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Secretory function of neutrophilic leukocytes of the patients with periodontal diseases

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SUMMARY

Aim of study was to examinate periodontal status among 20 44 year old patients and to study the secretory function of peripheral venous blood neutrophilic leukocytes (NL) exposed to various antigens and alkaline phosphatase (AP) activity in gingival crevicular fluid (GCF) in patients suffering from gingivitis and periodontitis. Clinicaly were determinted Russell's periodontal index (PI). Secretory function of NL affected by opsonized zymosane, non-opsonized E. coli was examinated in 77 patients with gingivitis and periodontitis, and in 35 donors, free of internal diseases, by means of β-glucuronidase (β-GD), lysozyme (LZ). NL secreted higher levels of β-GD in incubation medium in patients with periodontitis ($p \leq 0.001$) subject to degree of periodontal lesion. NL affected by various antigens secreted higher levels of LZ into non-cellular matrix in patients with gingivitis and periodontitis comparing to control environment in analogous groups. Data obtained from this study suggest that in patients with periodontitis response of NL to bacterial stimuli is specific and subject to the degree of periodontal lesion. Our study showed a significant difference of AP activity in GCF subject to pocket depth and degree of periodontal lesion. Once NL are exposed to corpuscules prone to phagocytosis, an increase in secretion of β-GD and LZ can be explained by overall increase in secretion of NL lysosomic enzymes, thus disclosing the mechanism of inflammatory periodontal tissue damage.

Key words: periodontal diseases, alkaline phosphatase, β-glucuronidase, lysozyme.

INTRODUCTION

Severe periodontal disease has been reported to affect from 5 to 20% of the population (1). Periodontal diseases are the result of the interaction of the periodontal microflora and the multifaceted host response to the infection, aspects of this complex interaction are being identified and evaluated for both their static relationship to disease severity and their association with future disease progression (2). The host reaction to gingival microorganisms is characterized in part by an influx of neutrophilic leukocytes (NL), which is one of the most important steps in host defense (3, 4). NL establish the first defense barrier against the microbial invasion (3, 4, 5), they contain the necessary material for killing pathogenic microorganisms (4). As a result of these interactions between the microbial flora and the host, a sequence of host immune mechanisms may be activated even at the expense of damaging the periodontal tissues (6).

Polymorphonuclear leukocytes contain a broad spectrum of acid hydrolases and neutral proteases.

Lytic enzyme release from NL such β-glucuronidase (β-GD) and elastase may contribute to the development of periodontal disease (7).

β-GD is a lysosomal acid hydrolase which has a significant role in connective tissue ground substance degradation, β-GD is involved in the degradation of glycosaminoglycans. Its action is on oligosaccharides generated by the action of hyaluronidase on the ground substance (8).

Studies on the relationship between periodontal disease and β-GD activity of crevicular NL have revealed, elevated levels of β-GD in gingival crevicular fluid (GCF) from local interaction of NL with endotoxin and other factors released from subgingival microorganisms (7). Levels of this enzyme are associated significantly with inflammation, pocket depth and alveolar bone loss (9, 10).

Lysozyme (LZ) is also found in crevicular fluid and saliva and acts against gram-positive and gram-negative bacteria (11). This enzyme acts on the beta-1, 4 glycosidic bonds of the peptido-glycans of bacterial cell walls and may be an important protective enzyme in periodontal diseases (12). Some studies have indicated a contradictory dependence between lysozyme levels and gingival inflammation (12). It is also found decreased concentrations of lysozyme in saliva of periodontitis patients but elevated levels in the crevicular fluid (11). Alkaline phosphatase (AP)-enzyme is enriched in the membranes of mineralizing tissue cells (osteoblasts) and is present in

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NL granules (12). AP is produced by many cells within the periodontal environment, the principal sources being, polymorphonuclear leukocytes, bacterial sources within supra-and sub-gingival plaque, and osteoblast and fibroblast activity, with a small contribution from serum (14). Preliminary data for AP levels in supra-gingival plaque measure and demonstrate extremely low levels of AP (14). If the source of GCF AP is primarily the NL, through secondary granule release, it would form a potentially powerful marker of inflammation (14). The immune response of the macro-organism to oral microbial flora and its toxins is ambiguous (13, 15).

The aim of the present study was to determine the β-GD and LZ activity in incubation medium of NL and AP in gingival crevicular fluid (GCF) taken from patients with gingivitis and periodontitis and donors with no inflammation of the periodontium.

MATERIALAND METHODS

Patients

Our study patients were selected from a large number of individuals with pathology of periodontal tissues who were examined clinically and radiographically and diagnosed as having gingivitis (gingival inflammation, bleeding detected during probing) or periodontitis (deep periodontal pockets, destruction of periodontal tissues surrounding the affected teeth and advanced bone loss). We included in our study only those patients with very marked signs of gingivitis and periodontitis using Russell's (16) periodontal index (PI): from 0.0 to 8.0 points.

The study was performed on 112 systemically healthy subjects within an age range of 18 to 44 years: 35 donors with intact periodontium, they were rated PI from 0.0 to 0.2 points, 40 patients with gingivitis, was rated according to PI from 0.3 to 2.0 points, 37 patients with periodontitis, was rated according to PI from 4.0 to 8.0 points.

Secretory function of peripheral venous blood neutrophilic leukocytes

The secretory function of peripheral venous blood NL was determined accordingly to technique described by Talstad et al (17). Peripheral blood (5 ml) was taken from subjects who had abstained from morning meals by means of a sterile vacuum test-tube containing heparine (20 IU/ml). Test-tubes were kept in a thermostat under an angle of 45 degrees at a temperature of 37^{O} C for one hour. Subsequently, plasma was pumped out and the leukocyte count was standardized to $1x10^9/l$ cells with the help of Hank's balanced salt solution. Every test-tube received 1.5 ml of plasma and 0.1 ml of stimuli of bacterial origin, the concentration of which in phosphate buffer was equal to 2 μg/ml. Prepared media were placed into a thermostat of 37^{O} C and kept for one hour. Phagocytes were activated by use of opsonized zymosane, non-opsonized *E. coli* ATCC 25922. Zymosane was opsonized accordingly to R. Zeiger et al (18).

Collection of gingival crevicular fluid

Small capillary tubes can be placed within or at the orifice the gingival crevice. By capillary action fluid enters the tube. The volume of fluid entering the tube can be precisely measured (19).

GCF was collected mesially and distally to each tooth after assessing the presence or absence of plaque, and registration of any other clinical parameters. The tooth was isolated with cotton rolls and dried with air.

Enzyme analysis

β-glucuronidase (β-GD) activity in incubation medium of NL was determined by J. Mead et al (20). Using 4-methylumbelliferyl-ß-D-glucuronide as a substrate, and a spectrofluorometer HITACHI MPF-2A (excitation wavelength -365 nm, emission wavelenght -450 nm) was utilized for that purpose. Alkaline phosphatase (AP) activity was determined in gingival crevicular fluid by use of automatic biochemical analyzer „Monarch" („Instrumentation Laboratory"). Lysozyme (LZ) was analysed using the spectrophotometric method (21), supplemented with *Micrococcus luteus 2665.*

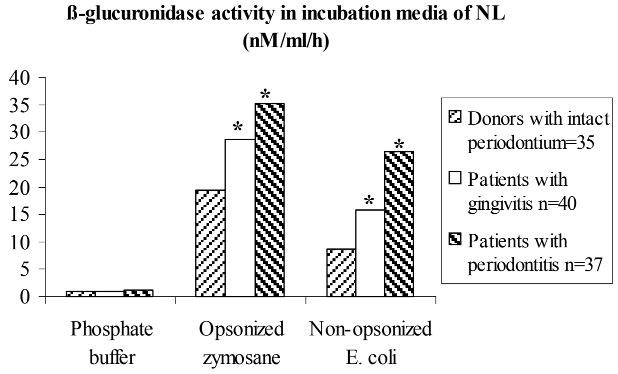
Reagents: 4-methylumbelliferyl-ß-D-glucuronide, 4-methylumbelliferone, Dulbeck buffer (pH 7.4), Hanks' balanced salt solution (pH 7.3) were purchased from Sigma Chemical Co (USA).

Statistical analysis

Results were calculated and statistic analysis performed by means of SPSS MS for Windows. The comparison of quantitative values was performed using Student's or Fisher's F criterion. Continuous variables were described as Mean ± SEM. A probability value of <0.05 was taken as the level of statistical significant. The study design and completion followed ethical guidelines for conducting studies at Kaunas University of Medicine.

RESULTS

Data in Figure 1 shows that β-GD activity in incubation media (without affectors) of NL obtained from peripheral venous blood was similar in healthy subjects and patients with gingivitis and periodontitis ($p>0.05$), was equal to 0.99 ± 0.01 nM/ml/h, 1.080.12 nM/ml/h, 1.250.11 nM/ml/h. However, when incubation media were affected with opsonized zymosane, β-GD activity highly increased in both groups of patients ($p \leq 0.001$) comparing to control environment, more markedly in patients with periodontitis, β-GD activity was dramatically higher in patients with periodontitis than in control group ($p \leq 0.001$)



* The were significant differences between donors with intact periodontium and patients with gingivitis and periodontitis in analogical incubation medium of NL ($p<0.05$)

Fig. 1. β-glucuronidase activity in incubation media of NL

and even significantly higher that in patients with gingivitis ($p \leq 0.01$). According to our data, β-GD activity highly increases ($p \leq 0.001$) when incubation media of NL is affected with non-opsonized *E. coli* both in control group and in gingivitis and periodontitis groups comparing to response of NL in incubation media with phosphate buffer. Once affected with non-opsonized *E. coli* β-GD activity was significantly higher in patients with gingivitis ($p \leq 0.001$) and periodontitis ($p \leq 0.001$) comparing to donors with intact periodontal tissues.

LZ activity in incubation media of NL significant rised (Figure 2) affecting with opsonized zymosane of patients with gingivitis and periodontitis, as well as of donors with intact periodontium ($p \leq 0.001$), comparing with control environments (phosphate buffer) in correspondent groups. LZ activity did not differ significantly in incubation media of NL of patients with gingivitis and periodontitis affected by opsonized zymosane (it was accordingly equal to 30.34.2 mg/L, 32.74.1mg/L) in comparison with the enzyme activity in analogues incubation media of NL of donors with intact periodontium. Significantly increased LZ activity was not determined in incubation media of NL with non-opsonized *E.coli*, of patients with gingivitis and periodontitis, as well as of donors with intact periodontium ($p > 0.05$), comparing with control environments (phosphate buffer). The increase in activity of NL secretion in non-cellular environment in patients with periodontitis was determined by exposing the incubation media to corpuscules prone to phagocytosis and bacterial toxins.

Data on AP activity in the samples of GCF given in Figure 3. According to our data AP activity in GCF patients with gingivitis and periodontitis was determined statistically significantly ($p \leq 0.001$) higher as compared to donors with intact periodontal tissues.

DISCUSSION

As has been shown by investigations in recent years (22) periodontal disease is initiated by subgingival infection with selective Gram-negative bacteria, but the presence of microorganisms alone is not the only factor responsible for periodontal destruction (3, 23). The responses of the host to periodontopathic microorganisms are thought to be critically important (24).

NL are the principal cells of the host defense system and the primary protective cells against periodontal diseases (25). Released granule components from infiltrating leukocytes, such as lysosomal enzymes and reactive oxygen species, which are normally intended to degrade ingested microbes, can also lead to tissue destruction and amplification of the inflammatory response (26). It is well known that the activity of gram-negative bacteria present in dental plaque results in release of contents of lysosomic granules into non-cellular environment by NL with subsequent suppression of bacterial adhesion and growth (27, 28) as well as destruction of bacteria in non-cellular environment. Stimulating factors induce an increase in NL activity which stops microbial invasion and can even damage the tissues of macro organism (29, 30, 31). In localized aggressive periodontitis, in particular, uncontrolled neutrophil recruitment and activation has been demonstrated to lead to the aberrant release of an array of noxious agents intended to fight the bacteria, with the potential for causing further tissue damage (32). Neutrophilic leukocytes have been implicated to play a destructive role in the periodontal tissue breakdown process due to high levels of lysosomal enzymes, generation of superoxides and reactive oxygen derivatives (33). Our findings support data from other studies showing the relationship of increase in β-GD activity in sulcus fluid and periodontal tissue lesions (34, 35). Differences were determined between control group and the group of patients with gingivitis and periodontitis in terms of neutrophilic leukocyte degranulation according to β-GD activity induced by opsonized zymosane, non-opsonized *E. coli*. Non-opsonized *E. coli* and particularly opsonized zymosane significantly ($p \leq 0.001$) induces β-GD activity that is dependent upon periodontal status. The highest activity was determined in patients with periodontitis and severe periodontal lesions, particularly when induced by opsonized zymosane. Lysozyme is found in crevicular fluid and saliva, and acts against gram-positive, and gram-negative bacteria (38), and is found decreased concentrations of lysozyme in saliva of periodontitis patients, but increase levels in the creicular fluid. No significant changes were observed neither in the crevicular fluid nor in the unstimulated or stimulated saliva (39). Our data show that LZ activity in incubation media of NL of patients with gingivitis and periodontitis was highly increased affecting with opsonized zymosane, comparing with control environments in correspondent groups. Recently Chapple et al (40) reported that monitoring AP activity in GCF permits prediction of periodontal attach-

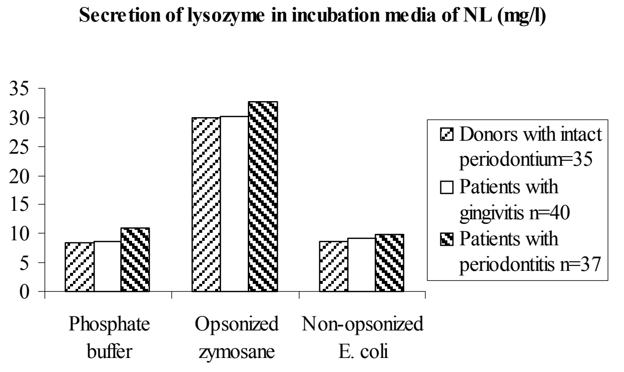


Fig. 2. Secretion of lysozyme in incubation media of NL

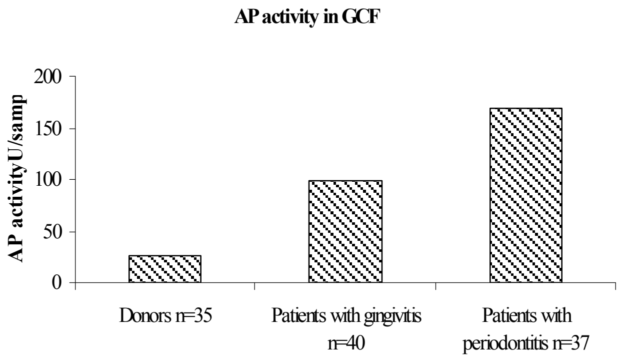


Fig. 3. AP activity in GCF

ment loss. AP level in sulcular fluid notably increases in cases of untreated periodontitis (36, 37). Increased AP activity in teeth bearing orthodontic appliances might be due in part to gingival inflammation produced by plaque-retentive appliances, independently of clinically detectable dental displacements (44). Our data determined highly increased dependence of AP activity in GCF on lesions severity of periodontal tissue. Having determined the increase AP in GCF and β-GD, LZ activity in NL under the action of various antigens, a discussion can be raised on the activation of secretion of the entire system of lysosomic enzymes in these cells. According to Wilton (43), non-cellular release of lysosomal factors by NL from gingival crevicular exudate is possibly more efficient than phagocytosis. Subsequently, proteolytic enzymes can be incorporated into the explanation of mechanism of development of periodontal disease and give scientific grounds for oral hygiene in prevention of inflammatory periodontal disease.

CONCLUSIONS

1. Peripheral venous blood NL excreted significantly higher levels of ß-glucuronidase in patients with gingivitis and periodontitis than in donors with healthy periodontium affected by opsonized zymosane and non-opsonized *E.coli* ($p \leq 0.001$).
2. Secretory activity of lysozyme was increased in patients with gingivitis and periodontitis comparing with control environment in analogous groups affected by opsonized zymosane ($p \leq 0.001$).
3. Alkaline phosphatase activity in gingival crevicular fluid highly increased dependence ($p \leq 0.001$) on lesions severity of periodontal tissue.
4. Proteolytic enzymes can be incorporated into the explanacion of mechanisms of development of periodontal disease and give scientific grounds for oral hygiene in prevention of inflammatory periodontal disease.

REFERENCES

1. Oliver RC, Brown LJ, Löe H. Periodontal Diseases in the United States population. J Periodontol 1998; 69: 269-78.
2. Lamster IB, Grbic JT. Diagnosis of periodontal disease based on analysis of the host response. Periodontology 2000; 7:83-99.
3. Seymour GJ. Importance to the host response in the periodontium. J Clin Periodontol 1991;18: 421-6.
4. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 2000; 80: 617-53.
5. Seymour GJ, Whyte GJ, Powell RN. Chemiluminescence in the assessment of the polymorphonuclear leukocyte function in chronic inflammatory periodontal disease. J Oral Pathol 1986; 15: 125-31.
6. Van Dyke IE, Serhan CN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. J Dent Res 2003; 82: 82-90.
7. Alpagot T, Duzgunes N, Wolff LF, Lee A. Risk factors for periodontitis in HIV patients. J Periodont Res 2004; 39: 149-57.
8. Podhrasky J, Jany Z, Velgos S. Beta glucuronidase activity in human gingiva in health and periodontal disease. Arch Oral Biol 1982; 27: 615-6.
9. Alpagot T, Wolff LF, Smith QT, Tran S. Risk indicators for periodontal disease in a racially diverse urban population. J Clin Periodontol 1996; 23: 982-8.
10. Lamster IB, Holmes LG, Gros KBW. The relationship of -glucuronidase activity in crevicular fluid to clinical parameters of periodontal disease. J Clin Periodontol 1994; 21: 118-27.
11. Syrjänen SM, Alakuijala P, Markkanen SO, Markkanen H. Gingival fluid, beta2 - microglobulin and protein levels as indicators of periodontal disease. Scand J Dent 1989; 97: 500-4.
12. McCulloch CAG. Host enzymes in gingival crevicular fluid as diagnostic indicators of periodontitid. J Clin Periodontol 1994; 21: 497-506.
13. Williams RC. A century of progress in understanding periodontal disease. Compend Contin Educ Dent 2002; 23(5 Suppl): 3-10.
14. Chapple ILC,Matthews JB, Thorpe GHG, Glenwrighl HD, Smith JM, Saxby MS. A new ultrasensitive chemiluminescent assay for the site-specific quantification of alkaline phosphatase in gingival crevicular fluid. J Periodontol Res 1993; 28: 266-73.
15. Fives - Taylor P, Meyer D, Mintz K. Virulence factors of the periodontopathogen Actinobacillus actinomycetem - comitans. J Periodontol 1996; 67: 291-7.
16. Russell A. A system of classification and scoring for prevalence surveys of periodontal disease.J Dent Res 1956; 35: 350-9.
17. Talstad I, Dalen H, Lehmann V. Degranulation and enzyme release during phagocytosis of inert particles and of bacteria by polymorphonuclear neutrophil granulocytes. Acta Pathol Microbiol Immunol Scand [C] 1983; 91: 403-11.
18. Zeiger RS, Twarog FJ, Colten HR. Histaminase release from human granulocytes.J Exp Med 1976; 144: 1049-61.
19. Mann WV. The correliation of gingivitis, pocket depth and exudates from the gingival crevice. J Periodontol 1963;34: 379-87.
20. Mead JA, Smith JN, Williamms RT. The biosynthesis of the glucuronidase of umbelliferone and 4-methylumbelliferone and their use in fluorometric determination of ā-glucuronidase. Biochem J 1955; 61: 569-74.
21. Parry R. M., Chandan R. C., Shahani K. M. A rapid and senditive assay of muramidase. Proc Soc Exp Biol Med 1965;119(4): 384-6.
22. Shelburne CE, Sandberg GP, Binsfeld CA, Wolff LF, Curry RA. Monoclonal antibodies to lipopolysaccharide of four oral bacteria associated with periodontal disease. J Periodontal Res 1993; 28: 1-9.
23. Ranney RR. Immunologic mechanisms of pathogenesis in periodontal diseases: an assessment. J Periodontal Res 1991; 26: 243-54.
24. Leone CW, Clark WB, McArthur WP. Host responses in the etiology and pathogenesis of periodontal disease. Curr Opin Dent 1991;1:29-36.
25. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 2000; 80: 617-53.
26. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal diseases: The challenge of anti-oxidants to free radicals and reactive oxygen species. Crit Rev Oral Biol Med 1999;10:458-76.
27. Buchmann R, Hasilik A, Nunn ME, Van Dyke TE, Lange DE. PMN responses in chronic periodontal disease: evaluation by gingival crevicular fluid enzymes and elastase - alpha - 1 - proteinase inhibitor complex. J Clin Periodontol 2002; 29: 563-572.
28. Labro MT. Host defense and infection.pp1-68. New York, etc.: Hoechst. M. Dekker Inc, 1994.
29. Levin MJ, Orechova LU. [The importance of immunological processes in pathogenesis of inflammatory periodontal diseases]. Paradontologija 1996; 65: 967-75. Russian.
30. Follin P, Wymann MP, Dewald B, Ceska M, Dahlgren C. Human neutrophil migration info skin chamber is associated with production of NAP - 1/IL8 and C5a. Eur J Haematol 1991;47:71-76.
31. Nurcombe HL, Bucknall RC, Edwards SW. Neutrophils isolated from the synovial fluid of patients with rheumatoid artritis, priming and activation in vivo. Ann Rheum Dis 1991; 50:147-53.
32. Buchmann R, Hasilik A, Van Dyke TE, Lange DE. Amplified crevicular leukocyte activity in aggressive periodontal disease. J Dent Res 2002;81:716-21.
33. Katz J, Sharabi Y, Chausho G. On the association between hypercholesterolemia, cardiovascular disease and severe periodontal disease. J Clin Periodontol 2001; 28: 865-68.
34. Layik M, Yamalik N, Caglayan F, Kilinc K, Etikan I, Eratalay K. Analysis of human gingival tissue and gingival crevicular fluid ā-glucuronidase activity in specific periodontal diseases. J Periodontol 2000; 71: 618-24.
35. Pouliot M, Clish CB, Petasis NA, Van Dyke TE, Serhan CN. Lipoxin A (4) analogues inhibit leukocyte recruitment to Porphyromonas gingivalis: a role for cycloxygenase-2 and lipoxins in periodontal disease. Biochemistry 2000; 39: 4761-8.
36. Dye BA, Vargas CM. The use of a modified CPITN approach to estimate periodontal treatment needs among adults aged 20-79 years by socio-demographic characteristics in the United States, 1988-94.Community Dent Health 2002; 19: 215-23.
37. Chapple IL, Garner I, Saxby MS, Moscrop H, Matthews JB. Prediction and diagnosis of attachment loss by enhanced chemilumi-

nescent assay of crevicular fluid alkaline phosphatase levels. J Clin Periodontol 1999; 26: 190-8.

38. Syrjanen S. M., Alakuijala P., Markkanen S. O., Markkanen H. Gingival fluid, beta - microglobulin and protein levels an indicators of periodontal diseases. Scand J Dent Res 1989; 97: 500-4.
39. Chapple IL, Garner I, Saxby MS, Moscrop H, Matthews JB. Prediction and diagnosis of attachment loss by enhanced chemiluminescent assay of crevicular fluid alkaline phosphatase levels. J Clin Periodontol 1999; 26: 190-8.
40. Chapple IL, Garner I, Saxby MS, Moscrop H, Matthews JB. Prediction and diagnosis of attachment loss by enhanced chemiluminescent assay of crevicular fluid alkaline phosphatase levels. J Clin Periodontol 1999; 26:190-8.
41. Jentsch H., Sievert Y., Göcke R. Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. J Clin Priodontol 2004; 31: 511-4.
42. Chapple ILC,Matthews JB, Thorpe GHG, Glenwrighl HD, Smith JM, Saxby MS. A new ultrasensitive chemiluminescent assay for the site-specific quantification of alkaline phosphatase in gingival crevicular fluid. J Periodontol Res 1993; 28: 266-273.
43. Wilton JMA. The role of the polymorphonuclear leukocyte in the control of subgingival plaque formation. J Periodontol Res 1982; 17: 506-8.
44. Perinetti G, Paolantonio M, D'Attilio M, D'Archivio D, Tripodi D. Femminella B, et al. Alkaline phosphatase activity in gingival crevicular fluid during human orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2002; 122: 548-56.

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