Detection of apoptosis in human periodontal ligament during orthodontic tooth movement

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ABSTRACT

Aim: To compare distribution of apoptotic cells in the Periodontium following Orthodontic force application, to study the apoptotic index and co-relate with different phases of tooth movement.

Material and methods: 100 patients, age 12-20 years, of class II div 1 malocclusion, were randomly divided into Groups I to V, requiring first premolars extraction with fixed Mechanotherapy. After leveling, canine was retracted using closed coil spring (100 gm forces). Surgical extraction of premolar was performed on 0, 3,7,14 & 21 days. Periodontium tissue was processed and apoptosis was evaluated by TUNEL assay. Apoptotic cells were counted from 4 different fields per slide and compared with the basal group.

Results: The mean Apoptotic Index increased from day 3 and peaked at day 7 for both compression and tension sides. On tension side, at days 14 & 21 apoptosis wasn't significant indicating earlier recovery. On compression side, there is more gradual decrease in apoptosis with lowest mean values at day 21, though not same as the basal level, indicating that the periodontal tissues require more than 21 days for complete recovery. The overall difference in apoptotic rate was statistically significant for both compression and tension sides.

Conclusion: Tissue response is a time-dependant normal physiological process where periodontal cells are cleared by apoptosis. The correlation coefficient value indicates the apoptotic activity increased with force on compression side & increased significantly on tension side too, signaling towards force-dependant direct relation between the two. Cells on tension side showed a more rapid rate of recovery as compared to compression side.

Keywords: Periodontium, Orthodontic, Mechanotherapy, Force-dependant

INTRODUCTION

The Mechanical force during tooth movement was reported to create compressed and cell-free areas, so called hyalinized tissue, in the periodontal membrane (Reitan and Rygh 1994). Hyalinized tissues were described as necrotic (Rygh 1972, 1973) or degenerating (Nakamura, Tanaka and Kuwahara, 1996) tissue from ultra-structural observations. The precise mechanism by which periodontal ligament cells disappear at the compressed area during tooth movement remains unclear. In the present study we examined whether periodontal ligament cells undergo apoptosis at the compressed area during tooth movement by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).

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OBJECTIVES

1. To study the Apoptotic Index in the periodontal tissue clearance during tooth movement.
2. To compare tissue changes in the Periodontium on the 3rd, 7th, 14th & 21st day after application of orthodontic force.
3. To correlate the above findings with different phases of orthodontic tooth movement.

MATERIAL AND METHODS

The study comprised of 100patients, age 12-20 years, having Class II division 1 malocclusion requiring first premolars extraction to be treated with Fixed Mechanotherapy using Standard edgewise Prescription (0.022"×0.028" slot). Leveling and alignment was done using .016", 018" and .020" stainless steel wires. Sentalloy closed coil springs were attached between canine & first premolars (200 grams force). Ethical clearance was obtained from the Ethics Committee of All India Institute of Medical Sciences, New Delhi with reference number (A-11-6/4/05) dated 8 August 2005. Written consent was obtained from each patient prior to their inclusion in the study. The patients were divided into five groups on the basis of the staging of the premolar extraction with respect to time. (Table 1)

1. Group I–no force was applied
2. Group II–force applied for 3 days
3. Group III–force applied for 7 days
4. Group IV–force applied for 14 day
5. Group V–force applied for 21 days

In Group I, the 1st premolar teeth were extracted prior to any force application. In the other groups, force was applied prior to 1st premolars extraction.

Procedure for Extraction of Premolar:
A Crevicular incision was made around the premolar tooth. Two vertical incisions were given on the buccal surface on the mesial and distal aspects of the tooth, followed by placement of two vertical osteotomy incisions with Tungsten Carbide Surgical burs, on the buccal surface in the mesial and distal aspect of premolar. Horizontal osteotomy cut joining the mesial & distal vertical cuts was given. During osteotomy, lingual cortical plate was preserved.

Tissue Sample Collection:
The extracted teeth, along with their Periodontium, were collected and were frozen at -70ºC and subsequently, fixed with Zambanis fixative solution. The fixed tissues were dehydrated with graded alcohol & embedded in paraffin. Sections of Periodontium, 6µ thick, were then cut & mounted on Polylysin-coated glass slides.

Henceforth, TUNEL assay was carried out to detect apoptosis. The Dead End™ Colorimetric TUNEL System is a non-radioactive system designed to provide simple, accurate and rapid detection of apoptotic cells in situ at single-cell level. The slides were observed under 20 X magnifications. TUNEL-positive cells were counted from 4 different fields on each slide, with 500 cells per field.

The Apoptotic Index, was determined using the formula

$$AI = \frac{\text{Total no. of cells showing apoptotic nuclei} \times 100}{500 \times 4}$$

STATISTICAL ANALYSIS
SPSS 12 software was used for all statistical analysis. The control group (group I) & the experimental groups (group II to V) were compared for the mean number of TUNEL- positive cells by applying one way ANOVA and Kruskal Wallis test. Statistically significant value was placed at $p<0.05$. Further the compression site was compared to the tension site in the experimental group by ANOVA test and $p<0.05$ was considered statistically significant.

RESULTS

Day 0, (Group I)
On compression side, the Mean Apoptotic Index was found to be 2.22±0.76 with a range of 1.87-2.5 (p value .001). On tension side, the Mean Apoptotic Index was found to be 1.22±0.46 with a range of 1.0-1.4(p value .014). The Mean Apoptotic Indices on compression & tension sides at day 0 had a direct correlation seen at .47 which was found to be statistically significant (p value .03).

Day 3, Group II
On compression side, the Mean Apoptotic Index was found to be 4.91±2.5with a range of 3.7-6.08 (p value .001). On tension side, the Mean Apoptotic Index was found to be 2.66±1.43 with a range of 1.9-3.3(p value .001). The Mean Apoptotic Indices on compression & tension sides at day 0 had a direct correlation seen at .63 which was found to be statistically significant (p value .003).

Day 7, Group III
On compression side, the Mean Apoptotic Index was found to be 12.75±2.9 with a range of 11.3-14.1 (p value .001). On tension side, the Mean Apoptotic Index was found to be 6.56±3.13 with a range of 5.1-8.03 (p value 14). The Mean Apoptotic Indices on compression & tension sides at day 0 had a direct correlation seen at .19 which was found to be statistically non- significant. (p value .41).

Day 14, Group IV
On compression side, the Mean Apoptotic Index ± S.D. was found to be 8.28±2.9 with a range of 6.9-9.6 (p value .001). On tension side, the Mean Apoptotic Index ± S.D. was found to be 3.53±1.77 with a range of 2.7-4.3 (p value .12). The Mean Apoptotic Indices on compression & tension sides at day 0 had a direct correlation seen at .67 which was found to be statistically significant (p value .001).

Day 21, Group V
On compression side, the Mean Apoptotic Index was found to be 5.47±1.23 with a range of 4.8-6.05 (at 95% confidence interval, p value .43). On tension side, the Mean Apoptotic Index ± S.D. was found to be 3.14±1.03 with a range of 2.6-3.6(p value .4). The Mean Apoptotic Indices on compression & tension sides at day 0 had a direct correlation seen at .46 which was found to be statistically significant (p value .04).

The Mean Apoptotic Index on compression side in control group at day 0 as 2.22±0.76. At day 3, the cell index had increased to 4.91±2.5 which was found to be statistically significant (p value .001). The Mean Apoptotic Count continued to increase and the

highest values were observed on day 7 (12.75±2.9). It decreased thereafter, as observed on day 14 (8.28±2.9) and day 21 (5.47±1.23).

The counts at day 7 & day 14 were statistically significant (p value .001) while the value observed at day 21 did not show a statistically significant difference when compared to day 0, although the count had not reached the observed count at day 0.

The Mean Apoptotic Index on tension side in control group at day 0 was 1.22±0.46. At day 3, the cell index had increased to 2.66±1.43 which was found to be statistically significant (p value < .05). The count continued to increase and the highest values were observed on day 7 (6.56±3.13) and decreased thereafter. The counts observed at day 14 (3.53±1.77) and day 21 (3.14±1.03) did not show any significant difference in their values and the mean indices were almost twice that at day 0 (1.22±0.46).

The cell count at day 7 was statistically significant (p value .001) but at day 14 & 21, no statistically significant difference (p value > .05) was observed when compared to day 0.

The overall apoptotic activity was found to be statistically significant for both compression and tension sides (p value < .05), irrespective of time.

A direct correlation of apoptotic activity between compression side & tension side was seen at day 3 (.63), day 14 (.67) & day 21 (.46), which was statistically significant (p value < .05).

At day7, the correlation had lower positive value (0.19) that wasn't statistically significant (p value >.05).

The overall correlation coefficient (0.73) of apoptotic activity on compression side with tension side was statistically significant (p value .001), signaling a direct relation between the two.

With respect to age and sex, no statistically significant difference in correlation of apoptosis on compression and tension sides was seen. (Table 2)

The apoptosis of periodontal cells on compression side with respect to time was statistically significantly (p value < .05) and on tension side was insignificant.(Fig 1)

Table 1: Distribution of sample into 5 groups

Group	Sample	Mean age	Duration after which force applied
Group I	10 M & 10 F	13.5 years	no force
Group II	10 M & 10 F	16 years	3 days
Group III	10 M & 10 F	14.29 years	7 days
Group IV	9 M & 11 F	14.2 years	14 days
Group V	11 M & 9 F	14.23 years	21 days

M: male, **F**: female

Table 2: Trend of average values of apoptotic index during the study on tension & compression sides

	0 days	3 days	7 days	14 days	21 days
Comp	2.2265	4.91	12.755	8.283	5.4715
Tens	1.2255	2.6605	6.569	3.5385	3.145

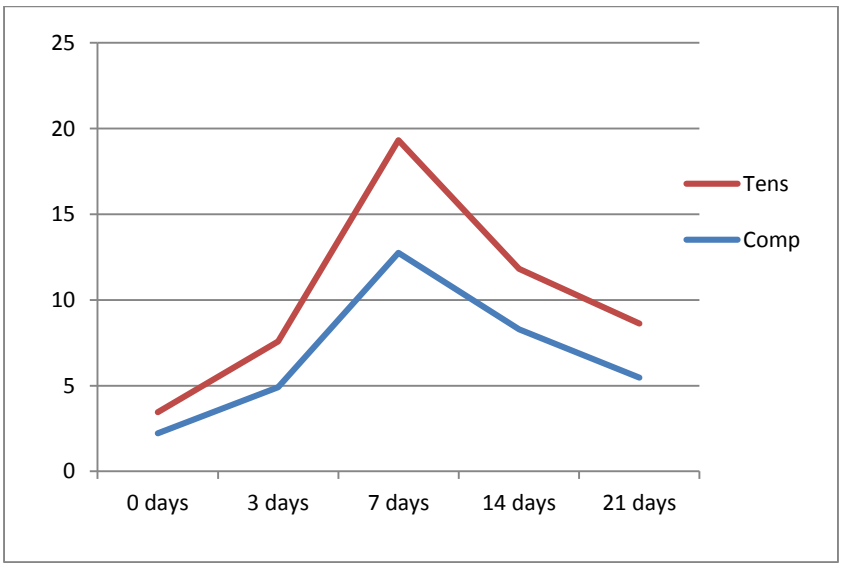

Fig. 1: Trend of average values of apoptotic index during the study on tension & compression sides

DISCUSSION

All connective tissues within the body are in a constant state of flux, synthesizing, degrading and reorganizing both the macro and micro molecular components of the matrix to maintain their structural and functional integrity. The state is considered to be of dynamic equilibrium wherein the catabolic and the anabolic processes act in synergy. This is of particular importance in the periodontal connective tissues, since the periodontal ligament is known to have a high cellular turnover rate and is under constant occlusal and non-occlusal loading forces.

The basis of orthodontic movement lies in the very fact that this dynamic equilibrium can be disturbed by the application of orthodontic forces which alter the local environment thus leading to selective areas of heightened cellular activity within the periodontal ligament which further leads to selective remodeling of the supporting alveolar bone. Under normal physiologic conditions, a tooth is considered to be at *'rest'* in its socket. Application of light orthodontic force is known to cause direct resorption of the adjacent bone interface as periodontal ligament vitality is preserved, thus allowing the osteoclasts to cause bone resorption from the adjacent vital periodontal ligament-bone interface. Thus, such forces are considered to be more physiologic and efficient in causing tooth movement. Thus, the control of orthodontic forces becomes extremely important and desirable to perform physiologic tooth movement.

One of the important indicators of direct resorption is the presence of apoptotic cells in the adjacent periodontal ligament bone interface. Studies have shown that apoptosis is marker of bone /connective tissue remodeling. Apoptosis plays a crucial role in developing and maintaining health by eliminating old, unnecessary and unhealthy cells without releasing harmful substances into the surrounding area.[1] The cell demise via apoptosis is a genetically controlled energy dependent, and takes place via a coordinated, predictable and predetermined pathway.

Jilka et al demonstrated that the missing osteoblasts die by apoptosis and those growth factors and cytokines produced in the bone microenvironment influence this process.[2,3,4] Drugs have also been known to effect apoptosis.[5,6]

In our study, we have tried to evaluate the efficacy of light orthodontic forces in causing direct remodeling of the adjacent bone surface studied by analyzing the number of apoptotic cells in the periodontal ligament of the teeth subjected to light forces at different time intervals, since apoptosis is an indicator of physiologic cell death and thus, would be more closely related to direct bone resorption. It has been already proved by W Zhong that cyclic stretching force induces early apoptosis of periodontal ligament cells.[7]

The main aim of our study was to determine whether apoptosis of periodontal cells occurs during orthodontic tooth movement and when apoptotic activity reaches a maximum level. It was found that significant apoptosis of cells does occur on both compression and tension sides, irrespective of time interval. Noxon et al reported that osteoclasts are at least cleared in part by apoptosis during experimental tooth movement in rats.[8]

As orthodontic force is applied, a signaling cascade ensues causing release of the biochemical molecules in periodontal ligament. These molecules in turn cause a transient inflammatory response and cell death leading to an increase in observed apoptosis. As we applied force over a period of 21 days, cell apoptosis started increasing from day 0 to day 3. It signifies that apoptotic changes start appearing in the early phase of orthodontic tooth movement and this time period varies from 0 to 3 days. Studies by Hamaya et al reveal that osteocytes showed apoptotic morphology at 6 hours, 12 hours and 1 day.[9] At 2 and 4 days, several osteocytes exhibited characteristics of necrosis and destructive images of the surrounding bone matrix. In similar studies by Hatai et al, TUNEL-positive staining of periodontal ligament cells began to appear at the compressed areas 12 hours after tooth movement in mice, being maximum at 24 hours and disappearing at 48 hours, with direct and undermining bone resorption beginning at the same area 72 hours after tooth movement.[10] PGE samples in alveolar bone peaked at 2 & 7 days (Joseph 1986), IL-1β and IL-6 was observed to reach a maximum on day 3 and to decline thereafter (Alhashimi et al, 2001).[11,12]

The biochemical signal molecules can also be detected in GCF during experimental tooth movement, provided plaque and other systemic conditions do not interfere with the cellular responses. The time period when the levels of these biomolecules are raised can be correlated with the onset of apoptosis (0 to 3 days in our study) and its peak activity (day 7 in our study). Of significance acid phosphatase is recognized as an important marker of osteoclast activity and bone resorption, whereas bone-specific alkaline phosphatase has been reported as a biomarker indicative of bone formation. Christenson [13] reported that alkaline phosphatase was observed to peak during the first 3 weeks of treatment, while acid phosphatase was seen to increase over the subsequent 3–6 weeks following initiation of treatment (Insoft *et al*).[13,14] IL-8 concentration in the GCF show gradual increase up to 10 days and declined on day 30 at the compression & tension sites (Tuncer et al).[15] Induction of IFN-gamma at both m-RNA and protein levels was significantly higher on day 3. The signal gradually became stronger on day 7 and remained high on day 10 (Alhashimi et al).[16] CD40 is a cell surface receptor

(expressed on monocytes, dendrite cells, and IL-6 or IL-8 secretion by ligation of endothelial cells, basophiles, epithelial cells, and fibroblasts) which belongs to the tumor necrosis receptor family (TNF-R). The strongest expression of $CD40^+$ was observed on day 3, decreased on day 7, and reached a low level on day 10 after application of orthodontic force. In contrast, in the treated animals CD40 ligand was expressed on day 3, the expression was enhanced on day 7, and was more pronounced on day 10. CD40L-expressing cells were found predominantly around hyalinized tissue in the resorption zone and the tension areas (Alhashimi et al).[17] Xiaozhe et al assessed the biological relevance between SFRP1 expression and the onset of apoptosis.[18] The number of TUNEL-positive fibroblasts gradually increased in the periodontal ligament 12 hours after the application of mechanical stress, sharply raised at 24 hours and peaked at 2 days. Simultaneously, an increased SFRP1 expression was seen in mice periodontal ligament during force-induced apoptosis.

As quoted in the above discussion, it can be seen that the initial phase varies between 0 to 3 days and peak between 4 to 7 days, these findings support our results of beginning of apoptosis and maximum apoptosis observed in our study.

Our study reveals that significant amount of apoptosis occurs at days 3, 7 and 14 on compression side and at days 3& 7 on tension side. Even Noxon et al, (2005) had reported that significant difference existed in the overall percentage of TRAP/ApopTag-positive nuclei between the control and the treatment groups at 3, 5, and 7 days.[4]

Maximum activity was seen at day 7 for both compression and tension sides. Rana et al suggested that maximum apoptosis occurs approximately 3 days after the insertion of appliance in the periapical tissue but the study was conducted in rats.[19] Though the peak apoptotic activity was usually observed around 2-3 days (as quoted in the above studies), but most of them were conducted in rats. The rate of metabolism varies in rats and humans the morphological changes, which take 2 days in humans to appear, are seen as early as 2hrs in rats. This might explain the variation in the time period of peak apoptosis observed.[20,21]

In our study, though significant apoptotic activity was seen at day 14 on compression side but it had started decreasing when compared to day 7 and as it approached day 21, no significant apoptosis occurred. Whereas on tension side, apoptotic activity had reduced at day 14 & 21, but it wasn't significant. This highlights upon the fact that removal of dead cells and their replacement on tension side begins after 7 days of force application but on compression side it starts after 14 days of force application These findings are supported in the study conducted by Mabuchi et al who investigated the cellular responses of periodontal ligaments during tooth movement and found that the ratios of PCNA-positive cells on the tension side 3 and 7 days after rubber block insertion were higher than those on the pressure side.[22] The ratios of PCNA-positive cells on the tension side were highest at day 3 after insertion and then decreased during the remainder of the experimental period. On the pressure side, the ratios of PCNA-positive cells increased up to day 10 post insertion, and then decreased from 14 to 28 days. The ratios of TUNEL-positive cells on both the tension and the pressure sides increased throughout the entire experimental period.

The level of activity on both compression and tension sides hadn't reached the basal level at day 21. Even Yijin et al revealed that maximum number of osteoclasts in PDL are seen from 2 weeks to 4 weeks during experimental tooth movement in rats with a positive correlation between the rate of tooth movement and osteoclast numbers, especially in young rats.[23] This clearly indicates that the cells take more time (i.e. >21 days) to recover to their state of physiologic equilibrium. Moreover, it is a well established fact that upon appliance activation, the stressed periodontal tissues need a period of at least 3-4 weeks for recovery. This again supports our findings why the mean apoptotic index could not reach the basal level at day 21.

A direct correlation between compression & tension was observed as increase in apoptotic activity with force on compression side led to its increase on tension side too, signaling towards a force-dependant direct relation between the two.

No correlation of apoptotic activity was seen with respect to age & sex which is in accordance to previous studies.

Present study is based on assumption that the morphology of alveolar bone is same in both the maxilla and mandible and hence, their physiological apoptotic activity will be same, regardless of whether maxillary or mandibular premolar is extracted.

CONCLUSION

- Orthodontic tooth movement is a physiologic process rather than pathologic that causes remodeling of tissues via apoptosis.
- As force is applied, significant apoptosis does occur with time on both compression and tension sides, with peak activity seen at 7 days.
- As apoptotic activity increased with force on compression side, the apoptotic activity increased significantly on tension side too, signaling towards a force-dependant direct relation between the two.
- The level of apoptosis on tension side starts reducing earlier indicating a more rapid recovery of cells as compared to compression side.

Hence, from our study we can conclude that tissue response to orthodontic tooth movement is a time-dependant normal physiological process; the periodontal cells are cleared by apoptosis.

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