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ABSTRACT:
Tobacco smoking is one of the major risk factors for periodontal diseases. Prolonged and heavy smoking can reduce gingival bleeding and therefore mask the clinical marker of bleeding on probing. Reduced gingival bleeding occurs in smokers than nonsmokers due to vasoconstriction of gingival vessels; it may also be attributed to the heavier keratinization of the gingiva in smokers. Although it is well established that smoking increases the proliferation of cells in healthy and dysplastic oral mucosa, the effect of smoking on the proliferative activity of gingival keratinocytes has not been extensively evaluated in periodontally disease patients. Therefore, the aim of our study was to determine the effects of smoking on the proliferation of gingival epithelium in subjects with gingivitis and those with periodontitis immunohistochemically using monoclonal antibodies to proliferating cell nuclear antigen (PCNA).

Key Words: Immunohistochemistry, Mitotic Index, Proliferating Cell Nuclear Antigen, Smokers gingiva, Non-smokers gingiva.

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INTRODUCTION:

Periodontal disease is an infectious disease resulting in inflammation within the supporting tissues of the teeth with progressive attachment loss and bone loss [1]. A clear association has been demonstrated between smoking and periodontal disease [2,3]. However the clinical signs of inflammation such as bleeding on probing and gingival inflammation are masked in smokers than non-smokers [4]. This could be due to nicotine and cotinine which are the components of cigarette. Nicotine stimulates the

sympathetic ganglia to produce neurotransmitters including Catecholamines affecting the α-receptors on blood vessels which in turn cause vasoconstriction whereas cotinine a nicotinic metabolic byproduct has a peripheral constrictive action on gingival vessels. The other possible reason could be due to the heavy keratinization of the gingiva in smokers [4,5].

The prevalence, severity of periodontal destruction and tooth loss is increased in smokers compare to non-smokers, which is due to altered neutrophil chemotaxis, phagocytosis and oxidative burst[6-9] There is also an increase in inflammatory components TNF-α, PGE2, collagenase and elastase in Gingival crevicular fluid (GCF) of smokers when compared to non-smokers. Nicotine stimulates IL-1α synthesis by human gingival keratinocytes because of which there is more periodontal destruction. Smokers have more pathogenic microflora but smoking has no effect on plaque accumulation [10].

Outer epithelial surface of gingiva is inflexible, tough and resistant to abrasion resulting from the formation of surface layer of keratin. This keratinization helps in forming an impermeable barrier thus limiting the penetration of toxins and antigens produced by microorganisms present in oral cavity. The permeability barrier is believed to consist of lipids derived from the membrane coating granules and the tight junctions formed by the intimate association of keratohyalin granules with tonofibrils [11]. Nicotine was shown to induce the proliferation of oral keratinocytes because it displaces the local cyto-transmitter acetylcholine from the nicotinic receptor (nAchRs) expressed by oral keratinocytes [12]. Van Oijen et al speculated that the increased proliferation index in smokers was due to the regenerative effect that was explained by the local regenerative response to compensate for increased cell loss or damage by tobacco as well as the other protracted effects of smoking [13]. Thickening of the gingival epithelium and rete proliferation could be due to an imbalance in homeostasis between cell division and cell loss [14,15].

The proliferative activity of the oral mucosal and gingival epithelium has been analyzed for many years by monoclonal antibodies to agents as proliferating cell nuclear antigen (PCNA). It is one of the nuclear antigens; it is a 36 KDa auxiliary protein to DNA polymerase delta. It is expressed in the nuclei of cells during the DNA synthesis phase of cell cycle. PCNA is clamped to DNA through the action of replication factor c (RFC) which is a hetero-pentameric member of the AAA+ class of ATPases. Expression of PCNA is elevated in the nucleus during the late G1 phase, immediately before the onset of DNA synthesis; expression becomes maximal during S phase before declining during the G2 and M phases. The level of PCNA correlates directly with rates of cellular proliferation and DNA synthesis [16].

Prolonged heavy smoking can reduce gingival bleeding and therefore mask the clinical marker of bleeding on probing often used by dentists to

monitor periodontal health. Reduced gingival bleeding in smokers than non-smokers due to vasoconstriction of gingival vessels may also be attributed to the heavier keratinization of the gingiva in smokers [17]. Therefore, the aim of our study was to determine the effects of smoking on the proliferation of gingival keratinocytes in subjects with gingivitis and those with periodontitis immunohistochemically using monoclonal antibodies to proliferating cell nuclear antigen (PCNA).

SUBJECTS AND METHODS:

A total of 60 subjects from the patient pool attending the outpatient clinic, Department of Periodontics, Vishnu Dental College and Hospital were enrolled in the study. The institutional ethical committee and review board approved the study protocol. Informed consent was obtained from each patient. Subjects exhibiting good general health with history of smoking with no history of periodontal treatment or antimicrobial therapy for the past 6 months were selected.

Subjects were placed into 6 groups of 10 subjects each. Non-smokers with clinically healthy gingiva (NSH; n=10), non-smokers with gingivitis (NSG; n=10), non-smokers with periodontitis (NSP; n=10), smokers with clinically healthy gingiva (SH; n=10), smokers with gingivitis (SG; n=10) and smokers with periodontitis (SP; n=10). The groups NSH and SH were recruited as the control with a gingival index score of 0 for the non-smoker and smoker groups respectively. The criteria for gingivitis were no attachment loss and a gingival index of >1. Periodontitis tissues were collected during modified Widman flap technique by giving internal bevel incision[18] The criteria for selecting smokers was individuals who had smoked 100 or more cigarettes over their life time and smoked at the time of interview (current smokers – Centers for disease control and prevention CDC).

Tissue collection:

All gingival biopsies (one per person) were collected. Biopsy samples were taken from the marginal gingiva in healthy and gingivitis groups during crown lengthening procedures and from periodontitis group during periodontal surgery and were fixed in 10% neutral buffer formalin and paraffin blocks were prepared.

Immunohistochemistry:

The tissues were deparaffinized by two changes in xylene (10 minutes) each, put in descending grades of alcohol and then rehydrated with water. They were then transferred to citrate buffer and antigen retrieval was done using microwave method 3 cycles 5 min each at power 450W. Then the slides were dipped in two changes of phosphate buffered saline (PBS) for 5 minutes and then wiped carefully with gauge to remove excess PBS. The slides were treated with 3% hydrogen peroxide for 10 minutes, and put in two

changes of PBS and then treated with power block for 20 minutes [19].

The primary antibody PC10 (Bio-Genex) were added to tissue samples and was incubated at room temperature for 1 hour. After one hour, the sections were taken out and washed in two changes of cold PBS (10minutes each), a drop of super enhancer was added and the slides were incubated for 30 minutes. The slides were then washed in two changes of PBS 10 minutes each. Then the secondary antibody was added and incubated for 30minutes. Then the sections were washed and carefully wiped to remove excess PBS and a drop of freshly prepared Diaminobenzidine (DAB) as added onto sections. Slide were then washed in running distilled water to remove excess DAB and counter stained with Harri's hematoxylin. They were washed with acid alcohol and xylene. The tissue sections were mounted with disterene dibutylphthalate (DPX) for microscopic examination at magnification 40x [19].

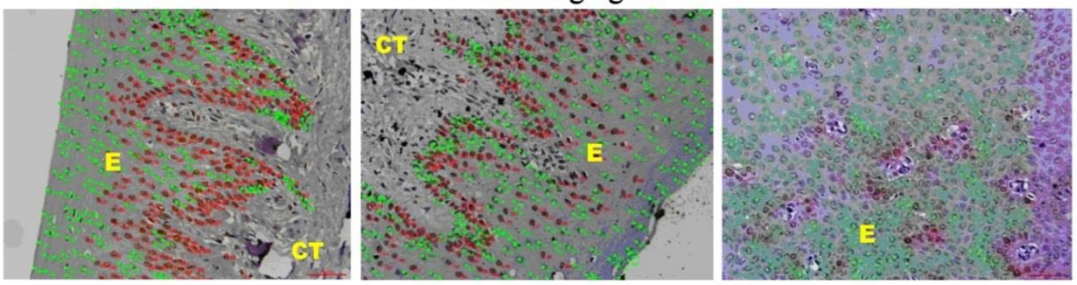
Evaluation:

The images were captured using BX 51 microscope with 0.5 adapter, 40X lens and DP 71 camera. 100 cells from the basal and parabasal layer were counted. The slide was divided into 5 grids and PC 10 expression was evaluated by counting the number of cells that were stained in brown color. The proliferation index was calculated by dividing the number of positively stained cells with the total number of cells in the magnified section. Data entry and descriptive analysis was performed using the SPSS statistical software program. Statistical analysis using paired T-Test was done.

RESULTS:

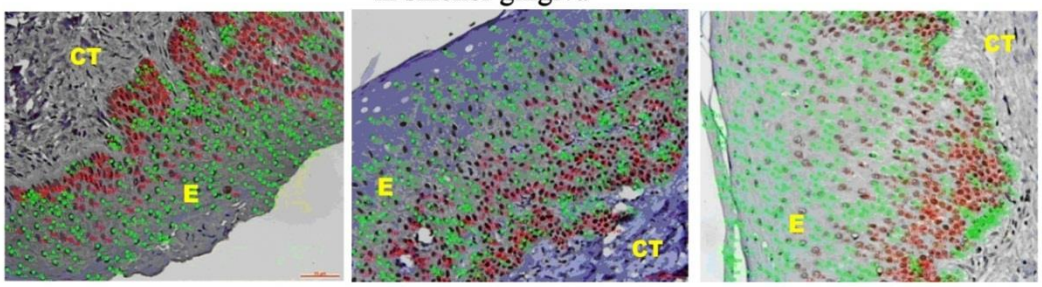
The proliferation index in non-smokers and smokers are shown in Figures 1. and 2. Cells with red nuclei were considered positively stained for PCNA. PCNA positive cells showed a uniformly dense red staining of nuclei, observed in basal and parabasal layers of the gingival epithelium. Highest numbers of PCNA positive cells are seen in smokers with healthy gingiva, least in non-smokers with periodontitis. Table1 shows comparison of proliferation index values within the non-smoker groups. Proliferation index values were statistically significant in NSH and NSG group compared to NSP group (P value < 0.05). Statistically significant difference was not observed between NSH and NSG groups. Table 2 shows comparison of proliferation index values within the smoker groups. Significant difference in the proliferation index values were seen in SH and SG group when compared to SP group (P value less than 0.05). However, no statistically significant difference was observed between SH and SG groups. Table-3 and figure-3 show highest proliferation index value in smokers gingiva compared to non-smokers gingiva (P value < 0.05)

Fig - 1 : PCNA staining in epithelium and connective tissue after cell counting in non-smoker gingiva



a - Normal gingiva b - Gingivitis c - Periodontis

Fig - 2 : PCNA staining in epithelium and connective tissue after cell counting in smoker gingiva



a - Normal gingiva b - Gingivitis c - Periodontis

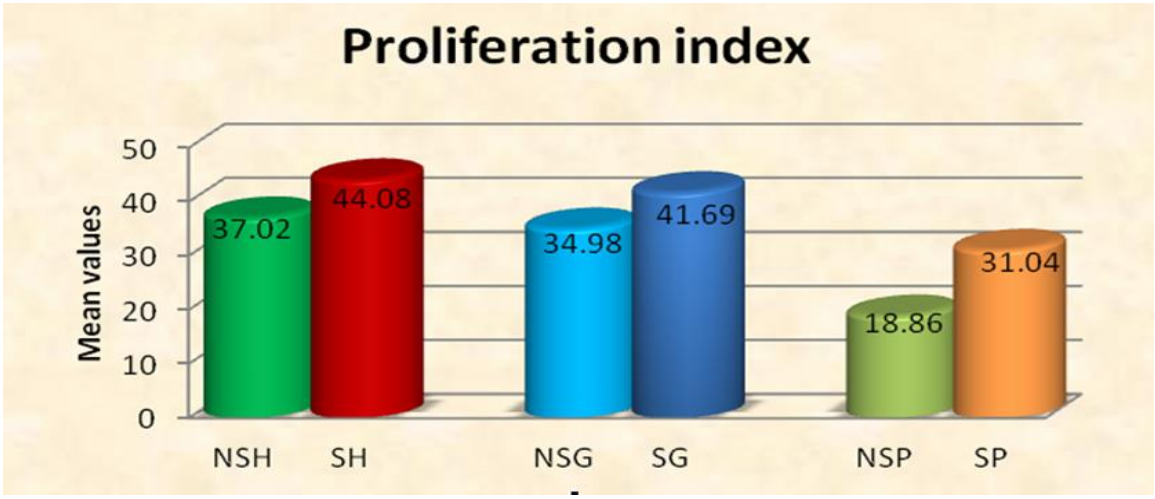


Figure 3: Proliferation index in smokers and nonsmokers gingiva
NSH: Non-Smokers with clinically Healthy gingiva; NSG: Non-Smokers with Gingivitis
NSP: Non-Smokers with Periodontitis; SH: Smokers with clinically Healthy gingiva
SG: Smokers with Gingivitis; SP: Smokers with Periodontitis

Table-1: PCNA INDEX Comparison within smoker and non-smoker groups

	N	Mean ± S.D	P VALUE
NSH	10	37.02 ± 5.44	0.044 Significant
SH	10	44.08 ± 5.99	
NSG	10	34.98 ± 4.79	0.007 Significant
SG	10	41.69 ± 7.42	
NSP	10	18.86 ± 10.07	0.019 Significant
SP	10	31.04 ± 8.65	

Statistically significant, if $p<0.05$

Table-2: Comparison between SH&SG, SH&SP and SG&SP.

	N	Mean ± S.D	P VALUE
SH	10	44.08 ± 5.99	0.417 Not Significant
SG	10	41.69 ± 7.42	
SH	10	44.08 ± 5.99	0.001 Significant
SP	10	31.04 ± 8.65	
SG	10	41.69 ± 7.42	0.012 Significant
SP	10	31.04 ± 8.65	

Statistically significant, if $p<0.05$

Table-3: PCNA index Comparison within non-smoker groups.

	N	Mean ± S.D	P VALUE
NSH	10	37.02 ± 5.44	0.331 Not Significant
NSG	10	34.98 ± 4.79	
NSH	10	37.02 ± 5.44	0.003 Significant
NSP	10	18.86 ± 10.07	
NSG	10	34.98 ± 4.79	0.002 Significant
NSP	10	18.86 ± 10.07	

Statistically significant, if $p<0.05$

DISCUSSION:

The present study revealed that proliferation index is higher in healthy group compared to gingivitis and periodontitis group. Table1 shows a significant difference in the proliferation index values between group NSH & NSP and NSG & NSP with a P value of <0.05 (figure3), which is mainly due to the inhibitory effect of infection on the proliferation of keratinocytes. Jarnbring et al [20] demonstrated fewer PC10 positive keratinocytes in the periodontitis group. This is due to the cell composition of the inflammatory infiltrate. T-cell and B-cell infiltrates could have both a negative effect on proliferation and a stimulating effect on apoptosis. T-cell products interferon-γ (IF-γ) and transforming growth factor-β (TGF-β) are both inhibitory for keratinocyte proliferation. Cytokines such as interleukin-4 (IL-4) and TGF-β have been shown to stimulate cell expression of P53 and promote apoptosis in the cell [20]

The proliferation index is highest for smokers with healthy gingiva (44.08 ± 5.99) table 2. The mean values were more in smoker group compared to non-smokers. Smoking alone had an effect on the proliferation of cells in the oral gingival epithelium regardless of the periodontal status of the subjects. Nicotine was shown to induce the proliferation of oral keratinocytes figure 4. Arredendo J et al [12] demonstrated that chronic stimulation of oral keratinocytes by nicotine alters the genetically determined program of the cell differentiation dependent expression of nAchRs subunits. Exposure of keratinocytes to nicotine also altered the mRNA and protein levels of cell cycle and cell differentiation marker PCNA. Therefore the downstream signaling from nAchRs expressed in the oral mucosa proceed via a pathway that up regulates the expression at transcriptional and translational levels of cell cycle progression regulators [12]. The findings of the present study are in accord with the study carried out by da Costa Filho et al [21] who detected an increase in the PCNA index in the gingival epithelium of smokers compared to non-smokers.

Analysis of the available data suggests that smokers may have a more pathogenic microflora. In that Bacteroides forsythus harbors subgingivally more in smokers than in non-smokers [22]. There was a tendency for Porphyromonas gingivalis, Prevotella intermedia, Eikenella corrodens, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans counts to be higher in smokers than non-smokers [23]. Plasma responses of smokers following lipopolysaccharide stimulation differed from those of non-smokers, in that smokers had significantly more TNF-α [24], IL-6, α2-macroglobulin, IL-1, IL-8 and monocyte chemotactic protein-1 levels [2]. This suggests that smokers have more severe disease compared to non-smokers. In this respect presence of high cell turnover in the keratinocytes, which are the major cell type of the

gingival epithelium is important for establishing and maintaining host-microorganism equilibrium. This may be the fact for higher mitotic index values in smokers compared to non-smokers in the present study.

Table 3 shows the mean values of proliferation index in non-smokers and smokers. The mean values were high in healthy group (NSH=37.02 ± 5.44, SH=44.08 ± 5.99) compared to gingivitis group (NSG= 34.98 ± 4.79, SG=41.69 ± 7.42) and periodontitis group (NSP=18.86 ± 10.07, SP=31.04 ± 8.65) figure 5. The results of the present study are supported by Gultekin SE et al [25] who demonstrated highest proliferation index for smokers with healthy gingiva and least for non-smokers with periodontits.

CONCLUSION:

Smoking induced the proliferation of gingival keratinocytes, regardless of the periodontal status of the subjects, because a significant increase in proliferation index was found in smokers compared to non-smokers. This heavy keratinization of gingiva in smokers can mask clinical signs of inflammation and reduce gingival bleeding which is an indicator of active tissue destruction. Hence clinical attachment level and probing pocket depth are reliable clinical findings for assessment of periodontal destruction in smokers.

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