ORTHODONTIC STRESS BCL-2 MODULATION AND HUMAN ODONTOBLAST'S SURVIVAL

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This study assessed the effect of orthodontic traction on Bcl-2 expression and apoptosis in human dental pulp. It also explored, in absence of noxious stimuli the regeneration of odontoblasts during the entire life of the tooth. Twenty young patients, with Class II malocclusion and severe to moderate crowding, were referred for orthodontic assessment. Whole pulps were removed. Half the pulps were fixed, paraffin-embedded and processed for histology and immunohistochemistry using anti Bcl-2, Caspase 9 cleaved and Caspase 9 not cleaved antibodies. The rest of the samples, both orthodontically treated and not treated dental pulps, were immediately frozen at -80ºC after the extraction and quantitative PCR was performed. Histology showed alterations in pulp microanatomy after 8 months of treatment. Immunohistochemistry depicted a decreasing expression of Bcl-2 in dental pulp over time in the non-treated while a very weak to absent Bcl-2 expression was detected in the orthodontically treated tissues. Active and non-active forms of Caspases, were expressed in both groups of dental pulp, however staining for the non active form was stronger than the corresponding cleaved form in all samples. The increased expression was detected mainly at nuclear level. Real time qPCR results correlated with those of immunohistochemistry and exhibited a decreasing expression of Bcl-2 in the treated samples. Orthodontic traction may inhibit the expression of Bcl-2, favoring the onset of apoptosis and leading us to conclude that the physical stress in the absence of noxious stimuli might make odontoblasts regeneration less likely.

Orthodontic treatment is becoming a very common dental cosmetic treatment not only in children but also in adults undergoing teeth alignment using orthodontic appliances. A large range of different types of orthodontic appliances exist nowadays in the market. Such a variety is mostly related to the degree of visibility after the braces are fitted.

Orthodontic treatment is based on the fact that teeth are moved because orthodontic forces are applied on them. However, the pathologies and alterations that could possibly result in the dental pulp as a consequence of such manipulations were

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not well studied. It was suggested about 30 years ago that forces applied on teeth cause changes on the dental pulp structure and probably effects its viability. Our group has undertaken this study to explore if long term orthodontic traction might cause dental pulp suffering or even pulp death leading to serious clinical implications. In addition, this study investigated the possibility of odontoblasts going into natural apoptosis even without noxious stimuli.

Apoptosis is a normal process of programmed cell death that occurs during organogenesis and adult life. Multiple pathways are involved in this process whereby the Bcl-2 family of proteins are key players (1). In addition, odontoblasts are known as post mitotic cells, and they could live the whole life of the tooth. However, the presence of pro-apoptotic molecules (Caspase -3 and -9) in odontoblasts cytoplasm and nuclei was suggested that odontoblasts may have a persisting turnover ability during the whole tooth life. Apoptosis is a controlled form of cell death that plays a fundamental role during embryonic development and in the maintenance of tissues including the tooth (2, 3). Defects in the regulation of apoptosis have been associated with the occurrence of many disease states including neuro-degeneration and cancer. Apoptotic cells show morphological and molecular characteristics such as cell shrinkage, membrane blabbing, chromatin condensation and DNA fragmentation (4-6). Apoptosis presents two main pathways which share the presence of caspases: intrinsic (mithocondrial) and extrinsic (receptors-related). In the intrinsic pathway Bcl-2 proteins, cytochrome c and Caspase 9 are involved, while in the extrinsic pathway, the FAS-FasL,FADD and Caspase 8 are involved. Both pathways therefore share the presence of Caspases. Caspases are cysteine proteases that cleave a set of proteins to initiate and promote apoptosis signaling. Caspase 8 participates in extrinsic pathway; it is activated by FAS-FASL signal transduction. Caspase 9 is the mediator of the intrinsic pathway, alteration in oxidative phosphorylation, hypoxia and oxidative stress can induce its activation (7). Once activated, Caspase-9 cleaves and activates downstream effectors Caspase 3 and 7, which target key regulatory and structural proteins for proteolysis leading to cell death. Caspase-9 is required in most scenarios of apoptotic cell death and its impaired activation leads to profound cellular consequences. The majority of caspase-9 deficient mice die prenatally due to severe morphological deformations of the brain which arise from excess cell numbers that accumulate during embryonic development. In addition, thymocytes isolated from caspase-9 deficient mice exhibited increased resistance to various pro-apoptotic stimuli, including genotoxic stress-inducing anti cancer drugs and gamma radiation (8). This apoptotic resistance also suggests that impaired Caspase-9 activation or loss of caspase-9 expression might be implicated in cancer development and tumor progression (9). Caspase 9 therefore plays an important role in initiating apoptosis execution in cells that need to be eliminated during early development stages, and is required for the continuous removal of damaged cells to suppress proliferative diseases during the entire lifetime of multi-cellular organism. Other members that participate in the intrinsic pathway are the Bcl-2 family proteins which are divided into anti-apoptotic and pro-apoptotic members (10-12).

On the other hand, the Bcl-2 family of proteins contain both pro-and antiapoptotic members that have a complex network of interaction both in the cytosol and on mitochondria. Such interactions play a key step in apoptosis and determine the fate of the cell (1). So far, it is not very well understood how the complex interaction network formed by the multiple Bcl-2 members present simultaneously in the cell orchestrates apoptosis signaling, it is also important to note that concerning the developing tooth, Bcl-2 proteins were reported to exist at the various stages of development. In addition, it was reported that in odontoblasts underlying cavity preparation, Bcl-2 expression was found to be increased significantly (13). These observations suggest that regulation of odontoblasts apoptosis by Bcl-2 may contribute to dentine homeostasis and damage-related dentine remodeling. Therefore, how Bcl-2 is related to these physiological and pathological processes is not entirely clear yet (14, 15).

MATERIALS AND METHODS

Patients

Twenty patients, 12 males and 8 females with an age range of 10 to 14 years were referred for orthodontic assessment, they had class II malocclusion and severe to moderate crowding, according to the Index of Orthodontic

Treatment Need, UK. In 6 cases, the extraction was performed after 3 months of treatment (Group 1). In another 6 cases, extraction was performed after 8 months of treatment (Group 2). In the remaining 8 cases the teeth were extracted before any orthodontic treatment began (Group 3 control). Treatments consisted of the straight wire technique using nickel-titanium archwires. The treatments always started with 0.12 Ø nickel-titanium arches followed by wires of increasing diameters (0.14, 0.16, 0.18, 0.16 x 0.16), and with an increasing pressure applied to the teeth. The adopted procedures conformed to the relevant ethical guidelines for human research. They were in agreement with the Helsinki Declaration of 1975 as revised in 1983, and were approved by the local Ethics Council. All subjects gave their informed consent to participate to the study.

Tissue biopsy collection

Pulps were extracted by incising the tooth longitudinally using a thin diamond bur under a water jet. The cut stopped 2 mm before the pulp cavity to avoid pulp damage due to the heat produced by the attrition between the bur and hard dental tissue. The tooth was split with a cutter and the quality of the pulp was checked on one side of the split tooth. Finally, the pulp was detached by a sharp instrument and then washed in a physiological saline solution.

Tissue processing

The pulps were fixed in a 10% buffered formalin solution (pH 7.4) and processed for routine microscopy according to standard procedures. Five micra sections were cut by a Leica microtome RM2145 and dried overnight at 37°C. One section for each sample was stained with haematoxylin and eosin for histological evaluation of pulpal tissues. Other sections were stored at room temperature until use.

Immunostaining

On the day of the experiment, the µm serial sections were de-waxed, rehydrated and treated for five min with Peroxidase Block and washed in phosphate buffered saline pH7.4-7.6 at room temperature. Immunohistochemistry was performed as described in the kit En-Vision + System HRP with AEC as substrate (Dako). Using the following mouse primary monoclonal antibodies: Bcl-2 clone 100 (Millipore, lot #. Jbc1881343; diluition 1:100); Caspase 9 cleaved (Cell Signal Inc, Cat. No. 9508; dilution 1:100); Caspase 9 (from Sigma-Aldrich lot-100k4852 diluition 1:40). All specimens were microwave-treated for antigen retrieving. The antibodies were diluted in a 0.1% BSA (Bovin Serum Albumin) solution. Appropriate positive and negative controls were run concomitantly. Caspase 9 antibody (Sigma-Aldrich USA) was able to recognize both precursors (pro-Caspase) and cleaved (Caspase) proteins, as described in manufacturer's datasheets. The sections were then incubated with Peroxidase labeled polymer conjugated to goat anti-rabbit immunoglobulin in Tris-HCl buffer, pH 7.4, containing stabilizing protein and an antimicrobial agent. Unbound polymer was removed by washing (2X with PBS, 5 minutes each) and subsequently AEC chromogen in substrate buffer for five minutes then stopped in distilled water. Slides were then cover-slipped using Dako Cytomation Faramount Aqueous Mounting Medium (Cat. No. S302580).

The immunohistochemical specimens were examined and photographed using a Leica Laborlux S Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by two different operators. The results were compared to an image analysis system obtained from digital JPG files. Adobe Photoshop CS6 extended (Adobe Systems Inc, San Jose, CA, USA) was used to elaborate images (16). We converted the image color profile from RGB to CMYK and selected the yellow channel since it has the best linear response to color intensity and thus to protein presence (17). In Photoshop, after inversion to obtain a negative image, the intensity of positivity was selected in all fields at 40x and obtained the media of gray scale value (0=white/255=black). The two subsets of values were compared with student *t*-test. The quantification of colorimetric staining has been represented using a score with values from 1+ (minimum) to 3+ (maximum) (16-17) (Table I).

mRNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

The dental pulps were collected from the premolar teeth according to approved guidelines, as follows: 15 normal human dental pulps from the 8 control patients,15 dental pulps from 3 month, and 15 dental pulps from 8 month orthodontically treated patients.

Sharp needles and excavators were used to release the dental pulp from the pulp chamber. They were washed in MSCBM medium (LONZA, Walkersville, MD USA), then stored at -80°C.

Extraction of mRNA was performed using RNeasy Mini Kit (50) from Qiagen and the RNA concentration was determined by measuring absorption at 260/280 nm. Dental pulps were rinsed twice with PBS and Medium. Once separated from the medium, the pulp cells were crushed, tripsinized, collected in a 15 ml Falcon tube, and centrifuged. The supernatant was removed and the cells were re-suspended with 600 µl of lysis buffer and followed the steps until the RNA extraction. RNA

was eluted in a final concentration of 50 µl of sterile nuclease-free water as recommended by the manufacturer and stored at -80 degrees. Reverse transcription was performed with a reverse transcription kit (Access RT-PCR, PROMEGA). Total volume of 25 µl was made mixing 9 µl of nuclease-free water, 5 µl of reaction buffer 5X, 1 µl of dNTP, 2 µl of $MgSO_4$, 0,5 µl AMV enzyme, 0,5 µl TFL DNA polymerase, 3 µl (300 nanograms) of RNA. Two microlitres of Forward and 2 µl Reverse primers were added to the final concentration. (GAPDH: 5'-ACCACAGTCCATGCCATCAC, 5'- TCCACCACCCTGTTGCTGTA-), (Bcl-2: 5'-TCACTTGCATTTCTTTGCCC, 5'-CCAACTCTTTTCCTCCCACC-). After 2 min of pre-incubation at 94°C, amplification was performed for 30 cycles (GAPDH, Bcl-2) consisting of denaturing at 94°C for 30 s, at 60°C for 30 s, and extension at 72°C for 1 min.

The products were loaded onto 2% agarose gels, visualized by ethidium-bromide staining and captured with an image analysis system(Transilluminator UVP, BioDoc-It, imaging system). RNA samples without reverse transcriptase were used as negative controls.

cDNA synthesis

A final volume of 20 µl of cDNA was synthesized by mixing 2 µl of dNTP, 4 µl of 5x reaction buffer, 1 µl of reverse transcriptase enzyme (for ImProm II), 1 µl of RNase Inhibitor, 8 µl of RNA (RNAconcentration of 0,1µg/µl), 2 µl of $MgCl_2$ (PROMEGA) and 2 µl of random primer (AMBION). After incubation at room temperature (RT) for 10 min the mixture was left at 37°C for one h and ten min at 70°C for enzymes inactivation. Concentration was determined from absorbance values at a wavelength of 260 nm using a BioPhotometer (Eppendorf, UK). cDNA template was used to perform qPCR mixing 5 µl of cDNA and 45 µl of nuclease free water.

RT-qPCR

A total volume of 10 µl was made with 5 µl of Sensimix-Syber green (Quantace SensiMix SYBR Kit), 0.2 µl F-primer, 0.2 µl R-primer and 4.6 µl of cDNA template. The experiments were performed in triplicate and the means of the results were normalized using as reference gene GAPDH. RT-qPCR was performed with the following conditions: 10 min at 95°C initial denaturation, cycling 40 for 15 s at 95°C, denaturation at 60°C for 30 s, primers, annealing/elongation and a final extension at 72°C for 30 s. A melting curve analysis was performed at each PCR by gradually increasing the temperature from 60° to 95°C. A single pick at the melting temperature of the PCR-product confirmed the primer specificity and RNA purity.

Primers from SIGMA were designed using the Primer Blast software from NCBI for the amplification of genes fragments of around 40 to 200 bp and an annealing temperature of 60°C. For the Quantitative analysis Rotor Gene Q-series software was used.

Statistical analysis

The data are presented as mean±SD. Analysis of variance (*t*-test) was performed to determine significance between the two sources of cells. A P-value of ≤0.05 was used to identify significance of differences.

RESULTS

The histological studies in the treated pulps, compared to the normal (not treated) control, did not show any significant morphological alterations before 3 months of orthodontic treatment. However, some significant structural modifications were observed after 8 months of treatment. These changes included significant vacuolization of the parenchyma, discontinuation of the odontoblasts monolayer, and vasodilatation or congestion of blood vessels (Fig. 1A, B, C)

The Immunohistochemistry results for Bcl-2 showed a marked difference between the treated and non-treated pulps. On the average, there was a very significant expression of Bcl-2 in the non-treated groups (Fig. 1D) compared to low expression at 3 months (Fig. 1E), or very low expression at 8 months in the treated pulps (Fig. 1F). The treatment decreased Bcl-2 expression significantly with time.

Regarding the Caspase 9, cleaved and non-cleaved, data showed that the expression of Caspase 9 not cleaved seemed to be more localized and concentrated in the nucleus, while no other differences were noted at 3 months and before treatment (Fig. 1G, H, I).

Concerning the cleaved Caspase 9 in the non-treated pulp, the expression was mostly in the nuclei, however in the treated pulps, it looked like being also cytoplasmic. Such an expression was markedly higher on the 8 months compared to the 3 months (Fig. 1L, M, N). The data emanating from the molecular study using quantitative PCR correlated very well with that of immunohistochemistry (Fig. 2). It depicted the relatively highest expression in the non-treated pulps (1.8±). Such an expression decreased over time, at 3 months it was 0.8 and 0.5 at 8 months (Fig. 3).

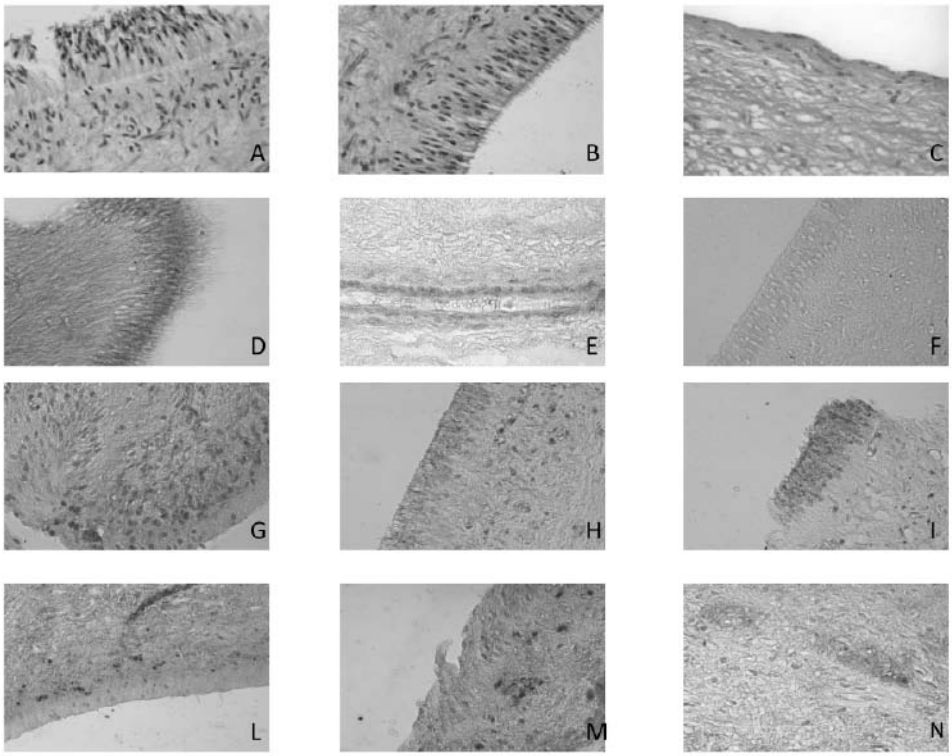

Fig. 1. *H&E staining 40X, control dental pulp (**A**), 3 months orthodontically treated dental pulp (**B**) and 8 months orthodontically treated dental pulp (**C**). Bcl-2 IHC staining 40X, control dental pulp (**D**), 3 months orthodontically treated dental pulp (**E**) and 8 months orthodontically treated dental pulp (**F**). Caspase-9 not cleaved IHC staining 40X, control dental pulp (**G**), 3 months orthodontically treated dental pulp (**H**) and 8 months orthodontically treated dental pulp (**I**). Caspase-9 cleaved IHC staining 40X, control dental pulp (**L**), 3 months orthodontically treated dental pulp (**M**) and 8 months orthodontically treated dental pulp (**N**).*

Table I. *The quantification of colorimetric staining using a score with values from 1+ (minimum) to 3+(maximum).*

	BCL2	Casp-9 cleaved	Casp-9 not cleaved
Not Treated	122.6 (+++)	124.55 (+++)	138.75 (+++)
Treated 3 m	32 (+)	70.2 (++)	77.5 (++)
Treated 8 m	4.5 (+)	75.4 (++)	84.2 (++)

** indicates that data are significant in 3 and 8 months orthodontically treated dental pulp if compared to the not treated.*

DISCUSSION

Studies based on inducible Bcl-2 over-expression in transgenic mice, reported a persistent Bcl-2 expression in the teeth of these mice while the levels of endogenous mouse Bcl-2 and Bax were similar between the wild type and transgenics. *In vitro* pulp cell cultures demonstrated a similar pattern of expression. Since, transgenic odontoblasts have a higher Bcl-2/Bax ratio, odontoblasts have a relative survival advantage if compared to the wild type cells. It is also shown that in transgenic mice, less odontoblasts apoptosis occurred at the basal level

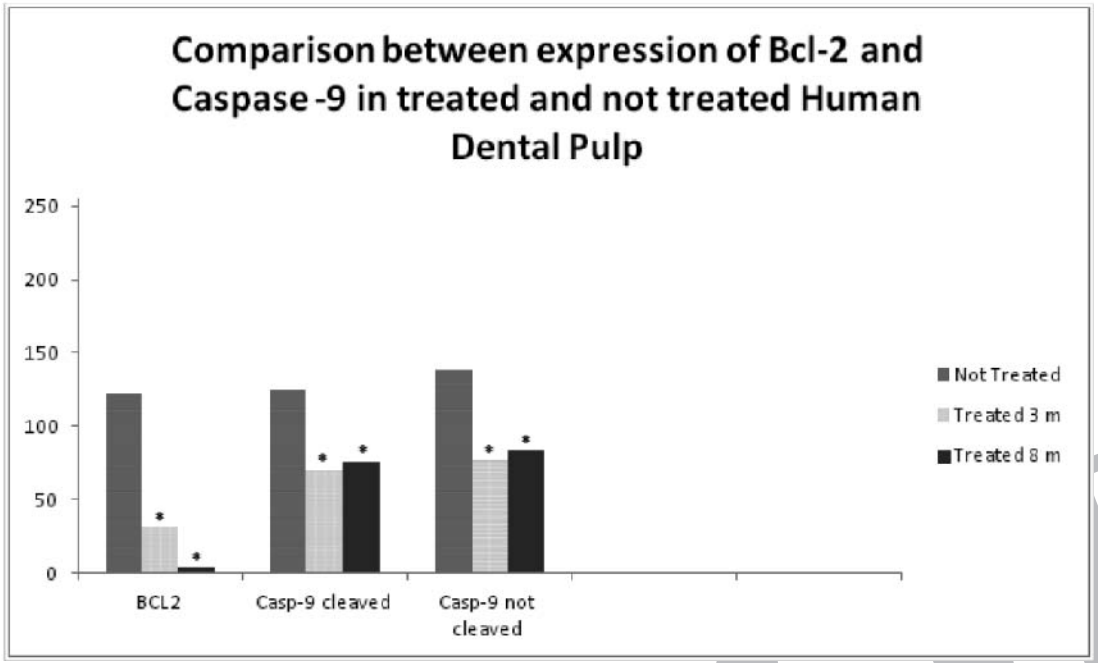

Fig. 2. *Image Analysis. Comparison between expression of Bcl-2 and Caspase -9 in treated and not treated Human Dental Pulp.*

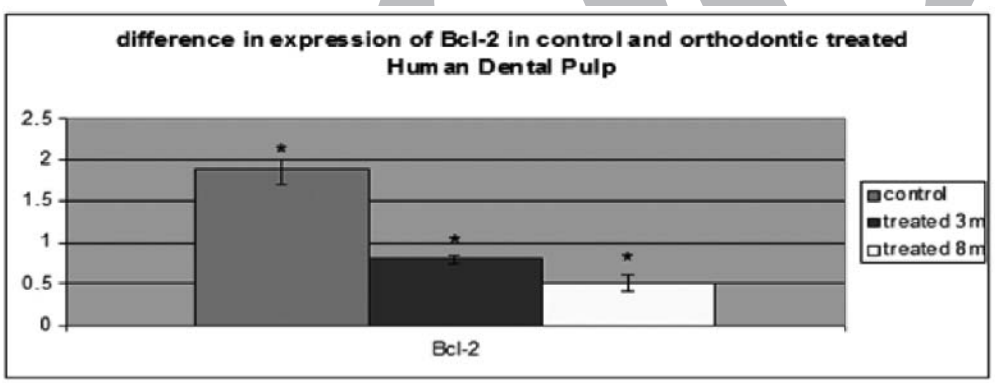

Fig. 3. *qPCR differences in Bcl-2 expression of 3 and 8 months orthodontically treated dental pulp compared to control dental pulp.*

after artificial cavity preparation in the teeth (18). Other studies reported more contiguous odontoblasts positive staining of Bcl-2, especially in those underlining the dental cavity (19-22). Apoptotic odontoblasts can release fragmented DNA into the surrounding mineralized dentine matrix which might contribute to a contiguous false positive staining for the matrix, especially if decalcification of the specimens is not sufficient. In addition, phagocytosis of the apoptotic odontoblasts by scavenger cells may also result in the discontinuous staining pattern. In this study, the odontoblasts, after 8 months of orthodontic treatment showed a similar pattern of discontinuity, a time dependent decrease in Bcl-2 expression correlating with a relative increase in Caspase-9 (cleaved and not cleaved). Orthodontic tooth movement is based on force-induced on the periodontal ligament (PDL) and alveolar bone movement (23, 24); such a process is described by the necrosis of PDL on the pressure side with formation of a cell-free hyaline zone followed by osteoclastic resorption of the neighboring alveolar bone and bone apposition by osteoblasts on the tension side. During these events, the local reaction is inflammatory. It

has been also reported in experimental studies that stress produced by orthodontic forces would cause a marked increase in the staining intensity of interleukin (IL-1) and IL1β in all cell types of PDL (25, 26). Meanwhile, alveolar bone remodeling depends not only on activation, differentiation, and maturation of osteoblasts and osteoclasts, but also on cell apoptosis and necrosis. Orthodontic tooth movement is induced by mechanical stimuli and facilitated by remodeling of the PDL and alveolar bone. A precondition for these remodeling activities, and ultimately for tooth displacement, is the occurrence of an inflammatory process. Vascular and cellular changes were the first events to be recognized and described, and a number of inflammatory mediators, growth factors, and neuropeptides have been demonstrated in periodontal supporting tissues (27, 28). Their increased levels during orthodontic tooth movement have led to the assumption that interactions between cells producing these substances, such as nerve, immune and endocrine system cells, regulate biologic responses following the application of orthodontic forces. Mechanical stress evokes biochemical responses and structural changes in a variety of cell types. In sites in which inflammation and tissue destruction have occurred, cells may communicate with one another through the interaction of cytokines and other related molecules. Thus, it is important to elucidate more completely the complex cytokine cascade flow associated with inflammation-mediated tissue destruction at the molecular level (29-33).

For some time our group has been studying the expression of various peptides and enzymes on mucosal tissue (dental pulp and Wharton's Jelly). In particular, we reported the expression of NOS (nitrossido- synthetase) on dental pulp orthodontically treated and not treated, showing a direct correlation between the time of treatment and the expression of NOS (34). Our group also reported that the orthodontic forces might accelerate the remodeling processes through the expression of MMPs (Matrix metalloproteinases) (35).

In this study it was demonstrated that orthodontic treatment down regulated Bcl-2 in the dental pulp, data showed that the longer the duration of treatment the greater was the downregulation. It is very likely that the traction forces inhibited the expression of the anti-apoptotic protein Bcl-2 thus favoring the predominance of apoptotic activity. The Bcl-2 data correlated very well with an up-regulation of Caspase 9 (cleaved and not cleaved) and histological alterations after 8 months of treatment. During the traction process, biochemical responses were likely to be evoked leading to delayed immunohistochemical and histological changes including decrease in number and less regeneration of Odontoblasts as well as the components of the extracellular matrix and MMP's. The hypothesis of how Bcl-2 is activated/deactivated is still controversial and raises many questions. Our findings showed negative regulation of Bcl-2 in those dental pulp subjected to orthodontic traction, suggesting that mechanical stress, as a physical entity, inhibits the binding of Bcl-2 to the apoptotic executioners like Bax and Bak, and with time the odontoblasts won't be able to get a replacement or regenerate.

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