HUMAN DENTAL PULP CELL APOPTOSIS: IMMUNOHISTOCHEMICAL STUDY AFTER APPLYING ORTHODONTIC TRACTION

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The aim of this study was to compare human dental pulp stress and programmed cell death after 3 and 6 months of orthodontic treatments by assessing the degree of apoptosis and related proteins. Human dental pulps were collected from twenty young patients orthodontically treated by Straight Wire technique. Samples were fixed, paraffin-embedded and processed for histology and immunohistochemistry using anti-heat shock protein 60 kDa (Hsp60), -caspase 3, -caspase 9, and -PCNA antibodies, as well as TUNEL reactions. Moreover, we performed immunoprecipitation for Hsp60 and caspase 3, and for Hsp60 and caspase 9, from paraffin extracted tissues. Increased levels of both caspases and Hsp60 occurred in 6-months treated samples; at the same time, we found increased levels of proliferating cell nuclear antigen and terminal deoxynucleotidyl transferase dUTP nick end labeling positive cells. Immunoprecipitation showed that Hsp60 forms a complex with both Pro-caspase 3 and Caspase 3, and this may accelerate Pro-caspase 3 activation, especially in the 6-months treated group. On the contrary, no complex between Hsp60 and Pro-caspase 9 was detected. The orthodontic tractions may be a cause of stress, apoptosis and proliferation in pulp tissue. These results suggest the need of further studies about the effects of long term orthodontic treatments on the dental pulp.

Dental pulp is a special type of loose connective tissue characterized by dental pulp cells and abundant amorphous extracellular matrix rich in proteoglycans and glycosaminoglycans, and poor in collagen fibres (1, 2). Moreover, dental pulp is rich of stem cells that are involved in tissue regeneration (3).

It is an open question whether the strength applied to teeth may cause a loss of pulpal viability (4-6). Indeed, it has been shown that dental pulp subjected to orthodontic traction (OT) shows morphological

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and structural changes, such as vacuolization, increase of extracellular matrix, and microcirculatory alterations just after an early treatment period (4, 5, 7). However, further tests are needed to confirm whether pulp remodelling after OT depends also on cell apoptosis.

In a previous paper by our team, we reported that dental pulp treated for 24 months and extracted 6 months after the end of OT showed irreversible damages, as demonstrated by a significant reduction of metalloproteinases MMP2 and MMP9 (8).

The present study covers a small population of patients subjected to OT with the aim to determine the possible m odifications of the levels of some of the most studied proteins in pulpal tissues involved in cell and tissue homeostasis, such as: (i) heat shock protein 60 kDa (Hsp60), one of the most important mitochondrial chaperone, crucial for cell survival after a number of stress events (9); (ii) Caspase 3 and Caspase 9, as two of the most studied members of the Caspase family, involved in apoptosis activation (10); (iii) proliferating cell nuclear antigen (PCNA), a marker for cell proliferation (11). In addition, the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed, as a well established method to determine the number of apoptotic cells in a tissue (12). In brief, this study investigated, mainly by employing immunomorphologic techniques, levels of cell stress, apoptosis, and proliferation of dental pulp cells after OT.

MATERIALS AND METHODS

Patients

Twenty patients 10 to 14 years old, (12 males and 8 females) with Class II malocclusion and severe to moderate crowding, according to the Index of OT Need, (UK), were referred for orthodontic assessment to Winchmore Hill Dental Practice in London (UK). In 5 cases, the extraction was performed before treatment (Group 1), in 8 cases the teeth were extracted after 3 months of applied orthodontic traction (Group 2), while in 7 cases extraction was performed after 6 months of orthodontic traction (Group 3). The treatments consisted of the straight wire technique using nickel-titanium archwires, with an increasing pressure applied to the teeth. The adopted procedures, conforming to the relevant ethical guidelines for human research, were in agreement with the Helsinki Declaration of 1975, as revised in 1983, and were approved by the local Ethics Council. A parental informed consent was obtained for each patient. In each case, a premolar tooth was extracted.

Tissue biopsy collection

Pulps were extracted, as Perinetti described (13, 14), by incising the tooth longitudinally using a thin diamond bur under a water jet. The cut stopped approximately 2mm before the pulp cavity to avoid pulp damaging due to the heat produced by the attrition between bur and hard dental tissue. The tooth was further split with a cutter and the pulp was maintained intact on one side of the split tooth.

Tissue processing

The extracted pulps were fixed in buffered formalin solution for 24 h. After fixation, the tissue was dehydrated in a graded series of alcohols, cleared in xylene, and embedded in paraffin. One section from each sample was stained with haematoxylin and eosin, for histological evaluation of the pulp tissue.

Immunostaining

Immunostaining by Streptavidin-Biotin Complex method (LSAB2 Kit peroxidase, DAKO, Cat. No. K0609) was performed, as previously described (15), using the following mouse primary monoclonal antibodies: Hsp60 (Sigma-Aldrich, Cat. No. H4149; dilution 1:300); Caspase 3 (Santacruz Biotechnology Inc., Cat. No. SC-7272; dilution 1:100); Caspase 9 (Cell Signal Inc, Cat. No. 9508; dilution 1:100); and PCNA (DAKO, Cat. No. M0879; dilution 1:100). The antibodies were diluted in a 0.1% bovine serum albumin (BSA) solution. The appropriate positive and negative controls were run concomitantly. Antibodies for Caspase 3 and Caspase 9 were able to recognize both precursors (pro-caspase) and cleaved (caspase) proteins, as described in manufacturer's datasheets. Fast red (Millipore), Amino Ethyl-Carbazole (AEC, DAKO, Cat. No. K346430) and 3,3'-Diaminobenzidine (DAB, DAKO, Cat. No. K0673) were used as revealing chromogens. Haematoxylin nuclear counterstaining was performed only for Caspase 3 and Hsp60 reactions. Slides were coverslipped using Dako Cytomation Faramount Aqueous Mounting Medium (Cat. No. S302580). Photography was performed by Leica Laborlux S Microscope with a Nikon DSL2 photo digital system.

TUNEL assay

The ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore, Cat. No. S7100) was used to label the free 3'-OH fragment DNA ends *in situ* with chemically labeled and unlabeled nucleotides (TdT-mediated). DNA fragments were labeled with the digoxigenin-nucleotide

and allowed, for 30 min in a humidified chamber at room temperature, to bind an anti-digoxigenin antibody conjugated to a peroxidase reported molecule. This bond was revealed using diamino benzidine (DAB) as a substrate. Finally, the specimens were washed with distilled water and mounted under a coverslip in an Aqueous Mounting Medium (Dako, Cat. No. S302580).

Staining evaluation

Each sample was analyzed with a "double-blind" system by two different operators. Moreover, the results were compared to an image analysis, obtained from digital TIF files, acquired with the multispectral system (16-18). To apply this method, sequential shots were made using CoKin® filters obtaining all the different color spectra. Adobe Photoshop® CS2 with image analysis tools was used to elaborate images (19, 20). Choosing the spectrum related to the chromogen, the image color profile was converted from RGB to CMYK. Then, the yellow channel was selected, as suggested by previous studies to be the one which has the best linear response to color intensity, and thus to protein expression (21). The quantification of colorimetric staining has been represented using a score with values from 1+ to 5+ (21). Numerical data obtained from image analysis have been statistically elaborated.

Immunoprecipitation

Immunoprecipitation to detect protein complexes (co-precipitating together) was performed, as previously described (22), in triplicate (three samples from different subjects for each group) after protein extraction from the paraffin sections, as previously described. The primary mouse monoclonal antibodies used were anti-human Hsp60 (SIGMA, Cat. No. H4149, dilution: 1:1000), anti-human caspase-3 (Santacruz Biotechnology Inc., Cat. No. SC-7272 dilution: 1:1000) and anti-human caspase 9 (Cell Signal Inc, Cat. No. 9508; dilution 1:1000). Briefly, antibody/protein complexes were immunoprecipitated with antibodies linked to sepharose A beads 4 fast few® (Amersham Biosciences, Cat. No. 17-0974-01). Non-specifically bound proteins were removed by repeatedly washing with isotonic lysis buffer.

Statistical analysis

Group data (mean +/-Standard Deviation) were obtained on the basis of the staining evaluation method described above. Differences between groups were analyzed using the student *t*-test. Correlation coefficients between caspases and Hsp60 results were evaluated using the Spearman rank method. Probability values of $P<05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Haematoxylin-Eosin staining showed vacuolization of dental pulp in the odontoblastic region (Ob) of Group 3 subjects (Fig. 1). By contrast, pulpal tissues from Groups 1 and 2 did not show any significant morphological alteration (Fig. 1).

The levels of Hsp60, caspases, TUNEL and PCNA positive cells in pulpal tissues were quantified. Statistical analysis data were summarized (Fig. 2 and Table I). In particular, levels of both caspases and Hsp60, as well as TUNEL positive nuclei, increased significantly after 6 months of treatment (Group 3), compared to other Groups (Fig. 2A). Moreover, we found a linear correlation between levels of Hsp60 and both Caspase 3 (Fig. 2B) and Caspase 9 (Fig. 2C), and between PCNA and TUNEL positive cells (Fig. 2D).

The immunohistochemical staining suggested that Hsp60, Caspase 3, Caspase 9, TUNEL and PCNA positive cells were present in all three groups but significantly increased in number in group 3 of dental pulp tissue. (Fig. 3). In addition, immunoprecipitation showed the presence of a complex between Hsp60 and both Pro-caspase 3 and Caspase 3 (cleaved form) in all examined conditions (Fig. 4A). Interestingly, the levels of the complex between Hsp60 and caspase 3 (activated form), in Group 3, dramatically increased, compared to Groups 1 and 2 (Fig. 4B). By contrast, we did not find any complex between Hsp60 and both Pro-caspase 9, and Caspase 9 (data not shown).

DISCUSSION

Apoptosis is one of the most studied mechanisms of cell death. It is a genetically regulated process of cell elimination, and plays important roles in both morphogenesis and pathogenesis of multi-cellular organisms (23, 24). It is activated by a variety of stress stimuli, including physical and mechanical ones (25, 26).

Caspase 9 is the initiator caspase induced by a number of stress signaling pathways that cause release of cytochrome c from mitochondria and activation of apoptosome that in turn cleaves the pro-enzyme of caspase 9 into the active form. The latter is able to activate other procaspases, such as pro-

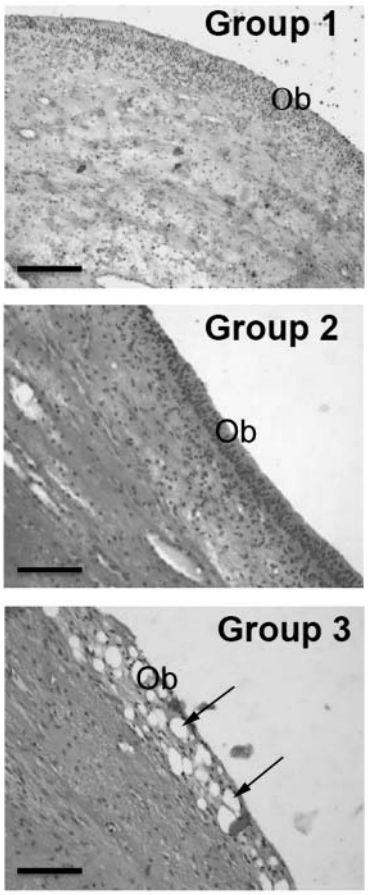

Fig. 1. *Haematoxylin-Eosin staining of dental pulp tissue in the three observed groups, showing clear vacuolization (arrows) of the dental pulp in the odontoblastic region (Ob) of group 3, while the dental pulp tissues from groups 1 and 2 did not show any significant morphological alteration. Bar: 100 micra*

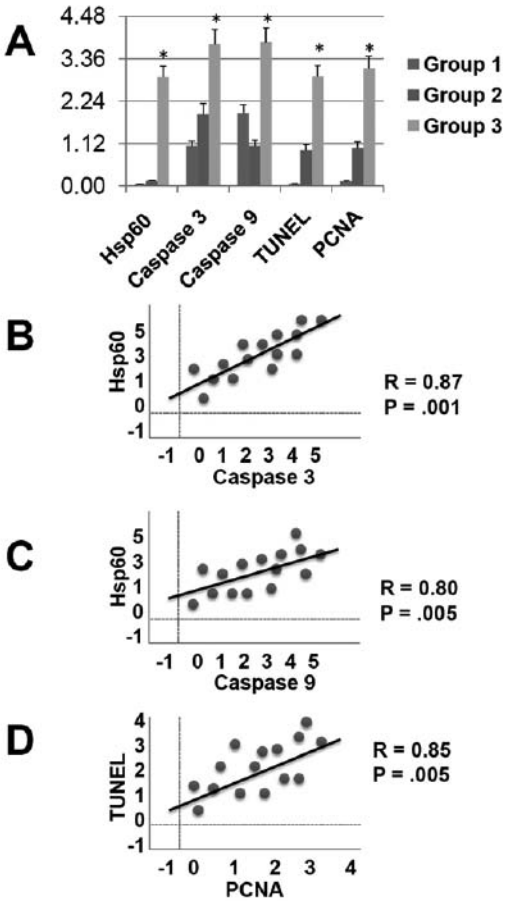

Fig. 2. *Graphical presentation of statistical analysis of immunohistochemical results showing significant increased levels of Hsp60, Caspase 3, Caspase 9, PCNA and TUNEL positive cells in group 3 compared to other groups (**A**). Linear regression analysis showed a correlation between Hsp60 levels and caspase 3 levels (**B**) and caspase 9 levels (**C**). The linear regression analyses showed as well a correlation between TUNEL and PCNA positive cells (**D**).*

caspase-3, that inactivates several cellular targets, such as poly-ADP-ribose polymerase, and induces DNA cleavage (27).

Several stress factors (e.g., hypoxia, chemotherapy, etc) induce mitochondrial release, not only of cytochrome c (28), but also of Hsp60 (29, 30). The latter accelerates pro-caspase 3 cleavage and apoptosis activation (31-34).

The mechanism of apoptosis is important for maintaining tissue homeostasis during tooth development. During enamel formation and dentinogenesis, apoptosis has been found in 50% of ameloblasts, odontoblasts and sub-odontoblastic regions (35). In relation to aging, apoptosis was found to be involved in pulpal chamber size decrease, due to secondary dentin deposition (36).These data shed some light on the effects of OT on pulpal cell apoptosis, an area that has still not been deeply investigated.

Moreover, PCNA is one of the most studied proliferation-associated protein. It was demonstrated to be a strong and sensitive indicator of cellular

Table I. *Means of evaluation and statistical analyses of immunohistochemical results.*

	Group 1	Group 2	Group 3	P (1 vs 2)	P (1 vs 3)	P (2 vs 3)
Hsp60	0.3+	0.9+	3.5+ (*)	n.s.	P<0.005	P<0.01
Caspase 3	1.8+	2.2+	4.1+ (*)	n.s.	P<0.05	P<0.05
Caspase 9	2.3+	1.8+	4.4+ (*)	n.s.	P<0.05	P<0.05
TUNEL	0.4+	1.1+	3.6+ (*)	n.s.	P<0.005	P<0.01
PCNA	0.8+	1.5+	3.2+ (*)	n.s.	P<0.05	P<0.01

Group data (mean ± Standard Deviation) were obtained after a staining evaluation was performed. Differences between groups were analyzed using the student t-test. Group 3 showed significantly higher values of immunopositivity compared to other groups (group 1 as a control group and group 2, where the patients were treated for 3 months by applied orthodontic traction before the dental pulp was extracted and evaluated) n.s.: not significant. (*): significant differences. Probability values of P <0.05 were considered significant.

growth fraction, because it is detectable throughout most of the cell cycle, during active DNA synthesis or cell division (11). Cell proliferation normally replaces cell death caused by necrosis or apoptosis, by regenerating the normal number of cells. Lost of the balance between cell proliferation and cell death may determine tumour failure or cancer development (11). In This study, data showed an increased expression of Hsp60 in patients treated for 6 months, compared not only to Group 1 (controls) but also to Group 2 (patients treated for only 3 months). This is an indication that a 6 months treatment constitutes more intense stress factor for the pulp tissue than a 3 months treatment. Clinically, after three months of treatment, teeth appear aligned. Consequently, we can conclude that the stress applied on the teeth should have been less. However, from the evidence obtained, it appears that the effect of the orthodontic traction for 6 months is a result of summation of multiple forces applied. Furthermore, it should be noted that each month the thickness of the wire increased, hence more pressure would have been applied on the teeth.

Caspase 3, Caspase 9 and TUNEL positivity were increased significantly after 6 months, indicating that such a stress was the cause of apoptosis of dental pulp cells. Concerning the caspase positivity detected in Groups 1 and 2 (whereby nuclear positivity by TUNEL was not significant), it might be due to the fact that the antibodies used were able to recognize both pro-enzymes and cleaved proteins, and the positivity was due to the presence of pro-enzyme in these tissues. Results of immunoprecipitation experiments for Hsp60 and caspase 3 were in agreement with such findings. There was a high quantity of pro-caspase 3 bound to Hsp60 in all groups, and high quantity of cleaved protein bound to Hsp60 in Group 3. Hence, we hypothesize that different time durations of treatment between Groups 2 and 3 were responsible for the incremental increase in the number of apoptotic events.

Results of linear regression analysis further confirm the hypothesis that increased levels of Hsp60 are associated to caspase activation. However, the search for a correlation between Hsp60 and TUNEL levels failed to give a significant result. This could be due to the limited number of cases included in the study.

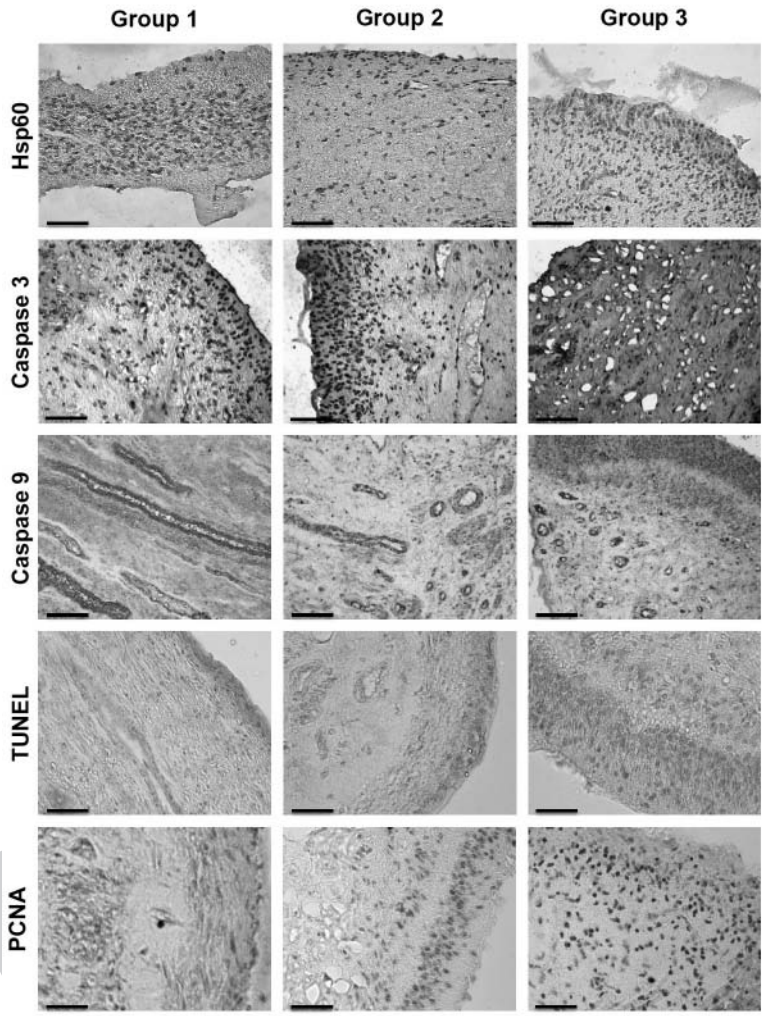

Fig. 3. *Panel of representative pictures of immunohistochemical staining for Hsp60, caspase 3, caspase 9, TUNEL and PCNA. All proteins were more abundantly present in group 3 specimens. Hsp60 was revealed by DAB chromogen and counterstained with haematoxylin. Caspase 3 was revealed by fast red chromogen and counterstained with haematoxylin. Caspase 9 was revealed by AEC chromogen and not counterstained (to reveal nuclear positivity, when present). PCNA and TUNEL were revealed by DAB chromogen and were not counterstained. Bar: 100 Micra*

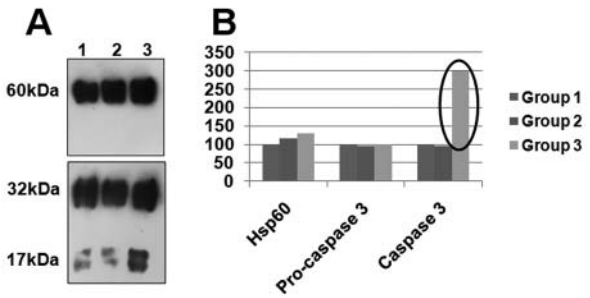

Fig. 4. ***A****: Immunoprecipitation of caspase 3 with Hsp60 antibody (above) and of Hsp60 with caspase 3 antidody (below); the latter recognizes both the precursor protein (pro-caspase 3, molecular weight: 32 kDa) and the cleaved, activated form (caspase 3, MW: 17 kDa).* ***B****: Semiquantitative band density measurement by arbitrary units showed that Hsp60/ caspase 3 levels of immunoprecipitate in group 3 specimens were higher (black circle) than in other groups.*

On the other hand, Hsp60 is one of the most studied member of the HSPs family, and is a highly conserved protein in the biosphere. It participates in mitochondrial protein folding, along with Hsp10 (37), but also in apoptosis, senescence and carcinogenesis. Activation of Caspase 3 by Hsp60 in our data was in agreement with what demonstrated by others (31, 32).

Finally, the PCNA results showed increased cell proliferation after 6-months of treatment. Interestingly, linear regression analysis showed a correlation between PCNA and TUNEL positivity. This is possibly due to the attempt of the dental pulp tissue to regenerate and replace dead cells.

In conclusion, our data supported the hypothesis that a 6-month OT is a stress capable of inducing both apoptosis and regeneration in dental pulp tissues. However, it is unclear which process was dominating. Hence, further studies are necessary to better understand the molecular mechanisms underlying the modifications in the dental pulp induced by orthodontic traction.

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