA3),

Experimental Adult 15 days (EA15), and Control Adult 15 days (CA15).

The first mandibular molars were extracted from 24 rats (12 experimental

and 12 controls), the PDL was carefully scraped from each root of the molar with a scalpel, and RNA extraction was performed. The mandibles of 16 rats (12 right semi-mandibles from experimental animals and both semi-mandibles from 4 control animals) were processed for fixation, decalcification, and immunohistochemical staining (Figure 1).

### RNA isolation

The PDL tissue was isolated and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA was eluted in 30  $\mu$ l of RNase-free sterile water (provided with the kit).

### Quantitative reverse-transcription polymerase chain reaction

Complementary DNA (cDNA) was synthesized from 10 ng of total RNA. SYBR Green assays were designed using the program Primer Express v 2.0 (Applied Biosystems, Foster City, CA, USA) with default parameters. Amplicon sequences were aligned against the *Rattus norvegicus* genome by BLAST to ensure that they were specific for the gene being tested. Oligonucleotides were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The primer sequences for  $\alpha$ -SMA

and control genes are shown in Table 1. The pre-amplification step was performed with TaqMan<sup>®</sup> PreAmp Master Mix (Applied Biosystems) using 6.5  $\mu$ l of cDNA.

Quantitative reverse-transcription polymerase chain reaction (PCR) was performed on an SDS 7900 HT instrument (Applied Biosystems) with the following parameters: 50°C for 2 min, 95°C for 10 min, and 45 cycles at 95°C for 15 s and 60°C for 1 min. Each reaction was performed in triplicate on a 384-well plate. Raw Ct values obtained with SDS 2.2 (Applied Biosystems) were imported into Excel, and the normalization factor and fold changes were calculated using the geNorm method.<sup>16</sup>

### Immunohistochemistry

Immunohistological staining was performed to detect the *in situ* protein expression  $\alpha$ -SMA and von Willebrand factor.

Eight young and eight adult mandibles were fixed in 4% paraformaldehyde (8.18715; Merck, Darmstadt, Germany) for 2 days, decalcified with a solution of 15% EDTA (pH 7.4, Fluka N° 03700; Sigma-Aldrich, St. Louis, MO, USA) and 0.5% paraformaldehyde for 12 weeks, embedded in paraffin, and sectioned at a thickness of 3  $\mu$ m.

The sections were either stained with  $\alpha$ -SMA mouse monoclonal antibody (clone 1A4, dilution 1:200<sup>13</sup>) or with von

**Table 1.** Primer sequences

$\alpha$ -SMA	Rat_Acta2_ex6-7-134F Rat_Acta2_ex6-7-189R	TGAGCGTGGCTATTCCTTCG TGATGTCACGGACGATCTCAC
Efla1	Rat_EF1A1_ex 4-5 F Rat_EF1A1_ex 4-5 R	CGCCAACTCGTCCAACCTGA CCAATGCCGCCAATTTTATAGA
Rps29	Rat_RPS29_ex 2-3 F Rat_RPS29_ex 2-3 R	GCTGAACATGTGCCGACAGT GGTCGCTTAGTCCAACCTAATGAAG
Tbp	Rat_TBP_ex1-2-150F Rat_TBP_ex1-2-221R	TGCCACACCAGCCTCTGA CCAAGATTCACGGTGGATACAA
Actin beta	Rat_ActinB_ex1-2-166F Rat_ActinB_ex1-2-237R	CGTGAAAAGATGACCCAGATCA CACAGCCTGGATGGCTACGTA

Willebrand factor rabbit polyclonal antibody (dilution 1:200; Sigma-Aldrich). All sections were incubated overnight with primary antibodies, which had been diluted in EnVision Flex Antibody diluent (Dako, Glostrup, Denmark) to achieve optimal staining. After incubation, EnVision+ Flex/HRP for mouse and rabbit (K8024; Dako) was applied for 30 min. EnVision Flex DAB (Dako) was used for color development according to the manufacturer's instructions. The sections were counterstained with hematoxylin according to Harris (No. 1092532500; Merck). Control sections were treated in the same manner but without primary antibodies. The specificity of the  $\alpha$ -SMA mouse monoclonal antibody against the acetylated NH<sub>2</sub>-terminal decapeptide of  $\alpha$ -SMA was previously tested by means of immunoblotting, enzyme-linked immunosorbent assay, immunohistochemistry, and immunofluorescence in various physiological and pathological situations.<sup>13,17-19</sup>

### Statistical analysis

The dependence between the responses and the independent variable was tested by three-way analysis of variance (ANOVA) (sum of type III), including third-level interactions. Multiple comparisons were made using Tukey's honestly significant difference methodology. The analyses were performed using Minitab 17 (Minitab Inc., State College, PA, USA).

Residual analysis of the model revealed a lack of homogeneity and normality (probability plots of the standardized residuals). To overcome this, the logarithm of  $\alpha$ -SMA was analyzed instead. The analysis of the standardized residuals showed no departure from the ANOVA validity conditions: the p-value of Levene's test for variance homogeneity was 0.998 (0.16), and the p-value of the Anderson-Darling test was 0.448 (0.345). We thus proceeded with the

logarithmic observations. Notably, the estimates of the differences are the differences in the logarithms and should be interpreted with some caution.

### Results

With respect to the health status of the 40 rats studied, no significant differences in weight gain were observed among the groups. Thus, the cutting of the right maxillary molars probably did not influence the animals' growth.

### qRT-PCR

Using qRT-PCR, we detected the expression of  $\alpha$ -SMA in periodontal tissue specimens obtained from the first mandibular molars. The expression level of  $\alpha$ -SMA in unopposed and control molars was determined relative to the internal control genes. Comparisons of the fold increase per group are shown in Table 2.

No difference in the expression of  $\alpha$ -SMA was found between the CY3 and CY15 groups or between the CA3 and CA15 groups.

The  $\alpha$ -SMA mRNA level was sharply upregulated in the PDL of the unopposed molars in the EY3 group compared with the EA3 group ( $p=0.008$ ). No other comparison among the eight groups showed a significant difference (Table 2).

### Immunohistochemistry

To distinguish positive immunohistochemical staining reactions for  $\alpha$ -SMA in the smooth muscle cells of vessels from myofibroblasts throughout the PDL, adjacent serial sections were stained for either  $\alpha$ -SMA or von Willebrand factor, a marker of endothelial cells. Vessels were identified by positive staining for both  $\alpha$ -SMA and von Willebrand factor and were excluded from the evaluation.

**Table 2.** Multiple-comparisons table of  $\alpha$ -SMA

Comparison	Estimate	SE	95% CI		p-value
			Lower bound	Upper bound	
EY3 – CY3	2.205	0.689	-0.084	4.494	0.065*
EY15 – CY15	-0.541	0.533	-2.314	1.232	0.967
EA3 – CA3	-0.582	0.551	-2.413	1.249	0.960
EA15 – CA15	-0.055	0.533	-1.828	1.718	1.000
EY3 – EY15	1.869	0.616	-0.178	3.916	0.091*
CY3 – CY15	-0.877	0.616	-2.925	1.170	0.837
EA3 – EA15	-0.428	0.616	-2.475	1.620	0.996
CA3 – CA15	0.099	0.457	-1.420	1.617	1.000
EY3 – EA3	2.544	0.616	0.497	4.591	0.008***
CY3 – CA3	-0.242	0.631	-2.340	1.855	1.000
EY15 – EA15	0.247	0.616	-1.800	2.295	1.000
CY15 – CA15	0.734	0.436	-0.714	2.181	0.697

\*\*\* $P < 0.01$ , \* $P < 0.10$

$\alpha$ -SMA, alpha-smooth muscle actin; CI, confidence interval; SE, standard error; EY3, Experimental Young 3 Days; CY3, Control Young 3 Days; EY15, Experimental Young 15 Days; CY15, Control Young 15 Days; EA3, Experimental Adult 3 Days; CA3, Control Adult 3 Days; EA15, Experimental Adult 15 Days; CA15, Control Adult 15 Days.

Multiple comparisons were made by Tukey's honestly significant difference test and calculation of confidence intervals. Expression of  $\alpha$ -SMA in the periodontal ligament of the first mandibular molars was evaluated. The expression level of  $\alpha$ -SMA in unopposed and control molars was determined relative to the internal control genes using quantitative reverse-transcription polymerase chain reaction.

PDL sections were observed and evaluated in the whole length of the root. The PDL along the roots of the first mandibular molars was imaged with a microscope (Axio Scan.Z1; Zeiss, Oberkochen, Germany) at 10 $\times$  and 40 $\times$  magnification in 8-bit TIFF images.

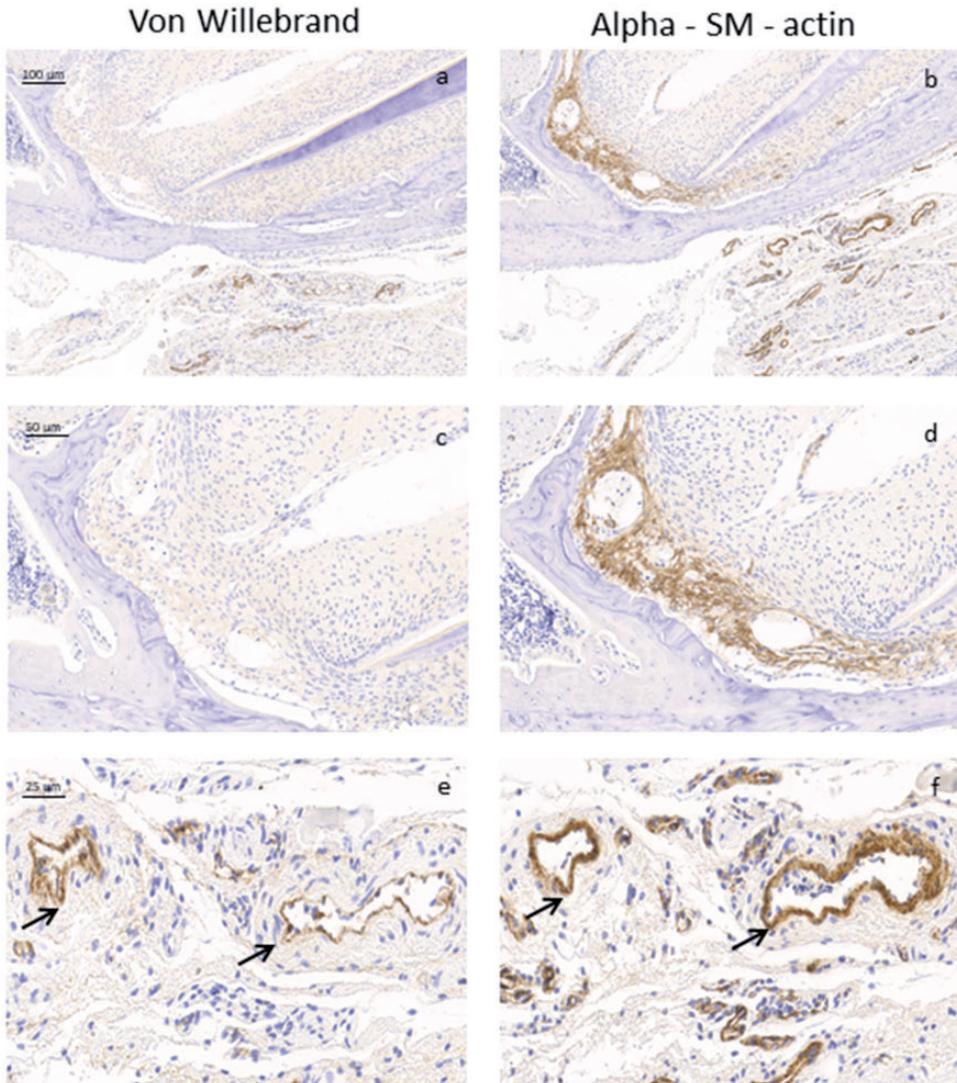
The smooth muscle and endothelium of blood vessels in the PDL in the whole length of the root expressed  $\alpha$ -SMA and von Willebrand factor, respectively. Both stainings were detected in close vicinity (Figure 2). The root apex of the unopposed molars in the EY3 group exhibited intense immunostaining mainly for  $\alpha$ -SMA, especially around Hertwig's epithelial root sheath (Figure 3). This immunoreactivity was detected within the cytoplasm of PDL cells possessing long cell processes.  $\alpha$ -SMA-positive cells were confined to the apical region in the remaining groups of young animals, but to a lesser extent.

Immunohistochemical localization of  $\alpha$ -SMA was weaker in the PDL of adult animals and was mainly detected at the apical region. In the cervical region of the PDL,  $\alpha$ -SMA was detected only in smooth muscle cells of blood vessels; it was not detected in the surrounding tissue. In the pulp tissue, the smooth muscle cells of the blood vessels were also positive for  $\alpha$ -SMA (Figure 3).

## Discussion

In the present study, we used a rodent model of teeth without antagonists, where no significant loading was transferred from the tooth to the PDL.

In all groups, the  $\alpha$ -SMA-positive region was mainly limited to blood vessels and to the apical root area of the first mandibular molar. However, scarce cells that were

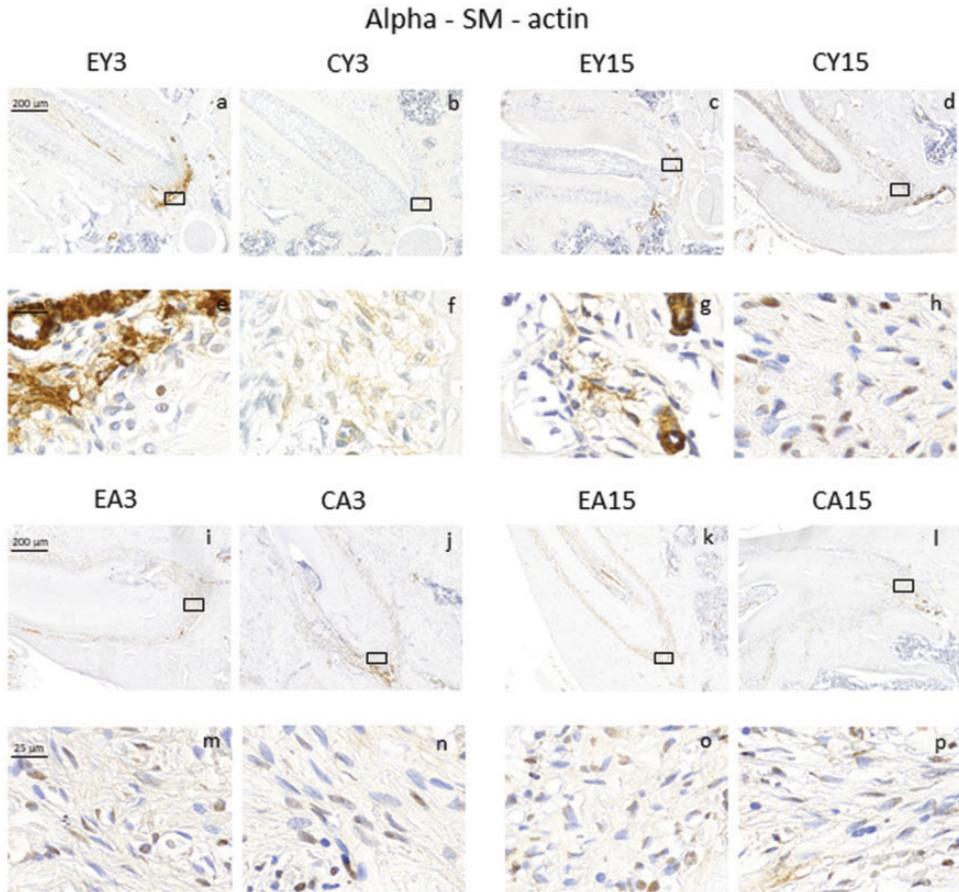


**Figure 2.** Immunohistochemical staining for (a, c, e) von Willebrand factor and (b, d, f) alpha smooth muscle actin ( $\alpha$ -SMA) on serial sections of the periodontal ligament of the first mandibular molar. (c–f) are higher magnifications of (a, b). In the apex region of the first mandibular molar, myofibroblasts expressed only  $\alpha$ -SMA, whereas blood vessels are labelled with both von Willebrand factor and  $\alpha$ -SMA antibodies. The black arrows indicate blood vessels.

immunopositive for  $\alpha$ -SMA were detected throughout the PDL.

Analysis of adjacent serial sections with von Willebrand factor and  $\alpha$ -SMA staining showed that in the apical region, the

immunohistochemically positive cells were mainly myofibroblasts and were unrelated to blood vessels. In the young animals, the number of  $\alpha$ -SMA-positive cells was elevated and concentrated in the apical region in



**Figure 3.** Immunohistochemical staining for alpha-smooth muscle actin in the periodontal ligament of the first mandibular molar. Images a–d and i–l are magnified 10 $\times$ , and images e–h and m–p are magnified 40 $\times$ . The black squares indicate the area viewed at higher magnification (40 $\times$ ).

EY3, Experimental Young 3 Days; CY3, Control Young 3 Days; EY15, Experimental Young 15 Days; CY15, Control Young 15 Days; EA3, Experimental Adult 3 Days; CA3, Control Adult 3 Days; EA15, Experimental Adult 15 Days; CA15, Control Adult 15 Days.

the 3-day experimental group; in the 15-day group, it decreased over time, showing less immunoreactivity with a similar localization pattern (concentrated in the apical root area). In adult animals, there was no difference in the expression level or immunoreactivity of  $\alpha$ -SMA. There was also no difference in the  $\alpha$ -SMA expression among the control groups of both age categories. In the young animals, these results may have been due to a time-dependent

variation in the intrinsic mechanical micro-environment within the PDL upon occlusal force alteration after loss of the antagonist teeth. In the adult animals, we suggest that due to aging of the PDL, the fibroblasts developed a lowered capacity to differentiate into myofibroblasts upon force stimulation.

Unopposed teeth can better express their eruptive potential because the occlusal forces exerted on them have been removed;

this is more obvious in younger animals.<sup>7</sup> This force alteration has an impact in the anatomy and metabolism of the PDL. Tooth loss reduces the number and diameter of Sharpey's fibers and the mineral density of the antagonist tooth.<sup>20</sup> The structure and metabolism of the collagen of the PDL is also affected by antagonist loss.<sup>21,22</sup> In unopposed teeth, the remodeling of the extracellular matrix of the PDL is altered.<sup>23</sup>

The PDL contains fibers and cells, mainly fibroblasts, which cannot produce force themselves. However, fibroblasts have the capacity to differentiate into myofibroblasts under mechanical stimulation. The main characteristic of myofibroblasts is their cytoskeleton, which is rich in  $\alpha$ -SMA, a mechanosensitive protein localized in the stress fibers of these cells.<sup>24</sup> The combined features of  $\alpha$ -SMA and the presence of stress fibers provide myofibroblasts with their contractile capacity, which they apply to the extracellular matrix via their cytoskeleton network. This process can contract the entire extracellular matrix and cell unit, provoking contraction of the whole PDL tissue.<sup>25,26</sup>

Moreover, the extracellular matrix of the PDL of a functioning tooth is adapted to withstand the occlusal forces.<sup>27</sup> The lack of occlusal forces changes the mechanical equilibrium in this previously balanced system, expressing tension forces.<sup>28</sup>

Expression of  $\alpha$ -SMA actin has also been detected during orthodontic tooth movement after mechanical stimulation of the PDL. In one study,  $\alpha$ -SMA expression was upregulated during the initial phase of tooth movement (1–3 days) and decreased during the lag phase (14–21 days) phase; tooth movement accelerated while  $\alpha$ -SMA expression increased and vice versa.<sup>15</sup> Under orthodontic forces, the expression of  $\alpha$ -SMA in myofibroblasts is upregulated on the tension side of the PDL and not on the compression side.<sup>24</sup> Under orthodontic tensile loading, myofibroblasts also

influence collagen synthesis and osteocalcin expression. Osteocalcin is an osteogenic marker of the later stages of osteoblast differentiation and bone formation, indicating an active role of myofibroblasts not only in remodeling of the PDL but also in remodeling of the neighboring alveolar bone.<sup>29</sup> Previous studies have shown that cells with a pericyte-myofibroblast phenotype have the potential to differentiate into functional osteoblasts and that osteoblasts can display contractile behavior through expression of  $\alpha$ -SMA.<sup>30,31</sup>

A possible explanation for the presence of myofibroblasts in the apical region of the tooth soon after antagonist removal could be that upon light mechanical loading of normal PDL tissue, the resistance of the collagen architecture protects embedded cells from stress-shielding. Mechanical loading coming from occlusal force alteration soon after tooth loss enhances  $\alpha$ -SMA expression in the PDL cells and induces their differentiation into myofibroblasts. Two weeks after tooth loss, the reconstructed extracellular matrix and collagen network architecture are adapted to the new mechanical environment; they take over the mechanical loading and protect the fibroblasts from stress, leading to downregulation of  $\alpha$ -SMA to normal levels.

Previous research has also shown that fibroblasts have an essential role in the reaction of the PDL due to mechanical force loading of the tooth by repairing and remodeling the affected matrix components of the PDL.<sup>32</sup> Therefore, expression of  $\alpha$ -SMA might play a role in the fibroblast contractile capacity and tooth eruption soon after loss of the antagonist tooth. After a few days, the eruptive movement ceases as a form of adaptation of the PDL in the new biomechanical environment.

However, based solely on the results of this study, we cannot say with certainty that the increased  $\alpha$ -SMA expression in the apex root area is the cause of the supra-osseous

tooth eruption or is a form of adaptation that develops during tissue formation to accommodate the vertical movement of the tooth and vascular adjustment as the apex is displaced toward the occlusal plane. Thus, the fact that we did not detect similar findings in the adult animals could be due to the minor vertical displacement measured in adult animals in previous studies.<sup>7</sup>

We kept the number of animals low for ethical reasons, and this might be why we did not detect a statistically significant difference in  $\alpha$ -SMA expression among all the studied groups. Due to lack of normality of the results, we proceeded with the analysis of the logarithm of  $\alpha$ -SMA expression; therefore, the differences should be interpreted with caution. Despite this compromise, we believe that the results of the qRT-PCR analysis are acceptable because they are in accordance with those of the immunohistochemistry analysis. Cells that were immunopositive for  $\alpha$ -SMA were detected in blood vessels and in scarce isolated cells throughout the PDL in all animal groups, and the expression was similar in all groups. However, in the young animals of the 3-day group, the number of  $\alpha$ -SMA-positive cells was significantly elevated and concentrated in the apical region in addition to the above-mentioned regions. Considering these observations, we conclude that the difference in  $\alpha$ -SMA expression as detected by qRT-PCR was associated with the myofibroblasts expressed in the root apex of the 3-day group of young animals.

Moreover, we detected no differences in  $\alpha$ -SMA expression in the adult animals, and this may be due to the short experimental period. The aging process may have caused the PDL of the adult animals to react more slowly in force equilibrium alterations.

In conclusion,  $\alpha$ -SMA-positive myofibroblasts were more numerous in the PDL of the unopposed molars in the 3-day group of young animals and were localized in the

root apex of the unopposed molars. The presence of myofibroblasts decreased with time after antagonist loss in the young animals. We suggest that  $\alpha$ -SMA-positive myofibroblasts in the PDL are implicated in the cellular and molecular process of post-emergent eruption of teeth without antagonists through their contractile activity; this process is age- and time-dependent.

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### Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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