J Clin Periodontol 2005; 32: 441–447 doi: 10.1111/j.1600-051X.2005.00691.x

Journal of Clinical Periodontology

Experimental gingivitis in cigarette smokers

A clinical and microbiological study

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Salvi GE, Ramseier CA, Kandylaki M, Sigrist L, Awedowa E, Lang NP. Experimental gingivitis in cigarette smokers. A clinical and microbiological study. J Clin Periodontol 2005; 32: 441–447. doi: 10.1111/j.1600-051X.2005.00691.x. © Blackwell Munksgaard, 2005.

Abstract

Objective: To assess clinical and microbiological changes during experimental gingivitis in cigarette smokers and non-smokers.

Material and Methods: Eleven current smokers ($\geqslant$5 years) and 11 (never-) non-smokers periodontally healthy or with gingivitis (PPD $\leqslant$ 4 mm) were included. According to the original model (Löe et al. 1965), an experimental 3-week plaque accumulation period resulting in experimental gingivitis and a subsequent 2-week period of optimal plaque control were performed. Subgingival plaque samples were collected at Days 0, 21 and 35 from one site per quadrant, pooled and analysed using checkerboard DNA–DNA hybridization.

Results: Smokers {mean age: 24.7 $\pm$ 2.2 [standard deviation (SD)] years, range 21–28 years} had a mean lifetime cigarette exposure of 6.4 $\pm$ 2.8 (SD) pack-years, while non-smokers presented with a mean age of 23.1 $\pm$ 2.2 (SD) years (range 21–29 years). Between Days 0, 21 and 35, no statistically significant differences in mean Plaque and mean Gingival Index scores were observed between smokers and non-smokers. In both smokers and non-smokers, mean total DNA probe counts did not significantly differ nor increase between Days 0 and 21. Also, between Days 21 and 35 they did not significantly differ nor decrease. This was also true for the various proportions of bacterial complexes.

Conclusion: Both current smokers and (never-) non-smokers reacted to experimental plaque accumulation with gingival inflammation. Periodontal and microbiological parameters assessed in current cigarette smokers did not significantly differ from those in non-smokers during an identical period of experimental plaque accumulation. Hence, the early host response to the bacterial challenge appears to be of similar magnitude and timing in both groups.

Key words: bacterial challenge, cigarette smoking, experimental gingivitis, gingiva, gingivitis, inflammation, pathogenesis, subgingival microbiota

Accepted for publication 23 August 2004

There is general consensus that cigarette smoking represents a true environmental risk factor in the pathogenesis of chronic periodontitis. The role of cigarette smoking has been extensively documented in case-control, cross-sectional, longitudinal and intervention studies (for review see Kinane & Chestnutt 2000). Multivariate analyses indicated that, after controlling for confounding variables such as oral hygiene, plaque, calculus, socio-economic and demographic factors, cigarette smoking still remained an independent risk factor for the development and progression of periodontal disease. This risk is associated with estimated odds ratios in the order of magnitude of 2.5–6.0 (Bergström & Preber 1994, Bergström 2003). Clinical signs of periodontal disease have been shown to be more severe among current smokers compared with former smokers with the lowest risk being observed in never smokers (Goultschin et al. 1990, Bergström et al. 1991, 2000, Bolin et al. 1993, Haber et al. 1993, Grossi et al. 1994, 1995, Linden & Mullally 1994, Martinez-Canut et al. 1995, Gonzalez et al. 1996, Machuca et al. 2000). Exposure to tobacco smoking is usually expressed as the product of cigarette consumption per time unit (i.e. cigarettes per day) and the duration of exposure (i.e. number of smoking years) yielding a lifetime cigarette exposure expressed as ''pack-years''.

Potential molecular and cellular mechanisms in the pathogenesis of smoking -associated periodontitis include immunosuppression, exaggerated inflammatory cell responses and impaired cell

functions in oral tissues. These aspects are well documented in special forms of periodontal tissue destruction such as necrotizing, refractory and generalized aggressive periodontitis (MacFarlane et al. 1992, Horning & Cohen 1995, Magnusson & Walker 1996, Quinn et al. 1996).

Studies have suggested that smokers with periodontal disease display less gingival inflammation (Preber & Bergström 1986) and gingival bleeding (Bergström & Floderus-Myrhed 1983, Preber & Bergström 1985, Bergström & Boström 2001) when compared with non-smokers. This may be explained by the fact that one of the numerous tobacco smoke byproducts, nicotine, exerts local and transient vasoconstriction reducing blood flow, oedema and clinical signs of inflammation.

Little difference in the level of plaque accumulation has been shown comparing smokers with non-smokers (Bergström & Eliasson 1987a,b, Haffajee & Socransky 2001). Controversial findings, however, have been reported on the association between cigarette smoking and the prevalence and relative proportions of specific periodontal pathogens. Stoltenberg et al. (1993) found no significant differences between smokers and non-smokers in the prevalence of five microorganisms commonly associated with periodontal disease (e.g. *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis*). On the other hand, Zambon et al. (1996) tested the hypothesis whether or not smokers were at greater risk than non-smokers for the subgingival colonization with putative periodontal pathogens. These investigators demonstrated in a dose-dependent manner that smokers harboured significantly higher proportions of *Tannerella forsythia* and elevated counts of *A. actinomycetemcomitans* compared with non-smokers. Using 16S rRNA polymerase chain reaction (PCR) analysis, Umeda et al. (1998) reported a significantly increased risk for current smokers to harbour *Treponema denticola* in periodontal pockets. In this context, cigarette smoking has been documented to decrease oxygen tension in untreated deep periodontal pockets (Hanioka et al. 2000), thereby creating a favourable subgingival environment for the colonization of Gram-negative anaerobic bacteria.

The experimental gingivitis model has been widely used to evaluate plaque accumulation and the development of gingival inflammation during a period of abolished oral hygiene practices. This model represents a well-controlled clinical set-up of short duration predictably leading to plaque accumulation on clean tooth surfaces and consequently, to gingival inflammation. Hence, the aim of the present study was to assess clinical and microbiological changes occurring during the development of experimental gingivitis in periodontally healthy young cigarette smokers and to compare these findings with those of periodontally healthy (never-) non-smokers.

Material and Methods

The study was designed and conducted as a controlled experimental gingivitis following the model proposed by Löe et al. (1965). The study protocol was submitted to and approved by the Ethical Committee of the Canton of Berne, Switzerland.

The outline of the experimental procedures is summarized in Fig. 1.

Patient selection

In order to be enrolled, subjects had to meet the following inclusion criteria:

- Age $\leqslant 30$ years
- Cigarette smoking $\geqslant 5$ years
- No relevant medical conditions
- A dentition with $\geqslant 24$ permanent teeth
- No pocket probing depth $\geqslant 4$ mm
- Willingness to comply with all study requirements and signing informed consent

Patients were excluded for one of the following conditions:

- Pregnant or lactating females
- Periodontal attachment loss anywhere in the dentition
- Incipient carious lesions that could potentially exacerbate during a period of oral hygiene abstinence
- Antibiotic therapy within 3 months of the baseline examination
- Chronic medication known to affect the periodontal status within 4 weeks of the baseline examination
- Alcohol or drug abuse
- Stomatological diseases

A control group of non-smokers (i.e. subjects who had never smoked) were recruited from the student body of the University of Berne, Switzerland.

Clinical assessments and procedures

After a prophylaxis procedure, including instruction in optimal oral hygiene practices and a thorough scaling and polishing of the entire dentition, smoking and non-smoking subjects were asked to perform optimal tooth cleaning procedures for a period of 3–4 weeks. The ability to perform proper plaque control was assessed for each patient before entering the experimental phase. At the end of this pre-experimental phase, subjects were evaluated for the presence of plaque and gingivitis, the objective being the achievement of low Plaque (PlI) and Gingival Index (GI) scores at baseline compatible with gingival health.

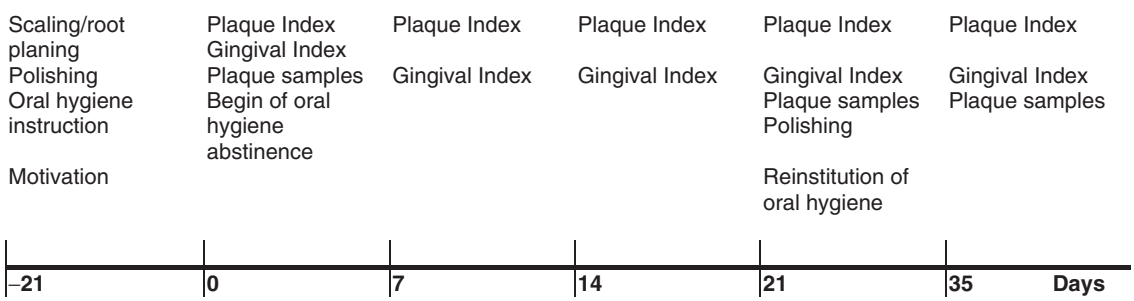

Fig. 1. Study design of experimental gingivitis (Löe et al. 1965)

Subsequently, the subjects were asked to refrain from all oral hygiene practices for a period of 21 days during which plaque was allowed to accumulate on all teeth surfaces and gingivae. Following this period of no oral hygiene, all subjects resumed optimal mechanical plaque control practices to reach pre-experimental levels of oral cleanliness and gingival health.

At baseline (Day 0), after 7, 14 and 21 days of undisturbed plaque accumulation and 14 days following the reinstitution of oral hygiene practices (i.e. Day 35), the following clinical parameters were assessed at six sites/tooth in the entire dentition using a calibrated manual periodontal probe (i.e. Michigan 0 periodontal probe):

- Supragingival plaque accumulation according to the criteria of the PlI system (Silness & Löe 1964).
- Gingival health or inflammation according to the criteria of the GI system (Löe & Silness 1963).

Third molars were excluded from the analysis

Subgingival bacterial sampling and analysis

After removal of supragingival plaque and isolation of the area with cotton rolls, subgingival plaque samples were collected with individual sterile Gracey curettes from pre-determined sites (i.e. disto-oral sites of first molars) in each quadrant. The four sites for plaque sampling were selected at baseline. Plaque samples were collected at baseline and again, at Days 21 and 35. The four subgingival samples were pooled in an Eppendorf tube containing 600 µl of TE solution (10 mMTris-HCl, 1 mM EDTA, pH 7.6) (Syed et al. 1980). After dispersion, 400 µl of 0.5 M NaOH were added.

Counts of 40 subgingival bacterial species were determined in each pooled plaque sample using a modification (Haffajee et al. 1997) of the checkerboard DNA–DNA hybridization technique (Socransky et al. 1994, 1998). The samples were lysed and the DNA placed in lanes on a nylon membrane using a minislot device (Immunetics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in a miniblotter 45 (Immunetics) with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labelled whole genomic DNA probes to 40 subgingival bacterial species were hybridized in individual lanes of the miniblotter. After hybridization, the membranes were washed at high stringency, and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and applying chemifluorescence detection. The probes and their source strains have been described previously (Ximénez-Fyvie et al. 2000). Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and a Storm Fluorimager 840 (Molecular Dynamics, Sunnyvale, CA, USA). Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each bacterial species. Signals were evaluated using the Storm Fluorimager 840 and converted to absolute counts by comparison with the standards on the same membrane.

Statistical analysis

The null hypothesis of no significant clinical and microbiological differences during an experimental gingivitis period between smokers and non-smokers was tested. Clinical data are presented as mean values ± 1 standard deviation (SD). Paired and unpaired *t*-tests were used for differences in PlI and GI scores. Differences in mean total DNA probe counts and relative proportions of the complex species were tested using the Wilcoxon rank sum test. The level of significance was set at $\alpha = 0.05$.

Results

All 22 subjects completed the final examination at Day 35. As shown in Table 1, no statistically significant differences between smokers and non-smokers with respect to age, race, gender and the number of teeth present were noted. The mean age was 24.7 ± 2.2 years (range 21–28 years) for the smoking and 23.1 ± 2.2 years (range 21–29 years) for the non-smoking cohorts, respectively. The mean daily cigarette consumption of the smokers amounted to 20 ± 4.9 cigarettes, and the self-reported smoking history yielded a 6.4 ± 2.8 pack-years exposure.

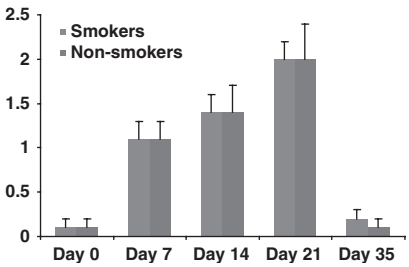

Fig. 2. Mean full-mouth Plaque Index scores with standard deviation bars at baseline (Day 0) and Days 7, 14 and 21 of experimental plaque accumulation and at Day 35 (after 2 weeks of re-institution of oral hygiene practices). No statistically significant differences were found between smokers and non-smokers.

Figure 2 illustrates mean full-mouth PlI ± SDs at baseline (Day 0) and on Days 7, 14, 21 and 35 in smokers (S) and non-smokers (NS), respectively. Both smokers and non-smokers displayed a statistically significant increase ($p < 0.05$) in the mean PlI during 3 weeks of undisturbed plaque accumulation, i.e. from baseline (S: 0.1 ± 0.1; NS: 0.1 ± 0.1) to Day 21(S: 2.0 ± 0.2; NS: 2.0 ± 0.4). Within both groups, a similar statistically significant decrease ($p < 0.05$) in the mean PlI was observed after re-institution of mechanical plaque control, i.e. from Day 21 to Day 35 (S: 0.2 ± 0.1; NS: 0.1 ± 0.1). No statistically significant differences, however, were observed when comparing the mean PlI of smokers with those of non-smokers at any observation periods.

Figure 3 shows mean full-mouth GI ± SDs at baseline (Day 0) and on Days 7, 14, 21 and 35 in smokers and non-smokers, respectively. Both smo-

Table 1. Demographic data of both current cigarette smokers and matched (never) non-somkers

	Smokers	Non-somkers
N	11	11
Age (years) ± SD	24.7 ± 2.2	23.1 ± 2.2
Age range (years)	21–28	21–29
Male/Female	3/8	4/7
Race	Caucasian	Caucasian
Teeth present ± SD	26.9 ± 1.9	27.3 ± 1.6
Cigarettes/day ± SD	20 ± 4.9	–
Pack-years ± SD	6.4 ± 2.8	–

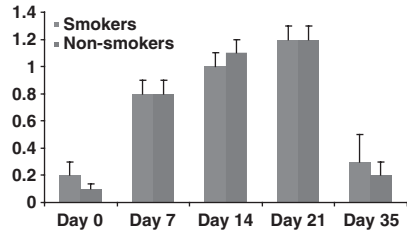

Fig. 3. Mean full-mouth Gingival Index scores with standard deviation bars at baseline (Day 0) and Days 7, 14 and 21 of experimental plaque accumulation and at Day 35 (after 2 weeks of re-institution of oral hygiene practices). No statistically significant differences were found between smokers and non-smokers.

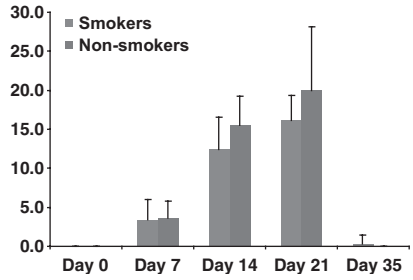

Fig. 4. Mean percentages of sites with Gingival Index scores $\geqslant 2$ with standard deviation bars at baseline (Day 0) and Days 7, 14 and 21 of experimental plaque accumulation and at Day 35 (after 2 weeks of re-institution of oral hygiene practices). No statistically significant differences were found between smokers and non-smokers.

kers and non-smokers displayed a statistically significant increase ($p<0.05$) in the mean GI from baseline (S: 0.2 ± 0.1; NS: 0.1 ± 0.04) to Day 21(S: 1.2 ± 0.1; NS: 1.2 ± 0.1). Within both groups, a similar statistically significant decrease ($p<0.05$) in the mean GI was observed during the resolution of gingival inflammation i.e. from Day 21 to Day 35 (S: 0.3 ± 0.2; NS: 0.2 ± 0.1).

Figure 4 illustrates mean percentages (%) $\pm$ SD of sites with GI scores $\geqslant 2$ (i.e. bleeding sites) at baseline (Day 0) and on Days 7, 14, 21 and 35 in smokers and non-smokers, respectively. In both smokers and non-smokers, a statistically significant increase ($p<0.05$) in the percentage of bleeding gingival sites was observed from baseline (S: 0.0 ± 0.0; NS: 0.0 ± 0.0) to Day 21(S: 16.1 ± 3.2; NS: 20.0 ± 8.1). Within both groups, a comparable statistically significant decrease ($p<0.05$) in the percentage of GI scores $\geqslant 2$ was observed from Day 21 to Day 35 (S: 0.3 ± 1.1; NS: 0.0 ± 0.0).

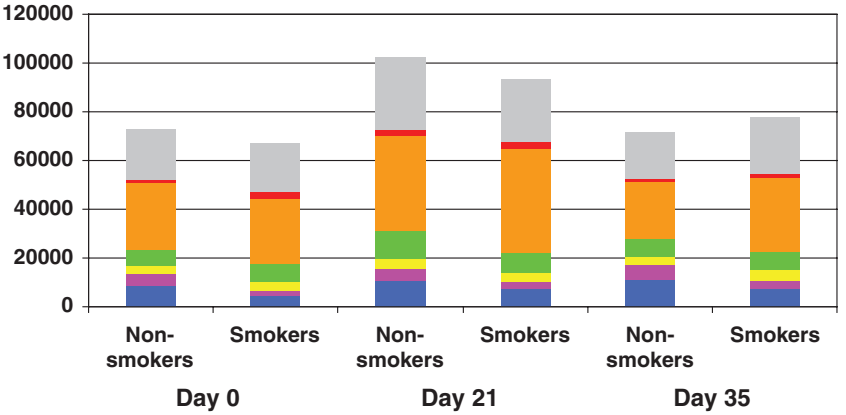

Fig. 5. Mean total DNA bacterial counts at baseline (Day 0) and Day 21 of experimental plaque accumulation and at Day 35. The seven different colours represent tightly related bacterial complexes in subgingival plaque as described by Socransky et al. (1998). From top to bottom: Grey: Additional microbial complexes not included in the analysis of the 40 bacterial taxa; Red: *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia*; Orange: *Prevotella intermedia*, *Prevotella nigrescens*, *Micromonas micros*, *Streptococcus constellatus*, *Campylobacter gracilis*, *Campylobacter rectus*, *Eubacterium nodatum*, *Campylobacter showae*, *Fusobacterium nucleatum* sp. *vincentii*, *Fusobacterium nucleatum* sp. *nucleatum, Fusobacterium nucleatum* sp. *polymorphum, Fusobacterium periodonticum*; Green: *Eikenella corrodens*, *Capnocytophagia gingivalis*, *Capnocytophagia sputigena*, *Capnocytophagia ochracea*, *Actinobacillus actinomycetemcomitans* serotype a; Yellow: *Streptococcus sanguis*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus intermedius*; Purple: *Veillonella parvula*, *Actinomyces odontolyticus*; Blue: *Actinomyces* species.

Figure 5 shows total DNA probe counts $\times 10^5$ from pooled subgingival plaque samples collected at baseline (Day 0) and on Days 21 and 35. No statistically significant differences were detected between smokers and non-smokers at baseline and at Days 21 and 35. In both smokers and non-smokers, no statistically significant increases ($p<0.05$) in total DNA probe counts were observed from baseline (S: $6.7 \times 10^4 \pm 4.7 \times 10^4$; NS: $7.3 \times 10^4 \pm 5.5 \times 10^4$) to Day 21 (S: $9.3 \times 10^4 \pm 4.7 \times 10^4$; NS: $10.2 \times 10^4 \pm 4.5 \times 10^4$). Within both groups, no statistically significant decreases ($p<0.05$) in total DNA probe counts were observed from Day 21 to Day 35 either (S: $7.7 \times 10^4 \pm 5.2 \times 10^4$; NS: $7.1 \times 10^4 \pm 5.32 \times 10^4$). At Day 35, however, samples from smokers harboured significantly fewer ($p<0.05$) DNA probe counts of red complex species compared to Day 21.

Figure 6 illustrates the proportions of bacterial complex species from pooled subgingival plaque samples collected at baseline (Day 0) and on Days 21 and 35. No statistically significant differences were detected between smokers and non-smokers at baseline and at Days 21 and 35. From baseline to Day 21, a statistically significant increase was observed for red complex species in non-smokers. Concomitantly, a statistically significant ($p<0.05$) decrease in purple complex species was observed in the non-smoking group. From Day 21 to Day 35, a statistically significant ($p<0.05$) decrease was noted for red complex species in both smokers and non-smokers. During the same time period, a statistically significant ($p<0.05$) increase in yellow complex species in smokers and in blue complex species in non-smokers was observed.

Discussion

The results of this clinical experiment demonstrated a reversible cause–effect relationship between the microbial challenge and the host response, irrespective of the smoking status of the participants. Thus, these results corroborated those of the original model described by Löe et al. (1965). The elimination of the bacterial biofilm resulted in complete resolution of marginal inflammation and the establishment of gingival health in both smokers and non-smokers. Because of the fact that both smokers and non-smokers reached pre-experimental levels of gingival health by meticulous plaque control, the basic concepts for prevention of gingival inflammation are applicable to smokers

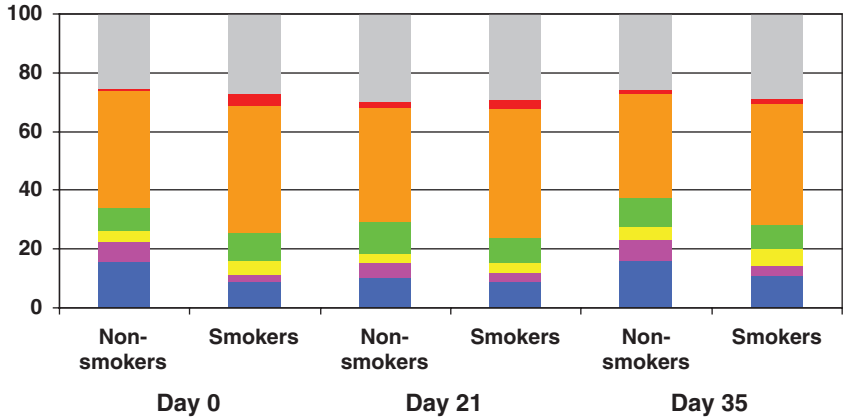

Fig. 6. Mean DNA proportions of bacterial complexes at baseline (Day 0) and Day 21 of experimental plaque accumulation and at Day 35. The seven different colours represent tightly related bacterial complexes in subgingival plaque as described by Socransky et al. (1998). From top to bottom: Grey: Additional microbial complexes not included in the analysis of the 40 bacterial taxa; Red: *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*; Orange: *Prevotella intermedia*, *Prevotella nigrescens*, *Micromonas micros*, *Streptococcus constellatus*, *Campylobacter gracilis*, *Campylobacter rectus*, *Eubacterium nodatum*, *Campylobacter showae*, *Fusobacterium nuc. vincentii*, *Fusobacterium nucleatum* sp. *nucleatum, Fusobacterium nucleatum* sp. *polymorphum, Fusobacterium periodonticum*; Green: *Eikenella corrodens*, *Capnocytophagia gingivalis*, *Capnocytophagia sputigena*, *Capnocytophagia ochracea*, *Actinobacillus actinomycetemcomitans* serotype a; Yellow: *Streptococcus sanguis*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus intermedius*; Purple: *Veillonella parvula*, *Actinomyces odontolyticus*; Blue: *Actinomyces* species.

as well. Compared with non-smokers, only a trend was observed for smokers to yield slightly less gingival bleeding sites (i.e. sites with GI $\geqslant$2) after 2 and 3 weeks of experimental plaque accumulation. This, however, could be attributed to the low number of subjects enrolled in the study.

The findings of the present study are in complete agreement with those reported by Bastiaan and Waite (1978). In that study, although of shorter duration (i.e. 10 days of abolished oral hygiene), a very similar protocol with a similar age group of participants as in the present study was followed. That study failed to show statistically significant differences between smokers and non-smokers with respect to experimental plaque accumulation and development of gingivitis. Other studies (Bergström & Preber, 1986, Danielsen et al. 1990, Lie et al. 1998a, Giannopoulou et al. 2003), however, have reported different clinical outcomes for smokers and non-smokers using the experimental gingivitis protocol. Although the latter studies showed that the rate of plaque formation was similar for smokers and non-smokers up to a period of 28 days of abolished oral hygiene practices, smokers consistently displayed less pronounced clinical signs of gingivitis. One reason for the discrepancy of the results of the present study with two of the studies mentioned (Bergström & Preber, 1986, Danielsen et al. 1990) may be the assessment of the status of gingival inflammation using electronic periodontal probes with a controlled pressure set at 0.6–0.65 N. In the present study, however, efforts were made to apply only an external pressure to the marginal gingiva as described by Löe and Silness (1963). The difference of the present results with those of other experimental gingivitis studies in smokers/non-smokers may be interpreted as an impaired sensitivity of the GI (Löe & Silness 1963) in detecting early differences in bleeding scores compared with bleeding on probing. Moreover, the comparison of two methods to assess gingival bleeding, i.e. probing to the bottom of the pocket and probing of the marginal gingiva resulted in significantly more bleeding in gingival health and experimental gingivitis when probing to the bottom of the pocket compared with probing at the gingival margin (Lie et al. 1998a).

The experimental outline of the study by Bergström and Preber (1986) further revealed that only mandibular anterior teeth were studied, while oral hygiene practices were maintained in the remaining parts of the dentition. It, therefore, cannot be excluded that the limited regions of the dentition exposed to experimental plaque accumulation influenced the outcome of the clinical development of gingivitis. In addition, smokers were asked to smoke one cigarette within 1 h before each examination in the latter study (Bergström & Preber 1986), a fact which may have influenced the results as well.

Furthermore, another explanation for the reduced signs of gingival inflammation noted in several experimental gingivitis studies discussed (Bergström & Preber, 1986, Danielsen et al. 1990, Lie et al. 1998a, Giannopoulou et al. 2003) may be attributed to the transient gingival vasoconstriction caused by nicotine. On the other hand, measurements of gingival blood flow by a laser Doppler fibreoptic probe have documented a transient increase during cigarette smoking followed by a rapid decrease towards baseline levels within 10 min. (Baab & Öberg 1987). Also, studies have shown that the density of the gingival vasculature was not significantly affected by smoking in young adults with healthy gingival conditions (Bergström et al. 1988, Danielsen et al. 1990, Persson & Bergström 1998). Based on these studies, it may be hypothesized that a similar gingival vasculature was present in our groups of smokers and non-smokers.

In the present study, PlI scores did not significantly differ between the two groups at any time point of the experimental protocol. Moreover, no significant differences were found with respect to the composition of the subgingival biofilm between smokers and non-smokers at baseline, Day 21 and 35. In this context, scarce evidence exists concerning the composition of the subgingival microbiota of smokers and non-smokers during experimental gingivitis (Lie et al. 1995, Lie et al. 1998b). The composition of the oral microbiota in subjects who had previously demonstrated to develop either a weak or a strong gingival inflammatory response to experimental plaque accumulation, respectively, was investigated (Lie et al. 1995). The results indicated that differences in response to experimental plaque accumulation were not caused by major differences in the composition of the subgingival microbiota. On the other hand, conflicting results have been reported on the prevalence of periodontal pathogens and the effects of periodontal therapy on the subgingival

microbiota in smokers and non-smokers with various forms of periodontal disease (Stoltenberg et al. 1993, Zambon et al. 1996, Haffajee et al. 1997, Renvert et al. 1998, Kamma et al. 1999, Darby et al. 2000, Boström et al. 2001, Haffajee & Socransky 2001). Nevertheless, recent data (Persson et al. in press) suggested that a long-term exposure (i.e. $\geqslant$26 years) to cigarette smoking compared with non smoking could not identify current or former smokers as a high risk group for the development of periodontitis or tooth loss. That study further demonstrated that a minimum of 30 years of smoking history may be required to detect a detrimental impact on periodontal conditions.

In conclusion, both smokers and non-smokers reacted to experimental plaque accumulation with a gingival inflammation of similar magnitude and timing. The composition of the subgingival microbial complexes did not differ significantly between smokers and non-smokers at any observation period. Resolution of experimental gingivitis occurred in comparable patterns and timing in both smokers and non-smokers.

Acknowledgements

This study was supported by the Clinical Research Foundation (CRF) for the Promotion of Oral Health, University of Berne, and by the Swiss Society of Odontology (SSO) Grant # 212. The competent laboratory assistance of Mrs. M. Weibel and Mrs. R. Hirschi Imfeld and the statistical advice of Mr. W. Bürgin, Biomed. Ing. are highly appreciated.

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