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Effect of smoking on crevicular polymorphonuclear neutrophil function in periodontally healthy subjects

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Background: Polymorphonuclear neutrophils (PMNs) represent the first line of cellular defences in the gingival crevice. Smoking, as probably the most important environmental risk factor for periodontitis, has been shown to adversely affect many neutrophil functions.

Objective: The aim of this study was to investigate the influence of smoking on PMN numbers and function in periodontally healthy smokers and non-smokers.

Methods: Sixty subjects were recruited: 15 non-smokers, 15 light smokers (< 5 cigarettes/day), 15 moderate smokers (5–15 cigarettes/day) and 15 heavy smokers (> 15 cigarettes/day). Full mouth plaque index, sulcus bleeding index and probing depths were measured. Crevicular washings were obtained from all subjects to harvest PMNs. Numbers of PMNs, percentage viability, and percentage phagocytosis of opsonized *Candida albicans* were recorded.

Results: Mean plaque scores and probing depths were (non-significantly) increased in smokers compared to non-smokers. Mean sulcus bleeding index scores were significantly lower in moderate (0.10 ± 0.10) and heavy (0.07 ± 0.11) smokers compared to non-smokers (0.14 ± 0.13) ($p < 0.05$). Compared to non-smokers ($1.73 \pm 1.08 \times 10^6$/ml), the numbers of PMNs were higher in light ($1.98 \pm 0.96 \times 10^6$/ml) and moderate ($2.03 \pm 1.43 \times 10^6$/ml) smokers and were lower in heavy smokers ($1.68 \pm 1.18 \times 10^6$/ml), though there were no significant differences in PMN counts between the groups ($p > 0.05$). Percentage viability of PMNs was significantly lower in light ($77.6 \pm 7.8\%$), moderate ($76.5 \pm 8.2\%$) and heavy ($75.0 \pm 6.5\%$) smokers compared to non-smokers ($85.5 \pm 6.0\%$) ($p < 0.05$). Furthermore, the ability of PMNs to phagocytose was significantly impaired in light ($58.3 \pm 4.1\%$), moderate ($51.9 \pm 2.33\%$) and heavy ($40.9 \pm 3.5\%$) smokers compared to non-smokers ($74.1 \pm 4.1\%$) ($p < 0.05$), with evidence of a dose–response effect.

Conclusion: Cigarette smoking adversely affected PMN viability and function in this periodontally healthy population.

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Periodontitis is a common complex disease with a primary bacterial aetiology that results in up-regulated immune-inflammatory responses in the periodontal tissues, leading to breakdown of the tissues and the clinical signs of disease. It is now clear that the host response plays a critical role in disease pathogenesis (1), and pathogenic processes are modified by environmental and acquired risk factors such as diabetes and smoking (2). For example, smokers exhibit 2.6–6 times more periodontal destruction than non-smokers (3), and many studies have shown a reduced response to treatment in smokers compared to non-smokers (4, 5). Furthermore, reduced success rates for implant placement have also been described for smokers (6).

It has been postulated that the subgingival flora may differ in smokers compared to non-smokers, and that this may explain the differences in periodontal disease expression in the two groups. Studies have shown that smokers have higher plaque levels (7) and/or a more pathogenic flora (8) characterized by the presence of species such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* (9). However, other studies have reported no differences in the elimination of the periodontal microflora in smokers and non-smokers (10). Yet more studies suggest that certain putative periodontal pathogens are more difficult to eradicate in smokers (11, 12).

It is becoming widely accepted that smoking has a direct effect on homeostatic mechanisms in the periodontium, as well as any possible influence on the periodontal microflora. Thus, smoking results in changes in periodontal tissue vascularity (13, 14), altered fibroblast attachment and function (15, 16), suppression of osteoblast proliferation (17, 18), stimulation of osteoclasts (19), increased gingival crevicular fluid flow (20), altered polymorphonuclear neutrophil (PMN) function (21–26), decreased production of sIgA (27) or IgG (28, 29), and decreased lymphocyte proliferation (30).

The PMN constitutes the first line of cellular host defences against bacteria in the gingival sulcus (1). The importance of the protective role of PMNs in periodontal diseases is supported by the finding that patients with severe periodontitis have reduced numbers of PMNs and/or impaired PMN function (31). Smoking also has many significant negative effects on PMN function, including impaired phagocytosis (26), super oxide and hydrogen peroxide generation (32), integrin expression (24), and protease inhibitor production (33). Furthermore, in a population of patients with refractory periodontitis, of whom 90% were smokers, a defect in peripheral blood PMN function was reported (26), further suggesting that tobacco use may adversely affect PMN function.

To date, there have been no studies that have investigated the effect of smoking on PMN function in the gingival crevice. The aim of this study was therefore to assess PMN function in crevicular washes in smokers and non-smokers who were periodontally healthy.

Material and methods

Sixty dental students of the University of Jena were recruited to the study. All subjects were periodontally and systemically healthy. Subjects were excluded if they had received systemic antibiotics within the last 6 months. The 60 subjects were divided into four groups, each containing 15 subjects. Smokers were categorized as light smokers ($<$ 5 cigarettes/day), moderate smokers (5–15 cigarettes/day) and heavy smokers ($>$ 15 cigarettes/day). Fifteen never smokers constituted the control group. Demographic data were recorded for all subjects. The study was approved by the local ethics commission prior to commencing.

All subjects received professional tooth cleaning 2 weeks prior to samples being obtained. In order to continue participation in the study at the sampling appointment, subjects were required to demonstrate good oral hygiene, demonstrated by a plaque score $<$ 25%.

The study was of cross-sectional design with clinical variables being recorded at one occasion only. Clinical parameters recorded included the plaque index (34), the sulcus bleeding index (35) and probing depths, all measured at six sites per tooth by a single calibrated examiner. The clinical measurements were obtained 1 day after obtaining the crevicular washes.

Crevicular washes were obtained using a previously described method (36). Briefly, crevicular washes were collected in the morning 2–3 h after breakfast using phosphate-buffered saline in a micropipette. In each case, 15 sequential washings of the gingival crevice were obtained. Washes were collected in 1 ml Eppendorf tubes and centrifuged at 600 ***g***. for 10 min. The numbers of cells and their viability were counted using a Neubauer chamber with trypan blue exclusion as an index of cell viability.

The measurement of phagocytosis of crevicular PMNs was first described by Skapski and Lehner (37) and was modified by Sigusch *et al.* (36). *Candida albicans* (Institute of Microbiology, Friedrich-Schiller-University, Jena, Germany) was used as indicator particles to determine the number of PMNs containing and adhering to the *Candida* cells. The analyses were performed with a light microscope using a magnification of $\times$1000 immediately after stopping the phagocytosis process. With a stain solution of 0.2% Eosin Y and 0.4% Trypan blue it was possible to differentiate the adhered (purple) from the ingested *C. albicans*.

Means and standard deviations were calculated for data obtained within groups. Comparisons between smoking groups were performed using the Mann–Whitney–Wilcoxon test (*U*-test).

Results

Demographic and baseline clinical data are presented in Table 1. There were no significant differences in the mean ages of the four groups. In the non-smoking group, males and females were equally represented. However, there was a (significant) tendency for the proportion of males to increase with increasing cigarette consumption ($p < 0.05$).

Table 1. Demographic and clinical data

	Control group (non-smokers) $n = 15$	Light smokers (< 5 cigarettes/day) $n = 15$	Moderate smokers (5–15 cigarettes/day) $n = 15$	Heavy smokers (> 15 cigarettes/day) $n = 15$
Mean (range) age (years)	23 (19–28)	23 (20–27)	25 (21–28)	24 (20–31)
Gender (m:f)	8:7	4:11	8:7	11:4
PI (mean ± SD)	0.47 ± 0.26	0.59 ± 0.25	0.63 ± 0.22	0.59 ± 0.24
SBI (mean ± SD)	0.14 ± 0.13	0.11 ± 0.12	0.10 ± 0.10*	0.07 ± 0.11*
PD (mean ± SD) (mm)	1.45 ± 0.21	1.51 ± 0.16	1.65 ± 0.29	1.79 ± 0.40

*Significantly different from controls ($p < 0.05$).

The lowest plaque scores were recorded in the non-smokers compared to the smoking subgroups. However, no statistically significant differences were identified in plaque scores between the groups ($p > 0.05$). The sulcus bleeding index decreased with increasing cigarette consumption (from 0.14 in the control group to 0.07 in the heavy smokers). Indeed, the sulcus bleeding index in both moderate and heavy smokers demonstrated a statistically significantly lower sulcus bleeding index compared to the non-smoking control group ($p < 0.05$). There were no statistically significant differences in mean probing depth between the groups, but the greatest mean probing depths were measured in the heavy smokers (1.79 ± 0.40 mm) and the lowest in the control group (1.45 ± 0.21 mm).

Table 2 presents data relating to PMN numbers, viability and phagocytosis. The number of PMNs increased from the non-smokers to the light and moderate smokers. In heavy smokers, there was a reduction in the number of PMNs to a value less than that of the non-smokers (no statistically significant difference). In the non-smokers, 85% of PMNs were vital cells, but by contrast, in all the smoking subgroups, the percentage viability of PMNs was significantly reduced (range 75–78%) compared to the controls ($p < 0.05$). It is also notable that there was a trend for percentage viability of PMNs to decrease progressively as cigarette consumption increased, although there were no significant differences in percentage viability within the smoking subgroups.

The ability of PMNs to phagocytose *C. albicans* was also significantly impaired by smoking. All three smoking subgroups had significantly lower percentage phagocytosis scores (41–58%) than the non-smokers (74%) ($p < 0.05$). Furthermore, the percentage phagocytosis scores decreased significantly from one smoking subgroup to the next as cigarette consumption increased (Table 2).

Discussion

This cross-sectional study evaluated PMN numbers and function and clinical periodontal status in a cohort of periodontally healthy young adults who were either non-smokers or light/moderate/heavy smokers. Smoking has been clearly shown to be a risk factor for periodontitis, and many studies have compared clinical status in smokers and non-smokers. For example, higher plaque scores have been reported in smokers compared to non-smokers (38). Gunsolley *et al.* (39) observed in 193 subjects (of whom 51 were smokers) a significantly higher plaque index in smokers (0.57 ± 0.07) than in non-smokers (0.43 ± 0.03), data that are similar to those reported in our study. However, it still remains to be established with certainty whether or not tobacco smoking results directly in a local environment that is more favourable for plaque accumulation, or whether the increased plaque scores in smokers simply represent a behavioural difference compared to non-smokers.

Notwithstanding the increases in plaque scores that were observed with increased smoking, we identified a reduction in bleeding tendency as cigarette consumption increased. Reduced bleeding in smokers compared to non-smokers has been reported previously. For example, in a study of 289 subjects, a lower percentage bleeding score was identified in smokers (33.3%) compared to never smokers (43%) (40). A dose–response effect of smoking on bleeding on probing has also been reported in a study of 12,385 individuals (41) in which never

Table 2. Polymorphonuclear neutrophils function, viability and phagocytosis by smoking status

	Control group (Non-smokers)	Light smokers (< 5 cigarettes/day)	Moderate smokers (5–15 cigarettes/day)	Heavy smokers (> 15 cigarettes/day)
Number (10^4/ml)	1.73 ± 1.08	1.98 ± 0.96	2.03 ± 1.43	1.68 ± 1.18
Viability (%)	85.5 ± 6.0	77.6 ± 7.8*	76.5 ± 8.2*	75.0 ± 6.5*
Phagocytosis (%)	74.1 ± 4.1	58.3 ± 4.1*#	51.9 ± 2.3*#	40.9 ± 3.5*#

*Significantly different from controls ($p < 0.05$).
#Significant difference between the groups ($p < 0.05$).

smokers had a mean bleeding-on-probing score of 8.5% compared to values of 6.3% in light smokers (≤ 10 cigarettes/day) and 4.7% in heavy smokers (> 10 cigarettes/day). Thus, reduced bleeding on probing in smokers is a consistent finding in periodontal studies, and is related to the effect of cigarette smoke on the periodontal vasculature (13, 14).

Given that the subjects in our study were periodontally healthy, we did not anticipate that there would be any significant differences in mean probing depths between the groups, and indeed, this was the case. However, there was a tendency for mean probing depths to increase with increasing cigarette consumption. Similar observations have been reported previously, with, for example, mean probing depths of 1.7 mm in never smokers, 1.9 mm in light smokers and 2.1 mm in heavy smokers (41).

The main aim of this study was to evaluate the effect of smoking on the function of crevicular PMNs. PMNs in the gingival crevice represent the first line of (cellular) defences against invading bacteria (1) and have fundamental importance in periodontal pathogenesis. If PMNs control the subgingival plaque bacteria, the patient is more likely to be resistant to periodontal breakdown. However, if PMN defences are overwhelmed by the infecting bacteria, then inflammation may spread deeper into the periodontal tissues. PMN function is therefore critical to the efficacy of the host defences.

The data from our study strongly suggest that smoking impairs PMN viability and function. In smokers, the percentage viability and percentage phagocytosis were significantly lower compared to non-smokers (Table 2). The numbers of PMNs in the crevice were also (non-significantly) lower in heavy smokers compared to non-smokers, though there were also higher numbers of PMNs in light and moderate smokers compared to non-smokers. It is possible that nicotine has a stimulatory effect on numbers of PMNs entering the gingival crevice in lower doses, but an inhibitory effect at higher doses, though this remains to be confirmed (42). However, the increases in numbers of PMNs in light and moderate smokers were not reflected by any increase in vitality or phagocytosis in these smoking subgroups. Rather, viability and percentage phagocytosis were significantly reduced in light and moderate smokers compared to non-smokers. That is to say, even though there were (non-significantly) increased numbers of PMNs in the samples from light and moderate smokers compared to non-smokers, the PMNs in these smoking groups had greatly impaired function and ability to phagocytose, supporting the detrimental effect of smoking on PMN function.

PMNs are the predominant phagocytic cells in bacterial infections (43). Intrinsic PMN defects are associated with localized infections (44) including aggressive forms of periodontal diseases (45). Smoking, as an environmental risk factor for periodontitis, adversely affects many PMN functions, resulting in impaired phagocytosis, inhibited respiratory burst (32), and decreased production and expression of cellular components such as integrin (24) and protease inhibitor (33). The negative effects of smoking on PMNs were first described by Eichel and Shahrik (22) who reported reduced function and mobility of PMNs after smoking one cigarette. Changes in neutrophil morphology and morphometry after smoking that resulted in reduced function and depressed ability to adhere have also been reported (46). Reduced ability to adhere could be one reason for the decreased phagocytosis in smokers that was observed in our study. Similar findings have also been reported in salivary PMNs after one single episode of smoking (21).

To summarize, in a group of periodontally healthy subjects, there was clear evidence of a deleterious effect of smoking on PMN function, including reduced viability and reduced phagocytosis. There was evidence of a dose–response effect, with the greatest reduction in PMN function being seen in heavy smokers (> 15 cigarettes/day) compared to moderate and light smokers (up to 15 cigarettes/day). It is possible that the impaired PMN function seen in smokers may contribute to an increased risk for periodontitis in later life. These data further support the importance of smoking as a risk factor for periodontitis. Future studies are required to investigate PMN function in smokers and non-smokers who have periodontitis.

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