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ESTIMATION OF KI-67 EXPRESSION IN GINGIVAL TISSUES: A CROSS-SECTIONAL STUDY OF SMOKERS AND NON-SMOKERS WITH AND WITHOUT CHRONIC PERIODONTITIS

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ABSTRACT

Introduction

Non-histone nuclear protein Ki-67 is expressed in proliferating cells during all active phases of the cell cycle; elevated expression of Ki-67 has been observed in several inflammatory and malignant conditions. It could potentially be considered the most precise markers for determining the tissue growth stage. Hence, the aim of this present study is to compare Ki-67 expression in gingival tissues of smokers and non-smokers with or without chronic periodontitis by immunohistochemistry.

Materials And Methods

Gingival biopsies from 40 participants, divided into four groups (non-smokers without chronic periodontitis (A), non-smokers with chronic periodontitis (B), smokers without chronic periodontitis (C), and smokers with chronic periodontitis (D)), were analysed. Ki-67 expression, inflammatory cell infiltrate, connective tissue density, and dysplasia features were assessed using immunohistochemistry and H&E staining. Mean and standard deviation were calculated, with $P < 0.05$ considered statistically significant.

Results

The smokers with periodontitis group had a greater mean number of Ki-67 positive cells per field. Comparison of Ki-67 cells per field between groups showed statistically significant differences between Group D and the first three groups. Additionally, H&E staining revealed epithelial dysplasia in Group D, supporting the immunohistochemistry findings.

Conclusion

The results indicate that smoking affects the cell cycle of the periodontium, with dysplastic features observed in patients with chronic periodontitis. Given these risks, it is crucial to implement comprehensive smoking cessation programs, including professional counselling and education on oral hygiene. Teaching patients how to maintain their oral health can help mitigate the adverse effects of smoking and improve periodontal health.

Keywords: Immunohistochemistry, Ki-67, Gingival Biopsy, Smoking, Chronic Periodontitis

INRODUCTION

Chronic periodontitis is a recognized global public health issue. It is defined as an infectious disease characterized by inflammation in the supporting tissues of the teeth, leading to progressive attachment loss and bone resorption. This condition results from the interaction between the host immune system and bacterial invasion. [1].

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The primary etiological factor is dental plaque, which, in conjunction with inflammatory and immunological responses, leads to destruction of the periodontium [2]. Smoking stands as a significant risk factor for periodontitis. It exerts its influence by modulating neutrophil activity, antibody formation, fibroblast functions, vascular factors, and the production of inflammatory mediators, thus stimulating the host immune response. Elevated smoking frequency has been associated with disturbances in both cellular and humoral immune responses, contributing to a deterioration in periodontal health. Moreover, the severity of periodontal disease tends to escalate with prolonged durations and increased intensities of smoking [3]. A fundamental secondary characteristic of periodontitis involves the loss of marginal alveolar bone, along with attachment loss facilitated by inflammatory mediators. [4]. One of the inflammatory mediators is ki-67.

Ki-67 acquired its nomenclature from its discovery in Kiel and the specific clone, 67th, observed in the initial 96-well plate. This protein serves as a marker for tumour grading, indicative of proliferation. Ki-67 expression is detectable across the G1, S, and G2 phases of the cell cycle, as well as in the G0 phase [5]. Paraffin-embedded sections undergo immunohistochemical (IHC) analysis to assess the Ki-67 labelling index. Clinical conditions exhibiting elevated Ki-67 proliferation often correlate with a poorer prognosis [6]. The region on human chromosome 10q26.2 is linked to the production of the non-histone nuclear and nucleolar protein Ki-67, which is encoded by the MKI-67 gene [7]. Ki-67 serves as a reliable marker for estimating tissue growth rates [8]. In patients with gingival overgrowth, both gingival epithelial cells and fibroblasts within the lamina propria exhibit Ki-67 expression [9]. The aggressiveness of tumour cells shows a stronger correlation with tumour cell proliferation rate, which in turn influences the clinical and behavioral outcomes. [5].

TGF-β distinctly boosts the expression of Ki-67. The mitogenic effect of PDGF on keratinocytes is evident in the increased expression of Ki-67, as observed in inflamed gingiva. In the context of chronic periodontitis, PDGF may play a role in upregulating Ki-67. Notably, nitric oxide acts as a significant modulator, contributing to the upregulation of Ki-67 in chronic periodontitis [10]. Smokers with chronic periodontitis experience increased tissue destruction, primarily due to cytokine imbalance. The free radicals present in tobacco smoke induce significant oxidative stress [10]. Considering the inflammatory nature of chronic periodontitis, characterized by elevated NF-κB levels, the concurrent presence of smoking is expected to further influence the proliferative activity of keratinocytes [10].

The aim of this study is to evaluate Ki-67 expression in gingival tissues of smokers and non-smokers, both with and without chronic periodontitis, by immunohistochemistry. Specifically, the objectives are: 1. To analyse and compare the differential expression of Ki-67 in the gingiva of non-smokers with and without chronic periodontitis.2. To analyse and compare the differential expression of Ki-67 in the gingiva of smokers with and without chronic periodontitis.3. To analyse and compare the expression of Ki-67 between smokers and non-smokers.4. To determine the role of Ki-67 in the pathogenesis of chronic periodontitis.

MATERIALS & METHODS

A total of 40 participants who attended the outpatient clinic of department of

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periodontics, at NIMS Dental college & Hospital were recruited for the study after giving their informed consent. The study was approved by the IEC of NIMS University NIMSUR/IEC/2022/292. The recruited subjects were divided into four distinct groups for the study.

Group A - Non-smokers without chronic periodontitis

Group B - Non-smokers with chronic periodontitis

Group C - Smokers without chronic periodontitis

Group D - Smokers with chronic periodontitis

Inclusion criteria

The study will involve systemically healthy individuals between the ages of 18 and 50 who have at least 20 erupted permanent teeth, excluding third molars, and are willing to participate. Our study particularly focuses on periodontitis patients who show clinical signs of inflammation related to local etiological factors, such as plaque and calculus & a gingival index score above 1, pocket probing depths greater than 4mm, attachment loss exceeding 3mm, and radiographic evidence of bone loss over 3mm.

Exclusion criteria

Patients with a history of systemic diseases such as diabetes, hypertension, cardiovascular diseases, anemia, and systemic disease-related periodontitis. Additionally, individuals with fixed prostheses or orthodontic appliances, as well as pregnant or lactating women and those who have used systemic drugs (such as antibiotics, steroids, anticoagulants, or immunosuppressive medications) in the past six months, will be excluded.

Sample collection and processing

Patients without systemic health issues requiring orthodontic extractions contribute to Group A samples, while samples for Groups B, C, and D are sourced from patients undergoing periodontal therapy at NIMS Dental College's Department of Periodontics. Using a surgical blade (*Figure 1*), the gingival papilla portion is excised and subsequently cleansed of blood with sterile saline before being fixed in a 10% buffered formalin solution for a full day. After fixation, the samples undergo block preparation, paraffin embedding, and dehydration in 100% alcohol. Following these steps, two 5 µm-thick slices are prepared: one for H&E staining and the other for immunohistochemistry. Analysis of inflammatory cell infiltrate grading [10], connective tissue density, and epithelial dysplasia [11] is conducted on the H&E-stained slides.

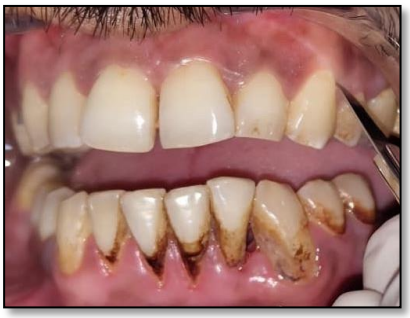

Figure 1: Gingival biopsy taken from interdental papilla region

Histopathological analysis

Immunohistochemistry

The Ki-67 detection method employs the streptavidin-biotin approach. To inhibit

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endogenous peroxide activity, a 3% hydrogen peroxide solution in tris buffered saline (TBS, pH 7.6) is applied for 10 minutes, followed by rinsing with TBS, after deparaffinization in xylene and rehydration in 96% ethanol. The sections are immersed in citrate buffer for 15 minutes to extract the antigen, then allowed to cool at room temperature. This process aims to reveal the antigen's epitope, as tissue fixation often leads to protein cross-linking, which can obscure the epitope. After cleaning with TBS, sections are incubated for 5 minutes to prevent alkaline phosphatase and peroxidase from reacting with nonspecific backgrounds. Primary antibodies against Ki-67 are then added, and the samples are incubated at room temperature for two hours. Following thorough rinsing with TBS, slides are covered with a secondary antibody for 10 minutes, and then streptavidin peroxidase is used for immune peroxidase labelling for another 10 minutes. Subsequently, a chromogen such as amino ethylene carbazole is utilized to visualize antibody binding. Finally, the sections are cleaned, mounted for inspection, and counterstained with Mayer's hematoxylin.

Statistical analysis

For every study group, the mean and standard deviations of clinical parameters & Ki-67 were calculated. One way ANOVA post hoc analysis was used to compare mean results between the various study groups. The level of significance in this investigation was determined at $P < 0.05$.

RESULTS

The mean age of participants in our study was 33.8 ± 8.6 years, with the youngest being 19 years old and the oldest 49 years old. Baseline clinical parameters were assessed for all groups. Descriptive statistics were performed to determine the mean plaque scores, gingival scores PPD (pocket probing depth) and clinical attachment level (CAL). The overall mean plaque score was higher in both smokers and non-smokers with chronic periodontitis (1.6 ± 0.20 and 1.59 ± 0.21, respectively). Similarly, the gingival scores (1.54 ± 0.25 and 1.45 ± 0.28), pocket probing depth (1.98 ± 0.21 and 2.1 ± 0.54), and clinical attachment level (1.98 ± 0.21 and 2.1 ± 0.54) were all relatively higher in Group B and Group D (*Table 1*).

Group (n)	Plaque score (Mean ±SD)	Gingival Score (Mean ±SD)	Pocket Probing Depth (Mean ±SD)	Clinical Attachment Level (Mean ±SD)
Group A: Non-smokers without Chronic Periodontitis (10)	0.38±0.10	0.50±0.16	1.40±0.11	1.4±0.11
Group B: Non-smokers with Chronic Periodontitis (10)	1.6±0.20	1.54±0.25	1.98±0.21	1.98±0.21
Group C Smokers without Chronic Periodontitis (10)	0.46±0.12	0.40±0.10	1.31±0.11	1.31±0.11
Group D: Smokers with Chronic Periodontitis (10)	1.59±0.21	1.45±0.28	2.1±0.54	2.1±0.54

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Table 1: Descriptives of clinical parameters among different groups

One-way ANOVA was performed to find the mean differences between the groups (*Table 2*). The mean Ki-67 cells were relatively higher among smoker patients with chronic periodontitis (Group D) at 60.1 ± 5.840, followed by non-smokers with chronic periodontitis (Group B) at 49.6 ± 8.872, smokers without chronic periodontitis (Group C) at 47.2 ± 6.268, and the lowest was observed in non-smokers without chronic periodontitis (Group A). Statistically significant differences were found between the groups.

Group(n)	N	Mean ±SD	F value	P value
Group A: Non-smokers without Chronic Periodontitis (10)	10	41.5±7.292	11.797	0.00*
Group B: Non-smokers with Chronic Periodontitis (10)	10	49.6±8.872		
Group C: Smokers without Chronic Periodontitis (10)	10	47.2±6.268		
Group D: Smokers with Chronic Periodontitis (10)	10	60.1±5.840		

Table 2: Description of mean Ki-67 cells among different groups

Post hoc comparisons were performed after the ANOVA (*Table 3*). The results showed a significant difference in the mean number of Ki-67 cells between Group A and Group D (p = 0.00), Group B and Group D (p = 0.014) & Group C and Group D (p = 0.002)

(I) Group	(J) Group	Mean Difference (I-J)	Sig.
Non-smokers without chronic periodontitis	Non-smokers with Chronic Periodontitis	-8.100	.096
	Smokers without Chronic Periodontitis	-5.700	.502
	Smokers with Chronic Periodontitis	-18.600*	.000
Non-smokers with chronic periodontitis	Non-smokers without Chronic Periodontitis	8.100	.096

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	Smokers without Chronic Periodontitis	2.400	1.000
	Smokers with Chronic Periodontitis	-10.500*	.014
Smokers without chronic periodontitis	Non-smokers without Chronic Periodontitis	5.700	.502
	Non-smokers with Chronic Periodontitis	-2.400	1.000
	Smokers with Chronic Periodontitis	-12.900*	.002
Smokers with chronic periodontitis	Non-smokers without Chronic Periodontitis	18.600*	.000
	Non-smokers with Chronic Periodontitis	10.500*	.014
	Smokers without Chronic Periodontitis	12.900*	.002

Table 3: Post hoc comparison among all groups

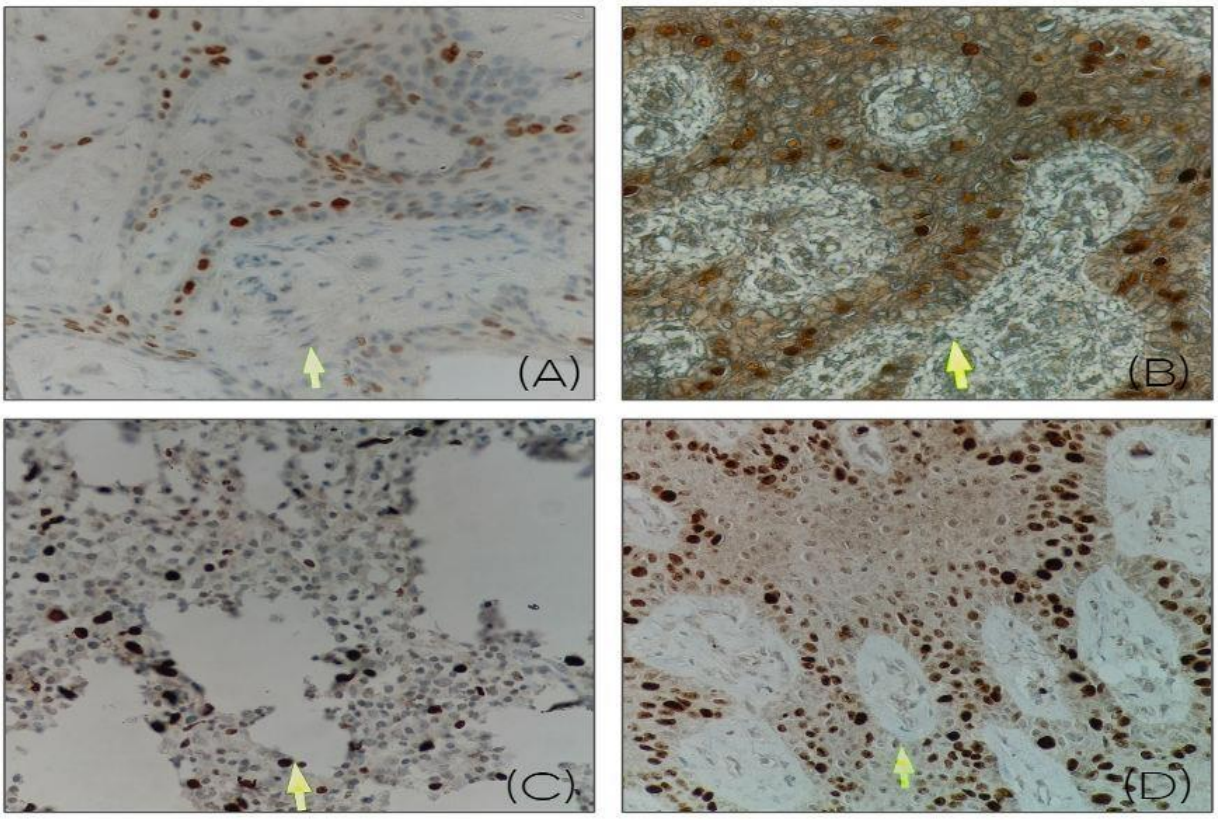

Figure 2: IHC staining of gingival tissues showing expression of Ki-67 among 4 different groups

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Sections stained by immunohistochemistry method showed positive results for Ki-67 cells found in the epithelium's basal and supra-basal layers in all four groups as in (*Figure 2A*) non-smokers without chronic periodontitis, (*Figure 2B*) non-smokers with chronic periodontitis, (*Figure 2C*) smokers without chronic periodontitis and (*Figure 2D*) smokers with chronic periodontitis.

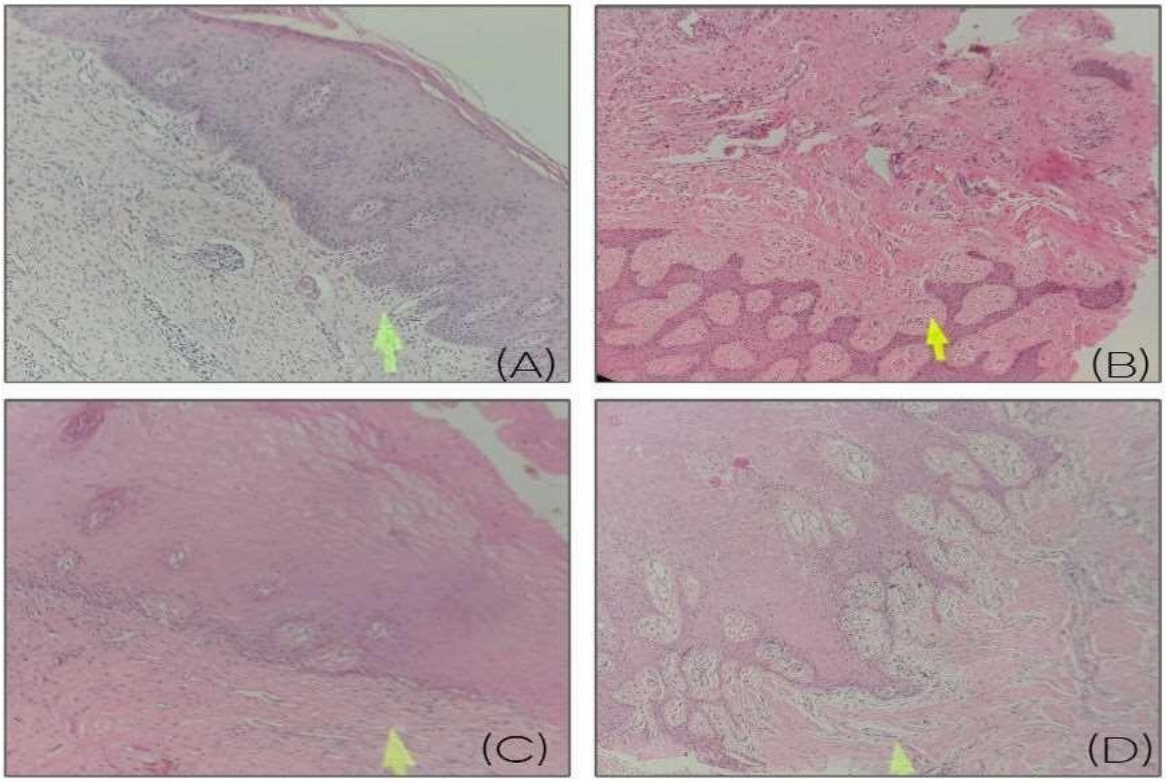

Figure 3: H&E staining of gingival tissues showing inflammatory cell infiltration and dysplasticfeatures among 4 different groups

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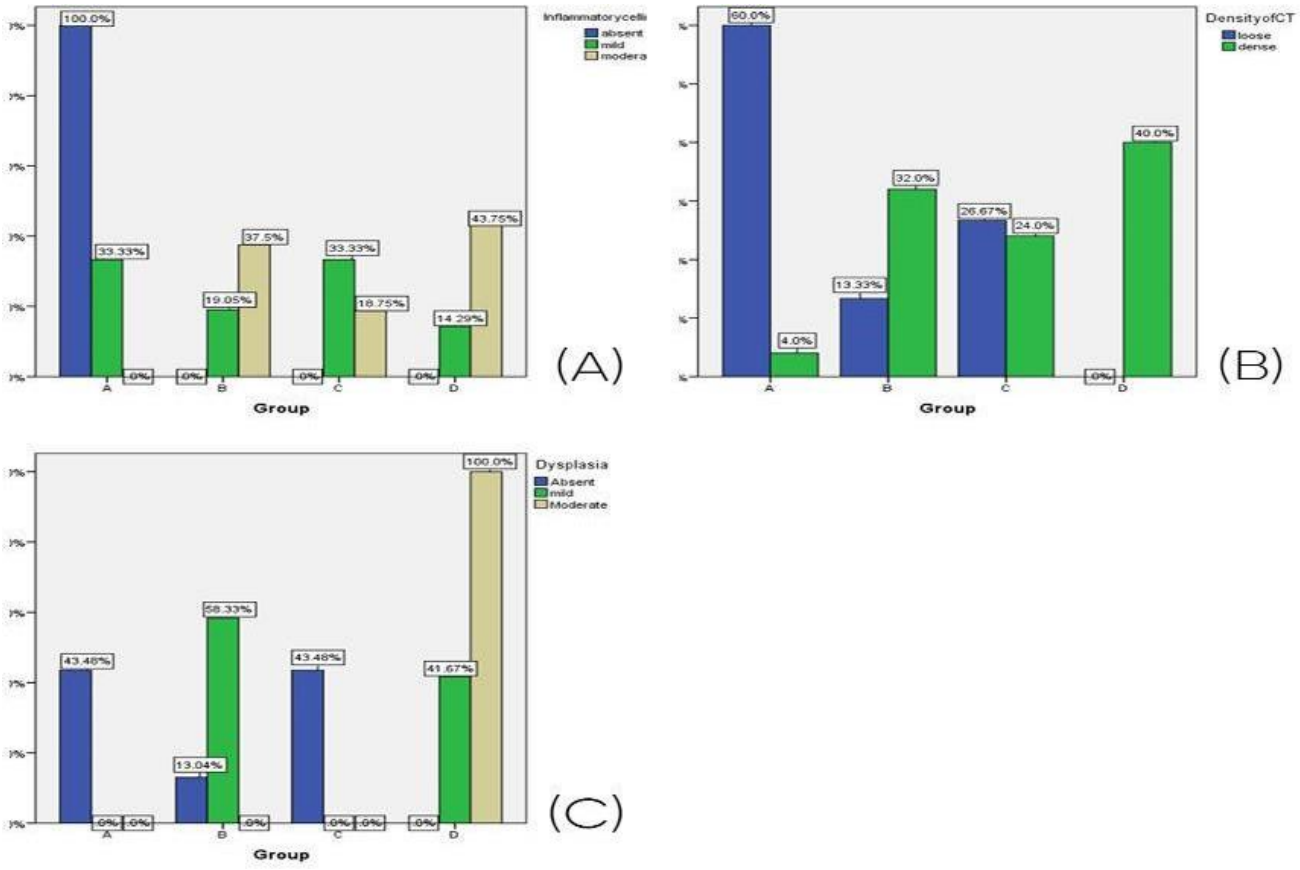

Figure 4: Graphical illustration of H&E staining among four different groups

The Ortho keratotic stratified squamous epithelium on the hematoxylin and eosin-stained slides had signs of increased melanin pigmentation and basal cell hyperplasia. Collagen fibers, dilated blood capillaries, and a few inflammatory cells were seen in the underlying connective tissue as in (Figure 3A) non-smokers without chronic periodontitis, (*Figure 3B*) non-smokers with chronic periodontitis, (*Figure 3C*) smokers without chronic periodontitis, (*Figure 3D*) smokers with chronic periodontitis.

Each bar graph illustrates the distribution of specific histological features (inflammatory cells, connective tissue fibre arrangement, and dysplastic features) among different groups of smokers and non-smokers with and without chronic periodontitis. The percentages are calculated based on the proportion of individuals within each group exhibiting the respective features and are visually represented in bar graphs for clear comparison.

Figure 4A Smokers showed the highest presence of inflammatory cells at 43.8%. Non-Smokers with Chronic Periodontitis exhibited a moderate number of inflammatory cells at 37.5%. Non-Smokers without Chronic Periodontitis, no inflammatory infiltrate was observed in this group.

Figure 4B depicts Connective tissue fibers were densely arranged in 40% of Chronic Periodontitis Smokers cases. Connective tissue fibers were densely arranged in 32%

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smokers. Non-Smokers without Chronic Periodontitis, Connective tissues were loosely arranged in 60% of cases.

Figure 4C illustrates that Mild dysplastic features were observed in 58.3% of Non-Smokers with Chronic Periodontitis cases & 41.7% of Smokers with Chronic Periodontitis cases. Moderate dysplastic features were observed in 100% of Smokers with Chronic Periodontitis cases, indicating a higher risk of malignant transformation in smokers with chronic periodontitis. This clearly shows that smokers exhibit a higher prevalence of dysplastic features, suggesting an increased risk of malignant transformation.

These figures collectively demonstrate the significant impact of smoking on inflammatory responses, connective tissue changes, and dysplasia in individuals with chronic periodontitis. Smokers show more severe inflammatory infiltration, denser connective tissue arrangement, and higher dysplastic changes, suggesting a greater risk of disease progression and potential malignant transformation. Non-smokers, particularly those without chronic periodontitis, exhibit fewer of these pathological features, indicating healthier tissue status.

DISCUSSION

The gingiva, an oral mucosal tissue, overlays the alveolar bone of the periodontium. Studies conducted by Narayanan et al. indicate the robust regenerative capacity inherent within gingival tissues [11]. Among various markers, Ki-67 stands out for its affordability, ease of use, and reliability [12]. Ki-67 immunolocalization was examined in gingival biopsies obtained from both non-smokers and smokers diagnosed with chronic periodontitis. Cellular division, proliferation, and mitosis represent prevalent aspects of periodontal healing and repair processes. Notably, Ki-67 emerges as one of the most effective markers for quantifying cell proliferation rates [10]. The aim of this study was to evaluate the differential expression of Ki-67 among four distinct types of gingival tissues.

Additionally, to ascertain whether tobacco use impacts cellular proliferation and periodontal healing within this group, smokers have been included in the study. The Ki-67 antigen was expressed in the epithelial basal and parabasal layers of all gingival tissue samples. The minimum expression seen in healthy (41.5cells/field) followed by smokers without periodontitis (47.2cells/field), non-smokers with chronic periodontitis (49.6cells/field), and smoker with periodontitis (60.1cells/field). Considering that normal gingiva exhibits a standard rate of cellular turnover compared to other tissues, Ki-67 expression was observed to be at its lowest level.

Based on the above findings, it is evident that tobacco exerts a significant influence on cell growth and proliferation. Research conducted by Arredondo et al. has documented that nicotine induces proliferation of oral keratinocytes [13] Van Oijen et al. conducted a notable study that suggests smokers exhibit a higher proliferative index, as assessed by Ki-67 expression, in comparison to non-smokers [14]. This phenomenon is likely attributed to the regenerative response of tissues, which serves to compensate for the increased cell loss induced by tobacco consumption.

In contrast to the two above mentioned groups, the chronic periodontitis group exhibited increased Ki-67 expression, attributed to the inflammatory cascade

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resulting in increased Ki-67 levels. Ambili et al.'s investigation revealed increased NF-κb levels in inflammatory periodontal tissues [15]. Moreover, Doger et al.'s research in cases of psoriatic dermatitis demonstrated a positive correlation between NF-κB, Ki-67 expression, and dermal cell turnover rate. It is conceivable that a similar mechanism underlying Ki-67 expression in healthy individuals may also be operative in periodontitis samples.
A similar study done by Preeti PL was conducted to know the expression of Ki-67 in four different groups. Smokers with periodontitis (52.73) exhibited the highest expression, with healthy individuals showing lowest expression of 24.61 cells/field [10]. The cellular proliferation by tobacco effect helps to explain this finding.

A study conducted by K. Kranti et al. [16] to analyse vasculo-endothelial growth factor (VEGF) and Ki-67 revealed that the periodontally healthy group exhibited negative staining, whereas the groups afflicted with periodontitis and periodontitis with managed type II DM displayed mild staining. Another study conducted by Sibel elif Gultekin [17] showed that the proliferating cell nuclear antigen (PCNA) percentage were higher than that of cells which are positive for Ki-67 in 4 types of groups to show regardless of periodontal state, the impact of smoking on cellular proliferation of epithelium of gingiva.

Smokers with chronic periodontitis have a higher amount of destruction due to cytokines dysregulation such as TNF-α and IL-β, even though they do not exhibit any of the classic inflammatory signs, such as colour alteration, consistency, or bleeding on probing. Numerous studies have demonstrated that tobacco smoke can result in noticeable oxidative stress and has a high concentration of free radicals [18].
A similar study proposed by Miguel A. Gonzalez-Moles et al [19], that the higher the severity of the dysplasia, the more frequently Ki-67 is expressed supra-basally, serving as an important marker for the existence of oral mucosa epithelial dysplasia.
In this present study, the degree of dysplasia was also analysed among the four different groups. In which smokers with chronic periodontitis (Group D) showed moderate dysplastic feature and no dysplastic feature was found in non-smokers without chronic periodontitis (Group A). This is observed because, certain cellular level alterations in the oral epithelium are caused on by tobacco use [20]. The degree of dysplasia may influence the rate of malignant transformation; lesions with high dysplasia may be 4.5 times more likely to experience this shift than lesions with mild dysplasia [21].

Furthermore, the study also examined the density of connective tissue fibers, revealing densely arranged fibres in smokers with chronic periodontitis (Group D) and loosely arranged fibres in non-smokers without chronic periodontitis (Group A). This disparity is attributed to the dose-dependent reduction of fibronectin and type 1 collagen formation induced by nicotine. Nicotine also impacts the proliferation and extracellular matrix of human gingival fibroblasts. Additionally, it has been proposed that nicotine may compromise the host's defences against periodontitis progression by diminishing gingival fibroblasts' ability to maintain gingival connective tissue integrity or facilitate wound healing during periodontal degradation or repair, while also promoting matrix destruction through increased fibroblast collagenase activity [24].

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In Sonmez et al.'s study [22], which utilized fibronectin as an indicator of nicotine's impact on fibroblast activity, no discernible differences were observed in the levels of fibronectin staining within the subepithelial connective tissue between smokers and non-smokers, or across subgroups.

In the present study, the count of inflammatory cell infiltrates was also analysed, revealing moderate inflammatory cell infiltration in smokers with chronic periodontitis (Group D) and mild to absent inflammatory cell infiltration in non-smokers without chronic periodontitis (Group A).According to a study by Sreedevi et al., a decrease in the number of inflammatory cells as demonstrated histo-pathologically may be the cause of the current study's decreased inflammatory symptoms.

Proinflammatory mediators and cytokines, including PGE2, have been associated to the destruction of periodontal tissue; nevertheless, inflamed periodontal tissues have also been shown to have higher amounts of growth factor [10]. TGF-β levels have been discovered to be significantly higher in chronic periodontitis, as Gurkan et al. have demonstrated [14]. Piekarska et al.'s study indicates that TGF β can significantly raise Ki-67 levels. It has also been demonstrated that HGF causes a rise in chronic periodontitis [10]. The research conducted by Kanayama et al. suggests that HGF can also regulate the cell cycle and Ki-67 expression [23] PDGF is another significant growth factor that aids in wound healing and periodontal regeneration. This substance affects many types of cells in the body in a mitogenic mechanism. According to a study by Rollman et al., gene transfer of PDGF causes keratinocytes to exhibit more Ki-67, indicating a mitogenic effect [24]. An immunohistochemistry investigation by Pinheiro et al. about periodontal disorders has revealed that inflamed gingiva, as opposed to healthy gingiva, expresses more PDGF [10]. This study provides evidence of the modulation of Ki-67 upregulation by locally produced PDGF in chronic periodontitis.

In comparison to the other three groups, smokers with periodontitis have the highest Ki-67 expression. The two causes of this are inflammation and the nicotinic impact [10]. In addition, H&E staining revealed features of epithelial dysplasia in smokers with chronic periodontitis (Group D) compared to other groups, supporting the findings of immunohistochemistry. Numerous studies have demonstrated that tobacco smoke can result in significant oxidative stress and produces a high concentration of free radicals [18]. Depletion of intracellular thiol compounds caused by locally produced ROS can induce oxidative damage and activate the extremely sensitive AP-1 and NF-b signaling pathways. As previously suggested, increased NF-b further accelerate cell division and increases Ki-67 level. Furthermore, the nicotinic effects of tobacco smoke on the neurotransmitter acetylcholine, and eventually on the activation of cell proliferation, should be recognized [19]. Hence, the compounded effects of smoking and inflammation on the tissues should be attributed to the elevated Ki-67 value observed in this group.

LIMITATIONS:

The study's constraints include solely focusing on smoking tobacco as a criterion, thus neglecting smokeless tobacco. Additionally, the sample size was limited; therefore, conducting the research with a larger sample size is considered for future research.

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CONCLUSION

The results indicate that smoking impacts the cell cycle of the periodontium, and dysplastic features are observed in patients with chronic periodontitis. Given these significant health risks, it is crucial to implement comprehensive smoking cessation programs for these patients. This should include professional counselling to support patients in quitting smoking, as well as thorough education on the importance of oral hygiene. Educating patients on how to effectively maintain their oral health can help mitigate the adverse effects of smoking and improve overall periodontal health.

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