Background: Whole salivary interleukin (IL)-1β, IL-6, matrix metalloproteinase (MMP)-8, and MMP-9 levels among habitual gutka chewers and non-chewers (controls) have not been investigated. The aim of the present study is to assess clinical periodontal parameters and whole salivary IL-1β, IL-6, MMP-8, and MMP-9 levels among habitual gutka chewers and controls.

Methods: Forty-five gutka chewers and 45 controls were included. Demographic information regarding age, sex, duration and daily frequency of gutka chewing, duration of gutka placement in the mouth, and daily toothbrushing habits were collected using a questionnaire. Periodontal parameters, including plaque index (PI), bleeding on probing (BOP), probing depth (PD) >3 mm, clinical attachment loss (AL), marginal bone loss (MBL), and number of missing teeth, were recorded. Unstimulated whole saliva samples were collected, and unstimulated whole salivary flow rate (UWSFR) was determined. Levels of IL-6, IL-1β, MMP-8, and MMP-9 were measured in UWS using an enzyme-linked immunosorbent assay.

Results: PI (P <0.01), BOP (P <0.01), PD >3 mm (P <0.01), and clinical AL (P <0.01) were significantly higher in gutka chewers than controls, as were whole salivary IL-6 (P <0.01), IL-1β (P <0.01), MMP-8 (P <0.01), and MMP-9 (P <0.01) concentrations. There was no significant difference in UWSFR, number of missing teeth, or MBL among habitual gutka chewers and controls.

Conclusion: Periodontal inflammatory conditions were worse, and whole salivary IL-6, IL-1β, MMP-8, and MMP-9 levels were higher among gutka chewers than non-chewers. J Periodontol 2015;86:689-695.

KEY WORDS
Cytokines; inflammation; interleukins; matrix metalloproteinases; saliva; tobacco, smokeless.
Gutka, a form of smokeless tobacco (ST), is a blend of areca nut, powdered tobacco, slaked lime (aqueous calcium hydroxide), and artificial fragrances (such as menthol).\textsuperscript{1,2} Gutka chewing is common in many southeast Asian countries (such as Bangladesh, India, Pakistan, and Sri Lanka);\textsuperscript{3-5} however, global export of gutka has made it accessible to several communities residing in North America and Europe.\textsuperscript{6-9} Gutka is commercially available in colorful and glittery sachets, most of which lack a health warning. Gutka is initially placed in the mouth and gently chewed and sucked. These contents are then held in the buccal vestibule for prolonged durations and continued to be chewed and sucked periodically. The contents may either be swallowed or spat out.

Studies have associated gutka chewing with the etiology of oral precancer and cancer.\textsuperscript{2,10,11} Moreover, periodontal inflammatory parameters (such as plaque index [PI], bleeding on probing [BOP], probing depth [PD], clinical attachment loss [AL], and marginal bone loss [MBL]) are also worse in gutka chewers than in individuals not using tobacco in any form (controls).\textsuperscript{1,4,12} Laboratory-based investigations have been used to assess the severity of periodontal inflammation among ST users.\textsuperscript{13} In a study by Jacob et al.,\textsuperscript{13} levels of interleukin (IL)-1\textbeta and IL-8 in the gingival crevicular fluid (GCF) were compared between gutka chewers and controls. However, the results showed no association between habitual gutka chewing and GCF IL-1\textbeta and IL-8.\textsuperscript{13}

Unstimulated whole saliva (UWS) is a complex oral fluid that can be used as an effective investigative tool for the assessment of periodontal inflammation.\textsuperscript{14,15} Under periodontal inflammatory conditions, proinflammatory cytokines (present in the GCF) leak into the oral cavity. It is also pertinent that compared with GCF collection, UWS samples can be collected non-invasively (by spitting into a funnel connected to a measuring cylinder) and do not require a skilled workforce.\textsuperscript{1,16} In addition, the process of UWS collection is relatively stress free, and multiple collections can be performed without imposing too much discomfort on the patient.\textsuperscript{14}

The present study is based on the hypothesis that periodontal inflammatory conditions are worse in gutka chewers compared with controls and that levels of proinflammatory cytokines (IL-6, IL-1\textbeta, matrix metalloproteinase [MMP]-8, and MMP-9) are also higher in gutka chewers compared with controls. Therefore, the aim of the present study is to assess clinical periodontal parameters and whole salivary IL-6, IL-1\textbeta, MMP-8, and MMP-9 levels among gutka chewers and controls.

**MATERIALS AND METHODS**

**Ethical Guidelines**

The study protocol was approved by the research ethics review committee of Jinah Hospital, Karachi, Pakistan (study protocol OR-46-2012). It was mandatory for all participants to read and sign the consent form before inclusion in the present investigation.

**Recruitment of Study Participants and Grouping**

The study was conducted from March 2012 to February 2013. In total, 90 participants (81 males and nine females), 45 gutka chewers (aged 39 to 58 years; mean age: 45.5 years) and 45 controls (aged 38 to 57 years; mean age: 42.1 years), were included in the present study. All participants were residents of Karachi, Pakistan, and were recruited from the Section of Dentistry, Jinah Hospital, Karachi, Pakistan. Individuals chewing ≥1 gutka sachet per day for ≥1 year were categorized as habitual gutka chewers (41 males and four females). Controls (40 males and five females) were defined as individuals who had never used any form of tobacco product. Exclusion criteria included: 1) self-reported habitual tobacco smoking, betel-nut chewing, betel-quad chewing, or alcohol drinking; 2) self-reported systemic diseases, such as diabetes mellitus, prediabetes, acquired immune deficiency syndrome, cardiovascular disorders, epilepsy, hepatic disorders, and renal disorders; 3) antibiotic and/or steroid intake within the past 3 months; 4) malocclusion (overlapping teeth); 5) lack of teeth; 6) periodontal treatment within the past 6 months; and 7) pregnancy.

**Questionnaire**

A standardized questionnaire was used to gather information regarding age (in years), sex, duration of gutka chewing (in minutes), daily frequency of gutka chewing (number of gutka sachets consumed daily), and duration of gutka placement in the buccal vestibule (in minutes). Data regarding daily toothbrushing habits was also recorded. All participants were asked about their daily toothbrushing frequency, last visit to the dentist, and oral rinsing after gutka consumption.

**Collection of UWS Samples**

To collect the UWS samples, patients were seen in the early morning hours. Patients were instructed not to eat or drink at least 2 hours before saliva collection. To collect the UWS samples, patient were seated on a dental chair and were requested to allow UWS to accumulate in their mouth for 5 continuous minutes, after which UWS was expectorated into a plastic funnel connected to a graded measuring cylinder.\textsuperscript{17} UWS flow rate (UWSFR) was determined by dividing the amount of saliva expectorated (in milliliters) by 5. Immediately after collection, UWS samples were placed in a container filled with crushed ice and aliquoted before freezing at −80°C. UWS samples were analyzed within 6 months of collection.

**Measurement of IL-1\textbeta, IL-6, MMP-8, and MMP-9**

Levels of IL-6, IL-1\textbeta, MMP-8, and MMP-9 were assessed according to the manufacturers' instructions.
using enzyme-linked immunosorbent assay (ELISA). For each cytokine investigated, a standard curve was constructed using standards provided with cytokine kits,** and protein concentrations were calculated. Using wells coated with a specific protein antibody, a total of 100-mL diluted standards with samples were dispensed in duplicate. The plates were incubated at room temperature for 60 minutes, after which they were washed three times. Conjugate solution (100 mL) was added, and the plates were incubated at room temperature for another 120 minutes. The wells were once again washed three times, and 100 mL substrate solution was added. The plates were incubated for another 20 minutes at room temperature, after which 50 mL stop solution was added to terminate color development. A spectrophotometer†† was used to determine the absorbance at 450 nm.

The overall sensitivity of ELISA for IL-1β, IL-6, MMP-8, and MMP-9 levels was 98.8% (95% confidence interval [CI] 93.6% to 100%), 99.2% (96.7% to 100%), 98.7% (94.4% to 100%), and 97.5% (95.9% to 100%), respectively, and the specificity was 100% (99.5% to 100%). All samples were diluted at a working dilution of 1:100 in phosphate-buffered saline.

Clinical Periodontal Examination
Clinical periodontal examinations were performed by a trained and calibrated investigator (FV), who was masked to the groups. The overall κ for intra-examiner reliability was 0.78. Full-mouth PI, BOP, PD >3 mm, and clinical AL were measured at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, disto-lingual/palatal, mid-lingual/palatal, and mesio-lingual/palatal) on all maxillary and mandibular teeth (excluding third molars). PD was measured to the nearest millimeter with a graded probe.‡‡ Fractured teeth with embedded root remnants were not investigated.

Measurement of MBL on Panoramic Radiographs
Panoramic radiographs were taken using a digital panoramic tomography machine§§ and viewed on a calibrated computer screen using a software program. To standardize radiographic assessment, all measurements were performed by one calibrated and trained investigator (FV). A head holder was used to stabilize the orientation of the patient’s head in both the vertical and horizontal planes. Standardized panoramic radiographs were taken at standard position, at an 8-degree downward tilt of the occusal plane compared with the standard position, at an 8-degree upward tilt of the anterior occlusal plane, and at a 10-degree downward tilt of the right and left sides. MBL was gauged as the vertical distance 2 mm below the cemento-enamel junction (CEJ) to the most apical part of marginal bone.²¹ MBL was measured on bilateral maxillary and mandibular premolars and molars.

Teeth surfaces where the CEJ and/or the bone crest were not clearly visible for technical reasons (such as dental restorations, interdental caries, overlapping of teeth, and/or poor radiographic quality) were excluded.

Statistical Analyses
Statistical analyses were performed using a software program. Clinical parameters and salivary cytokine concentrations (before and after stratification of data according to daily brushing habits) were assessed using one-way analysis of variance. For multiple comparisons, the Bonferroni post hoc test was used. P values <0.05 were considered statistically significant.

RESULTS

Characteristics of the Study Cohort
Mean ages of the gutka chewers and controls were comparable. Among gutka chewers, the mean daily amount of gutka chewing and duration of the chewing were 6.5 ± 1.2 sachets and 9.3 ± 1.2 years, respectively. The duration of gutka placement in the buccal vestibule was 40.7 ± 10.2 minutes. These results are shown in Table 1. The UWSFR was comparable among gutka chewers (0.53 ± 0.2 mL/minute) and controls (0.55 ± 0.3 mL/minute). Among gutka chewers and controls, the mean MBL was 2.1 ± 0.5 and 1.7 ± 0.3 mm, respectively.

Self-Reported Daily Toothbrushing Habits
Among gutka chewers, 95.5% and 4.5% individuals reported brushing their teeth once and twice a day, respectively. Among non-chewers, 84.4% and 15.6% individuals reported brushing their teeth once and twice a day, respectively. Fourteen (31.1%) gutka chewers reported rinsing their oral cavity with water after consuming this form of ST. In both groups, none of the individuals reported visiting an oral health care provider in the past 6 months (Table 1).

Clinical Periodontal Inflammatory Parameters
PI (P < 0.01), BOP (P < 0.01), PD ≥3 mm (P < 0.01), and clinical AL (P < 0.01) were significantly higher in gutka chewers than controls (Fig. 1). There was no significant difference in the number of missing teeth among habitual gutka chewers (5.2 ± 0.2) and controls (3.4 ± 0.7). After stratifying for brushing habits, there was no statistically significant intragroup

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** Human IL-1β, IL-6, MMP-8, and MMP-9, Quantikine ELISA Kit, R&D Systems, Minneapolis, MN.
†† EL800 Universal Microplate Reader, Bio-Tek Instruments, Winooski, VT.
‡‡ Hu-Friedy, Chicago, IL.
§§ Kodak 8000C System, Carestream Dental, Atlanta, GA.
¶¶ SPSS v.18, IBM, Chicago, IL.
difference in periodontal parameters among gutka chewers or controls who brushed their teeth either once or twice a day (Table 2).

**UWS IL-6, IL-1β, MMP-8, and MMP-9 Levels**
Whole salivary IL-6 (\(P<0.01\)), IL-1β (\(P<0.01\)), MMP-8 (\(P<0.01\)), and MMP-9 (\(P<0.01\)) levels were significantly higher among gutka chewers than controls (Table 3). After stratifying for brushing habits, there was no statistically significant intragroup difference in whole salivary IL-1β, IL-6, MMP-8, and MMP-9 levels among gutka chewers or controls who brushed either once or twice daily (Table 4).

**DISCUSSION**
To the authors’ knowledge from indexed literature, this is the first study in which pro-inflammatory cytokines (IL-6, IL-1β, MMP-8, and MMP-9) have been investigated in the UWS of gutka chewers. The present results demonstrate that levels of IL-6, IL-1β, MMP-8, and MMP-9 were significantly higher in gutka chewers compared with controls. An explanation in this regard may be that poor periodontal health status (as reflected by increased PI, BOP, PD, and clinical AL) among gutka chewers may have caused an increased accumulation of these cytokines in the GCF and hence leakage into UWS. These results, however, contradict a recent study by Jacob et al. in terms of cytokine profile. In that study, GCF levels of IL-1β and IL-8 were investigated among gutka chewers and controls, and the results showed no association among gutka chewing and GCF levels of IL-1β and IL-8. Although Jacob et al. reported a power of 80% for their study population, it is pertinent that GCF collection requires skillful training, as contamination of GCF strips with saliva may alter the levels of cytokines under investigation. Likewise, Lamster and Ahlo reported that methodologic concerns related to the collection and analysis of GCF are essential parameters that should be considered during GCF-based investigations. That study also emphasized that analysis of GCF-derived mediators in UWS are valuable and rapid tools in the assessment of periodontal inflammation. Because saliva collection is less sensitive to technique than GCF collection, UWS samples were used in the present study for assessing the levels of pro-inflammatory cytokines in UWS of gutka chewers and controls.

An interesting finding in the present investigation was that regardless of increased PI, PD, BOP, and clinical AL among gutka chewers versus non-chewers, there was no significant difference in the number of

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**Table 1. Characteristics of the Study Population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Gutka chewers (n = 45)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>45.5 ± 8.6</td>
<td>42.1 ± 6.3</td>
</tr>
<tr>
<td>Sex (males:females)</td>
<td>41:4</td>
<td>40:5</td>
</tr>
<tr>
<td>Duration of gutka chewing (years)</td>
<td>9.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Daily amount of gutka-chewing (no. of sachets)</td>
<td>6.5 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Duration of intraoral gutka placement (minutes)</td>
<td>40.7 ± 10.2</td>
<td></td>
</tr>
<tr>
<td>Daily toothbrushing (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Once</td>
<td>95.5</td>
<td>84.4</td>
</tr>
<tr>
<td>Twice</td>
<td>4.5</td>
<td>15.6</td>
</tr>
<tr>
<td>Have you visited a dentist in the past 6 months?</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 1.**
Periodontal inflammatory parameters among gutka chewers and non-chewers. *Significant difference (\(P<0.01\)) compared with control group.
missing teeth and MBL between the groups. These results may be associated with the age of the study population. It has been reported that periodontal inflammatory conditions are worse in elderly patients (>60 years) compared with relatively younger individuals (<49 years). In the present study, the mean ages of gutka chewers and non-chewers were 45.5 ± 8.6 and 42.1 ± 6.3 years, respectively. Because clinical AL precedes MBL, it is therefore hypothesized that gutka chewers aged >60 years may exhibit significantly higher clinical AL and MBL than controls in the same age group.

In the present study, whole salivary IL-1β, IL-6, MMP-8, and MMP-9 levels were significantly higher in gutka chewers (who were consuming ~6 gutka sachets per day for nearly a decade) than in controls. In addition, these individuals were placing gutka in their buccal vestibules for ~40 minutes before either swallowing or spitting out the contents. It may therefore be postulated that in comparison to the present results, individuals consuming high amounts of gutka (>6 sachets/day) for prolonged durations (>10 years) would express much higher levels of proinflammatory cytokines in UWS than individuals using gutka for shorter durations. According to Attin and Hornecker, toothbrushing once daily is sufficient to maintain oral health and prevent caries and periodontal diseases. The present authors agree, since there was no significant difference in periodontal conditions and UWS proinflammatory cytokines among gutka chewers and non-chewers after stratification of data for daily toothbrushing frequency (Tables 3 and 4). However, it is noteworthy that periodontal inflammatory parameters and whole-salivary proinflammatory cytokine levels were significantly higher among gutka chewers who brushed their teeth once a day compared with controls who also brushed once a day. It may be argued that gutka chewers were not able to achieve sufficient plaque removal by performing oral hygiene measures at home compared with controls. In addition, 70% of gutka chewers did not rinse their oral cavity with water after consuming this form of ST. This factor may have contributed to promoting plaque accumulation, thereby augmenting periodontal inflammation and causing raised levels of IL-1β, IL-6, MMP-8, and MMP-9 in UWS compared with controls. It is therefore speculated that gutka chewers who achieve adequate plaque control (by brushing at least once daily) have lower levels of periodontal inflammation and whole-salivary proinflammatory cytokines compared with gutka chewers who are

Table 2.
Periodontal Parameters Among Gutka Chewers and Controls After Stratification of Data for Daily Toothbrushing Habits

<table>
<thead>
<tr>
<th>Periodontal Parameter</th>
<th>Gutka chewers (n = 45)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brushing Once Daily (n = 43)</td>
<td>Brushing Twice Daily (n = 2)</td>
</tr>
<tr>
<td>PI (%)</td>
<td>82.5 ± 7.6*</td>
<td>77.1 ± 6.1†</td>
</tr>
<tr>
<td>BOP (%)</td>
<td>85.5 ± 4.7*</td>
<td>75.3 ± 9.6†</td>
</tr>
<tr>
<td>PD ≥3 mm (%)</td>
<td>38.9 ± 3.5*</td>
<td>30.9 ± 2.6†</td>
</tr>
<tr>
<td>Clinical AL (mm)</td>
<td>9.6 ± 5.2*</td>
<td>5.8 ± 1.6†</td>
</tr>
<tr>
<td>MBL (mm)</td>
<td>2.5 ± 0.6</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>Number of missing teeth</td>
<td>6 ± 0.4</td>
<td>4.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Significant difference compared with controls brushing their teeth once daily (P < 0.05).
† Significant difference compared with controls brushing their teeth twice daily (P < 0.05).

Table 3.
Whole Salivary IL-1β, IL-6, MMP-8, and MMP-9 Levels Among Habitual Gutka Chewers and Controls

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Gutka chewers (n = 45)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>54.7 ± 12.2*</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>108.6 ± 10.2*</td>
<td>12.5 ± 3.8</td>
</tr>
<tr>
<td>MMP-8 (µg/mL)</td>
<td>371 ± 28.7*</td>
<td>98.5 ± 14.4</td>
</tr>
<tr>
<td>MMP-9 (µg/mL)</td>
<td>592.5 ± 41.2*</td>
<td>89.1 ± 16.6</td>
</tr>
</tbody>
</table>

* Significant difference compared with controls (P < 0.01).
unable to achieve plaque control regardless of daily brushing frequency.

Questionnaires are an efficient approach to evaluate self-perceived oral health; nevertheless, they are less reliable about specific periodontal variables. Additionally, it has been reported that self-reported periodontal health is unreliable, particularly in situations when respondents with periodontal disease are unaware of their oral condition. Furthermore, the manner in which questions are administered to participants and education of respondents may also influence the outcome scores. An interesting finding in the present study is the lack of statistically significant difference in the severity of periodontal inflammatory parameters among gutka chewers who brushed their teeth either once or twice daily. Because data collection regarding daily toothbrushing habits was self-reported, and participants were aware that the interviewer was an oral healthcare provider, it is possible that reported daily toothbrushing regimens were biased. Another factor that could have influenced the periodontal health of gutka chewers brushing their teeth either once or twice daily is education status. It has been reported that the socioeconomic status and education status of habitual ST product users is significantly lower than those of individuals not using tobacco in any form. These factors (underprivileged socioeconomic status and poor education) may have prevented gutka chewers from seeking routine dental checkups/visits. This is supported by the results from a survey reporting that an increased prevalence of tobacco product use has been associated with low education levels.

A limitation of this study is that several of the demographic data were based on self-reporting. Moreover, no actual medical examination and/or test was applied to determine whether these individuals were systemically healthy. Areca nut is an integral component of gutka and is associated with the etiology of medical disorders such as metabolic syndrome. It is possible that latent systemic conditions may have contributed in aggravating periodontal inflammation in these individuals. Because UWSFR was comparable in gutka chewers and controls, the likelihood of contamination of UWS samples (by bacteria, epithelial cells, erythrocytes, leukocytes, food debris, or contamination from GCF) cannot be disregarded in both groups.

It is imperative for oral health care providers to be aware of the detrimental effects of gutka chewing on oral health. In addition, besides imposing a ban on such ST products, departments of health should emphasize the significance of routine medical and dental checkups in the community. Routine dental checkups and health consultation may help in the early diagnosis and treatment of periodontal inflammation among gutka chewers and non-chewers.

**CONCLUSION**

Within the limits of the present study, it is concluded that periodontal inflammatory conditions are worse and whole salivary IL-6, IL-1β, MMP-8, and MMP-9 levels are higher in habitual gutka chewers compared with non-chewers.

**ACKNOWLEDGMENTS**

The authors extend their appreciation to the Research Center, College of Applied Medical Sciences, and Deanship of Scientific Research at King Saud University for funding this research. The authors also thank the Visiting Professor Program at King Saud University, Saudi Arabia, for supporting this research project. The authors report no conflicts of interest related to this study.

**Table 4. Whole Salivary IL-1β, IL-6, MMP-8, and MMP-9 Levels Among Gutka Chewers and Controls After Stratification for Daily Toothbrushing Habits**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Gutka chewers (n = 45)</th>
<th>Controls (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brushing Once Daily (n = 43)</td>
<td>Brushing Twice Daily (n = 2)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>119.2 ± 23.1*</td>
<td>107.5 ± 14.7†</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>635 ± 19.6*</td>
<td>50.2 ± 20.7†</td>
</tr>
<tr>
<td>MMP-8 (μg/mL)</td>
<td>409.4 ± 21.1*</td>
<td>382.6 ± 16.5†</td>
</tr>
<tr>
<td>MMP-9 (μg/mL)</td>
<td>652.3 ± 31.2*</td>
<td>455.4 ± 27.1†</td>
</tr>
</tbody>
</table>

* Significant difference compared with controls brushing their teeth once daily (P < 0.05).
† Significant difference compared with controls brushing their teeth twice daily (P < 0.05).
REFERENCES


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