UNIVERSIDADE ESTADUAL PAULISTA "Júlio de Mesquita Filho"
FACULDADE DE ODONTOLOGIA DE ARARAQUARA

SABRINA CARVALHO GOMES

EFEITO DO CONTROLE DE PLACA BACTERIANA SUPRAGENGIVAL SOBRE PARÂMETROS SUBGENGIVAIS

ARARAQUARA
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EFEITO DO CONTROLE DE PLACA BACTERIANA SUPRAGENGIVAL SOBRE PARÂMETROS SUBGENGIVAIS

Tese apresentada ao Programa de Pós-Graduação em Periodontia da Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, para obtenção do Título de Doutor em Periodontia.

Orientadora:
Profa. Dra. Rosemary Adriana Chiérici Marcantonio

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Introdução

Biofilmes supra e subgengivais são reconhecidos fatores etiológicos das gengivites e periodontites, respectivamente (LÖE et al., 1965; LINDHE et al., 1975; DARVEAU et al., 1997; MULLER et al., 2002). Sugere-se que esses biofilmes possam manter inter-relações etiopatogênicas, dependentes do nível de infecção presente ou da expressão imune/inflamatória, e que, assim, a gengivite possa exercer importante papel na etiopatogênese da periodontite (PAGE et al., 1997; DARVEAU et al., 1997; LISTGARTEN, 1999). Os possíveis determinantes para esses mecanismos etiopatogênicos vêem sendo discutidos desde a década de 70.

Recentemente, Weidlich et al., 2001, mostraram que, à medida que ocorre o crescimento e desenvolvimento do biofilme supragengival, uma tênue área presente, entre a extensão apical do biofilme e a margem gengival, denominada zona livre de placa, desaparece. Nesse processo, há o contato do biofilme supragengival com a margem gengival. A manutenção de um ambiente anaeróbico, decorrente da inflamação marginal, permitiria o desenvolvimento de uma microbiota subgengival (LISTGARTEN, 1999). Essa microbiota estabelecida em pacientes susceptíveis poderia levar ao desenvolvimento da periodontite (PAGE et al., 1997).

Existem registros de que o controle do biofilme supragengival seja importante no diagnóstico clínico das periodontites, determinando a redução da profundidade de sondagem e do percentual de sítios com sangramento subgengival (CATÃO, 1999). Além de observações clínicas, alguns estudos avaliaram a alteração da microbiota subgengival como conseqüência desse controle supragengival. No entanto, estudos, como o realizado por Ximenez-Fyvie et al., 2000, associaram instrumentação subgengival, dificultando assim, uma interpretação adequada do efeito isolado do controle supragengival. Entre aqueles que se limitaram à instrumentação supragengival, encontramos alguns conflitos de resultados (SMULOW et al., 1983; KHO et al., 1985; BELTRAMI et al., 1987;

DAHLEN et al., 1992; KATSANOULAS et al., 1992; McNABB et al., 1992; AL-YAHFOUFI et al., 1995), tanto sob o ponto de vista clínico, quanto microbiológico. A ausência de critérios de inclusão claramente definidos, de um regime adequado de controle de placa bem como do monitoramento na execução do mesmo, a falta de controle de fatores modificadores e diferenças nos métodos de investigação microbiológica, possivelmente possam explicar as principais observações desses estudos.

Há evidências que o tabagismo interfere na expressão inflamatória marginal ou periodontal, bem como na perda de inserção clínica (HAFFAJEE e SOCRANSKY, 2001a; MULLER et al., 2002; ERDEMIR et al., 2004). Para alguns autores o fumo pode também estar associado a diferenças na microbiota periodontal embora essa não seja uma observação unânime (BÖSTROM et al., 2001; HAFFAJEE e SOCRANSKY, 2001b; VAN WINKELHOFF et al., 2001; VAN DER VELDEN et al., 2003).

Não existem relatos de como essas diferenças, entre pacientes periodontais fumantes e nunca fumantes, se expressariam em decorrência do controle do biofilme supragengival. Os estudos disponíveis, comparando esses grupos, avaliaram o efeito da formação do biofilme supragengival, mas, não da sua remoção, na manifestação clínica de gengivite (MULLER et al., 2002; SALVI et al., 2005). Da mesma forma, fizeram comparações após terapia supra e subgengival simultaneamente (HAFFAJEE e SOCRANSKY, 2001b; VAN DER VELDEN et al., 2003).

Considerando-se, portanto, que o efeito do controle de placa supragengival sobre parâmetros subgengivais é, ainda, uma questão aberta, decidiu-se investigar essa resposta e compará-la entre pacientes fumantes e nunca fumantes.

Proposição

O objetivo desse estudo foi avaliar o efeito do controle de placa supragengival sobre parâmetros clínicos e microbiológicos subgengivais e compará-lo entre pacientes fumantes e nunca fumantes.

Metodologia

O presente projeto foi aprovado pelo Comitê de Ética e Pesquisa (CEP, protocolo 32/03) da Universidade Estadual Paulista- Araraquara (Anexo 2).

Cálculo da amostra

O cálculo de amostra foi realizado a partir dos resultados obtidos por Catão, 1999. Estimou-se que 25 indivíduos deveriam compor cada um dos grupos experimentais (nunca fumantes e fumantes), para obter-se uma diferença média de 0.8mm de Profundidade de Sondagem, com um poder de 80% e intervalo de confiança de 95% entre os grupos.

Seleção da amostra

Aproximadamente 500 pacientes, encaminhados à Disciplina de Periodontia (Departamento de Odontologia Conservadora da Universidade Federal do Rio Grande do Sul- UFRGS), pelo serviço de Triagem da Faculdade de Odontologia desta Universidade (entre Julho 2003 e Agosto 2004), foram examinados para compor a amostra do presente estudo.

Aos pacientes interessados em participar do estudo, foi aplicado um questionário para coleta de informações sobre a saúde geral (Anexo 3). Os voluntários tornavam-se elegíveis se apresentassem:

- Ausência de condição sistêmica que interferisse com processo saúde-doença periodontal (diabetes, condições cardíacas que determinassem necessidade de profilaxia antibiótica, entre outras).
- História negativa de antibioticoterapia, nos seis meses predecessores, e de uso de antiinflamatórios, nos últimos três, ao exame clínico de inclusão.
- História negativa de tratamento periodontal prévio.
- História negativa de gestação ou uso de hormônios.

- Ausência de prescrição de agentes químicos de controle de placa bacteriana supragengival.
- Tivessem interesse em participar dos procedimentos experimentais de reavaliação semanal, durante todo o período experimental (6 meses).

Os pacientes interessados assinavam o Termo de Consentimento Livre e Esclarecido (TCLE- Anexo 4). Após, os pacientes elegíveis foram examinados clinicamente e incluídos se:

- Apresentassem, no mínimo, 12 dentes em boca.
- Diagnóstico clínico de gengivite associada à placa e periodontite crônica generalizada, de moderada a avançada (AAP, 1999).
- Pelo menos 4 dentes deveriam apresentar um sítio com Profundidade de Sondagem (PS) de 3-5mm e outros 4 dentes com, no mínimo, um sítio de PS de 6-10mm.
- Esses sítios, entre os quais foram randomicamente alocados os sítios experimentais para coleta de fluido e amostra microbiológica, deveriam apresentar: placa visível, diagnóstico de gengivite e diagnóstico de periodontite crônica.

Foram selecionados, de forma consecutiva, 50 pacientes, compondo uma amostra de conveniência de 25 pacientes nunca fumantes e 25 pacientes fumantes (Tabela 1/Anexo 1).

Durante o estudo, os seguintes critérios de exclusão foram aplicados:

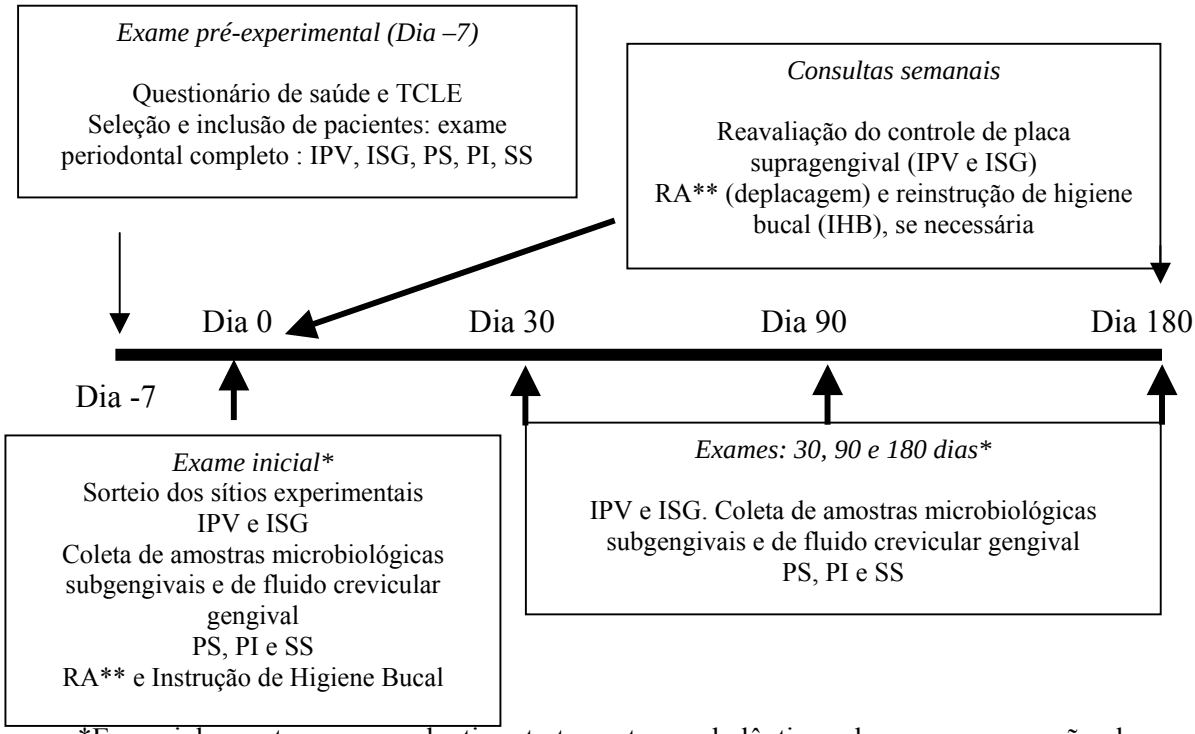
- Impossibilidade de seguir o regime de consulta semanal
- Desenvolvimento de alguma patologia que pudesse interferir com a condição periodontal
- Ingestão de antibióticos, antiinflamatórios ou hormônios.

Além disso, estabeleceu-se, que, durante o estudo, perda de inserção adicional de 2 milímetros, que fosse confirmada no exame subseqüente,

determinaria a exclusão do indivíduo dos procedimentos experimentais e encaminhamento para tratamento subgengival. Esse critério é uma adaptação do estabelecido por Haffajee et al., 1983. Seis pacientes foram excluídos do presente estudo e, na seqüência, substituídos. Na Tabela 2 (Anexo 1) estão sumarizadas as razões de exclusão. Nenhuma delas se prendeu ao critério de progressão de periodontite.

Desenho experimental

Esse estudo foi desenvolvido como um ensaio clínico não randomizado e não controlado. O controle de placa bacteriana supragengival foi considerado como intervenção experimental e o tabagismo foi avaliado como fator modificador de resposta periodontal. O desenvolvimento experimental deu-se de acordo com o seguinte fluxograma:



*Encaminhamento para exodontias, tratamentos endodônticos, bem com execução de procedimentos restauradores provisórios foram providenciados ao longo de todo o período experimental, de acordo com as necessidades dos participantes.

** Raspagem e alisamento supragengival, sem instrumentação subgengival. Deplacagem supragengival com curetas.

Calibragem do examinador

Nos períodos pré e trans-experimental, foram realizados os procedimentos de treinamento e calibragem do examinador, único responsável pelos exames clínicos durante o estudo. Os dados da calibragem estão na Tabela 3 (Anexo 1).

Exame clínico e amostragem de fluido crevicular e microbiota subgengival

Inicialmente, os pacientes foram submetidos aos exames compreendendo Índice de Placa (IPV) e Índice de Sangramento Gengival (ISG), de acordo com Ainamo e Bay, 1975. Após, foram realizados os procedimentos de coleta de amostras de microbiota subgengival, seguidos por amostragem de fluido.

Na seqüência, foram realizados os exames de Profundidade de Sondagem (PS), Perda de Inserção (PI) e Sangramento Subgengival (SS), de acordo com Armitage, 2004. Os valores de PS e PI foram arredondados para o milímetro superior mais próximo.

Seis sítios por dente foram examinados em todos os pacientes (mésio-vestibular, vestibular, disto-vestibular, disto-lingual, lingual, mésio-lingual). Os 3os. molares, dentes com lesão endo-periodontal, dentes com exodontias indicadas foram excluídos do exame, após avaliação dos dados clínicos e realização de exames complementares.

Dos 50 pacientes envolvidos no exame clínico periodontal, 45 contribuíram com amostras que foram submetidas à investigação do volume de fluido e avaliação microbiológica (Tabela 1/ Anexo 1).

Procedimentos de coleta microbiológica subgengival e de fluido crevicular gengival:

Os sítios experimentais, a serem submetidos à coleta de material microbiológico e fluido crevicular, foram randomicamente alocados entre aqueles que preenchiam os critérios de inclusão.

No total, 8 sítios experimentais (4 de cada categoria de sondagem: 3-5mm e 6-10mm), por paciente, foram submetidos à análise do volume de fluido

gengival (1440 amostras) e 4 (2 de cada categoria de sondagem: 3-5mm e 6-10mm) à análise microbiológica (720 amostras).

Todas as coletas microbiológicas foram realizadas por um único examinador. Brevemente, foi feita a remoção do biofilme supragengival do sítio experimental, seguida por lavagem leve, secagem (spray ar/água) e isolamento relativo (rolos de algodão). Inicialmente, um cone de papel absorvente estéril (# 30, EndoPoint, Paraíba do Sul, RJ) era inserido no sulco/ bolsa, estimando-se a extensão da penetração pelos dados anotados no exame de seleção da amostra, e mantidos por 30 segundos (HARTROTH et al., 1999). O cone foi conservado em tubos de ensaio tipo Eppendorf esterilizados, contendo 200µl de RTF (SYED e LOESCHE, 1972).

Novamente os dentes foram lavados e secos por 30 segundos. Aguardou-se, então, um intervalo (30s) para a inserção das tiras de papel Periopaper® (OraFlow, PlainView, New York) no sulco/bolsa. O Periopaper® foi mantido no sítio por 30 segundos e, imediatamente após (TÖZÜM et al., 2004), o fluido coletado era medido através do Periotrom 8000® (OraFlow, PlainView, New York). Periopaper® com a presença visível de sangue era descartado e o processo de coleta, refeito. Cada Periopaper® foi armazenado em eppendorf estéril a 20°Celsius negativos. Essa coleta de fluido crevicular gengival também foi realizada por um único examinador.

Método microbiológico

Foi realizada avaliação microbiológica através do método quantitativo da Reação de Polimerase em Cadeia: Real Time PCR (NONNENMACHER et al., 2004), na Phillipps Universitat (Marburg, Alemanha). Os primers e sondas utilizados para identificação bacteriana (Eubactérias, *A. actinomycetemcomitans*, *P.gingivalis*, *P.micros, D.pneumosintes)* foram baseados em seqüências específicas do gene 16S rRNA (Tabela 4/Anexo 1). O DNA bacteriano das amostras foi extraído de acordo com as instruções do fabricante (DNeasy Tissue Kit, Qiagen, Hilden, Alemanha).

A avaliação das amostras foi feita através de uma mistura de reação, contendo DNA da amostra subgengival em questão (template), 10x TaqMan Universal PCR Master Mix, $MgCl_2$, dNTP, forward primer e reverse primer (MWG, Munique) e a sonda TaqMan (Eurogentec, Bélgica). As condições cíclicas aplicadas foram as seguintes: 10 minutos a 95°Celsius, seguidos de 15 segundos a 95°Celsius e de 1 minuto a 60°Celsius.

A quantificação de DNA bacteriano presente nas amostras deu-se a partir da utilização de diluições em decimais seriados (10^{-1} a 10^{-8}) de cópias de plasmídios previamente quantificadas, em cada análise. Controles positivos e negativos foram utilizados em todas as reações.

As amostras e os controles foram avaliados em duplicatas. A seqüência de avaliação foi aleatória, em relação aos momentos experimentais. Ao final, 3600 reações foram realizadas.

O aparelho ABI Prism 7700 SDS (Applied Biosystems International) foi utilizado para monitorar a amplificação de PCR baseado na análise quantitativa de emissão de fluorescência.

Analise dos dados

A análise de dados (STATA software[*]) foi realizada considerando-se o indivíduo como unidade de estudo.

Para o IPV, ISG e SS, foi calculada a média do percentual de sítios positivos por paciente e, após, para o grupo.

Os valores médios de PS e PI, em milímetros, foram gerados para o indivíduo e, posteriormente, para o grupo, em cada momento experimental.

Curvas-padrão, geradas para as bactérias avaliadas, a partir de quantidades conhecidas de plasmídios (10^1 e 10^8), foram utilizadas para converter os Cts ("Cicle threshold") observados, em cada reação, em número de células bacterianas. Como essas variáveis não apresentavam distribuição normal, os dados foram, inicialmente, convertidos em função logarítmica. Foram calculadas as

[*] Stata 7.0 for Windows, Stata Corporation, College Station, TX, USA

médias por indivíduo e, na seqüência, para o grupo. Os resultados foram expressos em números absolutos.

O cálculo do volume do fluido crevicular gengival, em microlitros (μL), foi realizado através da conversão dos dados do Periotrom, a partir de uma curva padrão previamente constituída. Após, foi gerada a média por indivíduo, seguida pelo cálculo do grupo, para cada momento experimental.

Os dados anotados para todos os exames realizados foram comparados entre o momento inicial e final. O Teste de Wald foi utilizado e o *p* ajustado para múltiplas comparações. O nível de significância foi estabelecido em 5% e o intervalo de confiança (95%) foi calculado.

Resultado

Todos os índices clínicos periodontais avaliados, à exceção da PI, maior em fumantes, foram semelhantes entre os grupos no momento inicial do experimento (Tabela 5/Anexo 1). Após o tratamento, foi observada uma redução significante dos mesmos, para ambos os grupos (Tabela 5/ Anexo 1). No entanto, pacientes fumantes apresentaram menor percentual de sítios com sangramento marginal. Ainda que a PI final de fumantes tenha sido significativamente maior que para nunca fumantes os ganhos de inserção clínica ao final do estudo (redução da PI) foram de magnitude semelhante entre os grupos.

O volume do fluido gengival também reduziu, significantemente, ao longo dos 180 dias experimentais, independente do hábito de fumar. No entanto, maiores volumes de fluido apresentaram-se associados a pacientes nunca fumantes, em ambos os exames (Tabela 6/ Anexo 1).

A microbiota subgengival foi significativamente reduzida por meio do controle de placa bacteriano supragengival (Tabela 7/ Anexo 1), em ambos os grupos. No entanto, no exame final, pacientes fumantes apresentaram menor número de bactérias totais. As diferenças significantes observadas, inicialmente, para número de *P. micros* e *D. pneumosintes* em fumantes, desapareceram ao final do estudo ($p>0.05$).

Considerações finais

- O controle de placa bacteriana supragengival determina alterações clínicas relevantes supra e subgengivais
- Esse benefício é observado, também, em relação à microbiota subgengival determinando reduções significantes no número de bactérias.
- Ambos os grupos, nunca fumantes e fumantes, beneficiam-se do controle do biofilme supragengival.

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Resumo

No presente estudo foi avaliado o efeito do controle de placa bacteriana supragengival sobre parâmetros clínicos e microbiológicos subgengivais, comparando-o em pacientes nunca fumantes (NFU) e fumantes (F). Durante esse ensaio clínico (6 meses), 25 pacientes, de cada grupo, foram examinados clinicamente, por um examinador calibrado, no momento inicial (I) e aos 30, 90 e 180 (exame final F) dias. 45 desses indivíduos contribuíram com amostras para cálculo do volume de fluido e avaliação microbiológica.

As médias do percentual de sítios com IPV, ISG e SS e as médias da PS e PI (em milímetros), do volume de fluido (μl) e as médias do número de bactérias totais (Eubactéria, Eu), *Porphyromonas gingivalis* (Pg), *Peptostreptococcus micros* (Pm), *Dialister pneumosintes* (Dp), *Actinobacillus actinomycetemcomitans* (Aa) foram geradas para o indivíduo e, posteriormente, para os grupos. Dados do exame inicial e final foram comparados (teste Wald, p=0.05). Foi observado que o efeito do controle de placa bacteriano supragengival contribuiu para a redução significante de IPV (NFU- I: 91,1 e F: 8,7; FU- I: 88,5 e F: 6,4), ISG (NFU- I: 83,8 e F: 2,2; FU- I: 76,1 e F: 0,3), SS (NFU- I: 95,0 e F: 21,6; FU- I: 94,4 e F: 25,3), PS (NFU- I: 3,7 e F: 2,6; FU- I: 3,9 e F: 2,8), PI (NFU- I: 3,4 e 3.0; FU- I: 4., e F: 3,7), volume de fluido (NFU- I: 0,59 e F: 0,23; FU- I: 036 e F- 0,16) e número de Eubactérias (NFU- I: $1,09x10^5$; F: $2,3x10^1$; FU- I: $1,9x10^5$; F: $1,9x10^1$); Pg (NFU- I: $1,07x10^3$; F: $7,0x10^1$; FU- I: $1,4x10^3$; F: $9,4x10^1$); Pm (NFU- I: $2,1x10^5$; F: $0,3x10^5$; FU- I: $6,8x10^5$; F: $0,5x10^5$); Aa (NFU- I: $2,5x10^1$; F: $1,0x10^1$; FU- I: $1,7x10^1$; F: $0,76x10^1$) e Dp (NFU- I: $3,7x10^1$; F: $0,72x10^1$; FU- I: $9,6x10^1$; F: $0,83x10^1$). Ao final do estudo, pacientes fumantes apresentaram menor ISG e volume de fluido, maior PI e menor número de baterias totais. Conclui-se, no presente estudo, que o controle do biofilme supragengival determina alterações clínicas, do volume de fluido e do número de bactérias no

ambiente subgengival. Pacientes fumantes e nunca fumantes respondem a esse controle de forma expressiva.

Palavras-Chave: Higiene bucal, microbiologia, análise quantitativa, reação de polimerase em cadeia, tabagismo.

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Abstract

The present study aimed to investigate the effect of a supragingival plaque control regiment and to compare this effect between never smokers (NS) and smokers (S) periodontitis patients. During the 6 months experimental period 50 patients, 25 in each group, were clinically examined by a singular calibrated examiner in the baseline (I), 30, 90 and 180 days (F). 45 individuals contributed with GCF and microbiological sampling. Mean values of the percentage of sites VPI+, MBI+, BOP, the PPD and CAL measurements (mm), GCF volume (µl) and number of the Eubacteria (Eu); *Porphyromonas gingivalis* (Pg), *Peptostreptococcus micros* (Pm), *Dialister pneumosintes* (Dp), *Actinobacillus actinomycetemcomitans* (Aa), were calculated for the individual to compose the groups means. Data from baseline and final examinations were compared (test Wald p=0.05). It could be observed that a supragingival plaque control significantly contributed to VPI (NS- I: 91.1 and F: 8.7; S- I: 88.5 e F: 6.4), MBI (NS- I: 83.8 e F: 2.2; S- I: 76.1 e F: 0.3), BOP (NS- I: 95.0 e F: 21.6; S- I: 94.4 e F: 25.3), PPD (NS- I: 3.7 e F: 2.6; S- I: 3.9 e F: 2.8); CAL (NS- I: 3.4 e 3.0; S- I: 4.3 e F: 3.7), GCF (NS- I: 0.59e F: 0.23; S- I: 0.36 e F: 0.16) and number of Tb (NS- I: $1.09x10^5$; F: $2.3x10^1$; S- I: $1.9x10^5$; F: $1.9x10^1$); Pg (NS- I: $1.07x10^3$; F: $7.0x10^1$; S- I: $1.4x10^3$; F: $9.4x10^1$); Pm (NS- I: $2.1x10^5$; F: $0.3x10^5$; S- I: $6.8x10^5$; F: $0.5x10^5$); Aa (NS- I: $2.5x10^1$; F: $1.0x10^1$; S- I: $1.7x10^1$; F: $0.76x10^1$) and Dp (NS- I: $3.7x10^1$; F: $0.72x10^1$; S- I: $9.6x10^1$; F: $0.83x10^1$) reductions. At the end, smokers presented less marginal bleeding, CAL, GCF volume and lower numbers of Eubacteria then never smokers. It can be concluded that both never smokers and smokers periodontitis patients can benefit from a supragingival plaque control regimen, even though

smokers presented less marginal bleeding and less number of Eubacteria at the end.

Keywords: Oral hygiene, microbiota, quantitative analysis, reação de polimerase em cadeia, tobacco. .

Anexo 1

Tabela A1 - Descrição da amostra submetida somente à avaliação clínica e à avaliação clínica, amostragem de fluido crevicular gengival e microbiota, no grupo de nunca fumantes e fumantes. Araraquara, 2005

Avaliação		Nunca fumantes	Fumantes
Clínica	n	25	25
	Idade (média ± dp)	46.8 ± 7.1	45.9 ± 5.4
	Sexo (%M)	40	56
	Número de dentes (média ± dp)	21.4 ± 3.8	21.2 ± 3.7
	Número de ciagarros (média ± dp)	0 ± 0	19.4 ± 11.6
	Anos de hábito (média ± dp)	0 ± 0	24.8 ± 8.6
Clínica, FCG e microbiológico	n	24	21
	Idade (média ± dp)	47.3 ± 6.7	45.8 ± 5.1
	Sexo (%M)	41.7	55
	Número de dentes (média ± dp)	21.16± 3.7	20.85 ± 3.7
	Número de ciagarros (média ± dp)	0 ± 0	19.6 ± 11.8
	Anos de hábito (média ± dp)	0 ± 0	24.1 ± 8.7

n: número de indivíduos; dp: desvio padrão; M: gênero masculino

Tabela A2 - Pacientes excluídos, exames realizados até o momento de exclusão (momento experimental em meses), razão de exclusão, exposição ao fumo, idade (em anos) e gênero (m:masculino; f: feminino). Araraquara, 2005

Pacientes	Momento experimental	Razão da exclusão	Fumante	Idade/ gênero
1	0, 1 e 3	desconhecida	não	33m
2	0 e 1	antibiótico: mordida de aranha (recomendação médica)	sim	33 m
3	0 e 1	recursos financeiros	sim	40 m
4	0 e 1	desconhecida	sim	37 f
5	0 e 1	antibiótico: dor de garganta (por conta própria)	não	40 f
6	0 e 1	antibiótico: sinusite (recomendação médica)	sim	40 f

Tabela A3 - Valores do Índice Kappa em relação à profundidade de sondagem (PS) e perda de inserção (PI), de acordo com os períodos experimentais (número de indivíduos/sítios examinados). Araraquara, 2005

	Período Experimental			
Kappa	Pré-experimental (5/680)		Trans-experimental (4/486)	
	PS	PI	PS	PI
Não ponderado	0.65	0.51	0.52	0.60
Ponderado*	0.98	0.97	0.95	0.92

** (±1mm).*

Tabela A4 - Primers e sondas utilizados para investigação microbiológica

Primer e sonda	Sequência (5'-3')	Cepa	Número de acesso*
Universal			
Forward	TGGAGCATGTGGTTTAATTCGA		
Reverse	TGCGGGACTTAACCCAACA		
Probe	CACGAGCTGACGACA(AG)CCATGCA		
A. actinomycetemcomitans		MCCM 200	M75039
Forward	CAAGTGTGATTAGGTAGTTGGTGGG		
Reverse	CCTTCCTCATCACCGAAAGAA		
Probe	ATCGCTAGCTGGTCTGAGAGGATGGCC		
D. pneumosintes		ATCC 33048	X82500
Forward	GAGGGGTTTGCGACTGATTA		
Reverse	CCGTCAGACTTTCGTCCATT		
Probe	CACCAAGCCGACGATCAGTAGCCG		
P. micros		MCCM 3096	D14143
Forward	AAACGACGATTAATACCACATGAGAC		
Reverse	ACTGCTGCCTCCCGTAGGA		
Probe	TCAAAGATTTATCGGTGTAAGAAGGGCTCGC		
P. gingivalis		MCCM 3199	L16492
Forward	TGCAACTTGCCTTACAGAGGG		
Reverse	ACTCGTATCGCCCGTTATTC		
Probe	AGCTGTAAGATAGGCATGCGTCCCATTAGCTA		

*GenBank : número de acesso disponível em http://www.ncbi.nlm.nih.gov/entrez

Tabela A5 - Média (intervalo de confiança) do percentual de sítios com placa visível (IPV), sangramento marginal (ISG), sangramento subgengival (SS) e da profundidade de sondagem (PS) e Perda de Inserção (PI), em milímetros, no exame inicial (dia 0) e final (dia 180), em nunca fumantes e fumantes. Araraquara, 2005

	Nunca fumantes		Fumantes	
	Inicial	Final	Inicial	Final
IPV (%sítios)	91.1 (87.7-94.4) Aa	8.7 (4.7-12.7) Bb	88.5 (83.0-94.0) Aa	6.4 (3.7-9.0) Bb
ISG (%sítios)	83.8 (78.0-89.6) Aa	2.2 (1.0-3.5) Bb	76.1 (68.5-83.7) Aa	0.3 (0.1-0.7) Cb
SS (%sítios)	95.0 (92.1-97.8) Aa	21.6 (16.3-26.9) Bb	94.4 (89.4-99.3) Aa	25.3 (17.8-32.7) Bb
PS (mm)	3.7 (3.5-3.9) Aa	2.6 (2.4-2.8) Bb	3.9 (3.7-4.2) Aa	2.8 (2.5-3.1) Bb
PI (mm)	3.4 (3.1-3.7) Aa	3.0 (2.6-3.3) Cb*	4.3 (3.8-4.7) Ba	3.7 (3.3-4.2) Db*

Letras maiúsculas referem-se a comparações entre fumantes e nunca fumantes em cada período experimental: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).
Letras minúsculas referem-se a comparações entre diferentes períodos experimentais: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).
* redução sem diferenças significantes entre fumantes e nunca fumantes

Tabela A6 - Média (intervalo de confiança) dos valores do volume de fluido, em microlitros, no exame inicial (dia 0) e final (dia 180) em pacientes nunca fumantes e fumantes. Araraquara, 2005

Período experimental	Nunca fumantes	Fumantes	Média (95% IC)
Exame inicial	0.59 (0.52-0.69)Aa	0.36 (0.29-0.45)Ba	0.47 (0.41 – 0.55)a
Exame final	0.23 (0.18-0.29)Ad	0.16 (0.14-0.19)Bd	0.20 (0.17 – 0.23)d

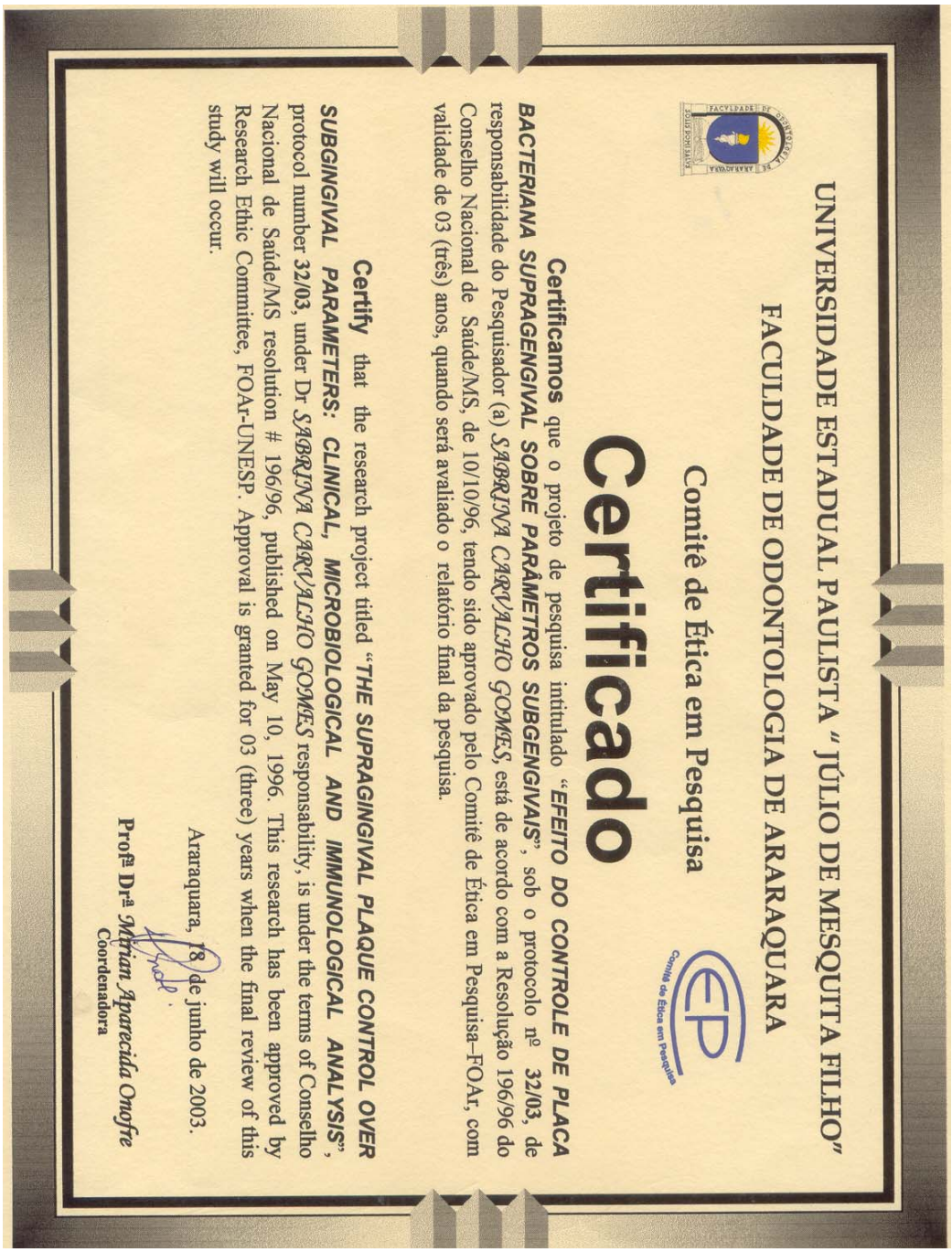
Letras maiúsculas referem-se a comparações entre fumantes e nunca fumantes: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).
Letras minúsculas referem-se a comparações entre diferentes períodos experimentais: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).

Tabela A7 - Média (intervalo de confiança) do número de bactérias totais e específicas avaliadas de acordo com os momentos experimentais (Inicial e final), para o grupo de nunca fumantes e fumantes. Araraquara, 2005

Bactérias	Nunca fumantes		Fumantes	
	Inicial	Final	Inicial	Final
Bactérias totais (em milhares)	109.3 (59.2-201.7)Aa	23.7 (11.7-47.9)Ba	199.3 (141.0-281.7)Ab	19.9 (10.7-36.9)Bb
Pmicros (em milhares)	2.1 (1.0-4.5)Aa	0.3 (0.1-0.7)Cb	6.8 (4.4-10.8)Ba	0.5 (0.3-1.1)Cb
P gingivalis	1071.5 (316.6-3626.7)Aa	70.4 (23.2-213.2)Bb	1475.1 (459.8-4731.9)Aa	94.0 (36.7-240.4)Bb
D pneumosintes	37.0 (22.7-60.2)Aa	7.2 (4.4-12.0)Cb	96.8 (56.4-166.1)Ba	8.3 (3.8-18.0)Cb
Aactinomicetencomitans	25.1 (11.3-56.0)Aa	10.3 (4.9-21.8)Ba	17.1 (8.4-34.6)Aa	7.6 (4.2-13.8)Ba

Letras maiúsculas referem-se a comparações entre fumantes e nunca fumantes em cada período experimental: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).
Letras minúsculas referem-se a comparações entre diferentes períodos experimentais: médias seguidas por letras semelhantes não diferem entre si ($p > 0.05$).

Anexo 2



UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado "***EFEITO DO CONTROLE DE PLACA BACTERIANA SUPRAGENGIVAL SOBRE PARÂMETROS SUBGENGIVAIS***", sob o protocolo nº **32/03**, de responsabilidade do Pesquisador (a) *SABRINA CARVALHO GOMES*, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 03 (três) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled "***THE SUPRAGINGIVAL PLAQUE CONTROL OVER SUBGINGIVAL PARAMETERS: CLINICAL, MICROBIOLOGICAL AND IMMUNOLOGICAL ANALYSIS***", protocol number **32/03**, under Dr *SABRINA CARVALHO GOMES* responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 03 (three) years when the final review of this study will occur.

Araraquara, 18 de junho de 2003.

Profª Drª *Miriam Aparecida Onofre*
Coordenadora

Anexo 3

Termo de Consentimento Livre e Esclarecido

Efeito do controle de placa bacteriana supragengival sobre parâmetros subgengivais: Análise clínica, microbiológica e imunológica
Dados do voluntário:
Nome completo:_____-
_____Idade_____
RG:_____Endereço_____
_____
__No. do prontuário: _____

1) Essa pesquisa tem o objetivo de verificar o resultado do tratamento da inflamação da gengiva (gengivite). Nessa pesquisa, inicialmente, faremos exames clínicos para a seleção dos voluntários. Será realizada, também, uma entrevista, comum a qualquer atendimento odontológico, com perguntas relacionadas à sua saúde geral e a seus hábitos de higiene bucal. Os possíveis desconfortos relacionados a essa seleção são aqueles decorrentes de um exame de rotina e do tratamento da gengiva. Serão tomadas todas as medidas para garantir que o exame e tratamento sejam seguros, tais como uso de materiais descartáveis e de instrumentais esterilizados.

2) Após esses primeiros exames, você poderá ser selecionado ou não para o estudo. Se você não for selecionado, os benefícios associados à sua participação nesse exame são o acesso a um diagnóstico preciso da sua condição bucal, bem como esclarecimento e encaminhamento para tratamento, de acordo com as suas necessidades. Fica, ainda, assegurado o direito ao sigilo de todos os dados coletados, dos participantes selecionados ou não, sendo que, em nenhum momento, será permitido acesso de outra pessoa a esses dados, além do pesquisador ou do próprio indivíduo. Se você for selecionado, e tendo aceito participar como voluntário, nesse mesmo dia será realizada uma moldagem dos seus dentes para fazermos um molde que facilite e melhore a qualidade dos exames clínicos a serem feitos ao longo do estudo. Após, será marcada nova data, a ser agendada pelo pesquisador responsável, para a realização de um exame radiográfico (radiografias) e exames clínicos para avaliação da inflamação (imunológico) e infecção presentes (microbiológico). Esses exames são absolutamente seguros e são comuns aos tratamentos de gengiva.

3) A partir do início do estudo, você será chamado a comparecer semanalmente, com horário agendado, por 6 meses, à clínica de periodontia (tratamento de gengiva) para receber os exames e tratamentos de gengiva necessários, bem como o acompanhamento adequado. O tratamento a ser realizado é conhecido, seguro e faz parte de todo os tratamentos de gengiva já existentes. Faz-se uma raspagem dos dentes (remoção do tártaro), polimento e instrução de higiene bucal.

Essa raspagem visa deixar os dentes mais lisos, facilitando a sua escovação. Você poderá apresentar alguma sensibilidade dos dentes após essa raspagem, o que é uma conseqüência de qualquer tratamento da gengiva, não sendo um resultado específico do tratamento que você vai receber. Em todas as visitas será tomado o cuidado com o seu controle de placa (limpeza da boca) para a garantia da sua saúde gengival. Esse tratamento resolve o problema de inflamação da gengiva (gengivite) e é uma fase necessária para o tratamento a ser realizado, posteriormente, na raiz do dente, direcionado para uma doença chamada periodontite. A periodontite, se não for tratada, pode levar a perda dos dentes. Todos os participantes do estudo receberão o tratamento da periodontite, uma vez completado o tratamento da gengivite. O tratamento da periodontite poderá ser realizado antes do período de 6 meses, caso seja julgado necessário para

proteger sua saúde. O tratamento da periodontite não faz parte do presente estudo, mas é um complemento necessário para o restabelecimento da sua saúde bucal e não difere em nada do tratamento preconizado para doenças como a sua. Você será encaminhado, também, para outras disciplinas (avaliação e/ou tratamento), quando necessário.
4) Os conhecimentos adquiridos, com o presente estudo, serão importantes, futuramente, para a prevenção e tratamento das doenças de gengiva, pois poderão contribuir para que melhores formas de tratamento da gengivite e da periodontite sejam desenvolvidas. É importante ressaltar que toda e qualquer dúvida a respeito desse estudo será esclarecida pelo pesquisador responsável e que você poderá requisitar esse esclarecimento a qualquer momento. Esperamos poder atendê-lo em todas as suas dúvidas e nos comprometemos a dar todas as informações que você precisar ou que tornarem-se necessárias no decorrer do estudo.
5) Sua participação no estudo é voluntária e você, a qualquer tempo, pode afastar-se dele, sem que isso implique em qualquer prejuízo ao atendimento que você precisa. Nessa pesquisa a sua identificação (nome, RG, endereço, telefone) será confidencial. Somente os dados dos exames serão utilizados para fins de pesquisa, sem a identificação dos voluntários, preservando, assim, a sua privacidade. Eventualmente, fotos dos seus dentes e suas gengivas poderão ser interessantes para registrar as mudanças que ocorrerão com o tratamento. Quando isso for necessário, será solicitada sua permissão. Uma negativa sua não implicará em nenhuma alteração nos cuidados à sua pessoa. Por outro lado, se você concordar que as fotos sejam tomadas, fica garantido que não será possível, sob qualquer circunstância, a associação da foto com sua pessoa, garantindo-se assim absoluto sigilo da sua identidade. As escovas de dente a serem utilizadas pelos voluntários serão cedidas pelo pesquisador responsável pelo estudo. Não haverá ressarcimento dos prováveis gastos que o voluntário possa ter ao participar do presente estudo.

Qualquer sugestão, informação adicional, problemas, dúvidas ou reclamações, ligue para nós:

- Sabrina Carvalho Gomes 235 5864
- Comitê de Ética em Pesquisa: (16) 201 6432/6434.

Por esse instrumento particular, declaro, para fins de Ética e Legislação em Pesquisa, que eu, _____, nascido (a) em _____ /_____/_____, portador do RG_____(Órgão Expedidor_____), residente à _____no._____ _, Bairro _____, cidade _____, Estado _____, que eu li e entendi as informações acima citadas e concordo em Participar da Pesquisa "participar da Pesquisa "**Efeito do controle de placa bacteriana supragengival sobre parâmetros subgengivais: Análise clínica, microbiológica e imunológica**", recebendo os exames (para análise clínica, microbiológica e imunológica) e tratamentos especificados.

_____, _____de _____de 2003 Assinatura do voluntário _____

Assinatura do pesquisador_____

Anexo 4

HISTÓRICO MÉDICO CONFIDENCIAL

Participante No. _____Iniciais _____Data de Nasc. _____Masc. / Fem.

Antes do início de qualquer estudo precisamos preencher e devolver esse formulário. Favor circular SIM ou NÃO. *A resposta sim não significa, necessariamente, que você não estará habilitado para o estudo. Marque com um X a sua resposta.*

1. Você está indo a ou recebendo tratamento de um médico? _____SIM NÃO
2. Está tomando ou usando remédios, comprimidos, cápsulas, ungüentos, injeções, hormônios, anticoncepcional ou qualquer outra droga, prescrita por médico ou por sua vontade? _____SIM NÃO

3. Fez uso de antibióticos nos últimos seis (6) meses ou de antiinflamatório nos últimos três (3) meses? SIM NÃO

4. Você é alérgico(a) ou já teve alguma reação de desconforto a algum medicamento, alimento ou outra substância?_SIM NÃO

5. Já teve alguma doença grave na infância ou depois de adulto(a) ?_____SIM NÃO
6. Você tem ou já teve algum problema cardíaco ou de pressão?_____SIM NÃO
7. Você já teve febre reumática?_____SIM NÃO
8. Você tem marca-passo cardíaco?_____ SIM NÃO
   Você já sofreu alguma cirurgia do coração _____SIM NÃO
9. Você tem algum problema respiratório ou no peito?_____SIM NÃO
10. Você já recebeu anestesia dentária? SIM NÃO Apresentou alguma reação alérgica ou algum problema relacionado a anestesia? SIM NÃO
11. Você sobre de desmaios, ataques súbitos ou convulsões?_____SIM NÃO
12. Você já teve hepatite, icterícia, doença de fígado ou renal?_____SIM NÃO
13. Você é diabético(a) ? _____SIM NÃO
14. Você já teve algum problema de amostra, doação ou transfusão de sangue? __SIM NÃO

15. Você tem algum cartão de saúde com advertências do seu médico? _____SIM NÃO
16. Existe alguma coisa a respeito da sua saúde que nós não perguntamos, mas você acha que nós deveríamos saber? SIM NÃO
17. Você fuma? SIM NÃO. Quantos cigarros por dia_____Há quantos anos? _____

Se você respondeu sim a qualquer das perguntas acima, por favor conte nos um pouco mais no verso (ATRÁS) desse formulário.

Favor informar imediatamente se houver qualquer mudança dessas informações.

Até onde eu saiba, essas informações estão corretas. Eu entendo que essas informações podem ser inspecionadas por indivíduos autorizados e que serão tratadas como estritamente confidenciais.

FAVOR ASSINAR AQUI: _____Data _____
Obrigada pela ***colaboração.***

Verificado por.....……...Data…….

Artigos

Artigo I: Gomes SC, Marcantonio RAC: Efeito do controle de placa bacteriana supragengival sobre indicadores clínicos e microbiológicos subgengivais: revisão de literatura. Revista Periodontia, 2004. v.14, p.45-52, julho.

Artigo II: Gomes SC, Oppermann RV, Piccinin F, Susin C, Nonnenmacher CI, Mutters R, Marcantonio RAC. Periodontal status in smokers and never smokers: clinical findings and real time PCR quantification of putative periodontal pathogens. Journal of Clinical Periodontology.

Artigo III: Gomes SC, Piccinin F, Susin C, Oppermann RV, Marcantonio RAC. Supragingival plaque control regimen in periodontitis patients: clinical effects over a 6 months period. Journal of Clinical Periodontology.

Artigo IV: Gomes SC, Susin C, Oppermann RV, Nonnenmacher C, Marcantonio RAC, Mutters R: Effect of supragingival plaque control regimen on subgingival microbiota in smokers and never smokers: longitudinal evaluation by Real Time PCR. Journal of Clinical Periodontology.

Artigo V: Gomes SC, Piccinin F, Susin C, Oppermann RV, Marcantonio RAC. Effect of supragingival plaque control on gingival crevicular fluid volume in smokers and never smokers with periodontitis: a 6-months clinical study . Journal of Clinical Periodontology.

Efeito do controle de placa bacteriana supragengival sobre indicadores clínicos e microbiológicos subgengivais: revisão de literatura

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Resumo

O objetivo do presente trabalho é, através de uma revisão sistemática da literatura, verificar se o controle de placa bacteriana supragengival, isoladamente, determina alterações clínicas e microbiológicas subgengivais, em pacientes periodontais**.** A literatura pertinente ao tema foi consultada e os artigos foram selecionados de acordo com critérios de inclusão previamente estabelecidos. Os resultados dessa investigação demonstram que frente a um adequado controle de placa bacteriana supragengival, alterações clínicas importantes, tais como redução da profundidade de sondagem e do percentual de sítios com sangramento subgengival, podem ser verificadas, independentemente da condição inflamatória inicial. Observa-se, também, que essas alterações clínicas estão associadas a mudanças na microbiota subgengival, sejam elas, quantitativas ou qualitativas. Dessa forma, pode-se concluir que um adequado controle de placa bacteriana supragengival é capaz de determinar alterações subgengivais relevantes em pacientes periodontais.

Unitermos: controle de placa supragengival, gengivite, periodontite

Abstract

The present literature review investigated the effect of the supragingival plaque control over subgingival clinical and microbiological parameters. Related literature articles were selected and included accordingly to specific inclusion criteria. The results showed that an adequate supragingival plaque control determined important inflammatory condition reduction and improved the subgingival microbiological composition independent of the initial inflammatory condition in the experimental sites. It can be concluded that a regular and adequate supragingival plaque control can determine clinical and microbiological subgingival alterations in periodontal patients.

Uniterms: supragingival plaque control, gingivitis, periodontitis.

Introdução

A partir da identificação da placa bacteriana como fator etiológico das doenças periodontais, autores vêm discutindo o efeito do seu controle, supra ou subgengival, sobre os tecidos periodontais. Recentemente, o conceito de biofilme

renovou essas discussões, existindo claras evidências da associação de diferentes tipos bacterianos com estados de saúde, gengivites e periodontites (RUDIGER et al, 2002; XIMENEZ-FYVIE et al, 2000b; XIMENEZ-FYVIE et al, 2000c). Além disso, está estabelecido que os biofilmes supra e subgengivais têm importância diferenciada para os eventos etiopatogênicos das gengivites e periodontites, respectivamente (PAGE, 1997). Alguns autores sugerem, também, que o estabelecimento de um biofilme subgengival possa depender da formação e crescimento do biofilme supragengival (PAGE, 1997; LISTGARTEN, 1999; XIMENEZ-FYVIE et al, 2000b; WEIDLISH et al, 2001; SBODORNE & BORTOLAIA, 2003; RANBERG et al, 2003).

Recentes publicações, utilizando avançadas técnicas de biologia molecular, permitiram a identificação microbiana periodontal (XIMENEZ-FYVIE et al, 2000b; XIMENEZ-FYVIE et al, 2000c; BOUTAGA et al, 2003). Através dessas técnicas, pode ser observado, também, o comportamento do perfil microbiano em pacientes sob tratamento e manutenção periodontal. No entanto, esses estudos limitaram-se à avaliação da composição microbiana após a instrumentação subgengival (XIMENEZ-FYVIE et al, 2000a), comprometendo dessa forma, a compreensão do efeito subgengival do controle de placa bacteriana restrito ao ambiente supragengival.

Estudos dessa natureza vêem sendo realizados desde a década de 60. No entanto, à semelhança de vários outros tópicos em periodontia, trazem resultados conflitantes, apontando tanto para a existência quanto para a ausência de benefícios, clínicos e microbiológicos, desse método terapêutico (SMULOV et al, 1983; KHO et al, 1985; BELTRAMI et al, 1987; DAHLEN et al, 1992; KATSANOULAS et al, 1992; MACNABB et al, 1992; AL-YAHFOUFI et al, 1995; HELLSTRÖM et al, 1996; WESFELT et al, 1998).

Dessa forma, com o objetivo de identificar ou esclarecer os possíveis efeitos do controle mecânico de placa bacteriana supragengival, isoladamente, sobre indicadores clínicos e microbiológicos subgengivais, foi realizada a presente revisão sistemática da literatura.

Materiais e Métodos

Inicialmente foram identificados artigos através do *medline (descritores: supragingival plaque control;* gingivitis; *periodontitis; periodontal therapy; periodontal treatment; mechanical plaque control and gingivitis, mechanical plaque control and periodontitis, oral biofilms)* e estudos consultados. Foram localizadas 1307 citações e, posteriormente, aplicados alguns limites, aceitando-se apenas: *estudos experimentais em humanos, em pacientes com gengivite e periodontite, com avaliação clínica e microbiológica do efeito do controle mecânico e limitado ao ambiente supragengival em, pelo menos, um lado teste* (n=9). Alguns estudos selecionados não contemplam critérios metodológicos importantes, mas, ainda assim, foram incluídos por conterem informações relevantes e historicamente valiosas para a validação de estratégias de pesquisa e prática clínicas, referentes ao controle de placa bacteriano supragengival.

Revisão da Literatura

KHO et al, 1985, realizaram um estudo clínico com o objetivo de verificar, clínica e microbiologicamente, o efeito do controle de placa supragengival no ambiente subgengival, em paciente periodontais (n=8). Sítios com Profundidade de Sondagem (PS) entre 7-9mm foram submetidos às avaliações. Após o período experimental, observou-se redução da média dos escores de placa e PS, porém, aumento nos escores de gengivite (Quadro 1). Na avaliação microbiológica foram observadas flutuações na proporção de 11 tipos bacterianos avaliados, associadas à presença ou ausência de sangramento gengival. Ao final do estudo, entretanto, não foi observada diferença no número e tipo de bactérias presentes quando comparados ao exame inicial. A partir dessas observações, os autores concluíram que o controle de placa bacteriana supragengival não tem impacto na microbiota subgengival em bolsas profundas.

Porém, é interessante observar que, nesse estudo, houve incremento na condição inflamatória nos sítios experimentais (fato não explicado pelos autores). Assim, a inferência da extensão da PS como responsável pelos resultados fica limitada. Ao contrário, o conceito atual de que a formação de biofilme

subgengival é favorecida pelo edema marginal (LISTGARTEN, 1999; RAMBERG et al, 2003), determina a necessidade de novas interpretações para os achados desse estudo.

No entanto, os resultados de BELTRAMI et al., 1987, corroboram esses achados. Através de um modelo experimental de boca dividida (n=8), 2 sítios (PS>6.5mm) randomicamente selecionados e em quadrantes opostos, foram submetidos (teste, T) ou não (controle, C) aos procedimentos de controle de placa bacteriana supragengival.

Ao final do período experimental houve maior redução da média dos escores de placa nos sítios teste, porém os demais índices (IG, PS e SS, Quadro 1), que denotam expressão inflamatória, não se modificaram. Da mesma forma, não foram observadas reduções no percentual dos morfotipos microbianos avaliados, para ambos os sítios. Assim, à semelhança dos resultados de KHO et al, 1985, houve relação entre ausência de redução de parâmetros inflamatórios e ausência de resultados microbiológicos. Além disso, de acordo com a metodologia empregada, os participantes não receberam instrução de higiene bucal durante o estudo e abstiveram-se do controle de placa bacteriana nos sítios experimentais, podendo ter influenciado os resultados. É importante destacar o papel da formação do biofilme supragengival, já aos 4 dias de acumulação, sobre a expressão inflamatória marginal (WEIDLISH et al, 2001).

Em 1992, KATSANOULAS et al, através de um estudo modelo de boca dividida (n=13), relatam redução significante de placa bacteriana supragengival no lado teste (deplacagem supragengival profissional 3xsemana), porém, sem alteração de outros índices clínicos (IG, PS, PI, SS, Quadro 1). Na avaliação microbiológica observou-se que a proporção de bactérias foi semelhante, entre os grupos, ao final do estudo. Nesse estudo, também, não houve instrução de higiene bucal e, principalmente, monitoramento da sua realização durante as consultas. Novamente, a ausência de redução dos parâmetros clínicos de inflamação, principalmente sangramento marginal, pode estar relacionada aos resultados microbiológicos observados.

Como alguns autores justificam a ausência de efeitos clínicos e microbiológicos como conseqüência da extensão da profundidade de sondagem, alguns estudos foram realizados com o objetivo específico de elucidar o efeito do controle de placa supragengival em diferentes categorias de PS.

AL-YAHFOUFI et al, 1995, avaliaram esse efeito em sítios com bolsas rasas (PS<=3.5mm) e alta prevalência de periodontopatógenos. Exames clínicos e microbiológicos foram realizados ao início e final do estudo (dia 30). Os resultados clínicos mostraram uma redução significante na média do Índice de Placa e do percentual de sangramento à sondagem. Mesmo havendo pouca redução da profundidade de sondagem, houve redução do percentual de bactérias e de sítios positivos para as mesmas (Quadro 1), sugerindo que a redução inflamatória marginal seja importante para o ambiente subgengival.

Mesmo comportamento dos indicadores clínicos e microbiológicos (total e proporção de bactérias) foi observado por MCNABB et al, (1992), através de um estudo de boca dividida (Quadro 1), realizado em 6 pacientes periodontais, com doença moderada (PS = 4 a 5mm).

Para avaliação do efeito do controle de placa supragengival, SMULOV et al., 1983, selecionou 56 sítios com bolsas profundas (PS>5mm), em 14 pacientes, que foram randomicamente alocados para os seguintes grupos (G) experimentais: *G1*: raspagem supra e subgengival; *G2*: apenas raspagem supragengival; *G3*: apenas raspagem subgengival; *G4*: nenhuma intervenção terapêutica. O controle de placa profissional (5x/semana) limitou-se aos grupos *G1* e *G2*. Nesse estudo foi relatada (valores não apresentados) a diminuição de sítios com placa e gengivite, sem diferenças entre os grupos. Porém, a redução significante PS foi observada, apenas, para *G1 e* G2 (Quadro 1), sendo acompanhada pela redução significante do número e percentual de bactérias subgengivais. Os demais (*G3 e G4*) não apresentaram reduções significantes, sendo semelhantes entre si e diferindo de *G1* e *G2.*

Verifica-se, corroborando as conclusões dos autores, que o controle de placa bacteriana supragengival alterou a composição microbiológica subgengival, mesmo em sítios nos quais a intervenção subgengival não foi realizada *(G2)* e,

principalmente que, na ausência desse controle, a instrumentação subgengival apresenta limitações clínicas e microbiológicas importantes. Essa observação, de certa forma, reforça a importância do controle supragengival na manutenção dos resultados terapêuticos periodontais, como mostrado em alguns estudos (LINDHE et al, 1982).

O estudo de WESTFELT et al. (1998) foi realizado para verificar, ao longo de 36 meses, o efeito do controle supragengival na microbiota subgengival e no padrão de progressão de doença periodontal (n=12). A presença de placa supragengival, SS, PS e PI (Quadro 1) foi avaliada. Grupos teste (controle de placa supragengival) e controle (instrumentação subgengival e controle de placa supragengival) foram constituídos e os resultados, posteriormente, comparados. Nesse estudo verificou-se que, muito embora, o controle de placa bacteriana supragengival tenha determinado a redução semelhante de placa (T e C), a redução do percentual de SS e PS foi significativamente maior no grupo controle. Além disso, o controle supra não foi capaz de impedir a PI. No entanto, esses resultados têm que ser avaliados com cautela, uma vez que 75% da amostra foram compostas por pacientes fumantes. Resultados (gráficos) ilustram a relação diretamente proporcional entre o percentual de sítios com progressão de doença (PI progressiva) e número de cigarros/dia. Esse estudo aponta para a possibilidade de que, embora possa haver alguma alteração subgengival associada ao controle supragengival, a sua magnitude pode variar de acordo com as circunstâncias clínicas e, possivelmente, ser modificadas por fatores de risco. Sendo o fumo um reconhecido fator de risco à doença periodontal destrutiva (ALBANDAR, 2002), pode-se questionar se, em uma amostra de pacientes não fumantes, seria observado o mesmo comportamento. Nesse estudo observou-se, também, maior redução microbiana no grupo controle. Ainda assim, é necessário individualizar os resultados de fumantes e não fumantes, uma vez que estudos recentes observaram pior comportamento microbiano, após terapia subgengival, em pacientes fumantes (VAN der VELDEN et al, 2003). Além disso, a diminuição numérica de bactérias, porém, a impossibilidade de eliminação das mesmas reforça o conceito de biofilme (XIMENEZ –FYVIE et al, 2000c).

DAHLÉN et al, 1992, avaliaram o efeito do controle de placa bacteriana supragengival sobre parâmetros subgengivais em pacientes (n=62) com sítios com diferentes expressões inflamatórias (PS) e história de doença destrutiva (PI) (Quadro 1). Exames clínicos (placa, PS e PI, Quadro 1) e microbiológicos foram realizados em bolsas rasas (sítios AB, PS=0-3mm) e profundas (sítios C, PS≥5mm) no início e final (24 meses) do estudo.

Os resultados mostraram um incremento positivo nos sítios com PS ≤ 3mm (valores não apresentados) e negativo do número de sítios com PS ≥ 4mm. Essas reduções na PS foram acompanhadas por redução do número de pacientes positivos as bactérias avaliadas. Sendo assim, é possível verificar que o benefício de um adequado controle de placa supragengival estendeu-se para todos os sítios, independente da profundidade de sondagem ou expressão de doença destrutiva iniciais.

Para verificar se o controle de placa bacteriana supragengival pode afetar, diferentemente, sítios com defeitos ósseos distintos, foi realizada uma investigação por HELLSTRÖM et al (1996). Doze pacientes foram submetidos aos exames clínicos e, após, foram selecionados de 6 a 8 sítios (PS>5mm), por paciente, sendo, pelo menos um sítio com defeito supra-ósseo, um com defeito infra-ósseo e um sítio com envolvimento de furca. Os exames clínicos e coletas microbiológicas foram realizados no exame inicial e 30 dias após.

Os resultados dos sítios experimentais mostraram redução do percentual de sítios com placa, sangramento à sondagem, do número total de bactérias viáveis e do percentual de P. gingivalis, independente do tipo de defeito ósseo e do valor inicial da profundidade de sondagem. Assim os autores concluem que o controle de placa bacteriana supragengival afeta, de maneira significativa, o ambiente subgengival, sem que esse resultado possa, necessariamente, ser explicado somente pela diminuição da profundidade de sondagem. Esses achados corroboram os resultados de alguns estudos anteriormente apresentados e que apontaram para os benefícios subgengivais, clínicos ou microbiológicos, de um controle de placa supragengival eficaz.

Discussão:

O objetivo da presente revisão sistemática de literatura foi avaliar o efeito do controle mecânico de placa bacteriana restrito ao ambiente supragengival, sobre indicadores clínicos e microbiológicos subgengivais. Procedeu-se à pesquisa dos artigos, limitando sua inclusão de acordo com critérios estabelecidos e relacionados ao objeto de estudo. Nesse sentido, foram excluídos artigos de revisão de literatura (HAFFAJEE et al, 2003; SBODORNE & BORTOLAIA, 2003) ou mesmo estudos experimentais que, a despeito da sua relevância científica, apresentam resultados de instrumentação mecânica, mas que não se restringiu ao ambiente supragengival (XIMENEZ et al, 2000a).

Nos estudos aqui revisados, critérios metodológicos importantes não foram, na sua maioria, contemplados. A calibragem dos examinadores, fator relacionado ao viés de aferição (JACOB, 2002), não foi realizada na maioria dos estudos, à exceção daquele realizado por KATSANOULAS et al, 1992. Mesmo assim, os valores desse procedimento não foram apresentados, comprometendo a interpretação dos resultados. Além disso, nos estudos revisados, procedimentos de cálculo de amostra não foram feitos, bem como não foram adequadamente especificados os critérios de seleção de pacientes e/ou dos sítios experimentais. Essas características, quando contempladas, conferem maior rigor metodológico aos estudos, melhorando, também a validade interna e externa dos mesmos (HAYES, 2002).

Da mesma forma, alguns desenhos experimentais não valorizaram os procedimentos de controle de placa, seja pela ausência de instrução de higiene bucal ou pela ausência de avaliação periódica e adequada (KHO et al, 1985; BELTRAMI et al, 1987; KATSANOULAS et al, 1992). Nesses estudos pouco ou nenhum efeito clínico e microbiológico subgengival pode ser observado. É interessante observar que autores correlacionaram a ausência de efeito no ambiente subgengival como decorrente da extensão da profundidade de sondagem inicial. Nesses casos, considerações sobre deficiências metodológicas existentes, associadas ao conhecimento atual da etiopatogênese das doenças periodontais, poderá ser o ponto de partida para interpretações contemporâneas dos resultados

observados. A base para esse questionamento encontra-se, exatamente, nos estudos que observaram resultados microbiológicos, independente da profundidade de sondagem inicial ou tipo de defeito ósseo existente (SMULOV et al, 1983; DAHLEN et al, 1992; MACNABB et al, 1992; AL-YAHFOUFI et al, 1995; HELLSTRÖM et al, 1996; WESFELT et al, 1998).

Outro aspecto a ser destacado diz respeito à técnica utilizada para avaliação microbiológica. À exceção do estudo de YAHFOUFI et al, 1995, que utilizou técnica baseada em biologia molecular, os demais estudos utilizaram microscopia de campo escuro ou técnica de cultura. O advento das técnicas microbiológicas baseadas em detecção molecular (Reação de Polimerase em Cadeia e DNA-DNA-hybridization) e sua reconhecida superioridade em relação à microscopia de campo escuro e técnica de cultura (PAPAPANOU et al, 1997; BOUTAGA et al, 2003; LOOMER, 2004) reascendeu as discussões a respeito do potencial de detecção microbiana das mesmas. Da mesma forma, incentivou novas discussões acerca da composição microbiana periodontal, da associação de grupos bacterianos com estados de saúde ou doença periodontal e, também do efeito das terapias periodontais, supra ou subgengivais (XIMENEZ-FYVIE et al, 2000b; XIMENEZ-FYVIE et al, 2000c; LYONS et al, 2003).

Conclusões

Assim, de acordo com as considerações feitas, pode-se concluir que à medida que um controle estrito de placa supragengival é realizado adequadamente, e avaliado em bases permanentes, melhoras clínicas e microbiológicas subgengivais podem ser esperadas. No entanto, o reconhecimento da existência de deficiências metodológicas importantes nos estudos existentes, somado às novas possibilidades de investigação microbiológica, faz da resposta subgengival ao controle de placa bacteriano restrito ao ambiente supragengival uma questão aberta e que deverá ser melhor investigada, através de estudos mais adequados.

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Quadro 1

QUADRO RESUMITIVO DOS ESTUDOS REVISADOS (N=9). ARARAQUARA, 2004.

	Smulov et al, 1983	Kho et al, 1985	Beltrami et al, 1987	Katsanoulas et al, 1992
Tipo de estudo	Exp., MBD, Long: 3 sem.	Exp., NC, Long: 18 sem.	Exp., MBD, Long: 3 sem.	Exp., MBD, Long: 3 sem.
Objetivo	Avaliar se o número de bactérias subgengivais se altera apenas com o controle de placa bacteriana supragengival.	Avaliar o efeito do controle de placa supragengival na microbiota subgengival.	Avaliar se o equilíbrio da microbiota sub- pode ser obtida através do controle de placa supragengival.	Avaliar o efeito do controle de placa supra na microbiota e condição clínica subgengival
Seleção pacientes (P) e sítios (S) bem estabelecidos?	P:Não S: Sim	P: Alguma informação S: Sim (PS > = 7mm)	P: Alguma informação S: Sim, 2 pares contralaterais, PS> 6.5mm IG>2; PO radiográfica	P: Alguma informação S: Sim (PS=4-6mm)
Amostra e faixa etária	14 (23-56 anos)	8 (30-60 anos)	8 (32-60 anos)	13 (27-77 anos)
Índices avaliados	(pi, IG, PS	(pi, SS, PS	(pi, IG, SS, PS	(pi, IG, PS, PI, SS
Calibragem examinador	NR	NR	NR	Sim; sem dados
Coleta /e técnica microbiológica	Curetas/cultura	Sonda terpada/cultura	Ponta papel/campo escuro	Cureta/campo escuro
Controle de placa	Grupo 1 e 2: sim, profissional, 5x/ semana	Sim: apenas semanas 1 e 2	T: 3x/semana C: sem intervenção supra	T: 3x/semana C: não
Resultados clínicos	Redução de (pi e (G : escores 1-3 (I) para 0-3 (f). Redução da PS observada, principalmente, para os grupos 1 (1 a 4mm em 71% dos sítios) e 2 (1 a 3mm em 79% dos sítios), sem dif. sig. entre ees. Grupo 3: redução de 1-2mm (50% dos sítios, NS), grupo 4 sem redução.	Dentes de Ramford: Redução de placa (1.2 para 0.4), gengivite (1.2 para 0.5) Sítios experimentais: diminuição de placa (1.1 para 0.4) e incremento de gengivite (1.2 para 1.4). 1mm redução de PS em todos os sítios .	T: redução sig. placa (2.6 para 0.4) Demais índices: sem dif. entre início e fina, para ambos os grupos.	(pi: redução sig. em todos os momentos exp. Redução NS para IG, SS (dados não mostrados) e PS: T: 4.65; C: 4.85 e PI: T: 8.62; C: 8.27.
Resultados microbiológicos	Diminuição da contagem de bactérias subgengivais, principalmente nos gru-pos 1 e 2 (SDE) Os demais grupos apresentaram diferenças, porém, sem a magnitude observada para grupos 1 e 2	Sem diferenças entre os exames inicial e final.	Sem diferenças, inter-grupos ao final do estudo.	Sem dif. entre os grupos T e C ao final do período experimental.
Conclusões dos autores	Controle de placa supragengival determina alterações microbiológicas subgengivais importantes. Na ausência desse, alterações microbiológicas significantes não são obtidas	O controle de placa bacteriana supragengival não determinou alterações significantes na microbiota subgengival em sítios profundos (PS> 7mm).	Sítios com PS maior não se beneficiam do controle de placa supragengival.	Em sítios com PS maior o controle de placa não é suficiente para determinar alterações clínicas e microbiológicas.

Legenda: Exp: experimental; NC: não controlado; MBD: modelo de boca dividida; Long: longitudinal (d: dia, sem: semanas, m: mês); Ipl: Índice de Placa de Silness e Löe, 1964; IG: Índice Gengival de Löe e Silness, 1963; PS: Profundidade de Sondagem r; PI: Perda de Inserção clínica; PO: Perda Óssea radiográfica; T: grupo teste; C: grupo controle; IHB: Instrução de Higiene Bucal; RAP: raspagem

Dahlen et al, 1992	McNabb et al, 1992	Al-Yahfoufi et al, 1995	Helstrom et al, 1996	Westfelt et al, 1998
Exp, NC, Long: 24 m.	**Exp., MBD, Long: 24m.**	**Exp., NC, Long: 37 d.**	**Exp., MBD, Long: 30sem.**	**Exp., MBD, Long: 36 m.**
Avaliar o efeito do controle de placa supragengival no ambiente subgengival.	Avaliar clínica e microbiologicamente o efeito do controle de placa supra em bolsas moderadas.	Avaliar presença de 3 periodontopatógenos e aspectos clínicos subgengivais após controle supra.	Avaliar o efeito do controle de placa supragengival em diferentes tipos de defeitos ósseos.	Avaliar o efeito do controle de placa na microbiota subgengival e padrão de progressão da doença.
P: alguma informação S: A e C: PS=0-3 mm S: B: PS > =5mm	P: Alguma informação S: PS = 4-5mm com 15% espiroquetas e 20% bacilos	P: Sim S: PS média = 3.5mm Menos de 5% / sítios com PS > 5mm	P: Sim S: Sim: PS > 5mm com defeitos supra e infra-ósseos e furca	P: Sim S: 3/quadrante, com PS>5mm
62 (30-59 anos)	6 (20-54 anos)	10 (22-39 anos)	12 (44-69 anos)	12 (40-65 anos)
PI visível, PS, PI, SS	Ipi, Ig, PS, PI, SS	Ipi, PS, PI, SS	Ipi, Ig, PS, PI	% placa corada, PS, PI, SS
NR	NR	NR	NR	NR
Ponta papel/cultura	Ponta papel/cultura/CE	Ponta papel/DNA-Hyb.	Ponta papel/cultura (Pg)	Ponta papel/cultura
Sim, nos 3 meses iniciais Depois: paciente	Sim, 3x semana, por 3 meses. Após, sem IHB.	Sim, na primeira semana	Sim, 2 a 3 X/semana e reforço na IHB	Sim, 2 a 3x/semana, nos 3 meses iniciais. T: RAP C: RAR
Redução de placa e gengivite: sítios A, B e C, sem diferenças inter-grupos. Redução de PS, principalmente em sítios com PS > =6,5mm.	Redução placa e gengivite para os grupos, sem. dif. 50% de redução do número de sítios com PS 4-5mm NS (valores expressos em gráficos).	Redução placa (de 1.30 para 0..39), de SS (68% dos sítios para 20%), de PS: 2.80 para 2.52, (NS). Sem alteração do PI (2.69-2.72).	Ipi e IG: redução sig para todos os sítios: 68% para 20% e 63% para 5% a 10%. Aumento do número de sítios PS = 0-3mm (de 58% para 71%), sem alterar percentual de sítios PS > 7mm Dif. Sig para todos os tipos de defeitos, à exceção dos infra-ósseos.	Redução NS de placa (T: 55.7% e C: 51.3%). Redução SIG de SS (T: 30.8% e C: 53%): PS 3-6mm: (T: 16.3% e C: 30.7%,). Redução em PS>6mm SIG para C: 10.7%. Maior % de sítios com PI em sítios com PS > 6mm (T: 32% e C: 2,7%). SIG para T.
Redução do número de Colônia (UFC) em todos os sítios (A, B e C), entre os exames inicial e final.	Redução periodontopatógenos, aumento nos gram-positivos.	Redução no número de sítios positivos para os microrganismos avaliados: A.a; Pg; PI.	Redução do número de UFC para Pg, para todos os sítios.	Intra-grupos: Dif. sig para ambos, entre 0 e 36 meses. Inter-grupos: redução, porém, sem diferenças aos 36 meses.
O controle de placa alterou a composição microbiana subgengival independente da PS e PI iniciais.	O controle de placa supra determina alterações microbiológicas em bolsas moderadas.	O controle de placa supragengival alterou número de bactérias subgengivais, favorecendo sua diminuição.	O controle supra determinou aumento no número de bolsas rasas e diminuiu o de profundas. Redução bacteriana ocorreu, independente da PS inicial.	O controle de placa supragengival diminui o TBV, mas não impede a perda de inserção clínica.

e alisamento supragengival; RAR: Raspagem Radicular, supra e subgengival simultaneamente; CE: campo escuro; TBV: Total de Bactérias Viáveis; UFC: Unidades formadoras de colônia, NS: não significante, Dif.sig: diferença significante; SIG: significativo; Pg: Porfiromonas gingivalis; Pi: Prevotela intermedia; A.a: Actinobacilus actinomicetencomitans.

Periodontal status in smokers and never-smokers: clinical findings and real time PCR quantification of putative periodontal pathogens.. Journal of Clinical Periodontology

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Abstract

Aim: The aim of the present study was to investigate the clinical condition and microbiological profile of patients with chronic periodontitis as related to the habit of smoking.

Materials and methods: 50 patients (age 33-59 years), 25 smokers (S) and 25 never smokers (NS), with chronic periodontitis constituted the study group. The following clinical were registered: Visible Plaque Index (VPI), Gingival Bleeding Index (GBI), Bleeding on Probing (BOP), Periodontal Probing Depth (PPD), Clinical Attachment Loss (CAL), Gingival Crevicular Fluid (GCF) volume (Periotrom®8000). Additionally, the number of *P.gingivalis, P.micros, D.pneumosintes, A.actinomycetemcomitans* and Eubacteria (Eu) were quantified in samples of the subgingival microbiota with the aid of Real Time PCR. Statistical analysis (test Wald, $p<0.05$) was performed using linear models adjusted for clustering of observations within individuals.

Results: Smokers and never-smokers showed similar values for VPI, GBI and BOP. Further analysis showed less GBI in heavy smokers. Smokers had deeper PPD in buccal/lingual sites and higher CAL independently of the tooth surface. Males and females differed to CAL and GCF. GCF volume was smaller is smokers, independent of the PPD. GCF volumes significantly associated to all bacteria, except to *A. actinomycetemcomitans*. *A. actinomycetemcomitans* showed the lowest counts. Similar amounts of Eu and *P.gingivalis* were observed for both groups. Significantly higher numbers of *D.pneumosintes* and *P.micros* were present in smokers and were associated with moderate and deep pockets. When heavy smokers were considered, higher counts of Eubacteria, *P.micros* and *D. pneumosintes* were observed. *Conclusions:* It can be concluded that the clinical differences between never smokers and smokers were related to PD, CAL and GCF volume. There is an association between mean number of bacteria to pockets depth and GCF volume. *Peptostreptococcus micros* and *Dialister pneumosintes* were significantly in higher numbers in smokers.

Keywords: Microbiology, quantitative evaluation, Polimerase Chain Reaction, periodontitis, smoking, cross-sectional

Running title: Clinical and subgingival microbiota features in smokers and never smokers

Introduction

Periodontitis is a destructive disease affecting the periodontal supporting tissues, which are associated with an imbalance between the subgingival biofilm and the host response (Page et al., 1997). Smoking is a known risk factor for destructive periodontal diseases and evidence exists of its role in the pathogenesis of periodontitis (Hujoel et al., 2003). Clinical and laboratorial studies have shown an association between deficiencies in the immune response, fibroblasts activity and smoking or tobacco products (Giannopoulou et al., 1999; Gamal et al., 2002; Giannopoulou et al., 2003; Rawlinson et al., 2003). Clinically, smoking has been associated with a reduced inflammatory response of the marginal gingiva, increased probing depth and greater attachment loss (Haffajee & Socransky, 2001a; Erdemir et al., 2004; Bajloon et al., 2005). Interestingly bleeding from the bottom of the periodontal pocket seems not to be affected by smoking (Müller et al., 2002; Haffajee & Socransky, 2001a).

Microbiota composition could explain, at least in part, the differences between smokers and non-smokers. However, while some authors have shown differences in the microbiota between smokers and nonsmokers (Shiloah et al., 2000, Haffajee & Socransky, 2001b; Van Winkelhoff et al., 2001; Van der Velden et al., 2003), several other studies have not been able to demonstrate relevant differences (Böstrom et al., 2001; Salvi et al., 2005; Natto et al., 2005; Buduneli et al., 2005; Apatzidou et al., 2005). Among the possible explanations for these conflicting findings are inherent limitations of some of the microbiological methods used. Bacterial culture is limited to those bacteria that are currently cultivable and DNA-DNA hybridization and PCR are semi-quantitative methods. Real Time PCR has been presented as an alternative method as it allows qualitative and quantitative evaluations (Boutaga et al., 2003; Sanz et al., 2004).

Aim

The aim of the present study was to investigate the clinical condition and microbiological profile of patients with chronic periodontitis as related to the habit of smoking.

Materials and Methods

Study design and sample: This cross-sectional study was performed with a convenience sample of consecutive patients seeking treatment (July 2003 and August 2004) from the pool of patients referred for treatment to the Department of Periodontology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. 50 patients (25 never smokers and 25 smokers) with an age range of 33-59 years were selected (Table 1). The study protocol was reviewed and approved by the Committee for Ethical Affairs of the State University of Sao Paulo at Araraquara, Federal University of Rio Grande do Sul and the National Council of Ethics in Research.

The participants were eligible for the study if they did not have unfavorable systemic conditions such as diabetes, heart or circulatory problems; previous periodontal treatment; anti-inflammatory or antibiotics therapy (previous 3 and 6 months respectively); use of chemical plaque control, pregnancy, hormones intake. Eligible patients willing to participate signed an informed consent. Eligible consenting patients were clinically screened and included in the study if they had 12 or more teeth present (excluding third molars, condemned or endo-periodontal lesions involved teeth). Four of them should have at least one site with PPD ranging between 3 and 5 mm, and another 4 at least one site with PPD 6 to10mm, with visible plaque, gingivitis and chronic periodontitis.

The sample size data derived from previous calculation to an interventional study.

Clinical and sampling procedures

Interview, clinical examinations and sampling collection were conducted one week after the screening examination. Participants filled out a questionnaire to gather demographic information and other health-related information including smoking habits.

Microbiological and Gingival Crevicular Fluid sampling

Initially, the presence of supragingival plaque and marginal gingival bleeding were registered, respectively with the Visible Plaque Index and Gingival Bleeding Index (VPI and GBI, Ainamo and Bay, 1975) in six sites per tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual and distolingual).

In sequence, microbiological samples were collected from 4 out of the 8 sites used to assess GCF (2 from each PPD sites category). Gingival Crevicular Fluid (GCF) was collected in 8 randomly selected from those meeting the inclusion criteria (4 with PPD 3-5mm and 4 with PPD 6-10mm, in different teeth).

Following careful removal of all supragingival plaque, the areas were washed with a water spray, isolated with cotton rolls and gently dried. A sterile paper point #30 (EndoPoint, Paraíba do Sul, RJ, Brasil) was inserted to the bottom of the periodontal pockets for 30 seconds (Hartroth et al., 1999). The paper points were then placed into sterile tubes containing 200µl of RTF (Syed and Loesche, 1972). The collected samples were stored at -20^0 C.

In sequence, the teeth were again washed (30s), the area isolated and gently dried (30s) to Crevicular Gingival Fluid (CGF) collection. CGF was collected with paper strips (PerioPaper ®Strips, OraFlow, PlainView, New York) inserted in the periodontal pocket to the point the examiner perceived slight resistance and maintained for 30 seconds (Tözüm et al., 2004). Immediately after, the amount of GCF was measured with the aid of a calibrated Periotrom 8000® (OraFlow, PlainView, New York). Strips with marks of blood were discarded. One examiner (SCG) performed all the microbial and GCF sampling.

Clinical examination

After, the periodontal examination was completed with the Periodontal Probing Depth (PPD), Clinical Attachment Loss (CAL) and Bleeding on Probing (BOP), measured with a PUNC-15 manual periodontal probe colour coded at 1 to 15mm (Neumar, São Paulo, Brazil). The nearest upper millimetre was considered.

Measurement reproducibility

One trained and calibrated periodontist (FP) performed all clinical examinations. The examiner was unaware of the patient's tobacco exposure dose. Approximately

10% of the patients prior to and during the examination period were re-examined, within one week, to assess reproducibility. The unweighted Kappa statistics at site level for PPD was 0.65 before the beginning the study and 0.52 during the study. Corresponding values for unweighted Kappa for CAL were 0.51 and 0.60, respectively. The weighted kappa (±1mm) at site level was 0.98 and 0.95 for PPD and 0.97 and 0.92 for CAL in pre and trans-experimental periods, respectively.

Real Time PCR Methodology

The methodology is published (Nonnenmacher et al., 2004). Briefly, oligonucleotide primers and TaqMan, based on species-specific conserved regions (16S rRNA gene) of *A. actinomycetemcomitans (Aa)*, *P. gingivalis (Pg)*, *P. micros (Pm)* and *D. pneumosintes (Dp)* (Table 5) were selected (Primer Express software V 1.0; Applied Biosystems –AB-International). The fluorescent dyes at the 5' and at the 3' ends of the probe were FAM (6-carboxyfluorescein; reporter) and TAMRA (6-carboxytetramethylrhodamine; quencher), respectively. Additionally, a universal bacterial primer pair was used to detect DNA from all Eubacteria species present in the samples. All primers and probes were checked for possible cross-hybridization with bacterial database (BLAST).

Using serial 10-fold dilutions from 10^1 to 10^8 copies of previously quantified plasmid standards the quantitative assay was performed. Plasmid standards and clinical samples were run in duplicates and the average values were used for calculation of the bacterial load.

Samples were assayed in duplicate in a 25-µl reaction mixture containing 2.5 µl of template DNA, 2.5 µl of 10xbuffer with ROX, 1.5 µl of 50mM $MgCl_2$, 1 µl dNTP (qPCR Core Kit, Eurogentec, Belgium), 12.5 pmol of forward primer and reverse primer (MWG, Munich), and 3.75 pmol of the probe (Eurogentec, Belgium). The cycling conditions used were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min each. During the annealing-extension step, the ABI Prism 7700 SDS (Applied Biosystems International) monitored real-time PCR amplification by quantitatively analysing fluorescence emissions. All PCRs were performed in duplicate. All the microbiological analysis was conducted blinded to clinical diagnosis.

Statistical analysis

Subjects included in the present study were either current smokers or have never smoked. Exposure to cigarette smoking was calculated for current smokers (number of cigarettes consumed per day x number of years of habit/ 20). The smoking cut-points were selected according to the median among current smokers. Heavy smokers were defined by the consumption of at least ≥20 packs/year or ≥7300 packs.

Data analysis was performed by STATA software[†] and using linear models. Measurements at tooth level were used and estimates were adjusted for the clustering of teeth into individuals using a robust variance estimator. Wald tests were used for comparisons, and the p-value was adjusted for multiple comparisons. The level of significance was set at 5%, and the 95% confidence intervals (CI) were calculated.

Raw Periotrom scores were converted to volume of GCF in μL following manufacturer's recommendations. Standard curves were used to convert Cycle Threshold (Ct) scores into number of bacterial cells using samples with known amounts of bacterial-specific DNA. These variables were not normally distributed, and to achieve normality the data was transformed using a logarithmic function. Results are reported in the back-transformed form.

Results

No significant differences were observed in the percentage of sites with visible plaque, marginal bleeding and bleeding on probing between smokers and never-smokers (Table 2). However, when smokers were further classified according to lifetime smoking exposure, heavy smokers had significantly fewer sites with marginal bleeding than never-smokers (66.88±4.70 vs. 45.86±6.85, p=0.02). No significant differences were observed between never-smokers and light smokers.

Smokers had significant greater attachment loss than never-smokers. Probing depth differences (p=0.006) were observed in buccal/lingual sites for smokers. Smokers showed significantly smaller amounts of GCF than never-smokers in 4-5mm PPD and PPD6+mm sites (Fig 1).

Marginal bleeding did not differ between males and females, even thought males showed a higher percentage (p=0.04) of plaque in buccal/lingual surfaces (Table 2). Bleeding on probing was significant greater in males than females, but the differences were small in magnitude. Mean probing depth and attachment loss in buccal/lingual sites and attachment loss in proximal sites were also significantly higher in males than females. After adjusting for age, smoking and dental plaque, probing depth differences between genders disappeared, whereas attachment loss remained significantly greater for males when compared to females.

No differences (p=0.09) in number of Eubacteria could be observed between smokers and never smokers (Fig 2). No significant differences were observed in the occurrence of *A..actinomycetemcomitans* and *P.gingivalis* regarding smoking status. *D.pneumosintes* and *P.micros* counts were significantly greater in smokers. Additional analysis showed that light smokers did not differ from never smokers in any of the studied bacteria, whereas heavy smokers had significantly higher total counts of bacteria as well as greater mean counts for *D. pneumosintes* and *P.micros*. No significant differences in bacteria load were observed between genders (data not shown).

In the multivariable analysis, the volume of GCF was significantly associated with females, never smokers, moderate and deep probing depths (Table 3). Total counts of bacteria, *P.gingivalis* and *A..actinomycetemcomitans* were only associated with probing depth, whereas *D.pneumosintes* and *P.micros* were significantly associated with smokers as well as moderate and deep pockets. No significant associations between bacteria and demographics (gender and age) were found.

An association between GCF and microbiota was observed, and increased amounts of GCF were significantly associated with increased total number of bacteria as well as increased counts of *P.gingivalis*, *D.pneumosintes* and *P.micros* (Table 4). This association could not be explained by differences in gender, age, smoking status and probing depth.

[†] Stata 7.0 for Windows, Stata Corporation, College Station, TX, USA

Discussion

The present cross-sectional investigation assessed the clinical condition and the subgingival microbiota in smokers as compared to never smokers. No differences were observed in the presence of supragingival plaque, marginal bleeding and bleeding on probing between the evaluated groups. However, when the level of exposure was considered, heavy smokers showed less marginal inflammation. Some studies show lower levels of marginal bleeding in smokers, but in these studies the level of exposure to tobacco is not always disclosed. Recently, Salvi et al., 2005, did not observe any difference on the incidence of plaque and gingivitis in a 35 days experimental gingivitis period in a group of smoker and non-smoker patients. However, heavy smokers were defined as consuming 6.4 ± 2.8 packs/year. In the present study, heavy smokers were defined by the consumption of at least ≥20 packs/year or ≥7300 packs.

The role of the smoking habit is established as a modifier factor for gingivitis and as a risk factor for periodontitis (Bergström et al., 1989; Albandar et al., 2002). Current epidemiological and clinical evaluations indicate that the level of exposure to the factor can explain differences observed in the inflammatory and destructive periodontal expression among smoker patients (Bergström, 2003; Susin et al., 2004). There is also an association between the level of exposure to the factor and different immune-inflammatory responses when smoker and non-smoker patients are compared (Petropoulos et al., 2004; Kamma et al., 2004).

Smokers showed larger probing depths than never smokers on buccal-lingual surfaces. These surfaces showed less plaque than proximal surfaces. Under these circumstances the effect of tobacco may be more easily detected in places where the plaque control measures are more effective than places where it is virtually absent. The role of smoking in periodontal destruction becomes more evident when the attachment loss is considered. Smokers showed a larger periodontal destruction than never smokers, both on buccal-lingual and proximal surfaces. Similar results are reported in the literature (Haffagee et al., 2001a; Bergström, 2004). It was also observed that males presented higher levels of attachment loss,

regardless of the smoking habit. Males and smokers are associated with larger probing depths and attachment loss (Susin et al., 2004; Susin et al., 2005).

A direct relationship between the amounts of fluid and probing depth was clearly shown. This observation is well established in the literature (Griffiths et al., 1992; Griffiths, 2003). The gingival fluid is considered an important inflammatory indicator (Griffiths, 2003). Smokers showed a lower volume of crevicular fluid in the experimental sites with significant differences starting from PPD>4mm on (Fig 1). These differences between the two groups are in contrast with the absence of significance of the other inflammatory signs, such as bleeding on probing. However, heavy smokers showed significantly less marginal bleeding than never smokers. This reduction of the inflammatory response to the presence of supragingival plaque would result, thus, in a smaller production of fluid. The observation of larger amounts of fluid in women cannot be explained by differences in probing depth or in the number of bacteria present in both genders. However, it must be considered that it can be associated with the higher number of men in the smokers group, without disregarding possible alterations for fluid related to the hormonal alterations observed in women (Holm-Pedersen & Löe, 1967).

In the present study, the microbiologic quantification of the total amount of bacteria present as well as the numbers of specific bacteria (*Porphyromonas gingivalis, Peptostreptococcus micros, Actinobacillus actinomycetemcomitans,* and *Dialister pneumosintes)* was performed by means of the real time PCR technique. The technique is both sensitive, specific and identifies from 10^1 to 10^8 cells present in the samples. Real Time PCR has been used in periodontal microbiological investigations (Lyons et al., 2000; Rudney et al., 2003; Yoshida et al., 2003; Boutaga et al., 2003; Kuboniwa et al., 2004; Lau et al., 2004; Morillo et al., 2004; Nonnenmacher et al., 2004; Suzuki et al., 2004; Sanz et al., 2004). *P.gingivalis, P.micros,* and *A.actinomycetemcomitans,* are recognized as potential periodontophatogens and represent, respectively, components of the red, orange, and green/blue groups (Socransky et al., 2004). Contreras et al., 2000, observed a

strong association between *D.pneumosintes* and severe periodontitis, suggesting its inclusion in the group of putative periodontal pathogen.

There were no differences between groups in the total number of bacteria (Eubacteria). There were no differences between smokers and never smokers concerning the amounts present of *A. actinomycetemcomitans* and *P. gingivalis.* Similar results concerning *P. gingivalis* were reported by Natto et al., 2005, suggesting that the composition of the subgingival microbiota may be independent from the smoking habit. Boström et al., 2001 have shown that smoking has little or no effect on the periodontal microbiota. In the present study, the number of *P.micros*, however, was significantly higher in smokers. This is in accordance with Van Winkelhoff et al., 2001 and Haffajee & Socransky, 2001b. *D. pneumosintes* was also present in larger numbers in smokers than never smokers. Differences among studies can be related to sensitivity issues of the used detection methods or, occasionally, as a result of differences observed in the microbial composition of distinct populations (Sanz et al., 2000; Haffajee et al., 2004).

The number of bacteria, with the exception of *A..actinomycetemcomitans*, was significantly associated with the volume of fluid. This association could not be explained by gender, age, smoking habits or probing depth. The mechanisms behind the observed associations are not clear. It is conceivable that those sites with higher levels of infection present a more severe inflammatory response and consequently a higher exudation of gingival fluid.

It was evident in the present study that smokers show a worse clinical periodontal condition than never smokers, especially in relation to overall attachment loss and probing depth in buccal-lingual surfaces. This observation cannot be explained by differences in the presence of visible plaque, marginal bleeding or bleeding on probing between both groups. The only difference that was observed was a smaller marginal bleeding when comparing heavy smokers to never-smokers. Crevicular fluid increased according to the probing depth, but it was significantly smaller in smokers than in never smokers with PPD > 4mm. In the microbiological analysis, the total of bacteria did not differ between smokers and never smokers, as did not the presence of *P. gingivalis* and

A..actinomycetemcomitans. However, *P.micros* and *D.pneumosintes* were significantly increased in smokers. Results indicate that specific microbial characteristics of the subgingival plaque may be associated with a worse periodontal clinical condition observed in smokers when compared to never smokers.

Conclusions

It can be concluded that the clinical differences between never smokers and smokers were restricted to CAL and GCF volume. There is an association between mean number of bacteria to pockets depth and GCF volume. *Peptostreptococcus micros* and *Dialister pneumosintes* were significantly in higher numbers in smokers. There is a possibility that these results may partially be explained by the degree of exposure.

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Table 1. Age, gender and smoking habits by experimental groups.

	Never smokers	Smokers
n	25	25
Age (mean ± SD)	46.8 ± 7.1	45.9 ± 5.4
Gender (%M)	40	56
N teeth (mean ± SD)	21.4 ± 3.8	21.2 ± 3.7
N Cigarettes per day (mean ± SD)	0 ± 0	19.4 ± 11.6
Years of habit (mean ± SD)	0 ± 0	24.8 ± 8.6

Table 2. Clinical parameters by gender and smoking status (mean±SE)

	Female	Male	p	Never smokers	Smokers	p
Buccal/lingual						
Visible plaque (%)	76.32±4.01	86.36±2.47	0.04	82.88±3.23	79.72±3.75	0.53
Marginal bleeding (%)	57.41±4.80	66.28±5.42	0.22	66.88±4.70	56.42±5.40	0.14
Bleeding on probing (%)	87.05±3.07	96.21±1.24	0.008	91.39±2.14	91.79±2.89	0.91
Probing depth (mm)	2.92±0.08	3.27±0.11	0.02	2.90±0.08	3.29±0.11	0.006
Attachment loss (mm)	3.02±0.15	4.10±0.22	0.0002	3.08±0.15	4.03±0.23	0.001
Mesial/distal						
Visible plaque (%)	91.94±2.16	96.12±1.44	0.11	95.14±1.16	92.88±0.24	0.40
Marginal bleeding (%)	86.81±3.56	91.34±2.19	0.28	91.73±2.29	85.99±0.35	0.15
Bleeding on probing (%)	93.52±2.36	98.91±0.58	0.03	96.73±1.12	95.66±0.23	0.68
Probing depth (mm)	4.00±0.11	4.32±0.17	0.10	4.06±0.13	4.26±0.15	0.32
Attachment loss (mm)	3.43±0.16	4.49±0.22	0.0003	3.54±0.18	4.37±0.22	0.006

Table 3. Multivariable analysis of the association between GCF volume and microbiota with Gender, Age, Smoking status and Probing depth

	Gingival crevicular fluid		Microorganisms									
			Total load		Pg		Dp		Pm		Aa	
	Log β	SE	Log β	SE	Log β	SE	Log β	SE	Log β	SE	Log β	SE
Gender												
Female												
Male	-0.22*	0.11	0.01	0.34	-0.79	0.84	0.30	0.34	0.04	0.40	0.07	0.53
Age	-0.01	0.01	0.04	0.03	0.01	0.07	0.05	0.03	0.05	0.03	0.00	0.04
Smoking status												
Never-smokers												
Smokers	-0.51**	0.11	0.44	0.34	0.16	0.84	0.87**	0.34	1.00**	0.40	-0.45	0.53
Probing depth												
≤3 mm												
4-5 mm	0.50**	0.10	1.90**	0.42	1.04	0.69	1.59**	0.47	2.06**	0.45	0.70	0.50
≥6 mm	0.86**	0.10	3.35**	0.41	2.92**	0.67	2.24**	0.46	3.21**	0.44	1.85**	0.49

* p< 0.05 ** p< 0.01

Table 4. Association between GCF volume and subgingival microbiota

	Log β*	SE	p
Total load	0.12	0.02	<0.001
Pg	0.04	0.01	0.003
Dp	0.07	0.02	0.001
Pm	0.09	0.02	<0.001
Aa	0.02	0.02	0.34

* Adjusted for gender, age, smoking, probing depth at baseline.

Table 5. Bacterial species, real-Time PCR primers and probes used

Primer and Probe	Sequence (5'-3')	Acession numbers
Universal *		
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA(AG)CCATGCA	
A. actinomycetemcomitans		M75039
Forward	CAAGTGTGATTAGGTAGTTGGTGGG	
Reverse	CCTTCCTCATCACCGAAAGAA	
Probe	ATCGCTAGCTGGTCTGAGAGGATGGCC	
D. pneumosintes		X82500
Forward	GAGGGGTTTGCGACTGATTA	
Reverse	CCGTCAGACTTTCGTCCATT	
Probe	CACCAAGCCGACGATCAGTAGCCG	
M. micros		D14143
Forward	AAACGACGATTAATACCACATGAGAC	
Reverse	ACTGCTGCCTCCCGTAGGA	
Probe	TCAAAGATTTATCGGTGTAAGAAGGGCTCGC	
P. gingivalis		L16492
Forward	TGCAACTTGCCTTACAGAGGG	
Reverse	ACTCGTATCGCCCGTTATTC	
Probe	AGCTGTAAGATAGGCATGCGTCCCATTAGCTA	

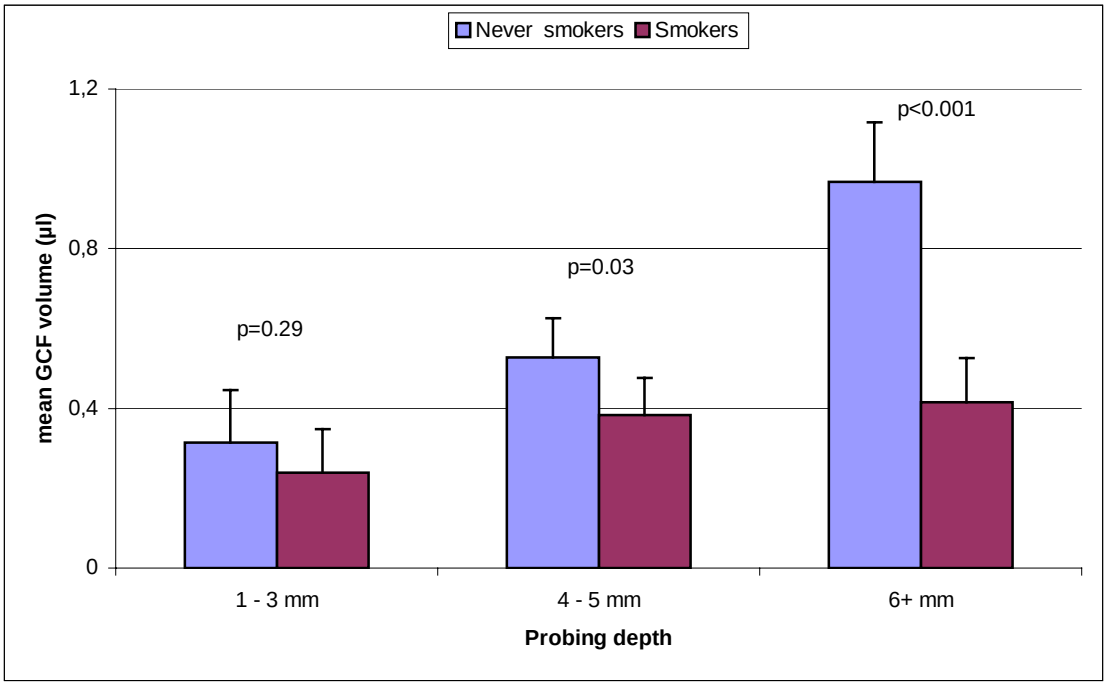

Figure 1. Gingival crevicular fluid volume (µL) by probing depth and smoking status (mean± 95%CI)

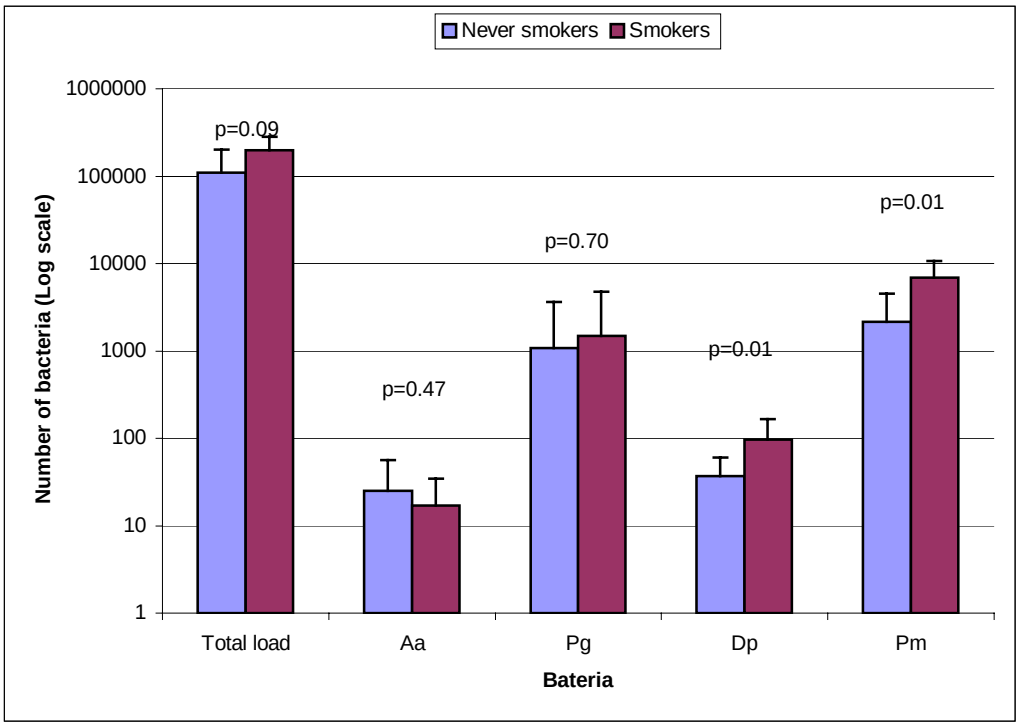

Figure 2. Number of Eubacteria (total load), *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Dialister Pneumosintes* (Dp) and *Peptostreptococcus micros* (Pm) by smoking status (mean± 95%CI).

Supragingival plaque control regimen in smokers and never smokers: clinical effects over a 6 months period. Journal of Clinical Periodontology

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Abstract

Aims: Evaluate the clinical effects of a supragingival plaque control regimen in periodontal patients and compare between smokers and never smokers. M*aterial and Methods*: 50 patients, 25 never smokers (40%males) and 25 smokers (56% males), with chronic periodontitis were selected. Visible Plaque Index (VPI), Gingival Bleeding Index (GBI), Bleeding on Probing (BOP), Periodontal Probing Depth (PPD), Clinical Attachment Loss (CAL) exams were performed at baseline, 30, 90 and 180 days. After baseline examinations supragingival scaling was performed. Oral hygiene enforcement and revaluation was carried out in a weekly basis during the experimental period. Statistical analysis was performed using linear models adjusted for clustering of observations within individuals. *Results*: Visible plaque reduced significantly for both groups, without differences inter-group. Marginal bleeding at baseline was similar between groups. At 30, 90 and 180 days, smokers had less marginal bleeding than never smokers (4.6% vs. 12.8%, 0.9% vs. 4.9%, 0.4% vs. 2.2%, respectively). Significant reductions were observed in PPD for shallow (1-3mm), moderate (4-5mm) and deep sites (6+mm) both for smokers and never smokers. At proximal sites pockets initially 6+mm showed 2.5mm and 2.2 mm reductions respectively for never smokers and smokers. Clinical attachment loss was significantly higher in smokers throughout the study, however, gains in attachment were similar for both groups (0.71mm to 1mm). BOP reductions in buccal/lingual and proximal sites was similar inter-group, but less expressive for deep pockets (range from 28.8%±7.7 to 39.7±6.5%). *Conclusions:* A supragingival plaque control regimen determined significant

changes in the clinical parameters associated if gingivitis and periodontitis. The benefits were extensive to smokers and never smoker patients.

Keywords: Supragingival scaling, oral hygiene, periodontitis, clinical trial, smoking.

Running title: Clinical effect of a supragingival plaque control in periodontal patients.

Introduction

It is generally recognized that an adequate control of the supragingival biofilm is essential to the maintenance of periodontal health following different therapeutic approaches (Rosling et al., 2001; Heasman et al., 2002; Schätzle et al., 2003; Axelsson et al., 2004). In fact adequate plaque control has been proposed both as the basis for the prevention of periodontal diseases as well as an initial requirement in the treatment of these diseases (Haffajee et al., 2003; Ower, 2003; Preshaw et al., 2005). The basis for the observed associations would be inhibition of development of subgingival biofilm in the presence of an adequate supragingival plaque control (Magnusson et al., 1984; Page, 2002; Mombelli, 2003).

A series of studies have investigated, with conflicting results, how the supragingival plaque control could affect the subgingival area in untreated periodontal patients. In general, studies relying on professional intervention and monitoring of patient oral hygiene performance have shown reductions in both clinical and microbiological subgingival parameters, the effect being less clear when the standards for dental plaque control are not well defined (Cerceck et al.,

1983; Smulov et al., 1983; Kho et al., 1985; Beltrami et al, 1987; Mcnabb et al., 1992; Katsanoulas et al., 1992; Al-Yahfoufi et al., 1995; Hellström et al., 1996). The same may also be applicable to studies that have monitored patients under maintenance care following treatment, as subgingival interventions will alter the local environment (Ximenez-Fyvie et al., 2000).

Tobacco is a recognized risk factor for periodontal disease. It has been shown that tobacco consumption changes the pattern of the clinical response. Bersgtröm, 1990 and Giannopoulou et al., 2003 reported a reduced marginal bleeding in smokers. Smokers showed less bleeding on probing, increased pocket depths and loss of attachment than non-smokers (Haffajee & Socransky, 2001; Müller et al., 2003; Erdemir et al., 2004; Susin et al., 2004). It has also been suggested that the periodontal response in smokers should be compared to that of patients who never smoked as ex-smokers may have residual effects of the habit (Spiekerman et al., 2003). As all this clinical parameters may be influenced by the smoking habits and by the supragingival plaque control, it is conceivable that smokers and never smokers may have a different response to this measure.

Aim

The aim of the present study was to evaluate the clinical effects of a supragingival plaque control regimen in periodontal patients and compare the results between smokers and never smokers.

Materials and Methods

Study design: A single-arm longitudinal clinical trial, considering the supragingival plaque control as intervention and smoking as an effect modifier

was conducted. Participants, selected according to the smoking status, were seen in a weekly basis. Clinical measurements were recorded at baseline, 30, 90 and 180 days.

Supragingival plaque control

Following baseline examinations the patients were enrolled in a full mouth program of a strict supragingival plaque control, including supragingival calculus removal with hand-curettes, extractions of condemned teeth, endodontic treatment, placement of glass ionomer temporary fillings and temporary removable prostheses.

The patients were instructed in daily plaque control according to individual needs. Weekly appointments for oral hygiene re-evaluation and re-instruction were conducted. At these occasions, supragingival calculus, either newly formed or visible due to gingival recession, was removed without ever reaching the subgingival area.

Toothbrushes and a non-therapeutic F-containing dentifrice were provided and replaced as needed throughout the experimental period (CloseUp, UNILEVER, Brazil). Dental floss, interdental and ended tufted brushes were also distributed according to individual needs.

Sample

The sample size was calculated based on previous information from our group (unpublished data). To detect a difference of 0.8mm in probing depth between groups (smokers and never smoker) with 80% power and 95% confidence interval, 25 subjects were estimated for each group.

Sample selection

The Committee for Ethical Affairs of the State University of Sao Paulo at Araraquara, Federal University of Rio Grande do Sul and the National Council of Ethics in Research approved all the study protocol.

A convenience sample of consecutive patients seeking treatment was selected (July 2003 and August 2004) from the pool of patients referred for treatment to the Department of Periodontology (Federal University of Rio Grande do Sul, Porto Alegre, Brazil). The volunteers filled out a questionnaire to gather demographic information and other health-related information including smoking habits.

Volunteers were submitted to a clinical screening exam if they had no unfavorable systemic conditions that can interfere with the periodontal condition (diabetes, heart or circulatory problems, among others) and no previous periodontal treatment. Patients undergoing anti-inflammatory (previous 3 months) or antibiotics (previous 6 months) therapy were rejected as well as those using chemical plaque control. Pregnant women or with hormone intake were not included. Patients willing to participate signed an informed consent.

Eligible participants were submitted to the screening periodontal exam and included if they had a minimum 12 teeth (excluding third molars, condemned or endo-periodontal lesions involved teeth) of which 4 with at least one site with PPD of 3-5 mm and another 4 with at least 1 site with 6-10 mm, with visible plaque and clinical diagnoses of gingivitis and chronic periodontitis A total of 50 patients (25 per group) were initially recruited (Table 1).

Anti-inflammatory or antibiotics intake during the experiment, impossibility of following the weekly professional appointment and the development of any systemic condition that could interfere with the periodontal condition determined the patient exclusion during the experimental period (Table 2). Substitutes patients were included according to the above-related criteria and enrolled in the study protocol.

Clinical procedures

Reproducibility measurements

One calibrated periodontist (FP) performed all clinical examinations. Pre and trans-experimental examinations were conducted to assess the examination reproducibility. 10% of the patients were reexamined in a week interval, in both pre and trans experimental period, before being enrolled in the study protocol. The unweighted and weighted (±1mm) Kappa statistics at site level for PPD were, respectively, 0.65 and 0.98 before the commencement of the study and 0.52 and 0.95 during the experiment. Corresponding values for CAL unweighted and weighted kappa were 0.51 and 0.97 in pre-experimental period and 0.60 and 0.92 for CAL during the study.

Experimental exams (baseline, 30, 90 and 180 days)

Baseline examinations were conducted one week after the screening procedure. A full-mouth periodontal exam (six sites per tooth: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, distolingual) was conducted in each experimental day (baseline, 30, 90 and 180 days). Visible Plaque and Gingival Bleeding Index (VPI and GBI, Ainamo and Bay, 1975) were evaluated.

Periodontal Probing Depth (PPD), Clinical Attachment Loss (CAL) and Bleeding on Probing (BOP) were measured with the aid of a PUNC-15 manual color-coded (1-15mm) periodontal probe (Neumar, São Paulo, Brazil). The nearest upper millimeter was considered.

Statistical analyses

Data analysis was performed by STATA software[‡] and using commands that take into account clustering of observations within subjects. Measurements at tooth level were used and estimates were adjusted for the clustering of teeth into individuals using a robust variance estimator. Generalizing estimating equations were used to model the relationship between clinical parameters and various factors. An exchangeable correlation structure was used to model correlation within-subjects and between time periods. Wald tests were used for comparisons, and the p-value was adjusted for multiple comparisons. The level of significance was set at 5%, and the 95% confidence intervals (CI) were calculated.

Results

The supragingival plaque control regimen was effective in decreasing the amount of dental plaque and marginal bleeding (Fig. 1). The percentage of sites with dental plaque decreased to approximately 20% - 30% in proximal and buccal/lingual sites at 30 days, respectively, leveling off and then slightly decreasing at 180 days. Gingival bleeding was observed in less than 10% of the sites after 30 days of plaque control and remained low throughout the experimental period. No significant differences were observed between smokers

[‡] Stata 7.0 for Windows, Stata Corporation, College Station, TX, USA

and never smokers with regards to dental plaque. No differences were observed between smokers and never smokers at baseline, however smokers showed significantly less gingival bleeding than never smokers at 30, 90 and 180 days (4.6% vs. 12.8%, 0.9% vs. 4.9%, 0.4% vs. 2.2%, respectively).

BOP decreased significantly overtime, and the greatest improvement was observed in shallow probing depths (Fig. 2). BOP decreased from approximately 100% of the sites to 32% and 62% of the sites with moderate and deep probing depth after 180 days of supragingival plaque control. The improvement in BOP was not significant different between smokers and never smokers (Table 3).

During the experimental period, mean probing depth decreased from 6.7mm to 4.4mm and from 4.6mm to 3.0mm in deep and moderate pockets at baseline, respectively (Fig 3). No significant differences were observed between smokers and never smokers regardless of the probing depth at the baseline (Table 3).

The average attachment gain over the experimental period was approximately 1mm for sites with moderate and deep probing depth at baseline (Fig 4). Smokers had significantly higher CAL than never smokers throughout the experimental period, but no differences in improvement were observed with regards to smoking status (Table 3).

In the multivariable longitudinal analysis, time and probing depth at baseline were significantly associated with BOP, PPD and CAL. Significant decrease in BOP and PPD as well as gain in CAL occurred overtime and in sites with moderate and deep probing depths at baseline. Gender and smoking did not show any significant association with the outcomes.

Discussion

The present study showed that supragingival plaque control, performed as a sole measure, determines important changes in the clinical parameters associated to gingivitis and periodontitis. It was also observed that smokers and never smokers were significantly benefited by this control.

In the beginning of the study, approximately 80% of the sites showed visible plaque, with slightly higher amounts for the proximal surfaces. The largest reductions were observed within the 30 initial days of the study with additional increments occurring until the end of the study. Reductions were observed both for free and proximal surfaces. Plaque is usually more frequently present in proximal surfaces even after instructions aiming at its specific control (Halla & Oppermann, 2004). The expressive reduction of plaque observed in the present study may be related to the intensity in which the patients were advised and motivated along the 6 months of study.

The gingival bleeding initially observed in smokers and never smoker was similar. The percentage observed is higher than the one reported in other studies (Brown & Löe, 1993; Renvert et al., 1998). Our results suggest that, facing large amounts of plaque, as in our group of patients, the difference between smokers and never smokers may not be expressed. Apparently, the difference in the gingival inflammatory response between these two groups is only expressed when a certain level of supragingival plaque control is present. In our study smokers showed significantly less gingival bleeding already after 30 days than never smokers with similar reductions in the presence of plaque. Lie et al, 1998, showed

in an experimental gingivitis model that smokers presented less gingival bleeding (15%) than non-smokers (30%). Giannopoulou et al., 2003 observed significant differences in the mean GI scores, smaller in smokers (1.292 versus 1.5), after a period of experimental gingivitis period. Interestingly, the amounts of plaque were low for smokers (1.758) and nonsmokers (1.569), without significant differences. Bergström, 1990, as well, showed less gingivitis in smokers, as compared to nonsmokers with similar amounts of plaque, after period of a supragingival plaque control.

Significant changes were observed in the subgingival clinical parameters along the study. Significant reductions in the bleeding on probing were observed for all categories of probing depth. The deeper the initial PPD, the smaller the level of reduction in BOP observed yet, approximately 40% of the initially deep pockets did not show bleeding on probing at the end of the study.

In the past, few interventional studies restricted to the supragingival environment made a clear distinction between supragingival and subgingival bleeding, among them Beltrami et al., 1987; Katnosoulas et al., 1992; McNabb et al., 1992; Moreira et al., 2001. More recent studies, independent of the nature of the intervention, have been concerned with distinguishing the gingival bleeding, associated to the supragingival plaque, from the bleeding on probing, possibly originated from the bottom of the pocket and associated to the presence of the subgingival biofilm (Ximenez-Fyvie et al., 2000; Carvalho et 2004; Apatzidou et al., 2005). Our results clearly show a diverse behavior between gingival and subgingival bleeding associated with a regimen of supragingival plaque control

carried out as the single intervention. The levels of reduction observed for the gingival bleeding were more pronounced than those observed for the bleeding of probing the subgingival area. The reasons for the differences of response for smokers and never smokers between the gingival and subgingival bleeding are not clear.

It was observed in the present study that never smokers and smoker patients showed similar initial BOP. Both groups showed also similar reductions in BOP with treatment. These results are in agreement with Apatzidou et al., 2005, even though the percentage of bleeding on probing sites initially present in their study were smaller than ours. Preshaw et al., 2005, in a follow-up study with patients quitting smoking also observed no differences in the presence of BOP between non-smokers, current smokers and those who ceased smoking.

The pattern reduction of probing depth was similar for smokers and never-smokers, independent of the initial probing depth. Lower reductions (ranging from 0.3mm to 0.7mm) were observed in shallow sites (PPD=1-3mm). Cugini et al., 2000, observed a less pronounced reduction in the mean PPD value (0.3mm) after 12 months of SRP, even thought the initial mean PPD was greater (3.2mm+-0.3) than that observed in the present study. On the other hand, sites with moderate PPD (4-5mm) showed reductions between 1.1 and 1.8mm while the reduction ranged between 1.9mm to 2.5mm for PPD+6mm. The fact that the larger reductions were observed in sites with higher initial probing depth has been reported earlier (Cercek et al., 1983; Badersten et al., 1984; Egelberg, 1989). Interestingly, the level of reduction observed in sites with initial PPD of 4-5mm

was similar to the one observed by Apatzidou et al., 2005, between smokers and nonsmokers after six months of SRP involving the subgingival area. Carvalho et al., 2004, observed comparable levels of reduction after subgingival scaling and root planning alone or in conjunction with the use of systemic metronidazole. The results of the present study suggest that part of the PPD reductions observed, after simultaneous supra and subgingival instrumentation, may be related to the effect of supragingival bacterial plaque control. Our results reinforce the notion that the subgingival therapy must be performed after the treatment of gingivitis, when the objective is to individualize the therapeutic effects associated to gingivitis and periodontitis.

The reductions on the PPD were similar for smokers and never-smokers in all categories of PPD. Although more recent studies have also observed similar responses between the two groups, some studies observed a more modest response for smokers in PPD>6mm (Preber et al., 1995). Differences may be due to the design of the studies, the evaluation time, the definition of the degree of exposure to tobacco, and, as in the case of the present study, the model of supragingival intervention.

The clinical attachment level was the only parameter for which differences were significant between smokers and never-smokers. Smokers presented higher CAL than never-smokers at baseline and during the entire experimental period. Significant gains in clinical attachment were observed with no differences between the two groups.

Our results confirmed previous observations that sites with higher probing depth at the beginning of the study tend to gain more attachment (Badersten et al., 1984). Our average gain of clinical attachment in moderately deep pockets is similar to that observed in studies where the subgingival intervention was also performed (Carvalho et al., 2004; Apatzidou et al., 2005). However, this is not the case when sites with deeper PPD were considered where the subgingival complement is associated with larger gains. It is evident in the multivariate analysis that those factors associated with the reduction of BOP, PPD, and gains in the CAL, were the observation time and the initial PPD whereas gender and smoking were not. These observations indicate that the control of the supragingival biofilm should be considered as an important intervention measure when considering the subgingival environment. It should not be forgotten that subgingival instrumentation is decisive for the treatment of periodontitis.

Smokers and never smoker were similarly benefited by the regimen of supragingival plaque control conducted in the present study. These results illustrate the fact that relatively simple measures, like accomplishing an appropriate supragingival plaque control, are able to modify in a significant way disease severity. In this way its implementation as a routine prior to subgingival interventions seems warranted.

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Table 1. Sample description by experimental group

	Never-smokers	Smokers
n	25	25
Age (mean ± SD)	46.8 ± 7.1	45.9 ± 5.4
Gender (%M)	40	56
N teeth (mean ± SD)	21.4 ± 3.8	21.2 ± 3.7
N Cigarettes per day (mean ± SD)	0 ± 0	19.4 ± 11.6
Years of habit (mean ± SD)	0 ± 0	24.8 ± 8.6

Table 2: Number of participants excluded, reasons for exclusion, age, gender, smoking habit.

Participants	Reason	Age/ gender	Smoker
n=6	Unknown	33 M	N
	Spider bite: antibiotics prescription	33 M	Y
	Economic difficulties	40 M	Y
	Unknown	37 F	Y
	Auto-medication	40 F	N
	Systemic infection: antibiotics prescription	40 F	Y

Table 3. Change in clinical parameters from baseline to 180 days

Parameters	Smoking status	Proximal sites Probing depth at baseline ≤3 mm		4-5 mm		≥6 mm		Buccal and lingual sites Probing depth at baseline ≤3 mm		4-5 mm		≥6 mm	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BOP	Never smokers	87.4	2.3	74.5	4.1	38.9	6.0	82.3	2.5	60.3	5.5	32.0	7.5
	Smokers	85.2	5.2	68.2	4.1	39.7	6.5	83.0	4.2	53.9	5.3	28.8	7.7
PPD	Never smokers	0.5	0.1	1.8	0.1	2.5	0.2	0.3	0.1	1.4	0.1	1.9	0.3
	Smokers	0.7	0.1	1.6	0.1	2.2	0.2	0.4	0.1	1.1	0.2	1.9	0.3
CAL	Never smokers	-0.2	0.1	-0.9	0.1	-1.0	0.2	-0.1	0.1	-0.5	0.1	-0.7	0.2
	Smokers	-0.2	0.1	-0.9	0.1	-1.0	0.2	-0.1	0.1	-0.6	0.1	-0.9	0.2

All clinical parameters had a significant improvement from baseline to 180 days ($p < 0.01$)
No significant differences were observed between never smokers and smokers

Table 4. Multivariable longitudinal analysis by experimental period, gender, smoking status and probing depth at baseline

		Clinical outcomes					
		Bleeding on Probing [a]		Periodontal Probing Depth [b]		Clinical Attachment Level [b]	
		β	SE	β	SE	β	SE
Experimental period	Baseline						
	30 days	-0.48**	0.01	-0.67**	0.02	-0.25**	0.02
	90 days	-0.63**	0.01	-0.86**	0.02	-0.35**	0.02
	180 days	-0.71**	0.01	-1.12**	0.02	-0.47**	0.02
Gender	Male						
	Female	0.03	0.03	0.08	0.07	0.33	0.08
Smoking status	Never smokers						
	Smokers	-0.01	0.03	0.10	0.07	0.13	0.08
Probing depth at baseline	≤3 mm						
	4-5 mm	0.24**	0.01	1.46**	0.02	0.25**	0.02
	≥6 mm	0.43**	0.01	3.16**	0.02	0.76**	0.03

[a] Model adjusted for experimental period, gender, smoking status, probing at the baseline and BOP at the baseline

[b] Model adjusted for experimental period, gender, smoking status, probing at the baseline

[c] Model adjusted for experimental period, gender, smoking status, probing at the baseline and CAL at the baseline

* $p < 0.05$ ** $p < 0.01$

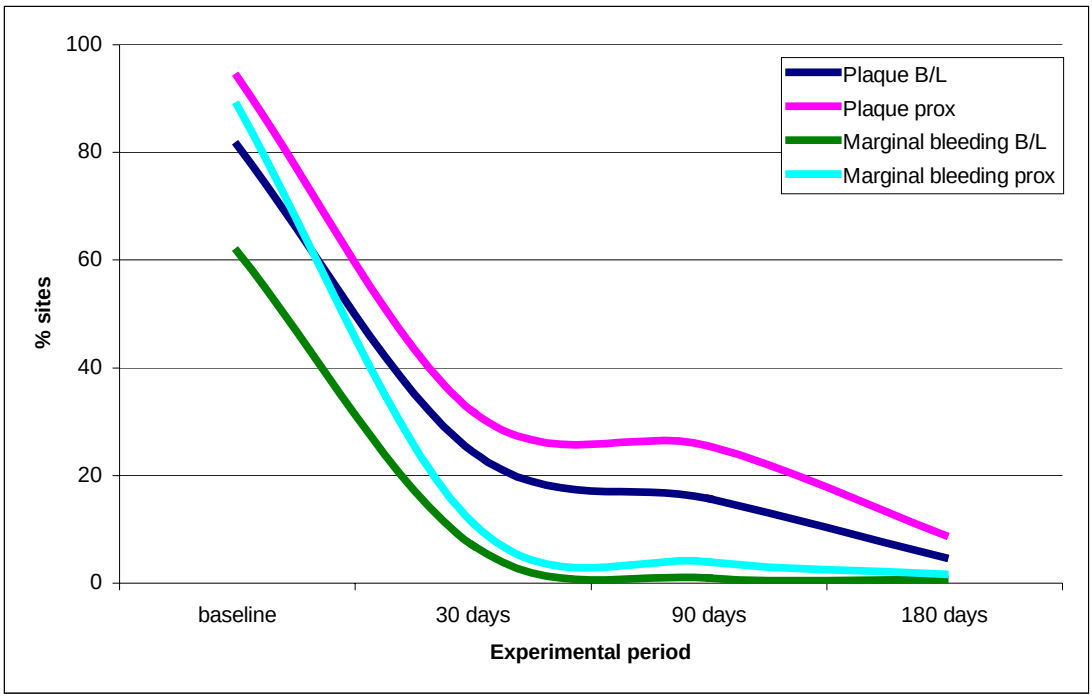

Figure 1. Mean percentage of sites positive to visible plaque and gingival bleeding at baseline and experimental period (30, 90 and 180 days).

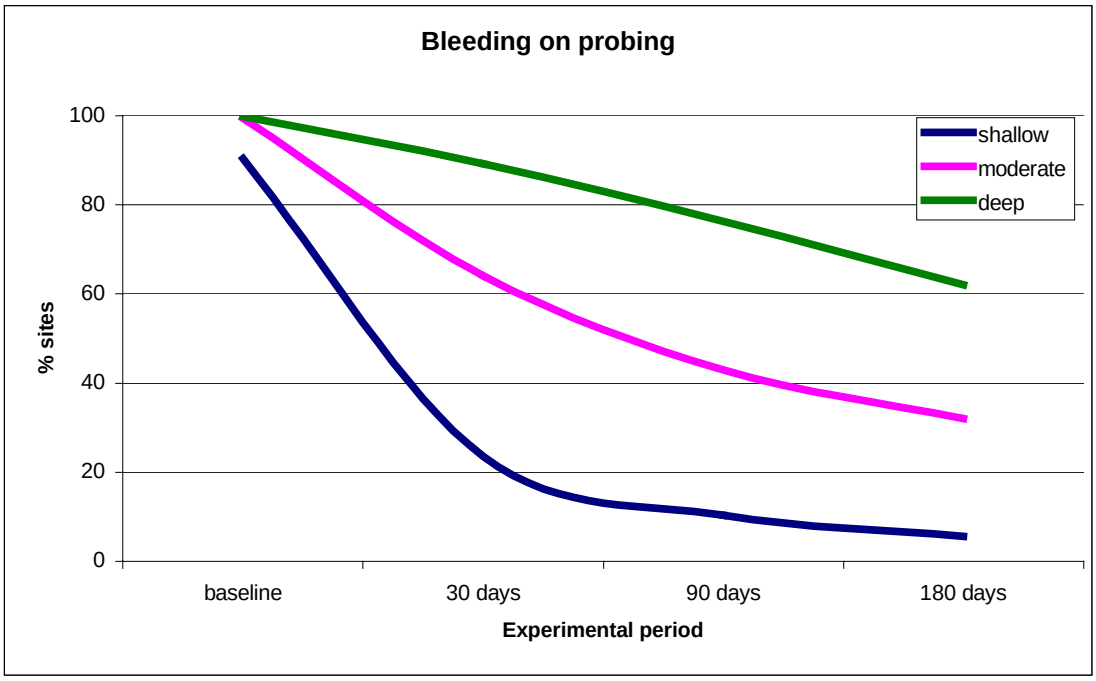

Figure 2. Mean percentage of sites positive to bleeding on probing, by periodontal pocket depth (shallow 0-3mm; moderate 4-5mm; deep 6+mm) at baseline and experimental period (30, 90 and 180 days).

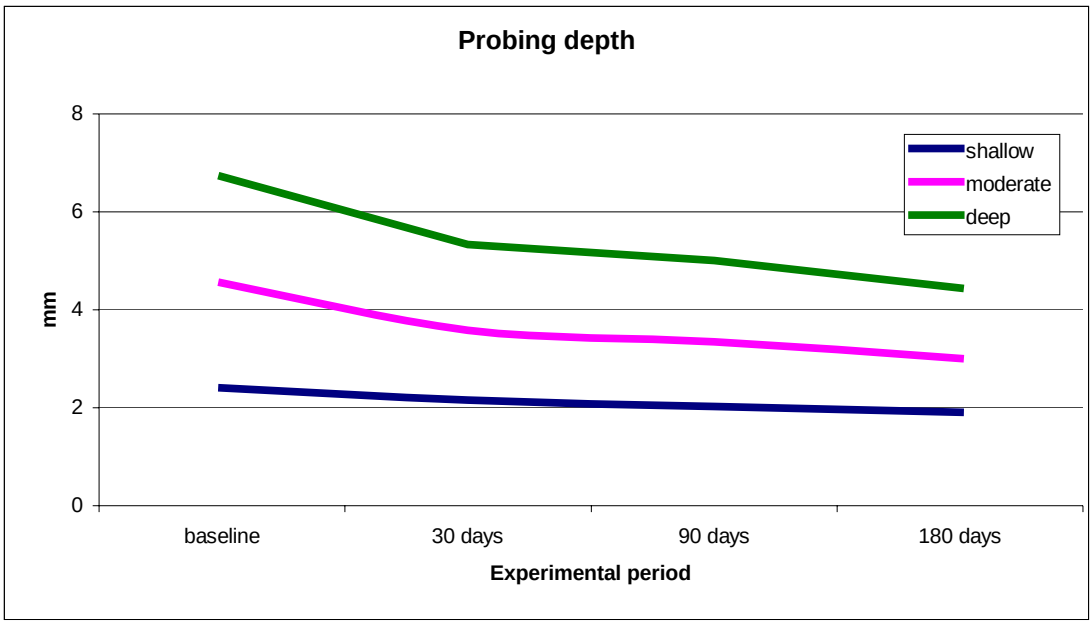

Figure 3. Mean probing depths values (in millimeters) by categories (shallow 0-3mm; moderate 4-5mm; deep 6+mm) at baseline and experimental period (30, 90 and 180 days).

Effect of supragingival plaque control regimen on the subgingival microbiota in smokers and never smokers: longitudinal evaluation by Real Time PCR. Journal of Clinical Periodontology.

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Abstract

Aim: To evaluate the effect of a strict supragingival plaque control regimen in the subgingival microbiota and to compare the effect in smokers and never- smokers patients. *Material and Methods*: 45 patients, 24 never smokers (42%males) and 21 smokers (55% males), with chronic periodontitis were selected. Supragingival debridment was performed at baseline and patients received oral hygiene instructions in a weekly basis for 180 days. Subgingival sampling in experimental sites, Bleeding on Probing (BOP) and Periodontal Probing Depth (PPD) exams were performed at baseline, 30, 90 and 180 days. Real Time PCR reaction (16SrRNA probes and primers and ABI Prism 7700®) quantified *Porphyromonas gingivalis*, *Peptostreptococcus micros*, *Dialister pneumosintes*, *Actinobacillus actinomycetemcomitans* as well as Eubacteria present (total bacteria). Statistical analysis was performed using linear models adjusted for clustering of observations within individuals *Results:* Smokers and never smoker patients showed a statistically reduction in the number of bacteria. Smokers showed a higher reduction in Eubacteria and *D.pneumosintes* in deep pockets. All over the experimental period, deep pockets harbored higher counts of bacteria. At the end, the number of bacteria in sites with initial PPD 6+mm harbored fewer bacteria than did at start those with PPD3-5mm. Higher numbers of bacteria were associated to BOP. *Conclusions*: The number of subgingival bacteria and putative periodontal pathogens was markedly affected by a supragingival plaque control. This effect was observed both for smokers and never-smokers.

Keywords: supragingival scaling, oral hygiene, quantitative, Polimerase chain reaction, subgingival, microbiology, clinical trial, smoking.

Running title: Effect of supragingival plaque control on subgingival microbiota

Introduction

It is well established that supragingival plaque control is essential in the treatment of gingivitis as well as in the maintenance of periodontal health following different forms of treatment (Axelsson et al, 2004). These results are associated directly to the removal of the supragingival deposits reducing the bacterial load at the gingival margin.

Listgarten, as early as 1975, suggested that supragingival plaque could modulate the presence of subgingival bacteria. In fact, it has been proposed that the presence of supragingival deposits and inflammation is associated with the formation and development of the subgingival biofilm (ten Napel et al., 1985; Weidlich et al., 2001; Ximenez-Fyvie et al., 2000; Haffajee et al., 2003) This observation has motivated different studies to investigate the influence of supragingival plaque control as such in the presence and composition of established subgingival biofilms. While some studies have not observed this effect (Kho et al., 1985; Beltrami et al., 1987) others have shown both quantitative and qualitative modifications in the subgingival environment (Al-Yahfoufi et al., 1995; Katsanoulas et al., 1992; Mcnabb et al., 1992; Hellström et al., 1996). These differences may be in part related to clinical results, to the supragingival plaque control regimen as well as to characteristics of the microbiology applied methods.

In this sense, the development of methods for bacterial identification based in molecular biology represented a new opportunity for evaluating the effects of periodontal therapy. DNA-DNA Hybridization as well as PCR has been recently applied to these investigations. It has been suggested that both techniques are semi quantitative and that Real-Time PCR could be an interesting alternative due to the possibility of absolute quantification of bacteria (Boutaga et al., 2003; Rudney et al., 2003; Morillo et al., 2004). Cross-sectional studies and a few number of longitudinal have shown the usefulness of the Real Time PCR for periodontal therapy investigations (Doungudomdacha et al., 2001; Morillo et al., 2004; Nonnenmacher et al., 2004). Until now, no longitudinal study has evaluated the supragingival plaque control regiment in the subgingival microbiota using the Q-PCR.

Tobacco is a well-recognized modifier factor related to periodontal disease expression (Bergström, 2004; Bergström et al., 2005). It is conceivable that it can modify the effect exerted on the subgingival microbiota by the supragingival plaque control.

Aim

The aim of the present study was to evaluate the effect of a strict supragingival plaque control regimen in the subgingival microbiota and to compare the effect in smokers and never- smokers patients.

Materials and Methods

Study design and experimental intervention

The present study was designed as a single-arm longitudinal clinical trial with supragingival plaque control being the intervention. Smoking was regarded as an effect modifier and participants were selected according to smoking status. Participants were seen in a weekly basis and clinical exams and microbiological sampling were performed at baseline, 30, 90 and 180 days.

A full mouth supragingival debridment was performed and patients received oral hygiene instructions by three trained periodontists all over the experimental period. Oral hygiene status was weekly checked and re-instructed accordingly to individual needs. Toothbrushes and a non-therapeutic F-containing dentifrice were provided and replaced as needed throughout the experimental period (CloseUp, UNILEVER, Brazil). Dental floss, interdental and ended tufted brushes were also distributed according to individual needs.

Study sample

The original data for sample calculation derived from an unpublished study from our group. As previously reported (Gomes et al., 2005) a convenient sample was drawn form the patients referred to periodontal treatment (Department of Periodontology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil). 50 patients (aged 33-59 years) were enrolled in the experimental procedures and 45 contributed to microbiological sampling (Table 1). The study protocol was reviewed and approved (Committee for Ethical Affairs of the State University of

Sao Paulo at Araraquara, Federal University of Rio Grande do Sul and the National Council of Ethics in Research)

Inclusion criteria are previously described (Gomes et al., 2005). Briefly, the volunteers were submitted to the clinical examination if they fulfilled the following statements: written informed consent, no unfavorable systemic conditions and pregnancy, no previous periodontal treatment, no anti-inflammatory and antibiotics intake in previous 3 and 6 months, respectively and no chemical plaque control prescription. Eligible patients were examined and included if they had: a minimum of 12 teeth (excluding third molars, condemned or endo-periodontal lesions involved teeth), presence of at least 4 teeth with one of the sites with PPD varying from of 3-5 mm and another 4 with one of the sites with 6-10 mm PPD. These sites should also present visible plaque, plaque associated gingivitis and chronic periodontitis. Baseline examinations were conducted one week after this screening.

Clinical and sampling procedures

Microbiological sampling

Sites to microbiological sampling were randomly selected from those meeting the inclusion criteria described above. The sampling session was performed before the clinical exams in each scheduled experimental day (baseline, 30, 90 and 180 days). A total of 4 sites (2 in the 3-5mm and 2 in the 6-10mm PPD categories) were sampled in each patient. The patients contributed with 180 samples in each exam (baseline, 30, 90 and 180 days). A total of 720 samples s were collected and submitted to Real Time PCR analyses.

Following careful supragingival plaque removal, the sites were gentle washed, dried and isolated with cotton rolls. A sterile paper point (#30, EndoPoint, Paraíba do Sul, RJ, Brasil) was inserted to the bottom of the periodontal pockets for 30 seconds. The paper points were then placed into sterile tubes containing 200µl of RTF (Syed & Loesche, 1972). The collected samples were stored at -20^0 C.

Clinical examination

A single examiner was calibrated to conduct all the clinical exams (Gomes et al., 2005). Periodontal Probing Depth (PPD) and Bleeding on Probing (BOP) were measured with a periodontal probe (PUNC-15 manual color-coded; Neumar, São Paulo, Brazil). 6 sites per tooth were evaluated (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, distolingual). A full-mouth (excluding third molars, condemned or endo-periodontal lesions involved teeth) periodontal exam was conducted in each experimental day (baseline, 30, 90 and 180 days). The nearest upper millimeter was considered. Baseline examinations occurred one week after the screening procedure.

Microbiological analyses: Real Time PCR methodology

The methodology is previously published (Nonnenmacher et al. 2004). *Oligonucleotide primers and TaqMan probes:* Primers and probes used for the detection and quantification of periodontophatogens (*A. actinomycetemcomitans*, *P. gingivalis*, *P.micros*, *D. pneumosintes*) are shown in Table 4. They were selected using Primer Express software V 1.0 (Applied Biosystems International) and based on species-specific conserved regions from the 16S rRNA gene. Identification of these regions was done by multiple sequence alignment

(ClustalW software). The fluorescent dyes at the 5' and at the 3' ends of the probe were FAM (6-carboxyfluorescein; reporter) and TAMRA (6-carboxytetramethylrhodamine; quencher), respectively. Species-specific probe and primer sets were designed based upon the variable regions of the 16S rRNAs of. Additionally, a universal bacterial primer pair was used to detect DNA from all Eubacterial (total bacteria) species present in the samples.

Quantitative assay: Initially, plasmid cloning was performed. Plasmids were then purified with MaxiPrep (Qiagen, Hilden, Germany) and quantified by spectrophotometer of multiple dilutions and based on the calculated molecular mass. Quantification of target DNA was achieved by using serial 10-fold dilutions from 10^1 to 10^8 plasmid copies of the previously quantified plasmid standards.

DNA from the clinical samples was extracted (Qiagem, DNeasy Tissue system, Hilden, Germany). Plasmid standards and clinical samples were then run in duplicates and the average values were used for calculation of the bacterial load.

Real-Time PCR: Clinical samples were assayed in a 25-µl reaction mixture containing 2.5 µl of template DNA, 2.5 µl of 10xbuffer with ROX, 1.5 µl of 50mM $MgCl_2$, 1 µl dNTP (qPCR Core Kit, Eurogentec, Belgium), 12.5 pmol of forward primer and reverse primer (MWG, Munich), and 3.75 pmol of the probe (Eurogentec, Belgium). The cycling conditions used were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15s and 60°C for 1 min each. During the annealing-extension step, the ABI Prism 7700 SDS (Applied Biosystems International) monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The reporter dye (FAM) signal was measured relative to

the reference dye (ROX) as present in the PCR master mix to normalize for non-PCR related fluorescence fluctuations occurring from well to well. All the microbiological analysis was conducted blinded to clinical diagnosis.

Statistical analysis

Standard curves were used to convert Ct scores into number of bacterial cells using samples with known amounts of bacterial-specific DNA. Cell counts were not normally distributed, thus this variable was transformed using a logarithmic function. Results in the figures are reported in the back-transformed form and coefficients for the multivariable analysis are reported in log scale.

Measurements at tooth level were used and estimates were adjusted for the clustering of teeth into individuals using a robust variance estimator. Wald tests were used for comparisons, and the p-value was adjusted for multiple comparisons. The level of significance was set at 5%, and the 95% confidence intervals (CI) were calculated.

Generalizing estimating equations were used to model the longitudinal relationship between bacterial counts and various factors, and took in consideration clustering of observations within subjects. An exchangeable correlation structure was used to model correlation within-subjects.

Results

Eubacteria load decreased significantly over the 6 months experimental period (Fig 1). The greatest decrease occurred in the first 30 days after supragingival plaque control was established leveling off after 90 days. Somewhat similar pattern of decrease in bacteria counts were observed for *P.gingivalis*,

D.pneumosintes and *P.micros*. *A.actinomycetemcomitans* counts were small at the baseline and slightly decreased over time. At 180 days the mean bacteria counts were relatively small for all bacteria studied.

Deep pockets (PPD=6-10mm) showed higher bacterial loads than PPD 3-5mm pockets at baseline ($p< 0.001$). The difference in Eubacterial counts between deep and moderate pockets decreased greatly during the study, but remained statistically significant different at 180 days ($p< 0.001$).

Never smokers consistently had higher counts of bacteria than smokers at baseline in deep sites (PPD=6-10mm) for Eubacteria ($p=0.001$) and Pg ($p=0.019$), whereas *D.pneumosintes* and P.micros were in higher counts in smoker ($p=0.001$ and $p=0.002$, respectively) (Figs 2-3). Considering the 3-5mm PPD pockets, smokers showed higher counts for the studied bacteria.

Significantly greater reductions in Eubacteria and *D.pneumosintes* occurred in <6 mm pockets for smokers when compared to never smokers (Table 2). The frequency of *A.actinomycetemcomitans* was small throughout the study and no clear pattern could be observed in smokers and never smokers (Data not shown). Small differences were observed between never smokers and smokers after 90 days of therapy for all studied bacteria.

When smokers were analyzed according to lifetime exposure to smoking, heavy smokers consistently had higher counts of bacteria than light and never smokers at baseline. Heavy smokers showed the greater decrease in the load of microorganisms after 30 days of professional plaque control. Differences between

never smokers, light and heavy smokers after 90 days of supragingival plaque control were minimal.

In the longitudinal multivariable analysis, time, probing depth at baseline and bleeding on probing were significantly associated with mean counts of various bacteria during supragingival plaque control (Table 3). Smokers had significantly higher occurrence of *P.micros* overtime than non-smokers, whereas no other significant differences were observed regarding smoking status. Sites with probing depth ≥6 mm at baseline had significantly more bacteria count than shallow sites, and this was consistent during the experimental study. Significantly higher counts of bacteria were observed in sites with bleeding on probing.

Discussion

The present study showed that the supragingival plaque control regimen significantly reduces the number of subgingival bacteria in periodontal patients. The benefit of this control was observed in both smokers and never-smoker patients, regardless of the initial probing depth of the experimental sites. Significant reductions were observed at 30 days of plaque control with additional reductions occurring up to 90 days.

Several studies have reported the benefits of mechanical periodontal therapy on the subgingival microbiota. Traditionally, this evaluation is performed after a simultaneous control of both supra and subgingival biofilms (Ximenez-Fyvie et al., 2000b; Cugini et al., 2000; Doungudomdacha et al., 2001; Van de Velden et al., 2003; Apatzidou et al., 2005). However, when the supragingival biofilm control is singly performed, controversial results are reported (Kho et al., 1985,

Smulov et al., 1983, Beltrami et al., 1987, Dahlén et al., 1992, MacNabb et al., 1992, Katnosoulas et al., 1992; Al-yahfoufi et al, 1995, Hellström et al, 1996, Westfelt et al, 1998, Moreira et al., 2001). Possibly the differences in the results may be explained by the absence of well defined inclusion criteria, of a proper control of modifying factors, a systematic investigation of the quality of the supragingival control and by differences in clinical results.

Our results showed that an appropriate control of the supragingival biofilm determines significant microbiological reductions, independent of the initial PPD. It was also shown that sites with persistent subgingival bleeding harbored higher numbers of bacteria. The initial PPD is believed to be a major factor in determining the possibility of changes in the subgingival microbiota in association with supragingival plaque control measures. Kho et al., 1983, and Beltrami et al., 1987 have not observed differences in the microbiota, specially of deep pockets ≥ 6mm, but they also have not observed significant changes in the clinical parameters. Our findings are in agreement with several other studies where significant clinical changes are observed (Ximenez-Fyvie et al., 2000b; Cugini et al., 2000; Doungudomdacha et al., 2001; Van de Velden et al., 2003; Apatzidou et al., 2005; Colombo et al., 2005).

Cross-sectional or longitudinal studies, independent of the therapy, showed higher bacterial counts in patients/sites with higher PPD (Ximenez-Fyvie et al., 2000a; Nonnenmacher et al., 2004; Kawada et al., 2004; Ciantar et al., 2005). This pattern was also observed in the present study for total and specific bacteria counts and was maintained until the final examinations. Direct comparisons of

microbiological findings among studies are difficult due to the differences in the prevalence of microbiota in distinct populations (Haffajee et al., 2004) sampling methods, number of evaluated samples, detection method, examined species and also to the differences in the evaluation and expression of data.

However, our study showed differences in the mean counts of bacteria in the experimental groups when PPD was considered. In sites with PPD ≥6mm at baseline, never smokers showed higher numbers of Eubacteria and *P.gingivalis*. *D.pneumosintes* and *P.micros* were in higher numbers in smokers, independent of the PPD. In moderate PPD (3-5mm) sites, smokers showed higher amounts of total and specific bacteria. Haffajee & Socransky, 2001, reported a higher prevalence of bacteria from the orange and red complex in PPD<4mm in smoker patients compared to past and never smokers.

Different results are reported regarding the prevalence of bacteria in smoker patients as compared to nonsmokers (Stoltenberg et al., 1993; Zambon et al., 1996; Darby et al. 2000; Shiloah et al., 2000; Böstrom et al., 2001; Haffajee & Socransky, 2001; Van Winkelhoff et al., 2001, Salvi et al., 2005; Apatzidou et al., 2005). Apatzidou et al., 2005, did not observe differences in *P.gingivalis, A.actinomycetemcomitans, P.intermedia, T.denticola, T.forsythensis* between smokers and nonsmokers after 6 months of a SRP therapy. Van der Velden et al., 2003, studying the prevalence of individuals positive to certain bacteria, reported similar reductions in positive individuals for *P.gingivalis* between smokers and non-smokers. However the prevalence of individuals positive for *P. micros* was higher in smokers, an observation also reported by Van Winkenhoff et al., 2001.

Our results showed that smokers presented higher numbers of *P.micros* at baseline, 30 and 90 days. However, these differences were not present at the end of the investigation. Also when the levels of reduction in the presence of *P. micros* were considered, no differences were observed between smokers and never-smokers. When Eubacteria and *D.pneumosintes* are considered, smokers showed a higher reduction than never-smokers in sites with PPD ≤ 6mm. Smokers and never-smokers presented no differences in the absolute numbers of Eubacteria and *D.pneumosintes* at the 6-month examination. When final and initial results were compared for the remaining bacteria and PPD categories, smokers and never-smokers showed similar reductions. The reasons for this behavior are not clear. Shiloah et al., 2000, suggest that the differences observed in the subgingival microbiota can result from the level of exposure to the smoking factor. In the present study heavy smokers showed higher numbers of total and specific bacteria than light and never-smokers at baseline (result not shown). Heavy smokers showed the greatest reductions after 30 days but no differences could be found between groups after 90 days.

The deeper sites, in our study, had a mean PPD of 6.6±0.1mm at baseline and were reduced to a mean of 4.5±0.2mm after 6 months. These sites harbored the highest numbers of bacteria at start and also showed the greatest reductions. It is interesting to observe that, at the end of the study, theses sites harbored smaller numbers of bacteria than did at start those sites that had comparable mean PPD (3-5mm). These results illustrate the magnitude of the subgingival microbiota reduction that might be expected when an appropriate regimen of plaque control is

established. Considering the nutritional requirements of the studied bacteria, strict anaerobic or facultative, it is supposed that the decrease of the probing depth may have contributed for this reduction.

The microbiological method employed in this investigation was published by Nonnenmacher et al., 2004. These authors compared the prevalence of periodontophatogens in both healthy and diseased patients, observing a relationship between clinical inflammatory conditions and the number of these bacteria. No cross-reactions could be observed related to the primers and probes used. Other methods of microbiological analysis have been investigated with potential advantages related to the quantitative analysis (Lyons et al., 2000; Yoshida et al., 2003; Boutaga et al., 2003; Morillo et al., 2004; Lau et al., 2004; Nonnenmacher et al., 2004; Kawada et al., 2004; Sanz et al., 2004).

Thus, it is concluded that the sole control of the supragingival biofilm benefits the subgingival environment, determining significant microbiological reductions, regardless of the present inflammatory characteristics or the smoking habit. The complex interaction between the supra and subgingival environment becomes evident from the results of the present study. In this way, the characterization of a subgingival microbiota must be carried out based in a clear definition of the conditions, both subgingival and supragingival, in which this microbiota is being studied.

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Table 1. Sample description by demographic data and smoking habit

	Never smokers	Smokers
n	24	21
Age (mean ± SD)	47.3 ± 6.7	45.8 ± 5.1
Gender (% Males)	41.7	55
N Cigarettes per day (mean ± SD)	0 ± 0	19.6 ± 11.8
Years of habit (mean ± SD)	0 ± 0	24.1 ± 8.7

Table 2. Reduction in mean bacterial counts from baseline to 180 days, by probing depth at baseline and smoking status

		Total load (thousands)		Pg		Dp		Pm		Aa	
Probing depth at baseline	Smoking status	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
<6mm	Never smokers	23.3	11.1	263	148	13	4	671	292	5	7
	Smokers	102.4	29.4	910	685	67	23	3797.0	1281.0	4	3
	p-values	0.01		0.40		0.04		0.20		0.81	
6+mm	Never smokers	523.8	205.1	7131.0	6552.0	90	42	8929.0	5044.0	39	30
	Smokers	335.7	88.9	2185.0	1345.0	123	66	11155.0	3457.0	25	19
	p-values	0.84		0.54		0.39		0.94		0.69	

Table 3. Multivariable analysis of the effect of experimental period, smoking status, probing depth at baseline, bleeding on probing in the number of bacteria

		Microorganisms									
		Total load Pg			Dp			Pm		Aa	
		Log β	SE	Log β	SE	Log β	SE	Log β	SE	Log β	SE
Experimental period	Baselime										
	30 days	-0.8**	0.2	-1.4**	0.3	-1.7**	0.2	-1.1**	0.3	0.3	0.3
	90 days	-1.3**	0.2	-2.2**	0.4	-2.0**	0.2	-1.9**	0.3	-0.2	0.3
	180 days	-1.4**	0.3	-2.2**	0.4	-1.9**	0.2	-1.7**	0.3	-0.4	0.3
Smoking status	Never smokers										
	Smokers	0.0	0.3	-0.1	0.6	0.4	0.3	1.1**	0.4	-0.2	0.4
Probing depth at baseline	≤μμ 3										
	4-5 mm	1.3**	0.2	1.1**	0.4	0.8**	0.2	1.5**	0.3	0.6*	0.3
	≥μμ 6	2.4**	0.3	2.5**	0.4	1.5**	0.2	2.4**	0.3	1.5**	0.3
Bleeding on probing	Present										
	Absent	0.7**	0.2	1.0**	0.3	0.2	0.2	0.9**	0.2	0.7**	0.3

* p< 0.05 ** p< 0.01

Table 4. Bacterial species, real-Time PCR primers and probes used

Primer and Probe	Sequence (5'-3')	Acession numbers
Universal *		
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA(AG)CCATGCA	
A. actinomycetemcomitans		M75039
Forward	CAAGTGTGATTAGGTAGTTGGTGGG	
Reverse	CCTTCCTCATCACCGAAAGAA	
Probe	ATCGCTAGCTGGTCTGAGAGGATGGCC	
D. pneumosintes		X82500
Forward	GAGGGGTTTGCGACTGATTA	
Reverse	CCGTCAGACTTTCGTCCATT	
Probe	CACCAAGCCGACGATCAGTAGCCG	
M. micros		D14143
Forward	AAACGACGATTAATACCACATGAGAC	
Reverse	ACTGCTGCCTCCCGTAGGA	
Probe	TCAAAGATTTATCGGTGTAAGAAGGGCTCGC	
P. gingivalis		L16492
Forward	TGCAACTTGCCTTACAGAGGG	
Reverse	ACTCGTATCGCCCGTTATTC	
Probe	AGCTGTAAGATAGGCATGCGTCCCATTAGCTA	

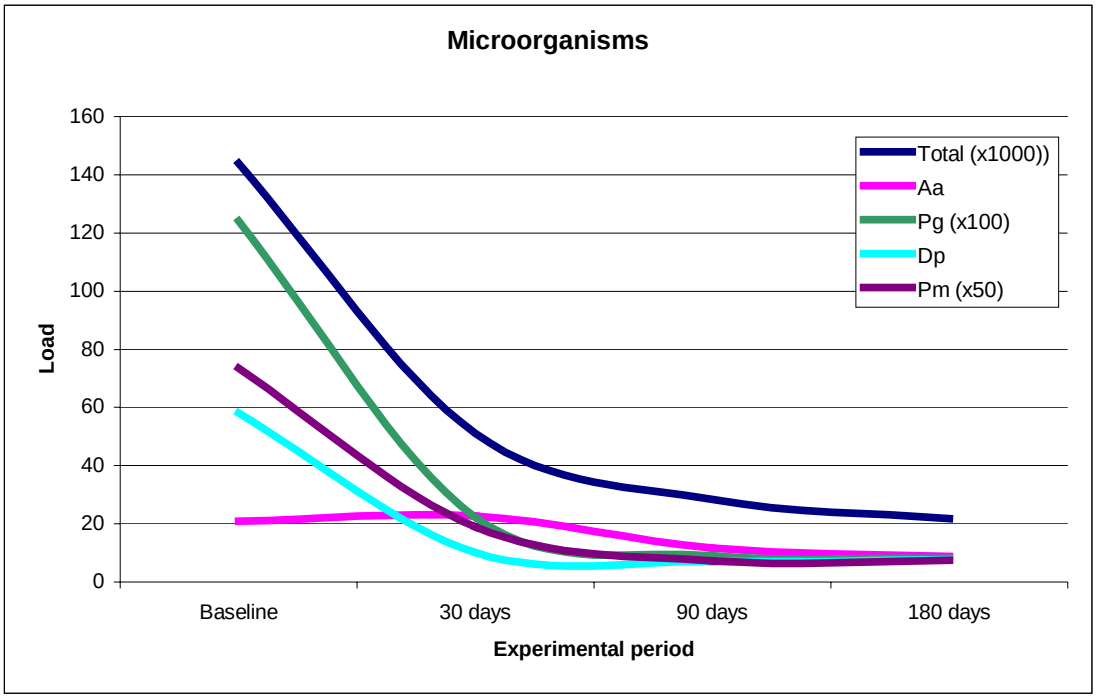

Figure 1. Mean number (load) *of Eubacteria, Porphyromonas gingivalis (Pg), Dialister pneumosintes (Dp), Peptostreptococcus micros (Pm); Actinobacillus actinomycetemcomitans (Aa)* by experimental period (baseline, 30, 90, 180 days)

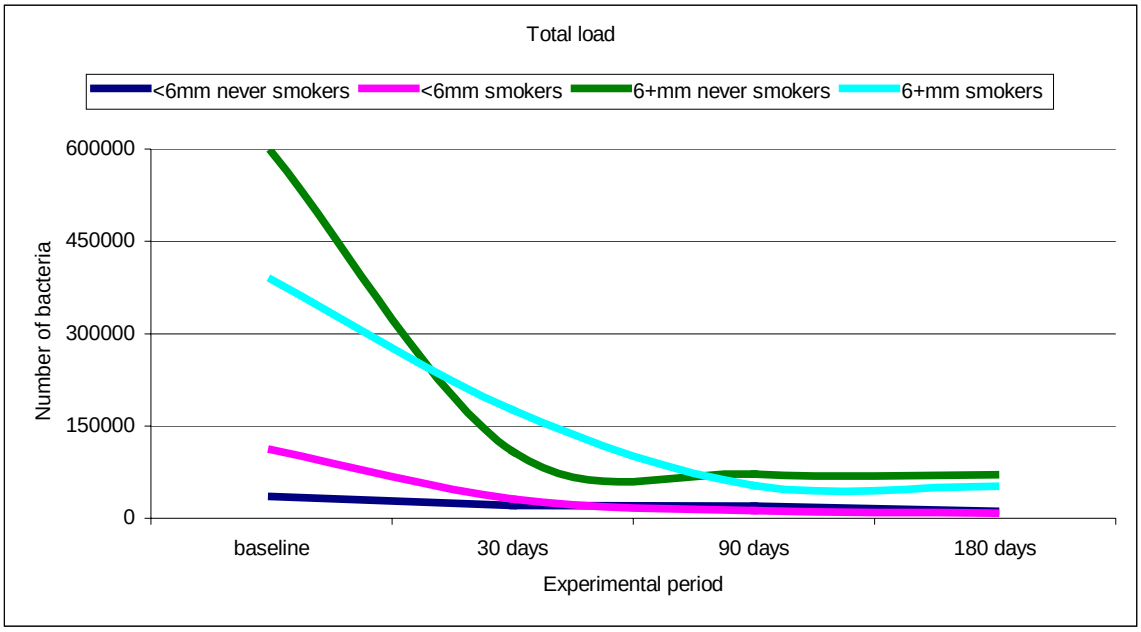

Figure 2. Mean number of Eubacteria (total load) by smoking status, probing depth (mm) and experimental period (baseline, 30, 90, 180 days)

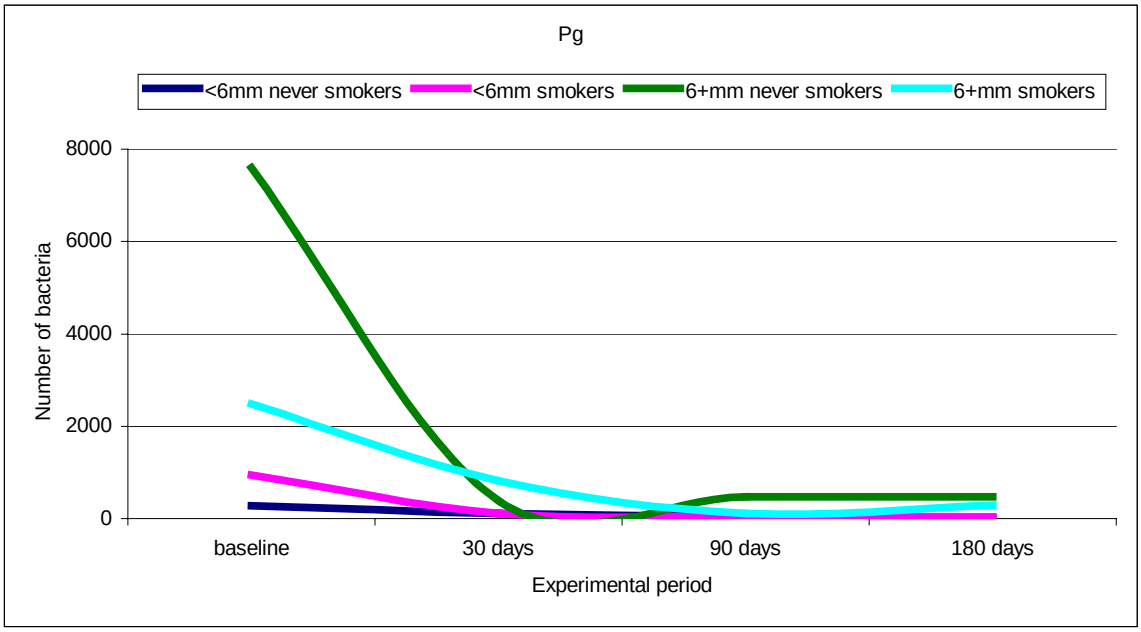

Figure 3. Mean number of *Porphyromonas gingivalis* by smoking status, probing depth (mm) and experimental period (baseline, 30, 90, 180 days)

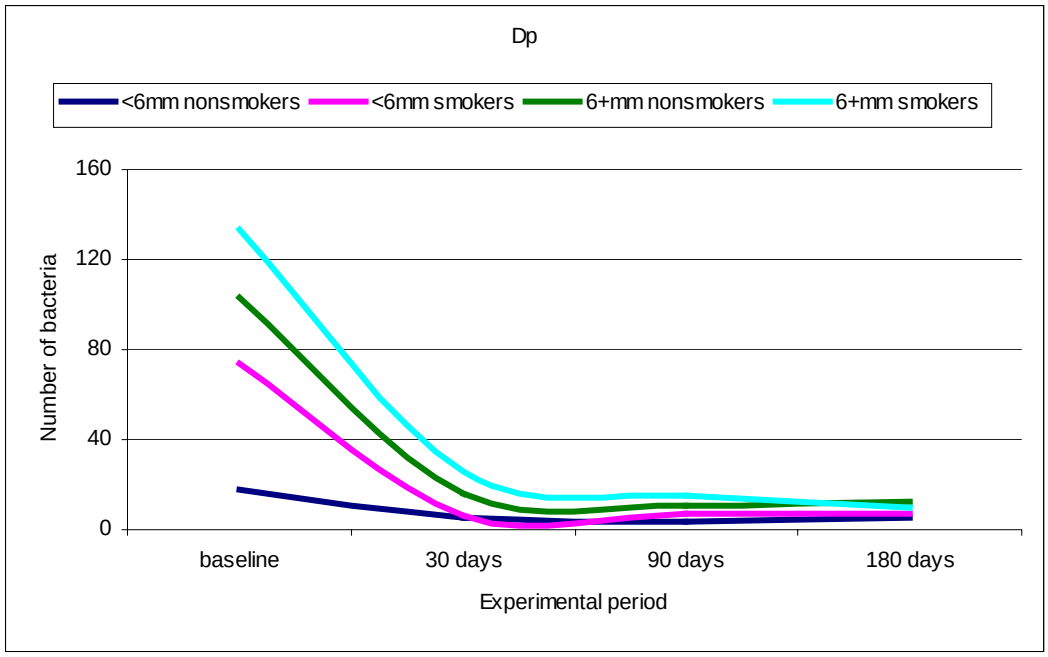

Figure 4. Mean number of *Dialister pneumosintes* by smoking status, probing depth (mm) and experimental period (baseline, 30, 90, 180 days)

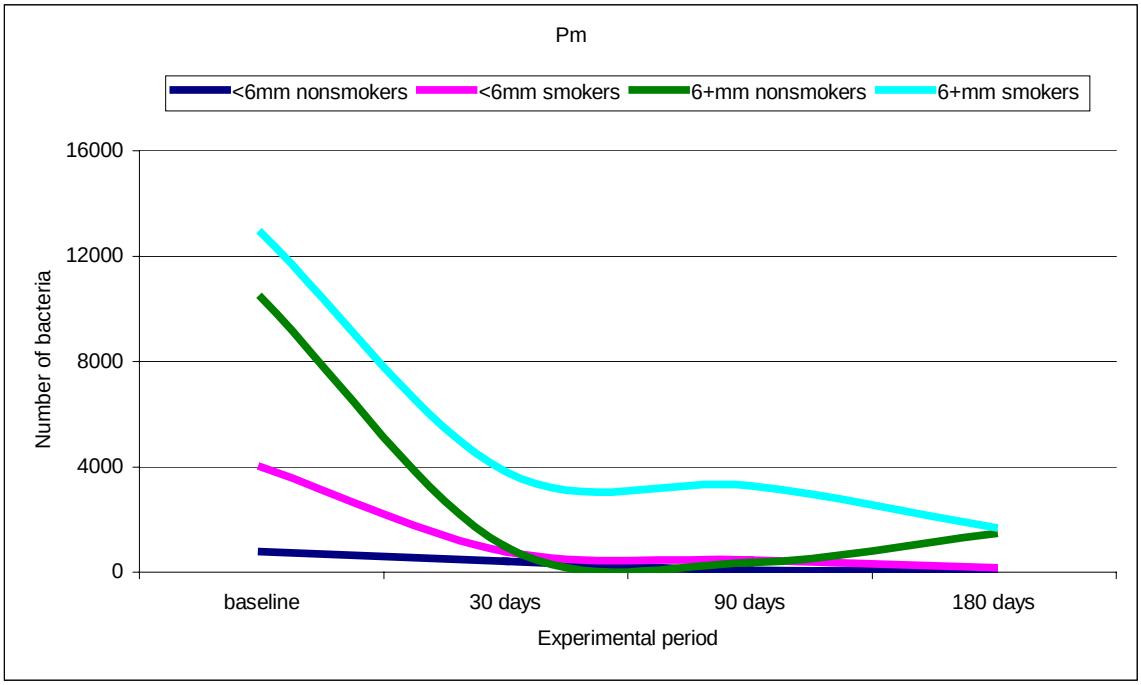

Figure 5. Mean number of *Peptostreptococcus micros* by smoking status, probing depth (mm) and experimental period (baseline, 30, 90, 180 days)

Effect of supragingival plaque control on gingival crevicular fluid volume in smokers and never-smokers with periodontitis: a 6-months clinical trial.. Journal of Clinical Periodontology

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Abstract

Aims: The aim of this interventional study was to evaluate the effect of supragingival plaque control over the gingival crevicular fluid volume in smokers and never smokers patients with periodontitis.

Material and methods: the sample included 45 patients diagnosed with chronic periodontitis (ages 33 to 59 years), 23 males and 22 females, 24 never smokers and 21 smokers. Following baseline scaling restricted to the supragingival area patients received oral hygiene instructions in a weekly basis for 180 days. Participants were clinically examined at baseline, 30, 90 and 180 days for Gingival Bleeding Index (GBI), Bleeding on Probing (BOP) and Periodontal Probing Depth (PPD) and the volume of gingival crevicular fluid (GCF). Statistical analysis was performed using linear models adjusted for clustering of observations within individuals *Results:* Never smokers showed greater GCF volume than smokers, and this difference was independent of gingival bleeding and bleeding on probing. Greater reductions in GCF volume occurred in the first 30 days. Additional (p=0.01), but limited, reductions were observed along the experimental period. Smokers and never-smokers showed similar reductions in the experimental period. Higher volumes of GCF were significantly associated with deeper pockets. Deep pockets (PPD 6+mm) in never smokers showed greater GCF volumes than deep pockets in smokers as well as shallow to moderate pockets in both never smokers and smokers. Multivariable analysis showed that smoking, gingival bleeding and PPD have significant independent effects on GCF volume. *Conclusions:* Supragingival plaque control executed as the sole intervention reduced significantly the volume of GCF in smokers and never smokers in spite of differences in volume between groups at start. Smoking status, gingival bleeding and PPD should be taken in account when GCG volume and components are under investigation.

Running title: Influence of a supragingival plaque control in GCF volume

Keywords: Supragingival scaling, oral hygiene, gingival crevicular fluid, clinical trial, smoking

Introduction

The gingival crevicular fluid (GCF) is a clinical expression of the inflammatory process present in the periodontal tissues. The association between the volume of GCF and periodontal health, gingivitis and periodontitis has been shown both clinically and histologically (Brill, 1960; Mann, 1963; Egelberg,1966a, Egelberg, 1966b; Rudin et al., 1970; Griffiths, 2003). Recent studies demonstrated increased amounts of the GCF during the course of an experimental gingivitis (Rüdiger et al., 2002; Zhang et al., 2002; Trombelli et al., 2004).

Exposure to tobacco may cause important vascular alterations in the periodontal tissues (Bergström et al., 1988; Mavropoulos et al., 2003). Peripheral vasoconstriction may explain the reported reduction in the amounts of GCF (Kinane & Radvar, 1997) and gingival gingival bleeding (Haffajee & Socransky, 2001; Erdemir et al., 2004; Bajloon et al., 2005) in smokers as compared to non-smokers. Studies comparing smokers and non-smokers usually place in the latter category both individuals who never smoked and former smokers. Recognizing that smoking may influence the periodontal condition also in former smokers, some authors suggest that, in order to disclose the effect of tobacco in its full extension, comparisons should be made between smokers and individuals who never smoked (Spiekerman et al., 2003).

Reductions in the volume of GCF have been observed during periodontal treatment after scaling and root planning (Apatzidou et al., 2005; Tüller et al, 2005). The lack of distinction between the treatment of the supra and subgingival environments prevents the evaluation of the relative importance of the supragingival plaque control component of the treatment even though it is known to influence the presence of GCF. Gomes et al 2005, have shown, in a 6-month observational study, significant reductions in gingival bleeding already 30 days of supragingival plaque control preformed as the sole intervention. Smokers and never smokers presented similar levels of gingival bleeding at start. However, larger reductions in gingival gingival bleeding were observed in smokers as compared to never-smokers throughout the study.

Monitoring GCF profiles in smokers and never-smokers associated with a supragingival plaque control regimen may constitute in a valuable source of information to the clinician. It would also be helpful when monitoring the expression of risk indicators associated with the development of gingivitis and periodontitis.

Aim

The aim of this interventional study was to evaluate the effect of supragingival plaque control over the gingival crevicular fluid volume in smokers and never smokers patients with periodontitis.

Materials and Methods

Study design

The present study was designed as a single-arm longitudinal clinical trial with supragingival plaque control being the intervention. Smoking was regarded as an effect modifier and participants were selected according to smoking status. Participants were seen in a weekly basis, and clinical and GCF measurements were recorded at baseline, 30, 90 and 180 days.

Supragingival plaque control

A full mouth supragingival debridment was performed and patients received oral hygiene instructions by three trained periodontists. Oral hygiene status was evaluated in a weekly basis and oral hygiene instructions were given regularly. Toothbrushes and dentifrice, without chemical agents that could interfere with supragingival plaque, were provided to participants as needed throughout the experimental period (CloseUp, UNILEVER, Brazil). Dental floss, interdental and ended tufted brushes were also distributed according to individual needs.

Study sample: Between July 2003 and August 2004 a convenience sample of consecutive patients seeking treatment was selected from the Department of Periodontology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. 45 patients, age 33 - 59 years, were selected (Table 1). The study protocol was reviewed and approved by following committees: Committee for Ethical Affairs of the State University of Sao Paulo at Araraquara, Research Committee at

Federal University of Rio Grande do Sul and the National Council of Ethics in Research.

The criteria for inclusion in the study were:

1. No known systemic disease
2. No previous periodontal treatment
3. No anti-inflammatory or antibiotics therapy in the previous 3 and 6 months respectively. No pregnancy and hormones intake
4. No use of chemical plaque control
5. At least 12 or more teeth present, excluding third molars
6. At least 4 teeth with one site with PPD ranging between 3-5 mm and 4 additional teeth with one site with PPD ranging between 6 and 10 mm. Willingness to return weekly and be enrolled in a plaque control regimen

The sample size was calculated based on previous information from an unpublished study from our group. We estimated that 25 subjects in each group would be necessary to achieve 80% power to detect a difference of 0.8mm in probing depth between smokers and non-smokers with a significance level of 5%.

Clinical and sampling procedures

At baseline, participants filled out a questionnaire to gather demographic information and other health-related information including smoking habits. Clinical examinations and sampling collection were conducted one week after the screening examination.

Clinical examination

A full-mouth, excluding third molars, periodontal examination was conducted at baseline, 30, 90 and 180 days. The presence of marginal bleeding was evaluated with the Gingival Bleeding Index (GBI, Ainamo and Bay, 1975). Periodontal Probing Depth (PPD) and Bleeding on Probing (BOP) were measured with a PUNC-15 manual periodontal probe color coded from 1 to 15 mm (Neumar, São Paulo, Brazil). The nearest upper millimeter was considered. Six sites in each tooth were examined: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, distolingual.

Gingival Crevicular Fluid sampling

Gingival Crevicular Fluid (GCF) was measured in 8 randomly selected sites in different teeth (4 with PPD 3-5mm and 4 with PPD 6-10mm and visible plaque, gingivitis and chronic periodontitis). Following careful removal of all supragingival plaque, the areas were washed with a water spray, isolated with cotton rolls and gently dried (30s) to Crevicular Gingival Fluid (CGF) collection. CGF was collected with paper strips (PerioPaper Strips, OraFlow, PlainView, New York) inserted in the periodontal pocket to the point the examiner perceived slight resistance and maintained for 30s (Tözum et al., 2004). Immediately after the amount of GCF was measured with the aid of a calibrated Periotrom 8000 (OraFlow, PlainView, New York). Strips with marks of blood were discarded. One examiner (SCG) performed all GCF sampling.

Measurement reproducibility

All clinical examinations were performed by one calibrated and masked periodontist (FP) prior to and during the experimental period. 9 patients were examined for reproducibility measurements. The unweighted Kappa statistics at site level for PPD was 0.65 before the beginning the study and 0.52 during the study. Corresponding values for unweighted Kappa for CAL were 0.51 and 0.60, respectively. Weighted kappa (±1mm) at site level was 0.98 and 0.95 for PPD and 0.97 and 0.92 for CAL in pre and trans-experimental periods, respectively.

Statistical analysis

Raw Periotrom scores were converted to volume of GCF in mL following manufacturer's recommendations. The variable GCF was not normally distributed, and to achieve normality this variable was transformed using a logarithmic function. Results are reported in the back-transformed form.

Measurements at tooth level were used and estimates were adjusted for the clustering of teeth into individuals using a robust variance estimator. Wald tests were used for comparisons, and the p-value was adjusted for multiple comparisons. The level of significance was set at 5%, and the 95% confidence intervals (CI) were calculated.

Generalizing estimating equations were used to model the relationship between GCF and various factors, and took in consideration clustering of observations within subjects. An exchangeable correlation structure was used to model correlation within-subjects.

Results

The volume of GCF significantly decreased over the 6 months experimental period (Table 2). Most decrease was observed within 30 days of supragingival treatment. Significant, but limited, additional decrease in GCF quantity was observed between 30 and 180 days. Never smokers consistently showed significantly greater GCF volume than smokers, and this difference was consistent over the 6 months period (Table 2).

The amount of GCF was clearly associated with PPD, and deep pockets showed significantly greater GCF than shallow pockets (Fig. 1). After the experimental period, no differences in mean GCF volume were observed regarding smoking status in shallow and moderate pockets (PPD≤5 mm), whereas never smokers had significantly greater volume of GCF than smokers in sites with probing depth ≥6 mm (Fig. 2).

During the first 30 days reductions in the GCF quantity seems to be associated with a decrease in marginal inflammation as expressed by marginal bleeding (Fig 3). Further decrease in GCF volume was probably associated with a reduction in periodontal inflammation as evidenced by occurrence of BOP. Never smokers showed greater GCF volume than smokers regardless of presence of marginal bleeding and bleeding on probing (Table 3).

The longitudinal multivariable analysis showed that smoking, marginal bleeding and PPD have significant independent effects on GCF volume (Table 4). Further analysis showed an interaction between probing depth at baseline and smoking status. Sites with PPD ≥6 mm in never smokers showed significantly greater mean GCF volume than sites with PPD ≥6 mm in smokers and sites PPD <6 mm in smokers and never smokers. This result is clearly shown in Fig. 2.

Discussion

The present study showed that the supragingival plaque control positively affects the inflammatory expression of sites with gingivitis and periodontitis, determining a significant reduction of the gingival crevicular fluid volume. The largest improvement was observed within thirty days of treatment for smokers and never smokers and additional reductions were observed along the investigation.

To our knowledge there are no studies assessing the effect of the mechanical control of the supragingival biofilm on the gingival crevicular fluid volume in sites with gingivitis and periodontitis. The accumulation of supragingival plaque determines an increase in the volume of fluid (Rüdiger et al., 2002; Zhang et al., 2002; Trombelli et al., 2004). Rosa et al., 2000, reported less gingival crevicular fluid in smokers patients after an experimental gingivitis period. Reductions of the fluid volume associated with subgingival instrumentation have been reported earlier (Linden et al., 2002; Figueredo et al., 2004, Tüller et al, 2005). Our results showed that the mechanical control of the biofilm restricted to the supragingival environment determined significant reductions in the volume of the fluid. The longitudinal multivariate analysis showed that marginal bleeding, smoking status and initial PPD have independent effects on the volume of GCF. The largest reductions in the volume of GCF were observed at the first month of treatment, possibly related to the decrease of the percentage of sites with marginal bleeding. As this bleeding was virtually absent for the remaining time of the study the additional reductions observed may be in part associated with the decrease in the bleeding on probing. The simultaneous presence of both bleedings were associated with increased GCF volumes as compared to the absence of bleeding independent of the PPD for both never-smokers and smokers.

Reduction in the volume of GCF was observed in all PPD categories and the largest reductions occurred in the deeper pockets. Reductions in volume of GCF were observed both for smokers and never-smokers along the entire period of the study Smokers showed significantly less GCF than never-smokers. When smoking was considered in the analysis by categories of PPD, never smokers showed significantly larger volumes of gingival fluid limited to deeper sites

(PPD+6mm). At the end of the study differences in volume were present only in these sites. The effect of smoking can be illustrated by the observation that the initial volumes of GCF at sites +6mm were similar to the volume observed for ≤5mm pockets in the never smokers. Along the experimental period, never smokers showed a faster reduction of this volume, although no differences were observed at 180 days. The reasons for these findings are not clear, but may be related with the observation that smokers show a slower response to periodontal therapy (Bergström, 2003).

There is a permanent interest in the composition and behavior of the components of the gingival fluid. The development of diagnostic methods, identification of risk indicators or markers, as well as predictors of future clinical attachment loss, became essential for the understanding of the periodontal disease pathogenesis (Figueredo et al., 2000; Preshaw & Heasman, 2002; Zhang et al., 2002; Giannopolou et al., 2003a; Giannopolou et al., 2003b; Erdemir et al., 2004; Hanioka et al., 2005, among others). Constituents of the fluid can be reported in absolute amounts (mg), concentration (mg/ml), or both, and by time of collection (pg/s). Giannopoulou et al., 2003a, observed that calculations of GCF may be inaccurate, since there may be contamination of the collecting media either by saliva or plaque, influencing results. On the other hand Zhan et al, 2002, reported that the initially observed differences for the concentration of PMN and IL-8 in the GCF disappeared when the volume was adjusted. Griffiths (2003) observes that in order to have a clearer understanding of these results the volume of GCF should be considered.

There is evidence that supragingival control may influence the subgingival environment, determining a decrease on the inflammation, even though this effect has not been evaluated in relation to the volume of GCF present (Katsanoulas et al., 1992; Mcnabb et al., 1992; Al-Yahfoufi et al., 1995; Gomes et al., 2005). Our results showed that the presence of marginal gingival inflammation is associated with increased volumes of GCF. Consequently, the control of the supragingival biofilm can differentiate the volumes of GCF related to gingivitis from those related to periodontitis. Gingivitis and periodontitis are currently considered two

different disease entities (Listgarten, 1999; Page et al., 1997). The observation that the volumes of fluid, identified with one or the other disease, are different may be of relevance when considering the origin of immune-inflammatory markers and/or indicators. These observations should be taken in account in investigations directed to the study the composition of the fluid and its impact on the diagnosis and treatment of periodontal diseases. They also suggest that a clear distinction should be made between smokers and never smokers, since smoking significantly interferes with the volume of crevicular fluid.

Conclusions

It can be concluded that the supragingival plaque control executed as the sole intervention reduced significantly the volume of GCF in smokers and never smokers. GCF volume is associated with smoking status, marginal bleeding and PPD, thus, these variables should be taken in account when GCG volume and components are under investigation.

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Table 1. Sociodemographic characteristics and smoking habits of the study population.

	Never smokers	Smokers
n	24	21
Age (mean ± SD)	47.3 ± 6.7	45.8 ± 5.1
Gender (% Males)	41.7	55
N Cigarettes per day (mean ± SD)	0 ± 0	19.6 ± 11.8
Years of habit (mean ± SD)	0 ± 0	24.1 ± 8.7

Table 2. Mean (CI) of the GCF volume, in microliters, according to experimental period and smoking habits.

Experimental periods	Never smokers	Smokers	Mean (95% CI)
Baseline	0.59 (0.52-0.69)Aa	0.36 (0.29-0.45)Ba	0.47 (0.41 – 0.55)a
30 days	0.33 (0.28-0.41)Ab	0.23 (0.19-0.29)Bb	0.29 (0.25 – 0.34)b
90 days	0.28 (0.23-0.34)Ac	0.21 (0.17-0.27)Ac	0.25 (0.21 – 0.29)c
180 days	0.23 (0.18-0.29)Ad	0.16 (0.14-0.19)Bd	0.20 (0.17 – 0.23)d

Lowercase letters refers to comparisons between different experimental periods: means followed by the same lowercase letters are not statistically different ($p > 0.05$)

Uppercase letters refers to comparisons between never smokers and smokers: means followed by the same uppercase letters are not statistically different ($p > 0.05$)

Table 3. Mean values comparison between never smokers and smokers GCF quantities, by marginal bleeding, bleeding on probing and probing depth.

Marginal bleeding	Bleeding on probing	Never smokers Mean	95%CI		Smokers Mean	95%CI		p
Probing depth<= 5								
Negative	Negative	0.36	0.22	–	0.17	0.13	–	0.008
			0.58			0.22		
Negative	Positive	0.49	0.38	–	0.26	0.21	–	0.0005
			0.62			0.33		
Positive	Positive	0.53	0.46	–	0.40	0.32	–	0.04
			0.63			0.50		
Probing depth 6+mm								
Negative*	Negative*	-	-		-	-		-
Negative	Positive	0.74	0.62	–	0.30	0.23	–	<0.0001
			0.89			0.40		
Positive	Positive	0.96	0.82	–	0.42	0.33	–	<0.0001
			1.09			0.53		

* Statistical analysis could not be performed due to small number of observations

Table 4. Multivariable longitudinal analysis of the effect of smoking, probing depth and experimental period in the volume of fluid

		Model without interaction			Model with interaction between smoking and probing depth at baseline		
Variable	Categories	Coefficient	95% CI	p	Coefficient	95% CI	p
Smoking	Never smokers	Reference			Reference		
	Smokers	-0.40	-0.61; -0.19	<0.001	-0.20	-0.54; 0.14	0.25
Probing depth at baseline	≤3 mm	Reference			Reference		
	4 –5 mm	0.50	0.30; 0.70	<0.001	0.47	0.18; 0.76	<0.001
	≥6 mm	0.99	0.76; 1.22	<0.001	1.22	0.93; 1.51	<0.001
Experimental period	Baseline	Reference			Reference		
	30 days	-0.53	-0.63; -0.42	<0.001	-0.53	-0.63; -0.42	<0.001
	90 days	-0.68	-1.02; -0.75	<0.001	-0.68	-0.80; -0.55	<0.001
	180 days	-0.88	-1.36; -0.95	<0.001	-0.88	-1.02; -0.75	<0.001
Interaction between smoking and probing depth at baseline	Smoking and probing depth 4–5 mm				0.03	-0.35; 0.42	0.87
	Smoking and probing depth ≥6 mm				-0.52	-0.92; -0.11	0.01

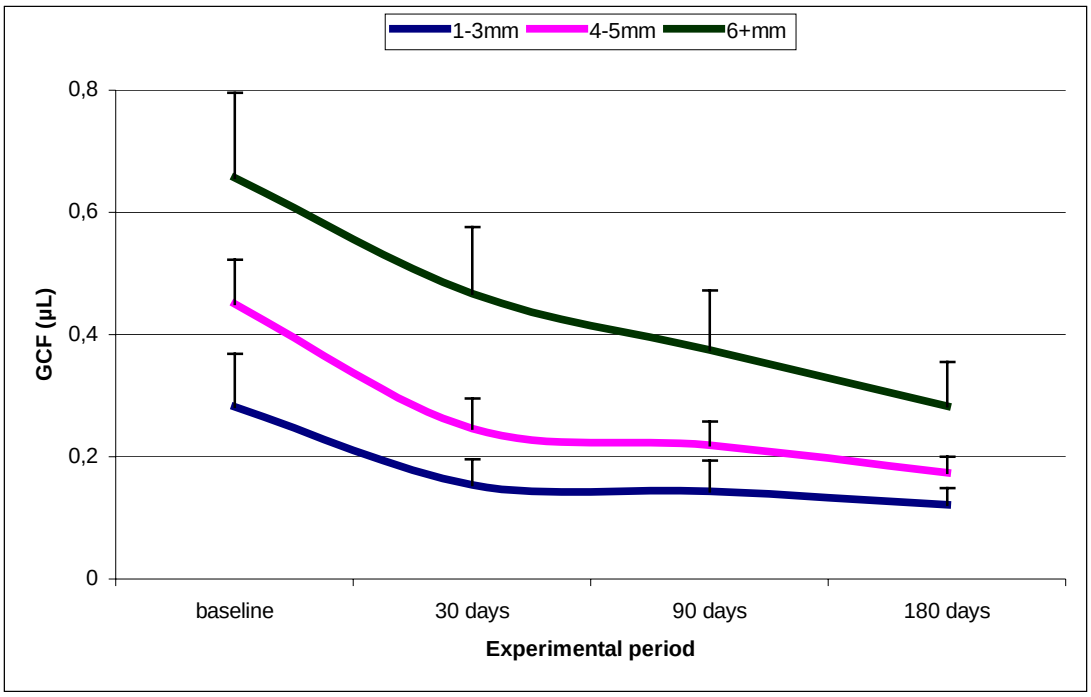

Figure 1. Mean GCF volume by probing depth during the experimental period (baseline 30, 90 and 180 days). Bars represent 95% confidence interval.

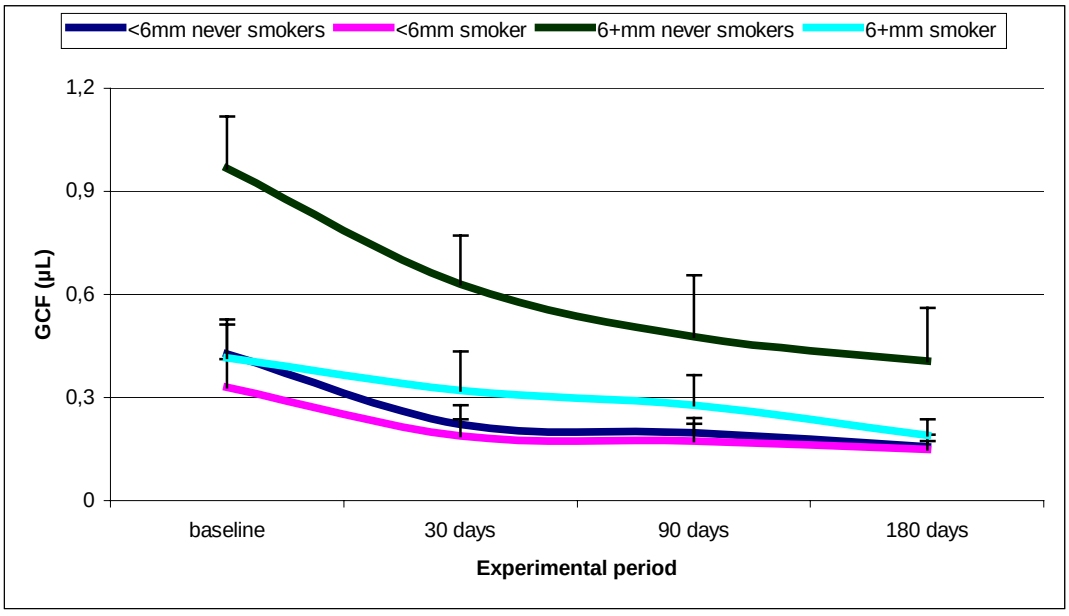

Figure 2. Mean GCF by smoking status and probing depth the experimental period (baseline, 30, 90 and 180 days). Bars represent 95% confidence interval.

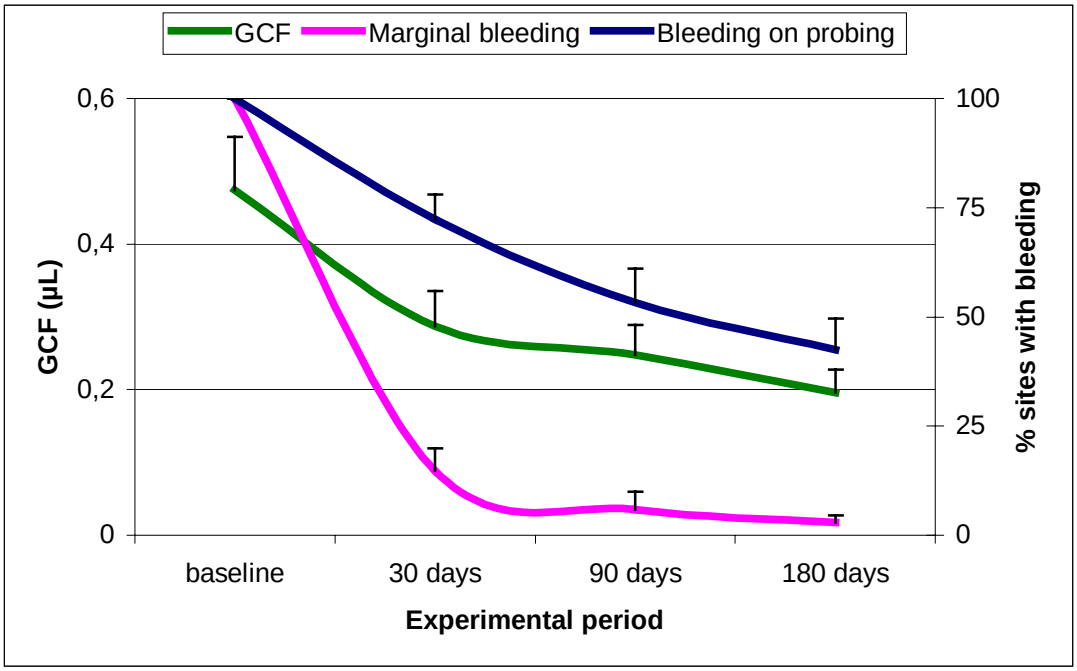

Figure 3. Mean GCF volume and percentage of sites with marginal bleeding and bleeding on probing during the experimental period (baseline, 30, 90 and 180 days). Bars represent 95% confidence interval.

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