# Proliferation and differentiation of periodontal ligament cells following short term tooth movement in the rat using different regimes of loading.

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meinen lieben Eltern

Giorgos und Tasula

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Zusammenfassung

ZIEL: Frühere Studien haben gezeigt, dass parodontale Ligamentzellen osteogenes Potential besitzen und die Fähigkeit zur osteoblastären Differenzierung durch mechanische Stimulation in vitro und in vivo über den ERK Signaltransduktionsweg. Ziel dieser Studie war es, diese regulatorischen Prozesse in einem tierexperimentellen Ansatz in der Ratte weiter zu untersuchen.

MATERIAL UND METHODE: Der rechte obere erste Molar von 25 anästhetisierten Ratten wurde mit einer Kraft belastet, um den Zahn mesial zu bewegen. Konstante Kräfte von 0.25 N und 0.5 N wurden über 4 Stunden an 5 Versuchstieren verwendet. Weiterhin wurden konstante Kräfte von 0.1 N über 2 Stunden in 10 weiteren Tieren angewandt und im Anschluß wurde der erste und zweite Molar permanent separiert mit Kunstoff. In diesen Tieren, wurde der Antagonist beschliffen und fünf Ratten aus der Gruppe wurden nach 1 Tag und fünf weitere nach 2 Tagen geopfert. Im letzten Versuchsansatz wurden intermittierende Kräfte von 0.1 N und 0.25 Hz in 5 verschiedenen Tieren über 4 Stunden appliziert. Die unbehandelte kontralaterale Seite wurde als Kontrolle verwendet. Paraffin eingebettete Schnitte wurden quantitativ immunhistochemisch auf die Faktoren PCNA, Runx2/Cbfa-1 und phosphorylierte ERK1/2 untersucht.

ERGEBNISSE UND DISKUSSION: In ausgewählten Abschnitten der Zugregionen war der Anteil von pERK1/2 positiven Zellen vergrößert verglichen mit den Abschnitten der Kontrollzähne in den verschiedenen Gruppen, dahingegen waren in ausgewählten Abschnitten der Druckzonen die Anteile nur dort vergrößert, in denen intermittierende Kräfte angewendet wurden. In representativen

Gebieten, in Druck und Zugzonen waren die Anteile Runx2 positiver Zellen vermindert nach Applikation konstanter Kräfte. Nach der Applikation konstanter Kräfte über 4 Stunden waren die Anteile PCNA positiver Zellen in der Zug und Druckzone geringer als in der Kontrollgruppe.

ZUSAMMENFASSUNG: pERK1/2, Runx2/Cbfa-1 and PCNA sind an der osteoblastären Differenzierung parodontaler Ligamentzellen unter verschiedener mechanischen Belastung beteiligt.

## Introduction

Periodontal ligament (PDL) cells are highly specialised cells that reside between tooth and alveolar bone and can differentiate into cementoblasts to synthesize alveolar tooth cementum or osteoblasts to synthesize alveolar bone for skeletal support of the tooth. In response to applied mechanical forces, osteoblast-like PDL cells perceive mechanical signals and respond to them via cellular events such as cell proliferation, differentiation, matrix synthesis and matrix degradation (Long et al., 2002; Davidovitch, 1991). These events, in turn, are controlled by sequential synthesis of neurotransmitters, cytokines, growth factors and arachidonic acid metabolites that regulate bone resorption at compression sites and bone synthesis at tensions sites (Krishnan and Davidovitch, 2006; Saito et al., 1991).

In vitro studies of cell cultures of different osteoblastic cell lines have demonstrated that the initial reaction of these cells to mechanical stress is partly mediated by deformation of the cytoskeleton (Sandy et al., 1993) via physical interaction of collagen type 1 and receptors of the integrin family (Calvalho et al., 1996). A key link between these membrane-bound receptors and changes in the pattern of gene expression has been shown to be the mitogen-activated protein (MAP) kinase pathways (Matsuda et al., 1998; Xiao et al., 2000).

Extra cellular signal-regulated kinases (ERKs), members of the MAPK family, have been shown to participate in diverse arrays of cell programs in a cell-type-specific mode. Mechanical stimuli have been shown to activate ERK1/2 in osteoblastic cells in vitro. In detail, the ERK1/2 signal pathway is involved in different cellular responses such as collagen synthesis (Chaudhary and Avioli, 2000), cyclo-oxygenase expression (Wadhwa et al., 2002) and osteopontin production (You et al., 2001). Ziros et al., (2002) have shown, that mechanical

stimuli led to increased expression of runt-related transcription factor 2 (Runx2/Cbfa1), a transcription factor that it is a pivotal regulator of osteoblast differentiation, via the ERK1/2 pathway. Runx2 binds to the osteoblast-specific cis-acting element 2 (OSE2), which is found in the promoter regions of many osteoblast-specific genes (i.e. osteocalcin, collagen type 1, bone sialoprotein, osteopontin, alkaline phosphatase and collagenase-3) and controls their expression (Ducy et al., 1997; Harada et al., 1999). Conceivably, Runx2 expression plays a key role during osteoblast differentiation and skeletogenesis (Karsenty et al., 1999). Kawarizadeh et al., (2005) have shown, that in the rat a short-term mechanical stimulus up-regulated Runx2 and that this regulation may be achieved via the ERK pathway.

The aim of this study was to expand previous in vitro and in vivo results by experiments applying various load regimes to the upper first molars of rats, and by quantitative analysis of immunohistochemical detection of ERK1/2 and Runx2 in regions representing the classic tension and pressure zones around the mesial root. In addition, we examined the relationship between cell differentiation and cell proliferation in the periodontal ligament during the early phase of tooth movement.

# Materials and methods

## Animals

Twenty-five 12 weeks old male Wistar rats, weighing 300-350 gr each (Harlan Winkelmann, Borchen, Germany) were used as experimental animals. They were provided with food and water ad libitum. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the local district government and the Animal Care Commissioner of the University of Bonn (Germany).

### Experimental Protocol

According to the experimental protocol of Kawarizadeh et al. (2005), the rats were anesthetized with 0.01 ml Rompun (Bayer, Leverkusen, Germany) and 0.24 ml Ketavet (Pharmacia & Upjohn, Erlangen, Germany). The animals were clamped onto a head-holding device and the occlusal surface of the maxillary right first molar was prepared by grinding of a small hole with a dental diamond bur. The tooth surface was then treated with self-etching bonding material (Xeno III, Dentsply DeTrey, Konstanz, Germany) for 60 seconds. An orthodontic appliance consisting of a T-loop (0.016 x 0.022-inch stainless steel wire, Ormco Corp., Glendora, CA, USA) was placed between the molar and a high-resolution 3D force/torque transducer (ATI, Industrial Automation, Garner, NC, USA), which had a resolution of 0.0125 N for force and 0.0625 Nmm for torque. The T-loop was fixed to the occlusal surface of the molar with light-curing composite (Tetric, Vivadent, Schaan, Liechtenstein). (Fig.1,2,3).

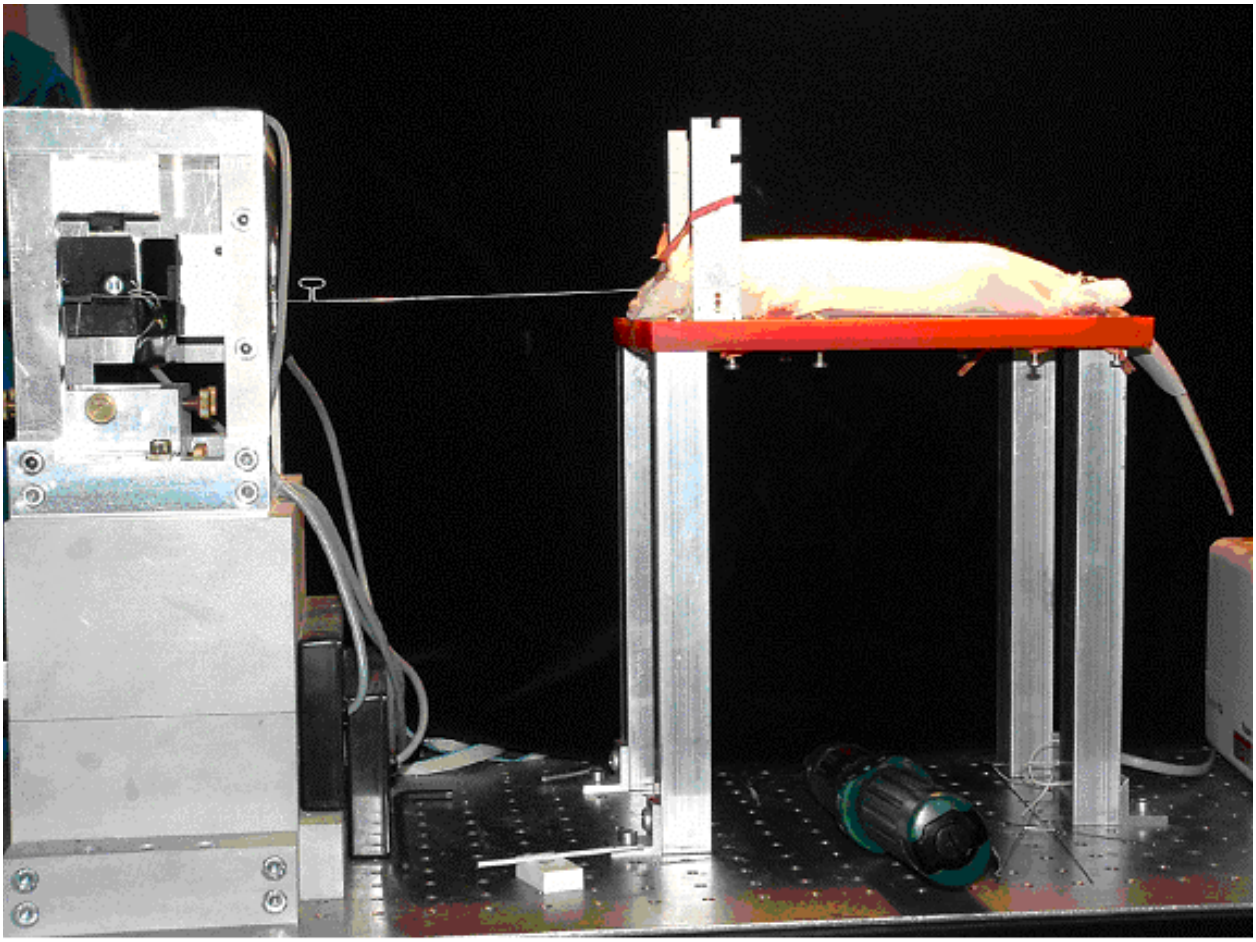

***Figure 1:*** *Experimental procedure for the application of the orthodontic force system. The rats were clamped onto a head holding device, in order to place an orthodontic appliance consisting of a T-Loop.*

***Figure 2****: Placement of the appliance in the oral cavity of the rat.*

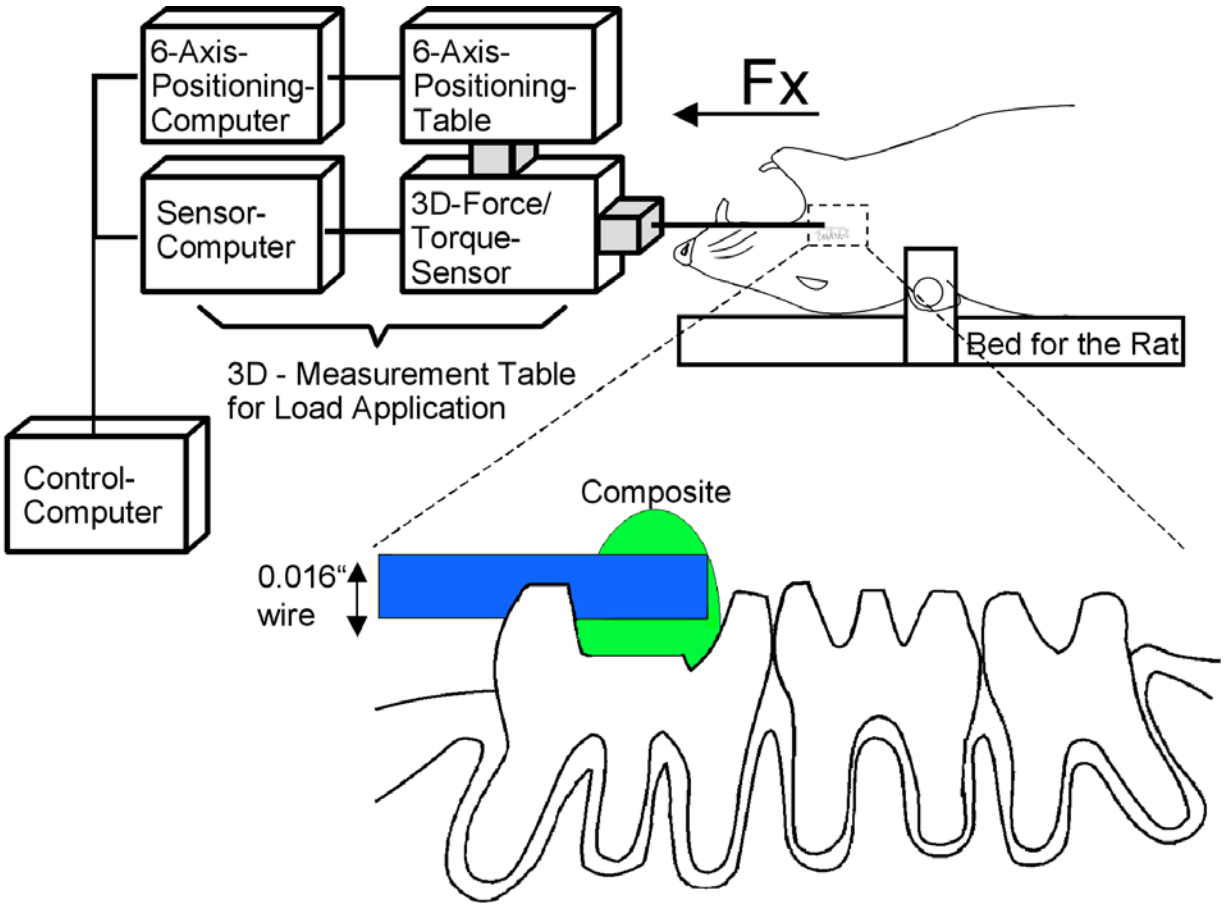


***Figure 3**: Experimental set up: Diagrammatic representation of the experimental procedure for the application of the orthodontic force system. The force system was applied and measured by a 3D force/torque transducer, and mounted onto a 6-axis-positioning table. By moving the transducer, we applied loads to the first molar.*

In a first group of animals, constant forces for 4 hours of 0.25 N and 0.5 N were applied in 5 animals each in order to move the molars mesially. In the second group, constant forces of 0.1 N were applied for 2 hours in 10 animals. Afterwards, the first and second molars were passively separated with composite and the occlusal contacts were eliminated by slicing the antagonistic lower molars. Five rats were killed after 1 day and five after 2 days. Finally, intermittent forces of 0.1 N and 0.25 Hz were applied for 4 hours in 5 animals. The applied forces were recorded continuously for the time of the experiment (Fig.4). The untreated contralateral molars in 5 rats served as controls.

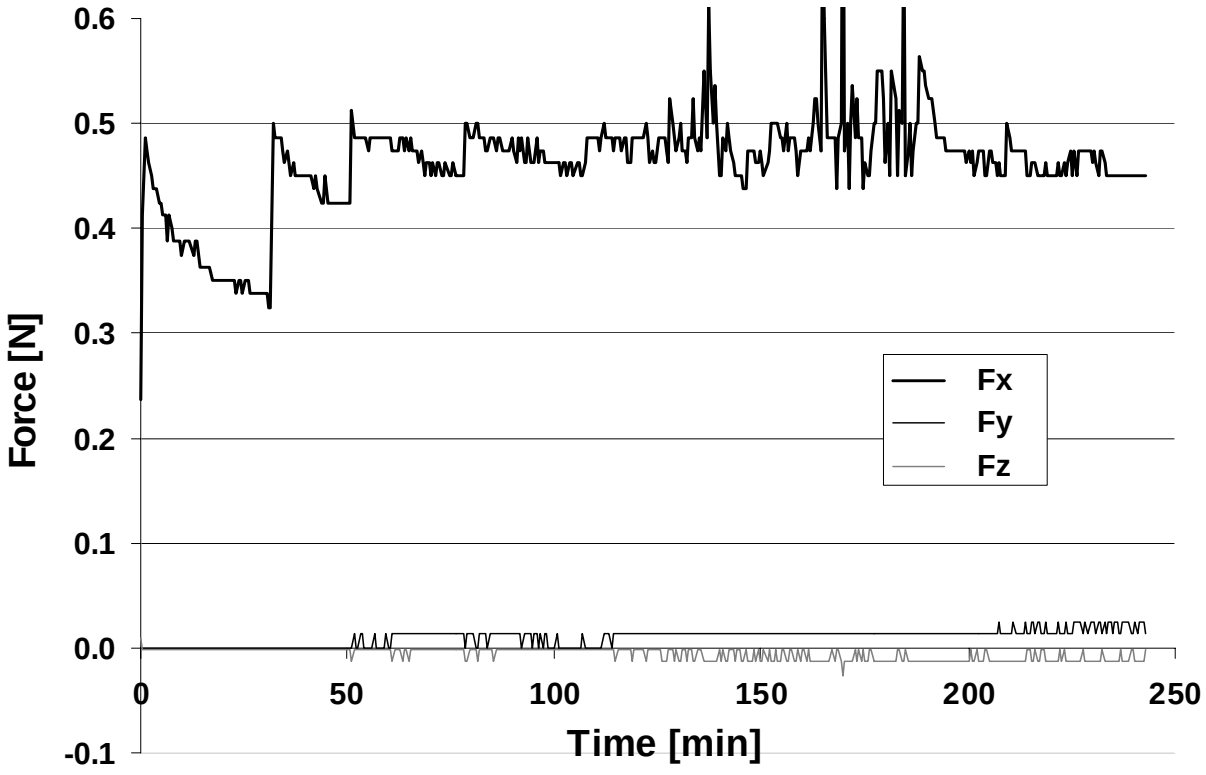


***Figure 4:*** *Presentation of a typical load-diagram: The measured force curves in the x-direction indicate a constant loading of the rat molar. The forces in the other directions were close to zero*

## Histology

Upon completion of the experiments, the anesthetized animals were killed by an intravenous application of 2 ml T61 (embutramid mebezonium iodide; Intervet, Unterschleissheim, Germany) and decapitated. The maxillae were removed and dissected into right and left halves. The soft tissues around the jaw bone, except for the gingiva, were removed. The specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) at room temperature for 6 weeks. After being dehydrated in ascending grades of alcohol, cleared in xylene, and paraffin-embedded, 5-µm serial sections were cut parasagittally on a microtome (HM 355 s; Microm Int., Walldorf, Germany) and mounted on glass slides (K. Roth, Karlsruhe, Germany). Selected sections were stained with haematoxylin-eosin.

## Immunohistochemistry

Immunohistochemical staining was carried out with anti-PCNA mouse monoclonal antibody (diluted at 1:500, ZYMED Laboratories, South San Francisco CA, USA), Runx2 goat polyclonal antibody (diluted at 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and pERK1/2 mouse monoclonal antibody (diluted at 1:50, US Biological, Swampscott, MA, USA). The sections were deparaffinized and rehydrated, rinsed with tris-hydroxymethyl aminomethane-buffered saline solution (TBS) at pH 7.4 for 10 minutes and then soaked in methanol/H2O2 for 20 minutes in the dark to block endogenous peroxidase activity.

In details the sections were treated with the following sequence:

| | |
|---|---|
| 1. xylol | 10 min. |
| 2. xylol | 10 min. |
| 3. 100% alcohol | 5 min. |
| 4. 100% alcohol | 5 min |
| 5. 90% alcohol | 5 min |
| 6. 70%alcohol | 5 min |
| 7. aqua dest | 5 min |
| 8. TBS | 10 min |
| 9. methanol/H2O2 | 20 min |

Anti-PCNA, Runx2 and pERK1/2 antibodies were used in TBS-BSA (4% bovine serum albumin) at 4°C overnight, diluted in a humidity chamber. Subsequently, sections were washed in TBS and incubated with suitable Envision+/HRP anti-mouse or anti-goat immune globulin/HRP (DakoCytomation, Hamburg, Germany) as secondary antibodies for 30 minutes in a humidity chamber at room temperature.

Antibody complexes were visualized using diaminobenzidine (DAB) for 10 minutes yielding a brown staining product. Thereafter, slides were rinsed, counterstained with Mayer's hematoxylin for 5 seconds, rinsed again, and mounted.

Again in details the mentioned sequence:

| | | |
|---|---|---|
| 10. primary antibodies: | | overnight |
| anti-PCNA mouse monoclonal antibody | 1:500 | |
| pERK1/2 mouse monoclonal antibody | 1:50 | |
| Runx2 goat polyclonal antibody | 1:50 | |
| 11. TBS | | 10 min. |
| 12. secondary antibodies | | 30 min |
| Envision+/HRP anti-mouse or | | |
| anti-goat immune globulin/HRP | | |
| 13. TBS | | 10 min |
| 14. Diaminobenzidine (DAB) | | 10 min |
| 15. TBS | | 10 min |

| | | |
|---|---|---|
| 16. | Mayer's hematoxylin | 1 sec |
| 17. | 100% alcohol | 2 min |
| 18. | 100% alcohol | 2 min |
| 19. | xylol | 2 min |
| 20. | xylol | 2 min |

Negative controls were prepared by omission of the primary antibodies from the staining procedures. The specifity of the used antibodies had been confirmed before by immunoblotting analysis (Ogata *et al.*, 1985) and by the manufacturer (US Biological, Swampscott, MA, USA), respectively.

## Morphometry

To establish representative regions of the periodontium and the adjacent alveolar bone, those sections were chosen that showed the third root of the right upper first molar in maximal length and with complete radicular pulp. From these, three sections at 30 µm intervals were taken for each quantitative analysis. Sections were scanned by means of a scanner camera (Axio Cam MRC; Zeiss, Göttingen, Germany) mounted on a light microscope (Axiophot 2; Zeiss, Göttingen, Germany), and viewed with imaging software (Axiovision; Zeiss, Göttingen, Germany) on a personal computer. Counting of the percentage of immuno-histochemically positive PDL cells was performed in two separate predefined areas of 750 x 375 µm each in every selected section. These areas were located mesio-coronally and disto-coronally of the mesial root. Counts were performed at a magnification of x 400. Means and standard deviations were calculated for each group of 5 animals and for the different regions. For statistical analysis, Student's t tests to determine differences between groups and with regard to the localization of counted positive cells were performed. The level of statistical significance was set at $p<0.05$. To evaluate the accuracy of the method, an intraobserver error of 3.9% (CV) was maximally calculated after the double-counting 30 randomly chosen sections.

## List of the chemicals

| | |
|---|---|
| Acetone | co.Otto Fischer,Saarbrücken,Germany |
| Alcohol (70-100%) | co.Merck,Münster,Germany |
| Embedding medium DePex | co.Serra,Heidelbrg,Germany |
| 3,3-diaminobenzidin(DAB) | co.Sigma,Steinheim,Germany |
| Ethylene diamine tetraacetic acid (EDTA) | co.Calbiochem,Darmstadt,germany |
| Eosin solution 1% | co.Merck,Darmstadt,Germany |
| Formic acid | co.Merck,Darmstadt,Germany |
| Formol 40% | co.Merck,Darmstadt,Germany |
| Hematoxylin | co.Merck,Darmstadt,Germany |
| Methanol | co.Merck,Darmstadt,Germany |
| Sodiumchloride | co.Merck,Darmstadt,Germany |
| Sodiumsulfate solution | co.Merck,Darmstadt,Germany |
| Paraffin-Histo-comp | co.Vogel,Giessen,Germany |
| Hydrochloride 2 mol/l | co.Merck,Darmstadt,Germany |
| Serumalbumin from bovine | co.Paesel and Lor,Frankfurt,Germany |
| Tris | co.ICN Biomedicals,Ohio,USA |
| Xylol original | co.Merck,Darmstadt,Germany |
| Xylol substitute XEM-200 | co.Vogel,Giessen,Germany |

# List of the reactors

**TBS-solution:**

-9.0 gr NaCl
-6.0 gr Tris
-1000 ml aqua dest. + 2 ml HCl  pH 7.6

**TBS-BSA-solution:**

-0.1 gr serumalbumin from bovine
-10 ml TBS-solution

**EDTA:**

-200 gr EDTA
-68 gr Tris
-2000 ml aqua dest

# List of the instruments

| | |
|---|---|
| Analysis scale | co.Sartorius,Göttingen,Germany |
| Thermo-cupboard | co.Memmert,Schwabach,Germany |
| Cover-glass | co.Engelbrecht,Edermunde,Germany |
| Digital-pH-Meter 197 | co.WTW,Vienna,Austria |
| Eppendorf-pipettes | co.Eppendorf,Hamburg,Germany |
| Light microscope Axioskop 2 | co.Zeiss,Jena,Germany |
| Practica Scan | co.Schneider,Feinwerktech.,Dresden |
| Rotation microtome HM 3559 | co.Microtom,Walldorf,Germany |
| Superfrost plus slides | co.Menzel,Braunschweig,Germany |
| Warm plate | co.Medax Nagel,Kiel,Germany |

## Results

### Histology

Orthodontic loading of the upper first molars resulted in a stretching of the periodontal fibers on the disto-coronal aspects of the mesial root and compression of the ligament on the mesio-coronal ones. Hence, zones of pressure and tension were formed (Ogata et al., 1985; Kawarizadeh et al., 2004) and could be clearly identified. In the pressure zone, obvious structural disturbance of PDL fibers was observed indicating initiation of hyalinization. Many fibroblasts showed pyknosis. In addition, vessel damage and extravasation was observed. In the areas under tension, the typical morphology with stretched PDL fibers was visible.
The morphological picture of the periodontal tissues was still characterized by the physiological distal drift of the molars in the rat. Apposition of bone on the mesial surfaces of the alveolar septa and of cementum on the distal root surfaces was seen. These surfaces were mostly covered by osteoblasts or cementoblasts, respectively.

### Immunohistichemistry and morphometry

#### Results for pERK1/2

In the selected areas under tension the proportion of pERK1/2 positive cells was higher than in control teeth in all types of loading, whereas these proportions in selected areas under pressure were increased after the application of the intermittent forces ($p<0.05$; Fig.5,6,7,8). In addition, we observed only in the tension zones a higher number of pERK1/2 positive cells within the same time interval after the application of higher constant forces (0.5 N) compared with the lower force (0.25 N) ($p<0.01$; Fig.5,7). There was no statistical difference concerning pERK1/2

positive cells between the tension and the pressure zones except for the third group, where constant forces for 2 hours of 0.1 N were applied and the animals were killed after 1 day ($p<0.05$).

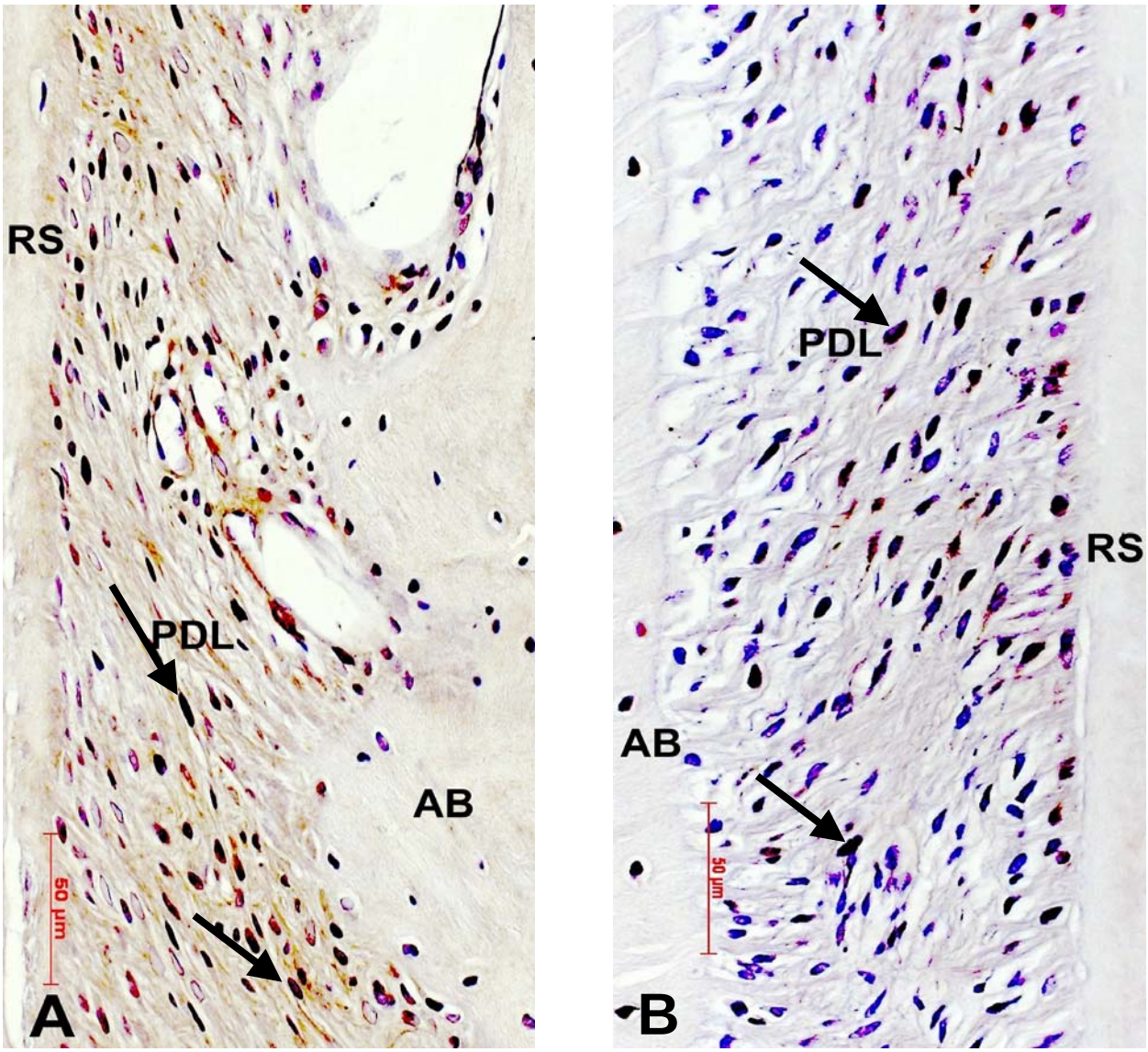


***Figure 5:*** *(A,B) Immunolocalization of pERK1/2 on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL after 4 hrs with application of a force of 0.5N. The immunostained cells appear brown (arrows). The labelled cells are regularly distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

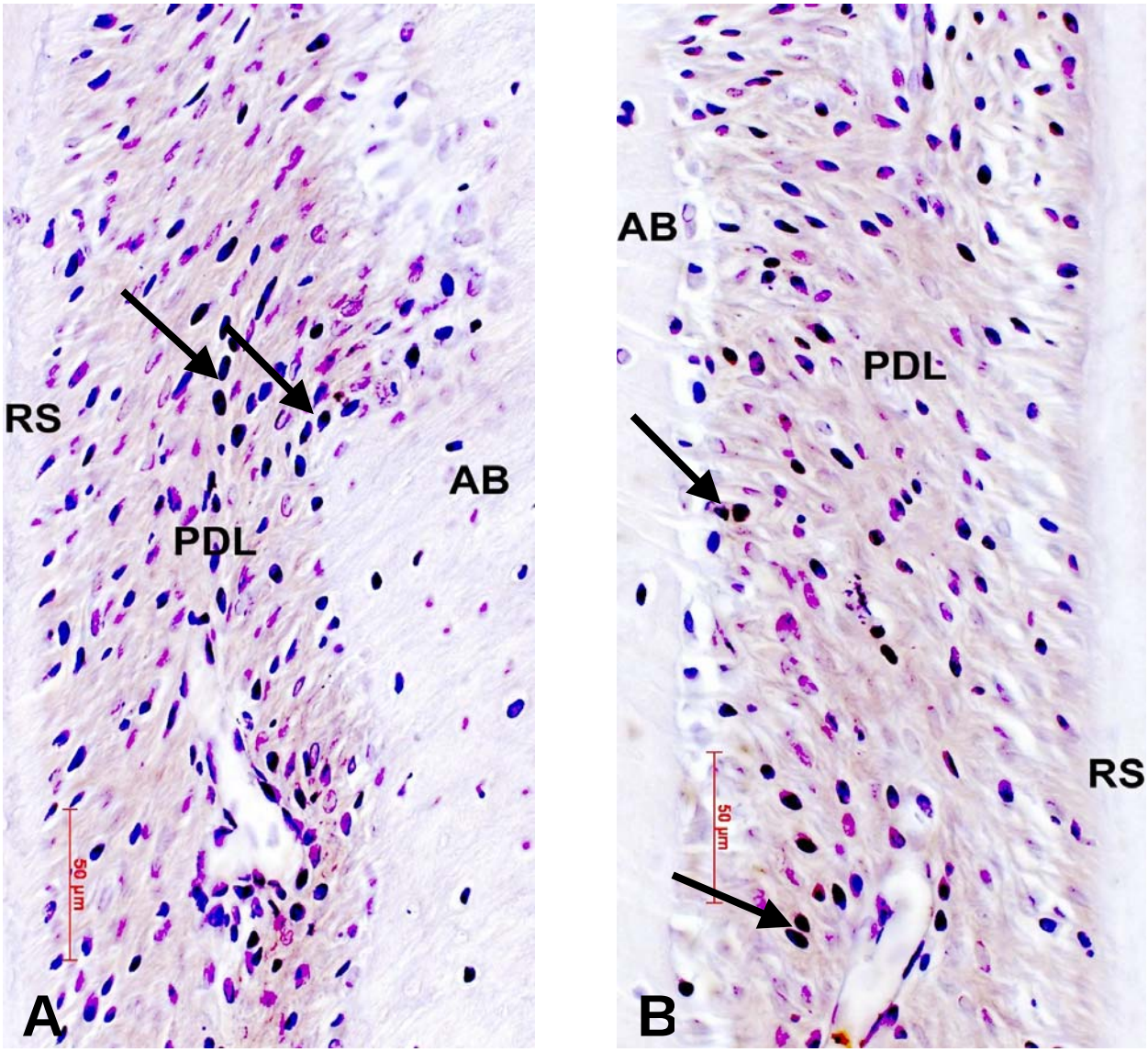


***Figure 6:*** *(A,B) Representative views of immunolabelling in control of pERK1/2 on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL. The immunostained cells appear brown (arrows). The labelled cells are regularly distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

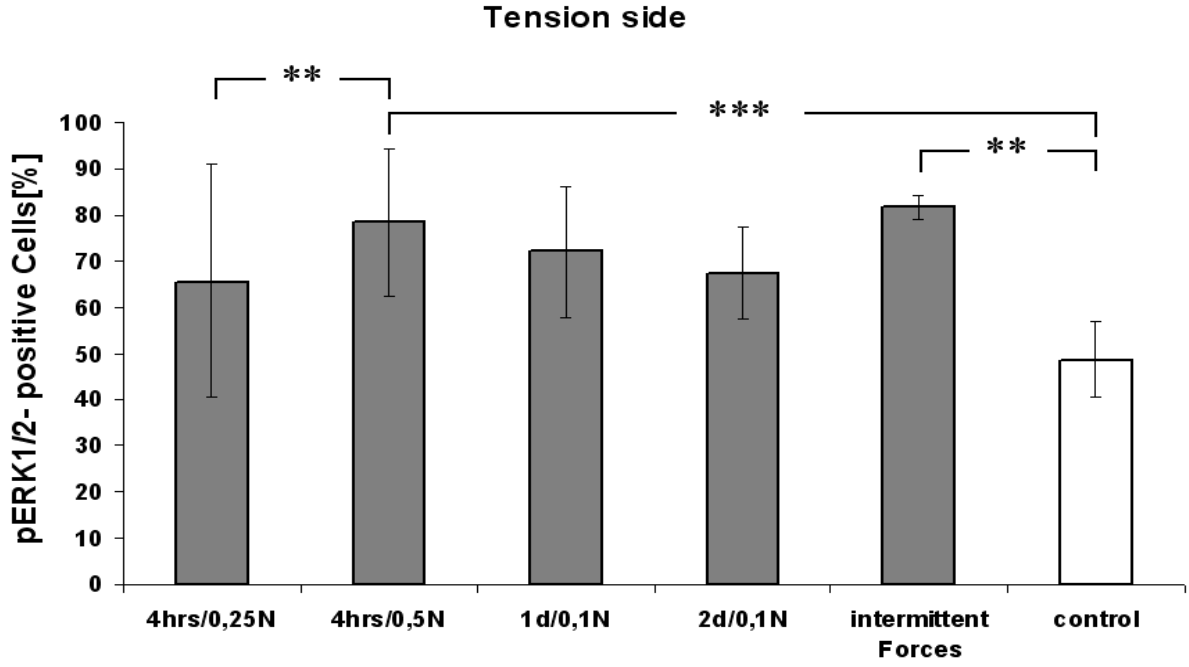


***Figure 7:*** *Quantification of pERK1/2 -positive cells disto-coronal. Each column represents a group of five animals treated with the mentioned loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

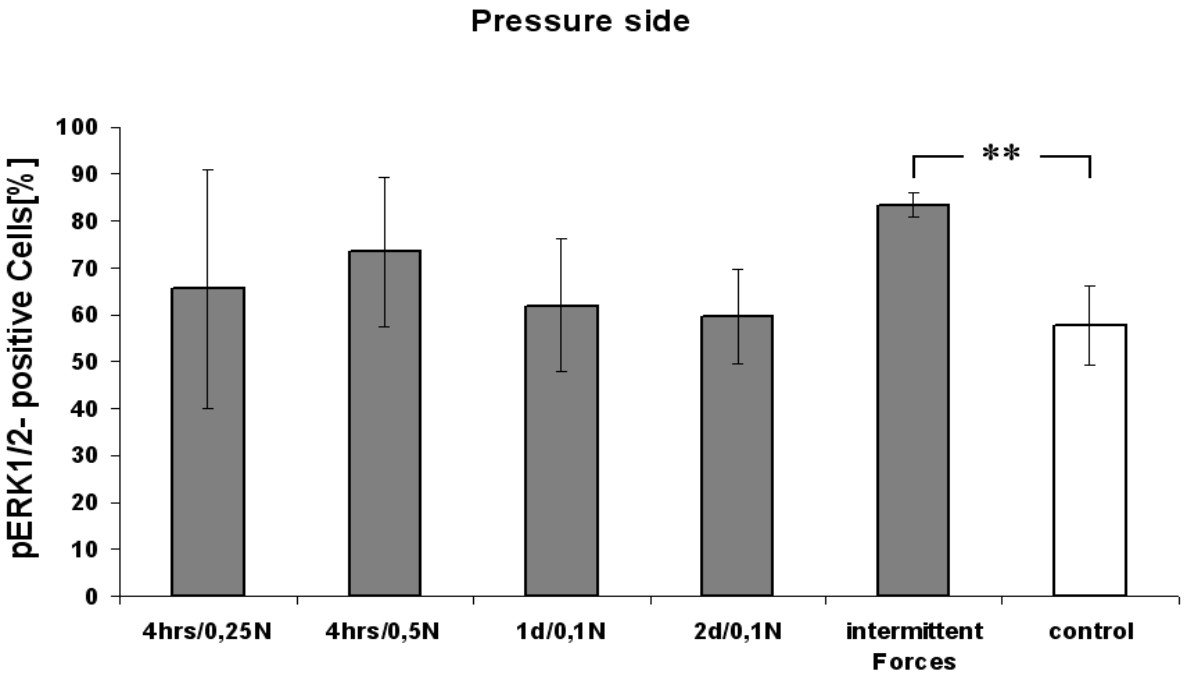


***Figure 8:*** *Quantification of pERK1/2 -positive cells mesio-coronal. Each column represents a group of five animals treated with the mentioned loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

**Results for Runx2**

In the representative areas, both under tension and pressure, the proportion of Runx2 positive cells decreased after the application of constant forces ($p<0.05$; Fig.9,10,11,12). Interestingly, in the same way as with pERK1/2 there was not any statistical difference with respect to Runx2 positive cells.

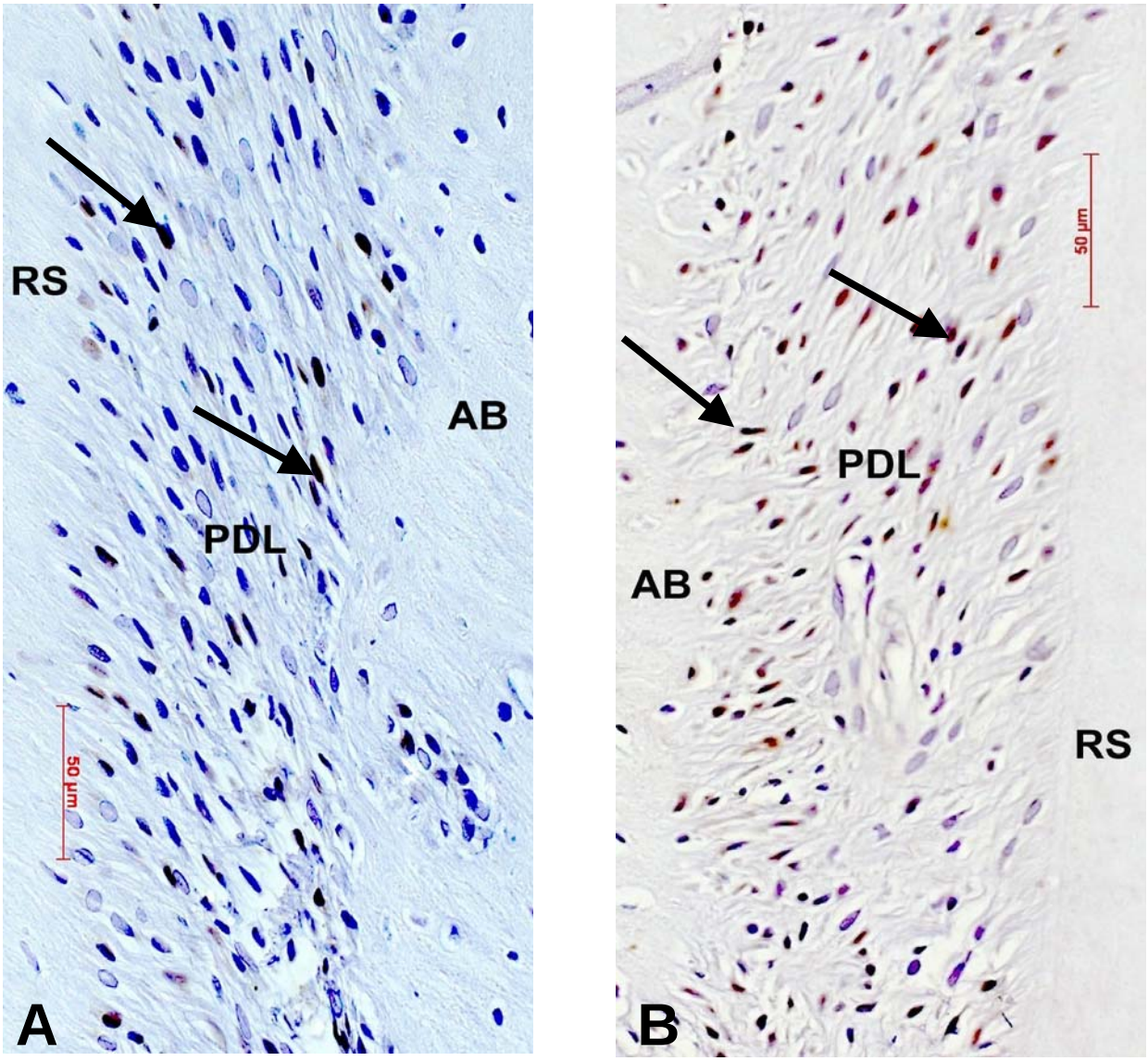


***Figure 9:*** *(A,B) Immunolocalization of cbfa-1 on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL after 4 hrs with application of force of 0.5N. The positive cells are immunostained and appear brown. The labelled cells are regular distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

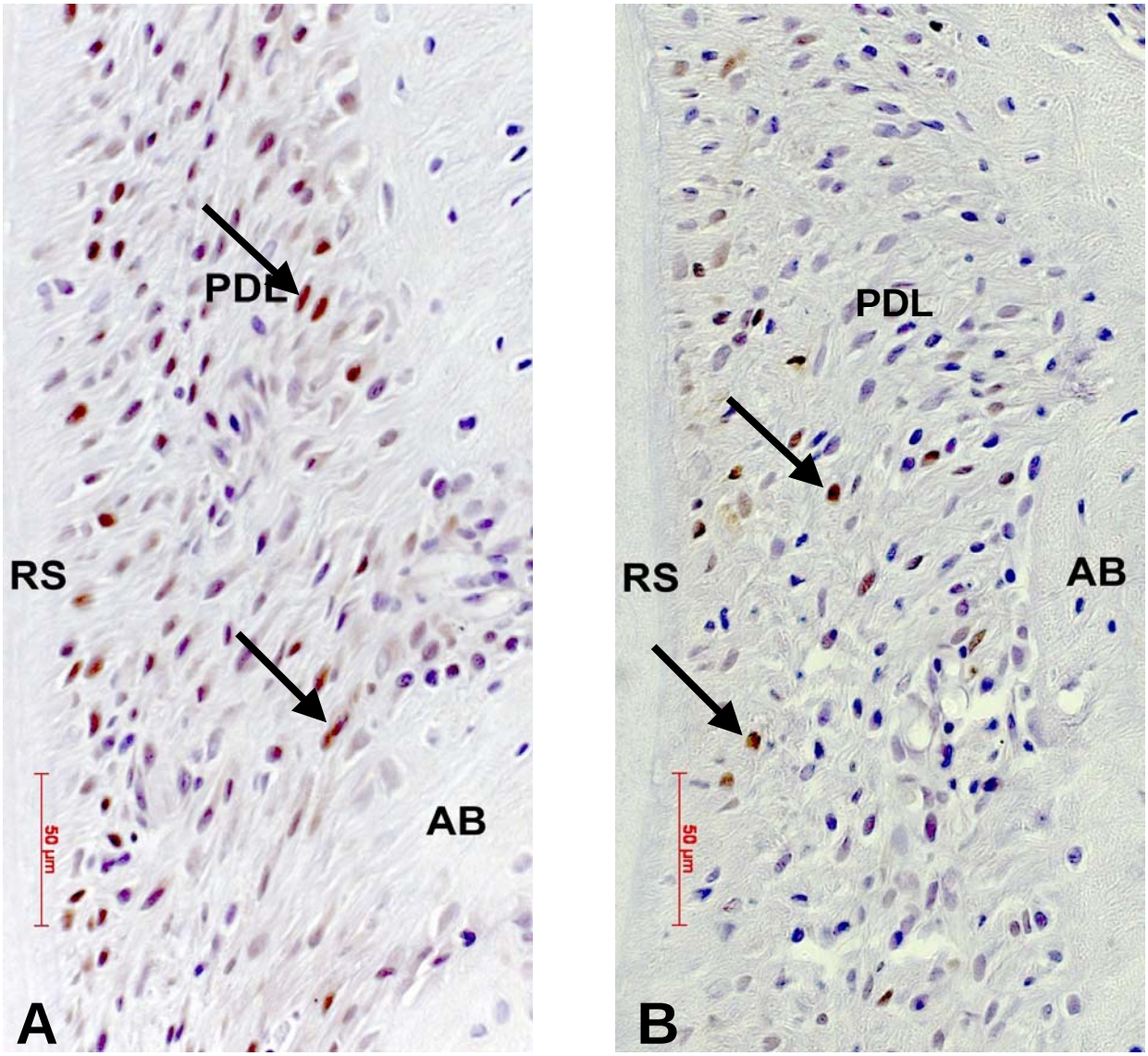


***Figure 10:*** *(A,B) Representative views of immunolabeling in control of cbfa-1 on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL. The positive immunostained cells appear brown (arrows). The labelled cells are regularly distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

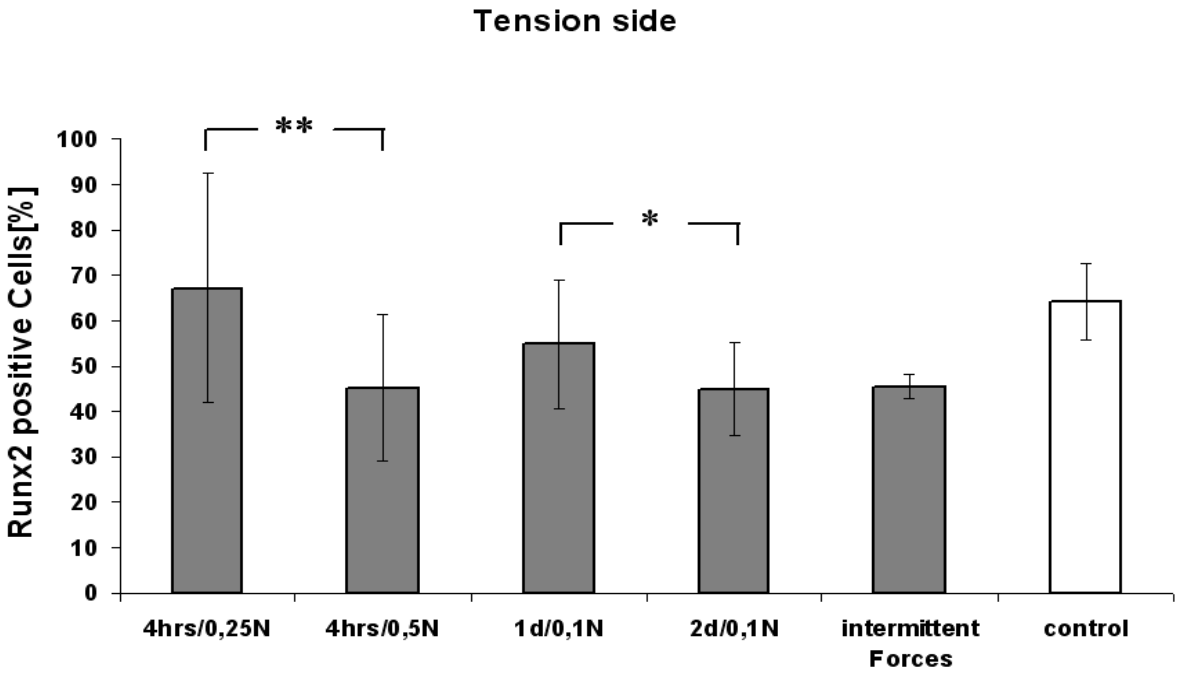


***Figure 11:*** *Quantification of Runx-2-positive cells distocoronal. Each column represents a group of five animals treated with the mentiond loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

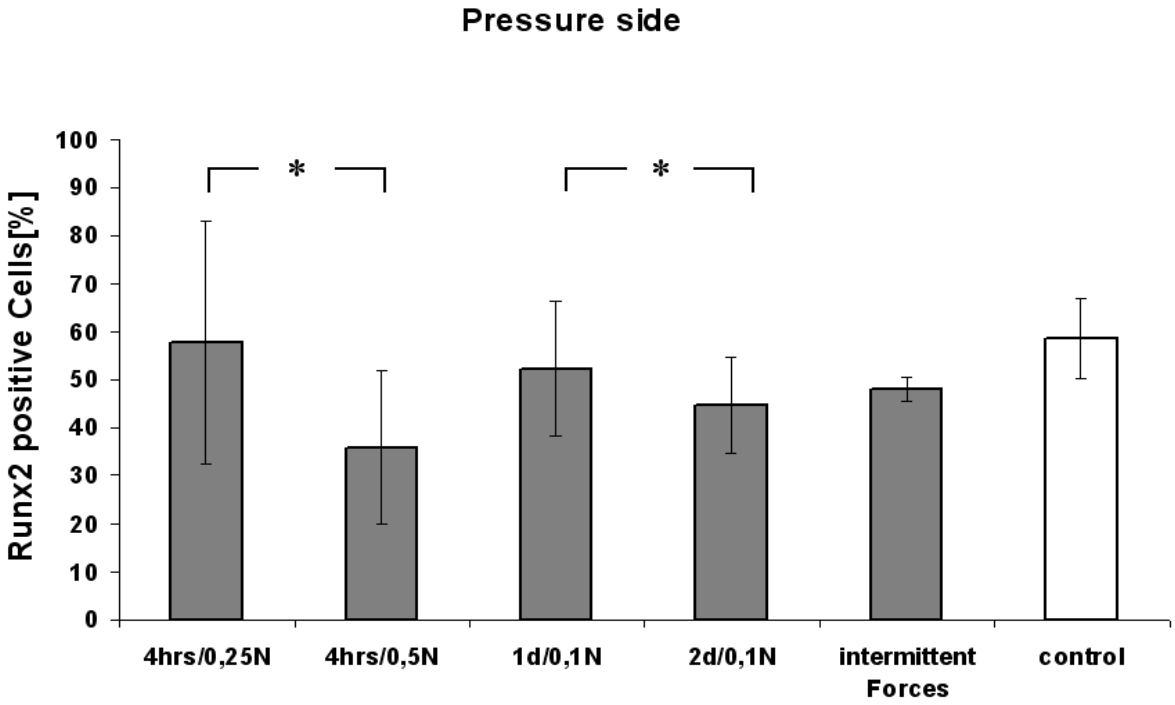


***Figure 12:*** *Quantification of Runx-2-positive cells mesiocoronal. Each column represents a group of five animals treated with the mentioned loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

Results for PCNA

In the selected areas, both under tension and pressure, the proportion of PCNA positive cells after the application of constant forces for 4 hours was lower than that in control teeth ($p<0.01$; Fig.13,14,15,16). In the fourth group, where constant forces for 2 hours of 0.1 N were applied and the animals were killed after 2 days, the proportion of PCNA positive cells under pressure was lower than on the tension side ($p<0.01$) and also lower than that in control teeth ($p<0.001$; Fig.16).

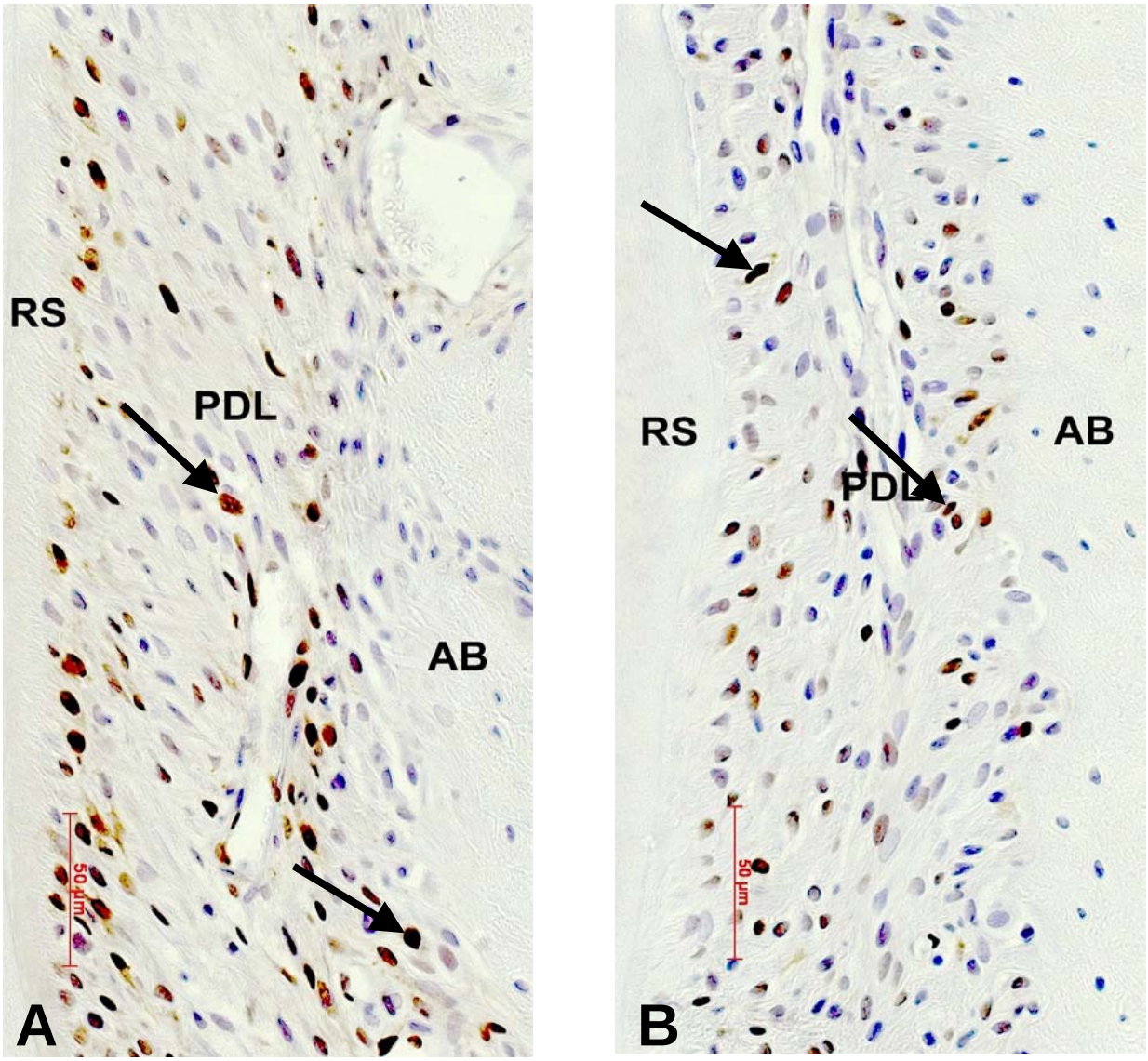


***Figure 13:*** *(A,B) Immunolocalization of PCNA on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL after 4 hrs with application of a force of 0.5N. The immunostained cells appear brown (arrows). The labelled cells are regular distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

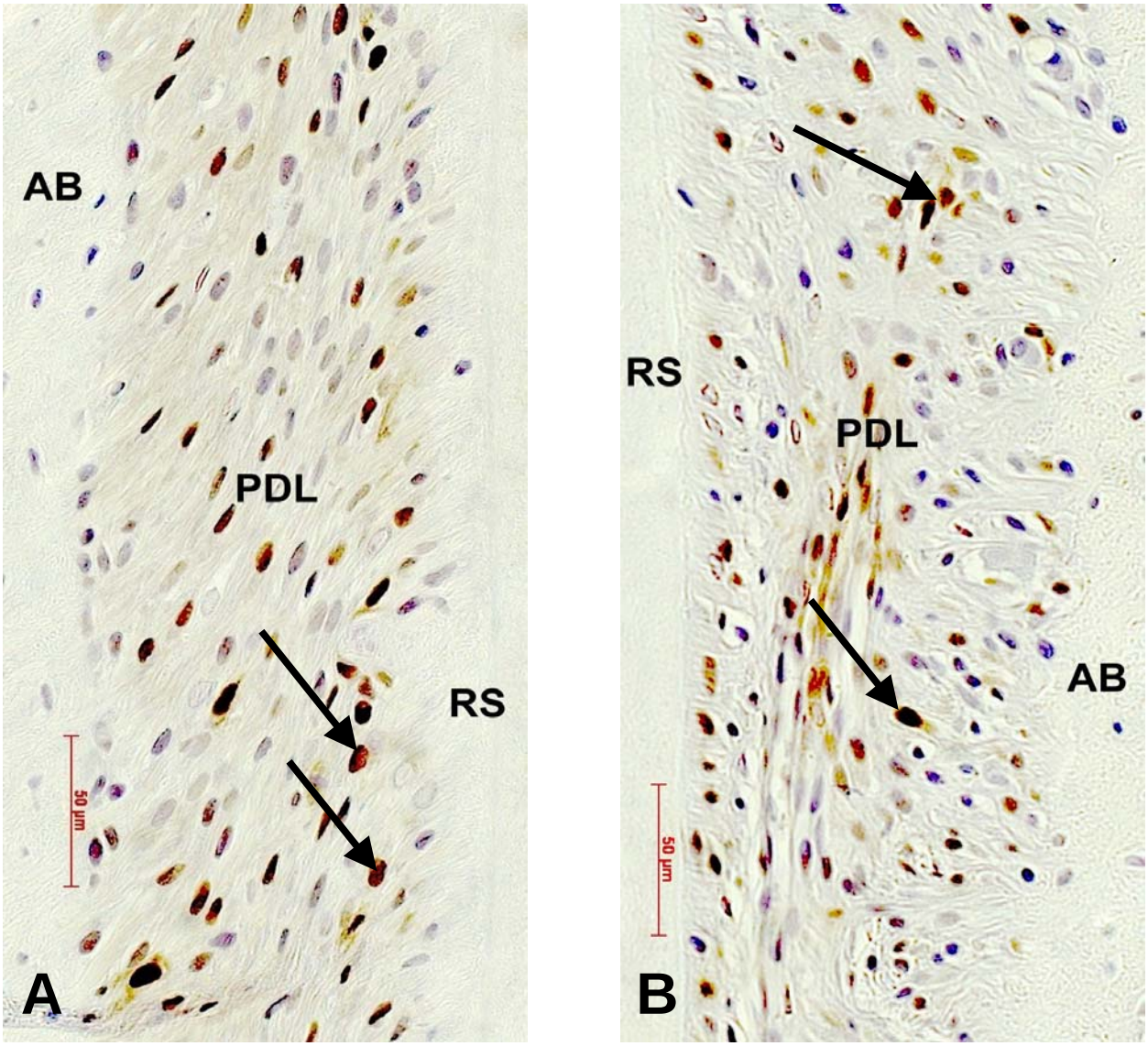


***Figure 14:*** *(A,B) Representative views of immunolabeling in control of PCNA on the disto-coronal (A) (tension side) and mesio-coronal (B) (pressure side) aspects of the mesial root in the PDL. The positive immunostained cells appear brown (arrows). The labelled cells are regular distributed in the periodontal ligament. Magnification 400; scale bars = 50 µm; AB, alveolar bone; PDL, periodontal ligament; RS, root surface; arrows, immunopositive cells.*

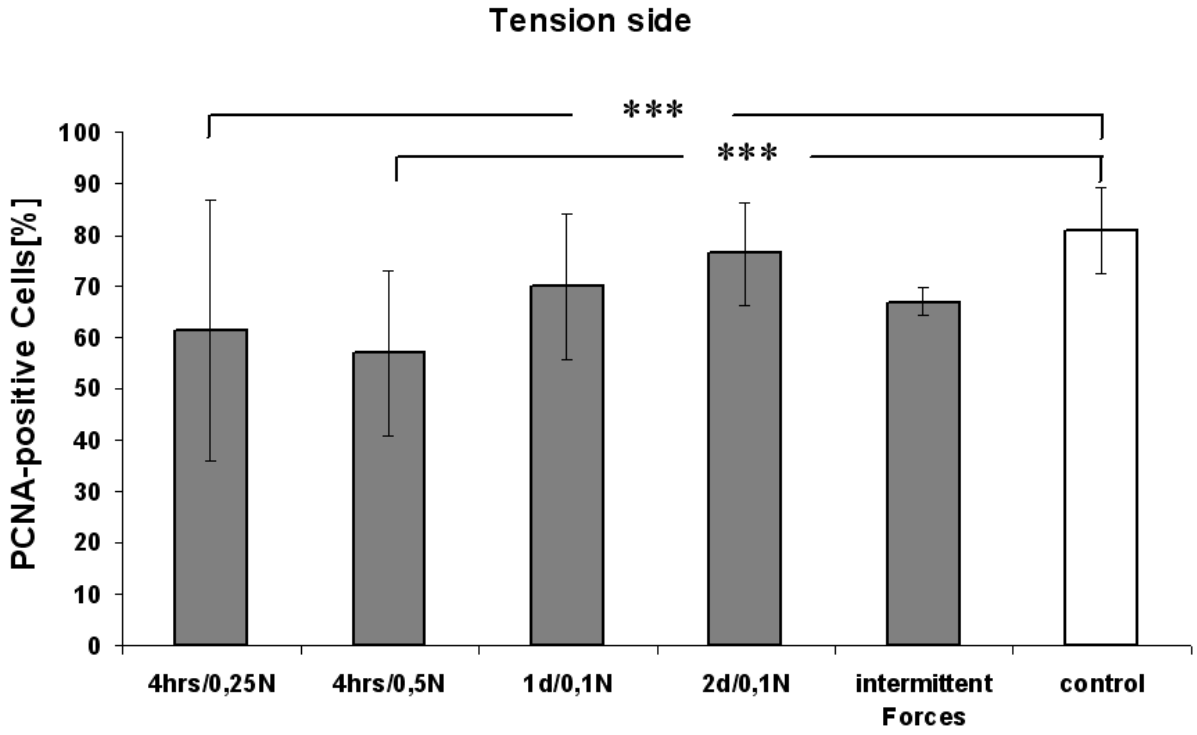


***Figure 15:*** *Quantification of PCNA-positive cells distocoronal. Each column represents a group of five animals treated with the mentioned loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

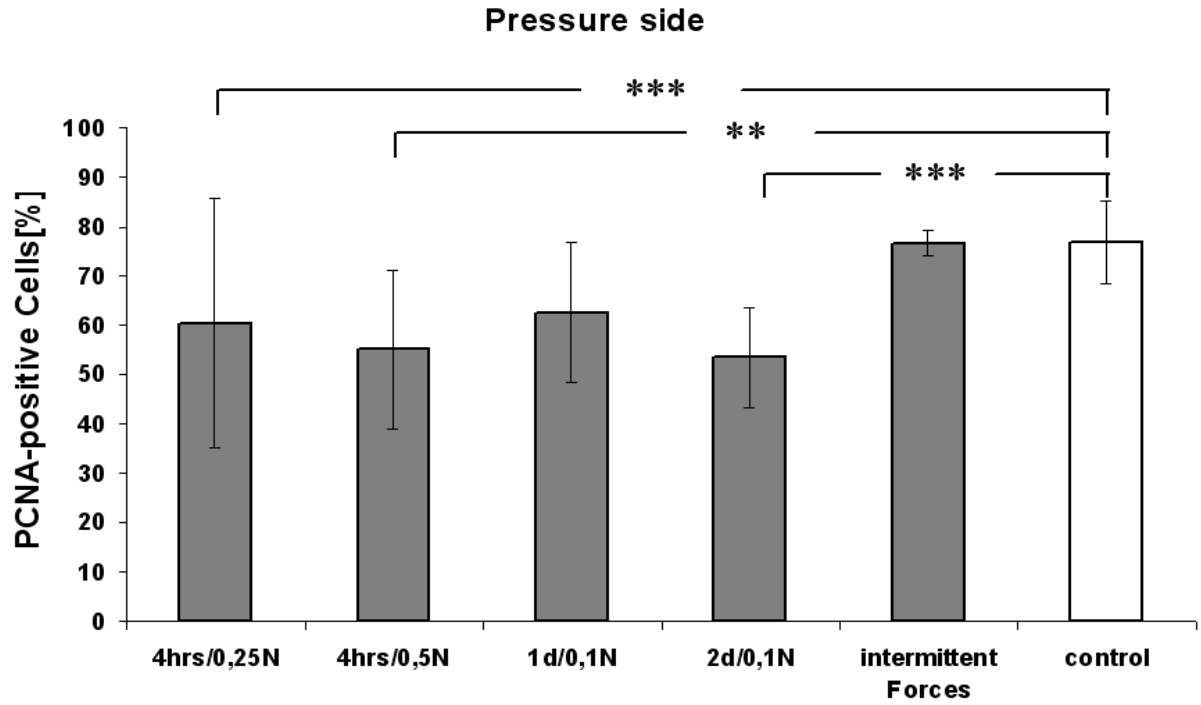


***Figure 16:*** *Quantification of PCNA-positive cells mesiocoronal. Each column represents a group of five animals treated with the mentioned loading regimes. The immunopositive cells were counted as percentages of the total number of cells. Results are representative of 3 sections of each animal. For each of the 5 specimens, a total of 2 fields in 3 sections was analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.001).*

| | 4hrs /0.25N | 4hrs /0,5N | 1d /0.1N | 2d /O.1N | Intermittent Forces |
|---|---|---|---|---|---|
| **pERK1/2** | ↑ | ↑ | ↑ | ↑ | ↑ |
| **Runx2** | n. s | n. s | n. s | n. s | n. s |
| **PCNA** | ↓ | ↓ | n. s | n. s | n. s |

***Table 1:*** *Summary of the modifications of the investigated factors after the different regimes of loading on the tension side; n.s, not significant.*

| | 4hrs /0.25N | 4hrs /0,5N | 1d /0.1N | 2d /O.1N | Intermittent Forces |
|---|---|---|---|---|---|
| **pERK1/2** | n. s | n. s | n. s | n. s | ↑ |
| **Runx2** | n. s | n. s | n. s | n. s | n. s |
| **PCNA** | ↓ | ↓ | n. s | ↓ | n. s |

***Table 2:*** *Summary of the modifications of the investigated factors after the different regimes of loading on the pressure side; n.s, not significant.*

# Discussion

Orthodontic tooth movement is induced by mechanical stimuli and realised by remodelling of the alveolar bone. Specifically, periodontal ligament cells bear the phenotypic characteristics of osteoblast-like cells and play a pivotal role in remodelling and repair (Meikle, 2006), but the exact sequence of events that take place during these processes remains an unresolved issue.

We performed the present study to investigate the responses of periodontal ligament cells to various mechanical loading. We applied constant forces of varying amount for different time periods. Moreover, it is the first time that intermittent forces were applied in a well-controlled animal model. We demonstrated that application of precise loading modifies the transcription of ERK1/2, Runx2 and PCNA, respectively.

Continuous mechanical stretching of human periodontal ligament cells in vitro has been shown to enhance ERK activity and increase the activator protein 1 (AP-1), a transcription factor that plays a pivotal role in controlling gene expression in osteoblast differentiation (Peverali et al., 2001). There are complex mechanisms for the interaction between AP-1 and Runx-2, including direct physical interactions (Franceschi and Xiao, 2003). In accordance with that, Ziros et al., (2002) have demonstrated in cell culture experiments that after mechanical stretching of human periodontal ligament cells, Runx2 was activated by means of ERK phosphorylation. These findings strongly implicate the ERK cascades in the stretch-elicited up-regulation of Runx2 activity.

Kawarizadeh et al., (2005) investigated short-term orthodontic loading in vivo in rats. The authors demonstrated that application of precise short-term loading activates ERK 1/2 and Runx2, respectively. In detail, they showed that in representative areas under tension, the proportions of Runx2- positive and pERK 1/2-positive cells increased within 8 hours of loading, whereas these proportions in

representative areas under pressure were significantly reduced in comparison with those in control teeth. These findings suggested that periodontal ligament cells undergo osteoblastic differentiation via the ERK pathway in the zones under tension.

In this study, rat molars were loaded orthodontically in the same way as Kawarizadeh et al., (2005) with a high-resolution 3D force/torque transducer for exact periods of time. Different force levels were applied and the animals were killed after 4 hours, 1 day and 2 days. An increased proportion of pERK1/2 positive cells in comparison with the control teeth in the tensions zones was found. This increase occurred in a force-dependent manner. In other words, the increase of the applied force from 0.25 N to 0.5 N during a constant time of 4 hours produced an increase of the pERK1/2 positive cells in comparison with the untreated animals. Conversely, after an experimental time increase of 1 on 2 days under a constant force of 0.1 N no statistically significant increase of pERK1/2 appeared in the tension zones. Respectively, in the pressures zones only the intermittent forces produced a higher proportion of pERK1/2 positive cells in comparison with the controls. On the other hand, differences between the tension and the pressure sides were not proven to be significant, which could be explained by the fact that there was already an obvious difference in the amount of positive cells between the tension and the pressure zone in the control teeth.

The proportion of Runx2 positive cells decreased after the application of constant forces in most of the teeth under investigation. This decrease on the pressure as well as on the tension side occurred in a time and force-dependent manner.

Finally, both under tension and pressure, the proportion of PCNA positive cells after the application of constant forces for 4 hours was lower than those in controls.

This study, in comparison with the study of Kawarizadeh et al., (2005), provides novel information concerning tooth movement. In detail, it was shown that the

expression of the involved factors was dependent on the amount of force that was applied. Thus, while there was a force dependent increase of the expression of pERK1/2 on the tension side, the opposite relationship was demonstrated for the expression of Runx2 on the pressure side. Kawarizadeh et al., (2005) showed similar results but in a time dependent manner. The reason for this discrepancy might be differences in the experimental protocol. First, we used the same amount of forces for a much longer period time and second Kawarizadeh et al. were used higher forces for the same time. This might also explain the insignificant differences between the tension and pressure sides, which are in contrast with the findings of Kawarizadeh et al., (2005).

In addition, a time dependent decrease was observed for the expression of Runx2 on both sides as well as for PCNA on the pressure side. Regarding PCNA, in vitro studies have shown that mechanical stress induces DNA synthesis in human periodontal ligament cells following 6 hours of stretching (Kletsas et al., 1998). In our study, in accordance with Kawarizadeh et al., (2005), an obvious increase in proliferation could not be observed. Thus, in vitro and in vivo findings are to be compared with caution. At last, intermittent forces resulted in a clear up-regulation of pERK1/2, showing that this stimulus can especially initiate differentiation of the PDL-cells.

Altogether, the findings of this study show that a mechanical stimulus induces differentiation of periodontal ligament cells toward osteoblasts via the ERK cascade, verifying the hypothesis of Roberts et al., (1982), that new osteoblasts are derived from periodontal ligament cells during orthodontically induced osteogenesis and also the report of Camilleri and McDonald, (2006), that the Runx2 gene is involved in the remodelling process of the alveolar bone.

A number of studies have identified other agents to effect bone remodelling and tooth movement. Increased immunoreactivity of Substance P has been

demonstrated in the PDL in the early phases of tooth movement (Davidovitch et al., 1988). This neuropeptide has been shown to cause vasodilation and increased vascular permeability, contributing to increased local blood flow that accompanies inflammation.

Cytokines and especially interleukin IL-1β and tumour necrosis factor-alpha (TNF-α) were found to affect bone metabolism by stimulating osteoclast development and activity. Systemic application of soluble receptors to IL-1 and TNF-a following experimental induction of tooth movement in the rat reduced the number of osteoclasts (Jäger et al., 2005).

In addition, the role of components of the RANKL/RANK/OPG system and that of NO in inducing bone remodelling was recently demonstrated (Ogasawara et al., 2004; Shirazi et al., 2002). RANKL is a regulator of osteoclast formation and activation, through which many hormones and cytocines produce their osteoresorptive effect. OPG is a decoy receptor produced by osteoblastic cells, which compete with RANK for RANKL binding.

Clinical and animal studies by various authors have identified the role of prostaglandins (PGE1 and PGE2) in stimulating bone resorption. Lee et al., 1990 have reported a direct action of prostaglandins on osteoclasts in increasing their numbers and their capacity to form a ruffled border and effect bone resorption.

Altogether, there is still need to further analyse the regulatory factors mentioned above for elucidating our understanding of remodelling processes following orthodontic tooth movement.

## Summary

Previous studies have indicated that periodontal ligament cells demonstrate osteogenic potential and osteoblastic differentiation via the ERK pathway under mechanical stress in vitro and in vivo. This study aimed to further analyse this regulatory process experimentally in the rat.

The right upper first molars of 25 anesthetised rats were loaded with forces in order to be moved mesially. Constant forces for 4 hours of 0.25 N and 0.5 N were applied in 5 animals each. Furthermore, constant forces for 2 hours of 0.1 N were applied in 10 animals and afterwards, the first and second molars were permanently separated with composite. In these animals, the antagonists were sliced and five rats were killed after 1 day and five ones after 2 days. At last, intermittent forces of 0.1 N and O.25 Hz were applied in 5 different animals for 4 hours. Untreated contralateral sides served as control. Parafin-embedded sections were analyzed quantitatively after immunohistochemistry for proliferating cell nuclear antigen (PCNA), runt-related transcription factor 2 (Runx2/Cbfa1) and phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2).

In selected areas under tension the proportion of pERK1/2 positive cells was increased compared with those in control teeth in all types of loading, whereas these proportions in selected areas under pressure were increased only after the application of intermittent forces. In representative areas, both, under tension and pressure the proportion of Runx2 positive cells decreased after the application of constant forces. After the application of constant forces for 4 hours in representative areas, both under tension and pressure the proportion of PCNA positive cells was lower than that in control teeth.

The involvement of pERK1/2, Runx2/cbfa-1 and PCNA in the reaction of periodontal ligament cells to different load regimes was verified.

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