Longitudinal study of volatile fatty acids in the gingival crevicular fluid of patients with periodontitis before and after nonsurgical therapy

L. Qiqiang[1,2], M. Huanxin[1], G. Xuejun[3]

[1]Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China, [2]Department of Periodontology, Capital Medical University School of Stomatology, Beijing, China and [3]Department of Cariology and Endodotology, Peking University School and Hospital of Stomatology, Beijing, China

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Background and Objective: Short-chain fatty acids (SCFAs) are important metabolic products of subgingival organisms and their concentrations are associated with the status of inflammation. The purpose of this study was to observe and analyze the change in concentration of SCFAs in the gingival crevicular fluid of patients with chronic periodontitis before and after periodontal treatment.

Material and Methods: Gingival crevicular fluid samples were taken from 21 patients with chronic periodontitis before periodontal treatment and 2 wk, and 2, 4 and 6 mo after treatment. The concentrations of six different SCFAs in the gingival crevicular fluid were measured using high-performance capillary electrophoresis. The presence of porphyromonas gingivalis in the same pretreatment gingival crevicular fluid samples used to measure SCFAs was analyzed using PCR amplification.

Results: Two weeks after periodontal treatment, the concentrations of lactic acid, propionic acid, butyric acid and isovaleric acid in the gingival crevicular fluid of patients with chronic periodontitis had decreased to the levels found in the healthy control group. However, the concentration of formic acid had increased. Statistically significant differences were found in the levels of these SCFAs before and after treatment. In the longitudinal observation, the concentrations of butyric acid and isovaleric acid in the gingival crevicular fluid had increased to a high level 2 mo after treatment. At the last two study time-points (4 and 6 mo after treatment), butyric acid and isovaleric acid were still present at a high level and showed a tendency to continue to increase. In contrast, the concentration of formic acid in gingival crevicular fluid showed a gradual decrease over the study period.

Conclusions: The concentration of formic acid in the gingival crevicular fluid has an inverse relationship with the severity of periodontitis, whereas the increased concentrations of butyric and isovelaric acids during the long-term observation period after therapy may indicate the status of recolonization of periodontal pathogens and reflect the subgingival ecology. These two fatty acids could be used as indicators for the development and progression of periodontitis.

Dr Meng Huanxin , BDS, MS, PhD, Department of Periodontoloty, Peking University School of Stomatology, 22 Zhongguancun Nandajie, Haidian District, Beijing 100081, China
Tel: +86 10 82195522
Fax: +86 10 62173402
e-mail: kqhxmeng@bjmu.edu.cn

Periodontitis is the most common oral disease in humans and has a severe effect on oral health. It is generally recognized that subgingival plaque is responsible for periodontal diseases. Bacteria present in subgingival plaque can produce a variety of virulence factors, such as proteases, lipopolysaccharides, fimbriae and metabolic products, which have a wide range of effects on organic tissue and the immune system. Short-chain fatty acids (SCFAs) are metabolic endproducts of anaerobic bacteria, are released into the microenvironment from infection sites and can diffuse across biological membranes (1–4). Their concentrations and composition in periodontal pockets are associated with the status of periodontal inflammation (5–7). *In vitro* research has demonstrated that, at certain concentrations, SCFAs can induce important biological effects on bacterial colonization and ecology, and on eukaryotic cell functions. For example, they can alter the proliferation of human gingival epithelial cells, endothelial cells, periodontal ligament fibroblasts, lymphocytes and human polymorphonuclear leukocytes (2,7–9). These findings are consistent with a pathogenic role of these molecules in periodontal diseases.

The progression of periodontal disease is characterized by intermittent periods of activity and inactivity in people of different ages and around specific teeth. For many years, scientists have searched for specific substances to indicate the presence of periodontal disease activity, attempted to understand periodontal disease activity and progression and endeavoured to determine factors with which to identify subjects at high risk for periodontal disease (1,10–12). Many investigations have reported that the main pathogenic microorganisms of periodontitis can produce various SCFAs, but the culture and identification of these bacteria *in vitro* are expensive and time-consuming, and generally produce unsatisfactory results (7,13–15). Therefore, if the identification of these bacteria could be replaced with detection of SCFAs, it would be much easier to determine changes in the proportions of these pathogenic bacteria and to predict the occurrence and progression of periodontal diseases. The aim of this study was to reveal whether SCFAs can be used as indicators of the development of periodontal diseases and which SCFAs could reflect the changes occurring in the disease process through a longitudinal observation.

Material and methods

Study groups

A total of 37 individuals were included in the study: 21 were patients with chronic periodontitis (14 men and seven women, 23–73 years of age; mean age ± standard deviation = 50.0 ± 5.4 years); and 16 were periodontally healthy controls (seven men and nine women, 22–45 years of age; mean age ± standard deviation = 28.9 ± 7.4 years). Patients with chronic periodontitis were chosen among individuals who were referred to the Department of Periodontology, Peking University School and Hospital of Stomatology, for periodontal problems or for routine periodontal treatment. The diagnostic criteria for chronic periodontitis were defined according to the classification proposed at the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999 (16) and were as follows: poor oral hygiene; at least eight teeth with a probing depth of > 5 mm and attachment loss of > 2 mm; and radiographic evidence of alveolar bone loss.

Individuals selected for this study fulfilled the following conditions: (i) good medical health; (ii) no periodontal therapy and no treatment with antibiotics during the preceding 12 mo; (iii) female subjects were not in the period of menses or gestation; (iv) no evident malocclusion and more than 24 teeth; and (v) no smoking habit.

Healthy controls were selected from the staff and students of Peking University School and Hospital of Stomatology. All individuals had good general health, practiced good oral hygiene, had no lost teeth and had almost no bleeding on probing, no probing depth of > 3 mm and no clinical attachment loss.

The study protocol was approved by the Ethics Committee of Peking University Health Center and the participants gave informed consent.

Clinical measurements and sample collection

Clinical measurements and samples were taken in the following order: plaque index (Silness & Loe 1964) (17); gingival index (Loe & Silness 1963) (18); gingival crevicular fluid sampling for SCFA analysis and *Porphyromonas gingivalis* detection; probing depth, measured at sample sites using a Williams periodontal probe; and bleeding index (Mazza, Newman & Sims 1981) (19). Ten nonstudy subjects were recruited and used for examiner calibration. The examiner was judged to be reproducible after meeting a percentage of agreement within ± 1 mm between repeated measurements of at least 95%.

At least one site in each quadrant with a probing depth of ≥ 4 mm was selected for gingival crevicular fluid sampling, and six to eight gingival crevicular fluid samples were collected from each patient. Samples were collected between 8 AM and 10 AM. After recording the plaque index, supragingival deposits were carefully removed by a curette. The sample sites were isolated with cotton rolls placed in the mucobuccal fold and were gently air dried before sampling. It was ensured that the samples were not contaminated with saliva. The gingival crevicular fluid sample was collected by placing a filter-paper strip (2 mm × 10 mm; Whatman, 3MM, London, UK) in the periodontal pocket until slight resistance was perceived, and the strip was then left in place for 15 s. Strips contaminated with visible blood were discarded. The filter-paper strip was weighed in a sterile Eppendorf (EP) tube, before sampling and half an hour after sampling, on an analytical balance (AE240S; Mettler, Zurich, Switzerland) in an airtight room. The difference between the weights was used to calculate the volume of gingival crevicular fluid. The weighing was

repeated and checked at least twice. In order to determine the volume of gingival crevicular fluid accurately, a calibration curve of serum was created before the investigation, as follows. Different volumes (0.1, 0.3, 0.5, 1.0, 1.5 and 2.5 µL) of human serum were dropped onto separate filter-paper strips [preweighed in an EP tube on an analytical balance (AE240S; Mettler)], and each filter-paper strip was then placed in an EP tube and weighed again on an analytical balance (AE240S; Mettler). The difference between the weights was calculated and used to establish a linear regression model between weight and volume ($r^2 = 0.9993$, $p < 0.001$) (Fig. 1).

In order to ensure the precision of the sampling method used in this study, we carried out a serial of trials to establish the stability and repeatability of the analytical balance.

Stability test – To verify the reliability of the analytical balance, 0.1, 0.3, 0.5, 1.0, 1.5 and 2.5 µL volumes of human serum were dropped onto separate filter-paper strips; each strip was then placed in an EP tube and weighed five times on the analytical balance. The results showed that the range of the relative standard deviation (RSD) of each EP tube with different filter-paper strips was 0.008–0.015%.
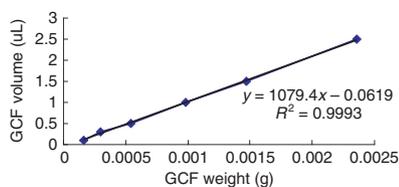

Fig. 1. Linear regression model between gingival crevicular fluid weight and volume. Different volumes (0.1, 0.3, 0.5, 1.0, 1.5 and 2.5 µL) of human serum were applied to a filter-paper strip, and each strip was placed in an Eppendorf tube and weighed on an analytical balance (AE240S; Mettler, Zurich, Switzerland). The weight of each paper strip was recorded before and after application of serum, and the difference between the weights was calculated to determine the serum weight. A linear regression model between weight and volume was established ($r^2 = 0.993$, $p < 0.001$).

Repeatability test – Five filter-paper strips were placed in five EP tubes and weighed on an analytical balance before and after the application of different volumes of serum. The difference between the weights was calculated and the measurement of each volume of serum was repeated four times in an identical manner. The results showed that the RSD of a 0.1-µL volume of serum was 17% and the RSD of the other volumes ranged from 5 to 7.2%.

The volume of gingival crevicular fluid samples obtained in this study ranged from 0.5 to 1.5 µL. The saturation of the filter paper used to collect the samples is 3.0 µL, so the paper and the sampling method used in this study were absolutely appropriate for the demands of our research.

Following weighing, the gingival crevicular fluid was eluted from the filter paper by immersion in a volume of deionized water equivalent to 100 times the sample volume. After washing, each EP tube containing the gingival crevicular fluid eluate was sealed and stored at −70°C. Gingival crevicular fluid samples were collected in the same way from the subjects of the control group.

After baseline measurements, all participants received a primary phase of nonsurgical treatment, including oral hygiene instruction, and scaling and root planing. Full-mouth supragingival professional teeth cleaning (scaling and polishing) was performed in a single session, and oral hygiene instructions were given at this time. One week later, nonsurgical treatment, consisting of subgingival debridement, was started and was performed three or four times within 1 mo. Gingival crevicular fluid samples were collected at baseline and at 2 wk, and 2, 4 and 6 mo following the intervention. Clinical data were recorded at baseline and at 4 and 6 mo after treatment in order not to disturb the subgingival environment at 2 wk and 2 mo after treatment. The treatment and all clinical examinations were performed by one of the authors. All patients completed the 6-mo trial. Because data were evaluated by matching any single site with its own preoperative site, 119

gingival crevicular fluid samples were collected at the start of the investigation and 2 wk after intervention. At the end of the trial, 74 gingival crevicular fluid samples remained that matched with their own preoperative site.

Analysis of SCFAs

The concentrations of all SCFAs were determined by high-performance capillary eletcrophoresis (Beckman, P/ACE system 5000; Beckman Coulter, Fullerton, CA, USA) equipped with a variable ultraviolet detector and a fused silica capillary, 57 cm in length and with an inside diameter of 75 µm. The separations were carried out at 20 kV with reversed polarity. Sample injection was by pressure (0.5 psi) at 6 nL/s for 10 s. The silica capillary was maintained at a constant temperature of 20 ± 0.1°C. The electrolyte contained 10 mM pathalic acid and 0.5 mM tetradecyl trimethyl ammonium bromide (T-TAB) and was adjusted to pH 5.7 with 0.1 mM lithium hydroxide. The difference in ultraviolet absorption (254 nm) between phthalate and the ions of interest was measured as a function of migration time (20,21).

Standard curves of SCFAs– Commercial fatty acids (formic, lactic, propionic, succinic, butyric and isovaleric) were obtained in a highly purified form from Beijing Chemical Co. (Beijing, China). Standard curves were obtained from the analysis of formic, lactic, propionic, butyric, succinic and isovaleric acid mixtures ranging from 20 to 100 mM in 2 mM pathalic acid buffer (pH 5.7 adjusted with lithium hydroxide). The linearity of the calibration curves for the six acids was excellent ($r^2 = 0.99$) within the concentration range of the standard.

Quality control of sample analysis

In order to ensure that the results of each analysis were comparable, buffer fluid and mixed standard acids fluid were prepared freshly before each experiment. In addition, different concentrations of mixed standard acids were analyzed at least five times and the standard curve of each acid was

calculated before sample analysis. We also carried out a serial of trials to ensure the stability, repeatability and recovery rate of the instrument used for detection.

Stability test– Two different concentrations of mixed standard acids solution were analyzed at baseline, and at 1, 2, 4, 6 and 8 h. The results showed that the range of the relative standard deviation (RSD) of each acid was 1.5–8.4%.

Repeatability test– To verify the reliability of the high-performance capillary electrophoresis, we took three different gingival crevicular fluid samples and analyzed the six acids in each sample three times. The range of RSD of different acids in gingival crevice fluid samples was 1.7–8.3%.

Recovery rate test– In order to verify the accuracy of the method used in the current experiment, we analyzed the concentrations of six acids in three different gingival crevicular fluid samples, then 5, 10 or 20 µL of 0.16 mM mixed standard acids was dropped into the gingival crevicular fluid samples and the combined samples were reanalyzed. The recovery rate of the six acids ranged from 79 to 132%.

Microbial sample collection and processing

Gingival crevicular fluid samples were collected from 21 patients with chronic periodontitis before treatment and from healthy controls. After removal of eluted gingival crevicular fluid samples for SCFA analysis, the deposits left in the EP tubes were used for detection of *P. gingivalis*. The deposits from gingival crevicular fluid were regarded as subgingival plaque; they were immersed in 500 µL of TE (10 mM/L Tris HCL, pH 7.6, 1 mM/L EDTA) buffer and mixed by vortexing, then the suspension was centrifuged at 10,000 g for 5 min and the resulting pellet was washed twice with 500 µL of TE (10 mM/L Tris HCL, pH 7.6, 1 mM/L EDTA) buffer. The samples were immediately stored at −20°C until required for DNA extraction.

DNA extraction and PCR amplification

Genomic DNA was isolated from gingival crevicular fluid deposits (including subgingival plaque) using a commercially available bacterial DNA mini kit (Watson Biotechnologies, Shanghai, China) following the manufacturer's instructions. DNA integrity was checked by electrophoresis on a 1.0% agarose gel. Bacterial 16S ribosomal DNA was amplified from the extracted DNA by the PCR, using the specific primers and PCR protocol of Ashimoto *et al*. (62); the PCR primers used in this study to detect *P. gingivalis* were as follows: upstream, 5′-AGG CAG CTT GCC ATA CTG CG-3′; and downstream, 5′-ACT GTT AGC AAC TAC CGA TGT-3′. The primer was synthesized on a Beckman DNA SM automated DNA synthesizer (SBS Genetech, Beijing, China).

The sensitivity of detection was 25–100 colony-forming units, as described by Ashimoto et al. (62). Briefly, 25 µL of PCR reaction mixture for *Porphyromonas gingivalis* contained 2 µL of sample, 2.5 µL of 10× PCR buffer, 1 unit of *Taq* DNA polymerase, 0.2 mM of each dNTP, 0.4 µM of each primer and 1.5 mM $MgCl_2$. PCR amplification was performed in the GeneAmp PCR system 2700 (ABI, Philadelphia, PA, USA). The PCR temperature profile for *P. gingivalis* included an initial denaturation step at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, a 1-min primer-annealing step at 60°C and an extension step at 72°C for 1 min, and then a final extension step at 72°C for 2 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The gel was stained with 50 µL/L of GoldViewTM (SBS genetechnology) and was then photographed under 300-nm wavelength ultraviolet light. DL 2000 (TaKaRa Biotechnology, Dalian, China) was used as the molecular weight marker.

Statistical analysis

Statistical analyses were performed with person as the unit of analysis. The normality of the data distribution was examined using the Kolmogorov–Smirnov test. All quantitative variables were normally distributed and data were reported as mean ± standard deviation. Data of rank (ordinal) scaled measures were reported as median (lower quartile to upper quartile). Changes of clinical parameters were analyzed using the Friedman test and the Wilcoxon test. Group comparison and the change of SCFA levels in the longitudinal investigation were analyzed using the *t*-test or the paired *t*-test and one-way analysis of variance. The difference in the prevalence of *P. gingivalis* between the group of 21 patients with chronic periodontitis before treatment and the healthy control group was tested using chi-square statistics; the difference in the concentrations of SCFAs between the *P. gingivalis*-positive and the *P. gingivalis*-negative subgroups in the group of 21 patients with chronic periodontitis before treatment was not analyzed as there were only a few patients in the *P. gingivalis*-negative group. Statistical significance was defined as $p < 0.05$. A software program (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA) was used for all calculations.

Results

Variation in clinical parameters before and after treatment

As a result of improved personal oral hygiene and professional supragingival and subgingival plaque control, the clinical parameters decreased significantly after treatment. However, comparison of clinical data between 4 and 6 mo after treatment found no statistical difference. The clinical parameters before and after treatment were analyzed using the *t*-test or the Friedman test and the Wilcoxon test, and the results are presented in Table 1.

Variation of SCFAs in gingival crevicular fluid before and 2 wk after treatment

Two weeks after periodontal treatment, the concentrations of lactic, propionic, butyric and isovaleric acids

Table 1. Clinical data of patients (*n* = 21) with chronic periodontitis at different examination time-points

Examination time-point	Probing depth (mm)	Bleeding index	Gingival index
Pretreatment	6.00 ± 1.57*	4 (3–4)*	2 (2–2)*
4 mo	4.14 ± 1.17	2 (2–2)	1 (1–1)
6 mo	4.79 ± 1.64	2 (2–3)	1 (1–1)

Values are given as mean ± standard deviation for probing depth, median (lower to upper quartile) for bleeding index and gingival index.
*$p < 0.05$ (*t*-test or the Friedman test and the Wilcoxon test) compared with the results at 4 mo and 6 mo.

in the gingival crevicular fluid of patients with periodontitis decreased significantly (19.69 mM vs. 10.51 mM, 11.68 mM vs. 6.00 mM, 3.11 mM vs. 1.25 mM and 3.11 mM vs. 1.03 mM, respectively; $p < 0.05$, pretreatment vs. 2 wk time-point). In contrast, the concentration of formic acid increased dramatically from pretreatment to the 2 wk time-point (3.85 mM vs. 11.16 mM, $p < 0.05$). The level of succinic acid also decreased (1.24 mM vs. 1.17 mM) at the same time-points after treatment, but no significant difference was found. The concentrations of SCFAs in gingival crevicular fluid before and 2 wk after treatment were analyzed using the paired *t*-test and the results are presented in Table 2,.

Comparison of SCFAs in gingival crevicular fluid between healthy controls and patients 2 wk after treatment

The inflammation status of patients with periodontitis was alleviated after thorough periodontal therapy. The concentrations of formic, lactic, propionic, butyric and isovaleric acids in the gingival crevicular fluid of the two groups were similar and no significant differences were found. It

was unexpected that the succinic acid concentration in the gingival crevicular fluid of patients 2 wk after treatment was higher than that in the healthy group. The data of both groups were analyzed using the *t*-test and the results are presented in Table 3,.

Variation of SCFAs in gingival crevicular fluid of patients with periodontitis at different time-points after treatment

In the longitudinal observation, we found a regular variation in the concentrations of formic, butyric and isovaleric acids in gingival crevicular fluid. Two months after treatment, the concentrations of butyric acid and isovaleric acid in the gingival crevicular fluid had increased compared with the concentrations observed 2 wk after treatment. At the last two visits (the 4 mo and 6 mo time-points after treatment), these two acids were still present at high concentrations and showed a tendency to continue to increase. In contrast, the pattern found for formic acid was completely different from that for butyric and isovaleric acids. Over time, the concentration of formic acid in the gingival crevicular

fluid decreased gradually in spite of a small increase at the second month. The change in succinic acid was dramatic. At 2 mo after treatment the concentration of this acid in gingival crevicular fluid reached a level even higher than pretreatment. Subsequently, the concentration decreased. There was a tendency for succinic acid to decrease over the duration of the study. The results are presented in Table 4. One-way analysis of variance was carried out to determine whether the levels of SCFAs in gingival crevicular fluid at different stages after periodontal treatment had significant differences.

Frequency of *P. gingivalis* in patients with chronic periodontitis and in healthy controls

The prevalence of *P. gingivalis* in subgingival plaque samples of the two study groups is summarized in Table 5. The frequency of *P. gingivalis* in subgingival plaque samples of patients with chronic periodontitis was significantly higher than that of healthy controls (81% vs. 6.3%, $p < 0.05$).

Variation of SCFAs in *P. gingivalis*-positive and *P. gingivalis*-negative groups

The group of patients with chronic periodontitis was subdivided, according to the presence or absence of *P. gingivalis* in the subgingival plaque, into two subgroups: the *P. gingivalis*-positive group and the *P. gingivalis*-negative group. The definition of *P. gingivalis* positive was that *P. gingivalis* could be detected at least in one of several samples from each patient, in other words, some samples from *P. gingivalis*-positive patients might

Table 2. Concentrations of short-chain fatty acids (SCFAs) in the gingival crevicular fluid of patients with chronic periodontitis, pretreatment and 2 wk after treatment

Study time-point	Formic acid	Succinic acid	Lactic acid	Propionic acid	Butyric acid	Isovaleric acid
Pretreatment	3.85 ± 2.91	1.24 ± 0.59	19.69 ± 9.76	11.68 ± 8.84	3.11 ± 1.86	3.11 ± 2.74
2 wk	11.16 ± 4.36*	1.17 ± 1.18	10.51 ± 5.29*	6.00 ± 2.82*	1.25 ± 0.97*	1.03 ± 0.50*

Values are given (in mM) as mean ± standard deviation. *n* = 21.
*$p < 0.05$ (paired *t*-test) vs. the concentration of SCFA in the gingival crevicular fluid pretreatment.

Table 3. Concentrations of short-chain fatty acids (SCFAs) in the gingival crevicular fluid of patients with chronic periodontitis 2 wk after treatment and in the healthy control group

Study group	Formic acid	Succinic acid	Lactic acid	Propionic acid	Butyric acid	Isovaleric acid
Chronic periodontitis ($n = 21$)	11.16 ± 4.36	1.17 ± 1.18	10.51 ± 5.29	6.00 ± 2.82	1.25 ± 0.97	1.03 ± 0.50
Healthy control ($n = 16$)	13.05 ± 11.17	0.68 ± 0.69	9.27 ± 6.36	5.87 ± 3.35	1.10 ± 0.87	1.56 ± 0.20

Values are given (in mM) as mean $\pm$ standard deviation.

Table 4. Concentrations of short-chain fatty acids (SCFAs) in the gingival crevicular fluid of patients with chronic periodontitis at different study time-points after periodontal treatment

Study time-point	Formic acid	Succinic acid	Lactic acid	Propionic acid	Butyric acid	Isovaleric acid
2 wk	11.16 ± 4.36	1.17 ± 1.18	10.51 ± 5.29	6.00 ± 2.82	1.25 ± 0.97	1.03 ± 0.50
2 mo	12.17 ± 3.71	$2.55 \pm 1.01*$	11.29 ± 4.35	$9.21 \pm 7.11*$	$3.57 \pm 1.66*$	$5.29 \pm 4.13*$
4 mo	10.77 ± 5.12	$2.18 \pm 1.52*$	12.08 ± 5.66	5.36 ± 3.34	$4.03 \pm 2.47*$	$6.07 \pm 4.46*$
6 mo	$9.39 \pm 4.67\#$	$1.15 \pm 0.76\#$	9.34 ± 4.93	5.25 ± 3.54	$5.03 \pm 2.87*$	$7.43 \pm 1.57*$

Values are given (in mM) as mean $\pm$ standard deviation. $n = 21$.
*$p < 0.05$ (one-way analysis of variance) vs. 2 wk after treatment.
#$p < 0.05$ (one-way analysis of variance) vs. 2 mo and 4 mo after treatment.

Table 5. Comparison of *Porphyromonas gingivalis* detection frequency in subgingival plaque samples between patients with chronic periodontitis before treatment and the healthy control group

Porphyromonas gingivalis status	Healthy control group		Chronic periodontitis group	
	Subjects ($n = 16$)	Sites ($n = 40$)	Patients ($n = 21$)	Sites ($n = 167$)
Positive	1 (6.3)		18 (81.0)*	
Negative	15 (93.7)		3 (19.0)	

Data are given as n (%).
*$p < 0.05$ (chi-square test) compared with the healthy control group.

Table 6. Comparison of the concentrations of short-chain fatty acids (SCFAs) in gingival crevicular fluid between the *Porphyromonas gingivalis*-positive and *P. gingivalis*-negative subgroups of patients with chronic periodontitis

Study group	Formic acid	Succinic acid	Lactic acid	Propionic acid	Butyric acid	Isovaleric acid
P. gingivalis negative ($n = 3$)	2.45 ± 1.72	1.36 ± 0.65	15.18 ± 4.82	7.74 ± 5.83	2.40 ± 1.03	1.78 ± 1.06
P. gingivalis positive ($n = 18$)	3.79 ± 2.42	1.51 ± 0.54	18.69 ± 9.38	12.83 ± 9.13	3.42 ± 1.71	3.62 ± 2.46

Values are given (in mM) as mean $\pm$ standard deviation.

not contain *P. gingivalis*; 18 patients were placed in the *P. gingivalis*-positive group according to this definition. The definition of *P. gingivalis* negative was that no *P. gingivalis* was detected in any sample from a patient. According to this definition, only three patients were placed in the *P. gingivalis*-negative group. Table 6 shows that the concentrations of SCFAs varied between the *P. gingivalis*-positive group and the *P. gingivalis*-negative group. However, as only three patients were present in the *P. gingivalis*-negative group, between-group differences in the concentration of each acid in the gingival crevicular fluid were not statistically analyzed.

Discussion

Gram-negative anaerobic bacteria, the main pathogenic microorganisms in periodontitis, produce many toxic

metabolic products. Research has revealed that SCFAs, metabolic end-products of anaerobic organisms, have a significant association with the inflammation status of periodontal tissue (2,8,22). Of these SCFAs, butyric acid has been suggested as an indicator for periodontitis. But, to date, there has been no report on the variation of these substances in gingival crevicular fluid before and after periodontal therapy. Therefore, the primary purpose of this study was to observe the effect of nonsurgical therapy on the concentration of these SCFAs in a longitudinal investigation. In the current investigation we selected a group of patients with chronic periodontitis to observe the changes of SCFAs in gingival crevicular fluid before periodontal treatment and 2 wk, and 2, 4 and 6 mo after treatment. The same sites of the patients within the chronic periodontitis group were selected as multiple sampling sites before and after treatment in order to exclude the effect of other factors; hence, the multiple comparisons of the SCFA levels at different study time-points were well controlled. Meanwhile, healthy controls were selected among the staff and students of Peking University School and Hospital of Stomatology. It is commonly known that the prevalence of periodontitis is higher in the adult population and therefore it was very difficult to find individuals with healthy periodontal tissues who were of a similar age as the group of patients with periodontitis. On the other hand, elderly subjects often have problems with their dentition or with systemic diseases. Therefore, the age of the individuals selected as healthy controls was younger than that of patients with chronic periodotitis in this study.

It has been reported that aging is a risk factor for severe periodontal disease and that the prevalence of periodontitis increases with age (23–26). However, Abdellatif et al. (23) showed that the increase in prevalence was much more pronounced in the poor oral-hygiene stratum than in the good oral-hygiene stratum when the data were stratified by oral hygiene status; he concluded that the effect of age on the progression of periodontitis could

be considered negligible when good oral hygiene was maintained. In our study, 21 patients with chronic periodontitis, referred to the outpatients section of the Department of Periodontology, Peking University School and Hospital of Stomatology, for periodontal problems, had poor oral hygiene and had not received any periodontal therapy or prescribed antibiotics during the 12 mo preceding the start of the study. Many studies have determined risk indicators for oral colonization by putative periodontal pathogens in different age stages (27,28). Hamlet *et al.* (29) described the natural distribution of the three putative periodontopathogens *P. gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* in an Australian population. They found no significant differences in the detection frequencies of the three species of bacteria for different age groups, but the presence of bacteria was strongly associated with pocket depth for both *A. actinomycetemcomitans* and *P. gingivalis*. In our previous investigation, we selected sample sites with different pocket depths and found that the concentration of different acids in the gingival crevicular fluid was actually associated with pocket depth, even in the same patient (5,6); for example, the concentrations of propionic acid and butyric acid were significantly lower in shallow pockets (pocket depth = 4 mm) than in deep pockets (pocket depth > 6mm) of patients with chronic periodontitis. In contrast, Slots *et al.* revealed that *A. actinomycetemcomitans*, mainly detected in juvenile patients with localized periodontitis, occurred at a higher prevalence in patients < 25 years of age than in adult and geriatric patients. However, *Bacteroides intermedius* showed no predilection for any age group (30,31). The other study of our group also showed that patients, younger than 30 years of age, with aggressive periodontitis were more likely to present with *A. actinomycetemcomitans* in whole saliva than were patients older than 30 years (odds ratio = 3.23, *p* < 0.05) (32). The reasons for these age differences might be that different clinical categories existed

in the individuals included in these studies. In other words, clinical categories should be analyzed individually so that there are no differences in the clinical characteristics of the sample sites.

Another uncertainty is the influence of gender on periodontal conditions and on the distribution of putative periodontopathogens in the subgingival microflora. Clinical attachment loss of all levels of severity is generally more prevalent in male subjects than in female subjects (33–35). Schenkein *et al.* (36) found no significant differences in the subgingival microflora between male subjects and female subjects, but the research of Sirinian *et al.* (37) showed that girls had a higher risk of harboring *P. gingivalis*, and that the male gender was a risk factor for harboring *P. intermedia* in periodontal pockets. The reasons for these gender differences have not been explored in detail, but are thought to be related to poorer oral hygiene and dental-visit behavior among male subjects than to any genetic factors. The patients selected for this study had poor oral hygiene and never accepted any periodontal therapy or took any prescribed antibiotics during the 12 mo preceding the study start. As we know, male subjects usually exhibit poorer oral hygiene and compliance than female subjects (23) and this explains why there were more male subjects than female subjects in our study. Future studies will be necessary to help understand the small, but definite, differences in periodontal disease seen between genders. These studies may reveal important destructive or protective mechanisms related to male or female gender.

In our study, after periodontal therapy, the clinical data of the patients showed an initial clinical improvement followed by a period of periodontal stability. The results indicated that the periodontal therapy was effective. With the condition of inflammation of each patient being eliminated, the concentration of formic acid in the gingival crevicular fluid increased significantly and approached that of the healthy group. This result confirmed our previous discovery that

formic acid had an inverse relationship with periodontal inflammation (5,6). By contrast, the levels of lactic, propionic, butyric and isovaleric acids in gingival crevicular fluid decreased significantly after therapy and statistically significant differences were found when we compared the levels of these acids in gingival crevicular fluid 2 wk after treatment with those before treatment. The concentrations of propionic, butyric and isovaleric acids in the gingival crevicular fluid of patients 2 wk after treatment had decreased almost to the concentrations of these acids in the healthy control group. These findings illustrate that propionic, butyric and isovaleric acids are associated with the inflammation found in periodontal disease. In addition, the concentration of lactic acid in gingival crevicular fluid decreased after treatment, possibly because of a reduction in the total amounts of bacteria in periodontal pockets or a reduction in the production of toxic metabolic products at local sites.

It is commonly recognized that the composition of the original biofilm in subgingival crevice would be destroyed with the application of periodontal therapy (38–40). Following reduction of the numbers of gram-negative anaerobic bacteria in periodontal pockets, there was a rapid increase in bacteria beneficial to the periodontium (41–43). It has been reported that the majority of bacteria associated with periodontitis are capable of generating succinic acid during culture media (44). Among these bacteria, *A. actinomycetemcomitans* and *Bacteroides* produced higher levels of succinic acid. Furthermore, studies have suggested that mechanical treatment alone is not effective in eliminating the pathogens *A. actinomycetemcomitans* and *Bacteroides* (45–47). According to these results, our finding, that the concentration of succinic acid in gingival crevicular fluid had increased dramatically at 2 mo after treatment and reached a level even higher than pretreatment, might indicate that bacteria related to periodontitis were recolonizing at that time. However, further investigations are needed to explain whether the increased concentration of

succinic acid observed after treatment in our current study was associated with these bacteria.

In the 6-month longitudinal observation, we found that the concentrations of butyric acid and isovelaric acid in gingival crevicular fluid reached a high level 2 mo after treatment. Moreover, the high level was maintained and displayed a tendency to increase in the long-term observation. Further correlation analysis indicated that the change in concentration of propionic acid was related to the change of probing depth (before treatment and 6 mo after treatment, $r = 0.448$, $p < 0.01$), and that the change in concentration of butyric acid was related to the change of bleeding index ($r = 0.298$, $p < 0.05$, data not shown). Many researchers have claimed that during a short period after complete periodontal therapy the composition of microorganisms in the gingival crevicular fluid of patients with periodontitis is similar to that of healthy persons (15,48,49) but returns to the original status 2 mo after treatment if no professional oral hygiene instruction and interruption are applied (46,47,50–52). Meanwhile, in *in vitro* experiments it was shown that *P. gingivalis* and *Prevotella loescheii* spent media contained high concentrations of butyric and isovaleric acids and that *Fusobacterium nucleatum* spent medium contained high concentrations of butyric and acetic acids (3). The variation of butyric and isovelaric acids during the long-term observation period in the present investigation perhaps indicates the recolonization of those putative periodontal pathogens (50,53,54).

There exist complex interactions among bacteria living in subgingival plaque. It may be co-operating or competition (55–58). Researchers have reported that although *P. gingivalis* could not grow in culture media without hemin, the situation changed completely when *P. gingivalis* was cultured with *Treponema denticola*. Through experiments it was found that the growth of *P. gingivalis* was dependent on the succinic acid generated by *T. denticola* and that the isovaletic acid produced by *P. gingivalis* also stimulated the growth of *T. denticola* (43,59–61). From these results we presumed

that succinic acid is an important energy source in the growth process of *P. gingivalis*. In our 6-month longitudinal study, the concentration of succinic acid showed a gradual decrease after treatment. Whether this reflected the growth of *P. gingivalis* in the subgingival crevice during this period requires verification. Through the verification, we would be able to identify which SCFA in gingival crevicular fluid could be used to reflect the variation of bacteria in subgingival plaque; moreover, the answers would provide us with a more appropriate basis on which to schedule appointment times in clinical practice, and perhaps even a better method for judging the susceptibility to periodontitis of individual subjects.

It should be mentioned that, in the present study, bacterial genomic DNA was isolated from the pellets of gingival crevicular fluid, *P. gingivalis* was detected from the same gingival crevicular fluid samples of patients with periodontitis and heathy controls from which the SCFAs were analyzed and that the concentrations of different SCFAs may directly reflect the bacteria present *in situ*. The results showed that the detection frequency of *P. gingivalis* in patients was 81%, which was significantly higher than that of healthy controls. In our study, gingival crevicular fluid samples were collected by 2 mm × 10 mm Whatman 3MM paper; after removal of eluted gingival crevicular fluid, the gingival crevicular fluid deposits remaining in the EP tube were used for *P. gingivalis* detection. Compared with the traditional method of sampling with a sterile Gracey curette at the apical extent of the pocket, the amounts of bacteria obtained in our study were lower, but the results were similar to other reports (62,63–65). Two subgroups were created, according to whether or not *P. gingivalis* was detected in the subgingival plaque of patients with periodontitis; however, as only three patients were present in the *P. gingivalis*-negative group, the difference in concentration of each acid in the gingival crevicular fluid between the two groups could not be analyzed. A greater number of subjects is required for future studies.

Altered composition and fluctuation in the concentrations of volatile fatty acids in gingival crevicular fluid reflect the activity of subgingival microbes. This study was a preliminary investigation of the probable relationship between volatile fatty acids and *P. gingivalis* in the subgingival environment. It is well known that periodontal diseases are multibacterial infections and that a certain combination of periodontopathogens, such as *Porphyromonas*, *Prevotellae* and *Fusobacterium*, seem to be important in the pathogenesis of the disease (66). These bacteria, except for *P. gingivalis*, also have the ability to produce virulence factors – volatile fatty acids – which have a wide range of effects on organic tissue and immune system. The relationships between these bacteria and volatile fatty acids in the gingival crevicular fluid are still unclear. Whether the change in concentrations of different SCFAs (especially butyric and isovaleric acids) in gingival crevicular fluid in longitudinal observations accurately reflect the change in prevalence of *P. gingivalis* need verification in further studies. Our challenge will be to dissect out the relationship between different bacteria and different SCFAs in gingival crevicular fluid, and to determine which species of bacteria in the subgingival plaque could be linked to the change in concentration of butyric acid and isovaletic acid in the gingival crevicular fluid.

Acknowledgements

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