AJDM

ALTAMASH JOURNAL OF DENTISTRY AND MEDICINE

Original Article

In-Vivo anti-Microbial activity of Subgingival Salvadora Persica in Chronic Periodontitis

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Abstract.

Objective: To evaluate the antimicrobial activity of locally delivered (SP) gel in nonsurgical therapy of chronic periodontitis.

Materials and methods: Sixty-six subjects (n 33/group) with untreated chronic periodontitis were randomized to receive either scaling and root planning (SRP) alone or SRP combined with SP gel. At baseline and 6 weeks the subgingival plaque samples were collected and assessed for the quantity of bacteria Porphyromonas Gingivalis (Pg) using Real Time Polymerase Chain Reaction (RT-PCR).

Results: At 6 weeks, the test group (SRP + SP) had a statistically significant lower Pg count compared to the control group (SRP only) (U=252, $p \leq 0.0015$).

Conclusion: Local delivery of SP gel into periodontal pockets acted as a beneficial adjunct to SRP and reduced bacteria Pg level in the short follow-up time.

Keywords: Miswak, scaling and root planning, porphyromonas gingivalis

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Chronic periodontitis is characterized by destructive inflammation of the supporting tissues of the teeth or the “periodontium” that results in loss of attachment, tooth mobility and if left untreated, eventually leads to tooth loss.[1] Though it is generally accepted as a multi-factorial disease, small number of gram-negative bacteria which are more pathogenic are associated with diseased states [2,3] Colonization and overgrowth of a consortium of gram negative bacteria referred as the “red complex bacteria” (*Pg, Td, Tf*), are strongly implicated in the progression of periodontal disease.[4,5] Owing to their strong virulence factors and their invasion and host immune response evasion abilities, these gram-negative bacteria, along with Aggregatibacter actinomycetemcomitans (Aa), colonize the gingival sulcus and induce inflammation and tissue destruction.[6,7]

Conventional therapy revolves around complete elimination of periodontopathogens and calculus, with scaling and root planning (SRP) being the “gold standard” for treatment of CP. It is generally accepted, however, that the benefits of SRP are limited in areas with poor access to mechanical debridement, such as deep periodontal pockets and furcation areas, necessitating additional or adjunctive treatment.[8,9] Antibiotics are a common adjunctive therapy, augmenting the benefits of mechanical debridement of the periodontal pockets by suppressing any remaining periodontopathogens. They are, however, associated with increased drug resistance and may cause side effects such as nausea and vomiting as well as taste disturbances. Additionally, their efficacy is dependent upon patient compliance.[10] An alternative to systemic antibiotics is placement of local antimicrobials directly into the periodontal pockets, which reduces any systemic side effects and eliminates the need for patient compliance.[11, 12]

A renewed interest in plant-derived medicines has been fueled by their profound therapeutic benefits as well as their advantages of higher safety levels, lack of side effects and greater affordability when compared to the synthetic alternatives. Roots and twig of an evergreen shrub Salvadora Persica (SP) (known as “siwak” or “miswak” in Arabic and “Peelu” in Urdu) have been used as an oral hygiene tool since thousands of years. The efficacy of SP as an oral hygiene aid arises from a combination of the mechanical cleansing effects and its anti-plaque and anti-caries activity,[13-15] with studies showing regular users exhibiting lower gingival indices .[16-18]

The antibacterial activity of SP extracts are well-documented in literature,[19] with investigations reporting higher efficacy against common periodontopathogens including Pg, Td, Aa, Prevotella melaninogenica (Tm), Prevotella intermedia (Pi), Fusobacterium nucleatum (Fn) and Eikenella Corrodens (Ec).[15, 20, 21] In an in vitro study whole, non-extracted pieces of miswak embedded in agar plates were shown to exert strong antimicrobial activity against periodontopathogenic and cariogenic bacteria (Streptococcus mutans, Lactobacillus acidophilus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Haemophilus influenzae).[15] Following up on this study, Sofrata et al. discovered that the primary antibacterial component of SP was its constituent benzylisothiocynate (BITC), which is particularly effective against Porphyromonas Gingivalis and Aggregatibacter actinomycetemcomitans .[22]

AbdElRahman et al assessed the antibacterial effects on selected pathogens (Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Actinomyces naeslundii, Prevotella intermedia, Streptococcus mutans, Lactobacillus acidophilus, Candida albicans) using SP extracts in four separate mediums: sterile distilled water, 96% ethanol, 2% acetic acid, and ethyl acetate as solvents. The study had two important conclusions: first, antibacterial activity of SP was strongest in ethanolic extracts; and second, antimicrobial activity of crude miswak extracts was comparable with conventionally used antimicrobial agents, such as 0.2% aqueous chlorhexidine.[23] In a study on methanolic extracts Alali et al. concluded that SP inhibited the growth against both Gram positive and negative bacteria, but showed greater effects on gram positive.[24] Amoian et al reported that miswak containing chewing gums caused an improvement in plaque index, gingival index and bleeding index, indicating an anti-plaque activity.[25] Despite its known antimicrobial and anti-inflammatory activity, to

the best of the authors' knowledge there is no in vivo study

investigating the antimicrobial action of SP on bacteria in pathologically deepened gingival sulcus or the periodontal pocket, which is a dynamic environment. The aim of this study is, therefore, to assess the antimicrobial effects of SP gel following SRP in moderate periodontal pockets of chronic periodontitis.

Materials and methods

The ethical approval for the current study was attained from the Ethical Review Committee (ERC) at Ziauddin University (reference code: 0860319THOM). The clinical trial has also been registered under clinical trials.gov, holding the Identifier number: NCT03749369. The study was conducted in the department of Periodontology, Ziauddin College of Dentistry. Sixty-six subjects diagnosed with untreated chronic periodontitis, following Armitage's criteria [26] of presence of moderate (PD of 5 to 6mm or clinical attachment loss CAL of 4 to 6 mm) or deep pockets (PPD ≥7 mm or CAL ≥ 6 mm) and intra-bony defect ≥ 3 mm on intraoral periapical radiograph, having no history of systemic disease affecting the progression of periodontitis and with a minimum of 20 natural teeth were included in the study. Pregnant and lactating women were excluded on basis of ethical matters as well as an altered response to plaque and increased likelihood of progress of periodontal disease, as were smokers and those who were unable to give informed consent. Subjects were selected by convenience sampling methods and were randomly assigned, through the flip of a coin, into either the Test group (SRP and SP gel) or Control group (SRP only).The SP gel was formulated and developed by Ziauddin Pharmacy College, utilizing the methodology described by Al-Ayed[27] and Asani.[28] One hundred grams of SP were first washed and cut into small pieces with a sharp knife. After being left to dry at ambient temperature for 15 days, the SP pieces were ground to powder using an electrical grinder. The powder produced was mixed with 95% ethanol (Merck, Germany) and allowed to stand for 48 hours. A Whatman filter paper was used to filter the mixture, and the filtrate was dried, forming SP extract.To form the gel, powder of potassium sorbate was dissolved in water and heated to 80°C. Hydroxypropyl methylcellulose (HPMC) was gradually added and mixed by constant stirring. Drops of water were added and mixed until a homogenous transparent gel of the desired consistency was formed. The gel was stored in refrigerator overnight. The SP extract was mixed to Polyethylene glycol 400 (Sigma Aldrich, Germany) and gradually added to the gel.

On week 0, full mouth 6-point periodontal charting was performed using a University of North Carolina 15 (UNC 15) probe. Following the protocol set by Moneira.[29] four non-contiguous interproximal sites with a periodontal pocket depth (PPD) between 4 and 6 mm were selected for monitoring and collection of subgingival plaque samples in each subject. In the selected sites, a sterilized Gracey curette was inserted subgingivally and the plaque was collected using a single stroke. The curettes containing the plaque samples were immediately placed in a clean plastic Eppendorf tube (Elkay, Costelloe, Ireland) containing 0.15 mL of buffer solution (Tris-HCl and EDTA, pH 7.6). In each of the four selected sites, a sterile paper point was also inserted, left undisturbed for 15 seconds each and then inserted into the same Eppendorf tube. The mixture was dispersed using a vortex mixture and stored at 4 Celsius until DNA extraction was performed.

Each subject underwent SRP, the endpoint of which was considered to be smooth surfaces of crown and root. Root planning was performed under local anesthesia. Debridement was performed using a combination both ultrasonic piezoelectric scalers and hand instruments (Gracey curettes and sickle scalers). For each subject all sites with 4 mm or greater PPD were selected and marked on the chart. On completion of nonsurgical periodontal treatment, the pocket in these sites received either the SP gel using a blunt cannula attached to the syringe (in the test group) or were irrigated with a syringe containing distilled water (in the control group) for 30–60 s. Subjects received oral hygiene instructions and were instructed to not eat and drink for the next hour to allow maximum absorption of the constituents. On week 6, once again subgingival plaque sampling was performed using a sterilized Gracey curette and paper point in the same points as the baseline. Microbial testing was performed using Real-time Polymerase Chain Reaction (RT-PCR),

which has been documented in literature as being a sensitive, specific, reproducible, efficient and rapid detection and quantification method of *Pg*[30] The first step was DNA extraction, which was performed using QIAGEN Qiamp mini kit, following the instructions provided by the manufacturer. The tubes were stored in the freezer till amplification. For the quantification process, three reaction mixes were prepared, according to Table 1.

Table 1: Reaction mixes for DNA Quantification

A	10 µLMastermix	1 µL PPC	5 µL DNA	4 µLwater
B	10 µL mastermix	1 µL *Pg*	5 µL DNA	4 µL water
C	10 µL mastermix	1 µL Pg	2 µL positive control	7 µL water

The PCR cycler were programmed as described in the catalogue. First, PCR activation was carried at 95°C for 10 minutes and comprised of 1 cycle. This was followed by a 2-step cycling: step 1 was denaturing which was performed at 95°C for 15 seconds and comprised of 40 cycles; step 2 was annealing and extension which were 40 cycles carried at 60°C for 2 minutes. The PCR tubes were placed into the real-time thermal cycler and secured with the Rotor-Disc 100 Locking Ring. The threshold cycle (CT) for each well was calculated using the cycler's software. The resulting threshold cycle values for all wells were exported to a blank Excel spreadsheet for data analysis for use with Multi-Assay Kit Data Analysis Template Excel Software.

Data was analyzed using SPSS version 23. Normality of the numeric data such as age, PPD, PG was assessed using Shapiro-Wilk test. Frequency and percentage were reported for categorical data as gender. The baseline PPD and PG were compared between both groups using Mann-Whitney U test/independent sample t-test. Difference was calculated for PG by subtracting baseline values with follow-up values. Comparison of difference in PPD and PG were done using independent sample t-test/Mann Whitney U test. A $p \leq 0.05$ was considered as statistically significant.

Results

A total of sixty-six participants were recruited in this study. Overall mean age of the patients was 48.68±9.12 years (range: 30 to 70 years). Fifty-eight of sixty-six participants completed the study. In the test group three failed to follow-up and in the control group a total of five participants failed to follow-up.Out of the 66 recruited participants, 38 were males (57.6%) and 28 were females (42.4%). Majority of the participants in test group gender and groups were

compared by using chi-square test. Chi-square test (X^2=0.992, df=1) revealed that statistically there was no significant difference between gender in the groups (*p*=0.319). At baseline there was no statistically significant difference in mean PPD level between test and control groups at baseline, with *p*=0.164 (Table 2).

Table 2: Comparison of periodontal pocket depth (PPD) of included patients at baseline between test and control group

	Test group (n=33)		**Control group (n=33)**		***p*-value**
	Median (IQR)	**Mean Rank**	**Median (IQR)**	**Mean Rank**	
PPD (mm)	4.21 (3.48-4.91)	30.21	4.43 (4.12-5.28)	36.79	0.164

Non-parametric distribution between groups; Mann-Whitney U test was applied
Insignificant at 0.05 level of significance
PPD= Periodontal pocket depth

Pg at baseline and 6th week were compared between the test group and control group using Mann-Whitney U test (Table 3). There were statistically insignificant differences observed for *Pg* between test group and control group at baseline (U=482, *p*=0.422). At 6 weeks, the test group (n=31) had significantly lesser *Pg* than control group (U=252, *p*=0.0015). The difference of *Pg* (*Pg* at 6th week-*Pg* at baseline) was also compared between groups by using Mann-Whitney U test. Mann-Whitney U test revealed that the test group had significantly lower *Pg* as compared to control group (U=147.5, *p*=0.001), pointing towards an inhibitory action of SP on the growth of *Pg*.

Table 3: Comparison of cycle threshold (CT) value of *Pg* at baseline, 6th week follow and difference (from baseline to 6th week) between test and control group

	Test group		**Control group**		***p*-value**
	Median (IQR)	**Mean Rank**	**Median (IQR)**	**Mean Rank**	
Pg (baseline)	16.40 (12.90-20.00)	35.39	16.10 (12.40-18.55)	31.61	0.422
Pg (6 weeks)	2.40 (0.001-4.100)	24.13	8.400 (0.001-11.15)	36.50	0.005
Difference in Pg	-12.70 (-11.80 to -14.45)	20.76	-7.95 (-6.80 to -10.75)	40.23	0.001

non-parametric distribution, Mann-Whitney U test was applied
Significant at 0.05 level of significance

Discussion

In the current study, we evaluated the antimicrobial effects of adjunctive use of subgingival SP gel in patients with moderate to severe CP and found a significant amount of periodontopathogen *Pg* reduction compared to SRP alone**.** Scaling and root planning alone often serves as definitive treatment of CP, causing cessation of the disease process and restoring the periodontium's health and function. The ultimate goal of SRP is to render the root surface completely free of plaque and calculus deposits, however studies indicate that this goal is not always attainable.[31] Despite some residual plaque and deposits, however, SRP causes an elimination in the signs of inflammation (bleeding and plaque score), reduction in PPD and the number of periodontopathogens and a gain in CAL,[32-34] which has led to the hypothesis that there is a certain threshold of bacteria that is required to cause disease; a level lower than this threshold causes an infection that the host can overcome.[35] This shifts our focus from the unrealistic goal of attaining absolutely zero amount of periodontopathogens to having an amount that is compatible with health. Subgingival colonization by *Pg* is an established risk factor for periodontitis,[36] promoting thedysbiotic environment that results in chronic inflammatory changes characteristic of periodontitis. Theoretically this makes presence of *Pg* a suitable screening biomarker of periodontitis.[37] Several studies have utilized culture techniques to estimate the bacterial cell numbers in subgingival plaque samples,[38,38] however growth and quantification of *Pg* has been shown to be difficult as compared to other bacterial species.[40] On the other hand, the PCR technique can theoretically detect as low as a single copy of the target DNA. In an experimental study the sensitivity, specificity, and positive and negative predictive values of the real-time PCR were observed to be 100, 94, 94, and 100%, respectively, effectively concluding that real-time PCR provides a rapid, sensitive and accurate confirmation of counts of *Pg* in subgingival plaque samples.

In the present clinical trial, a statistically significant decrease in *Pg* levels was observed in both the test and control group, which are indicative of the improvements achieved by the treatment protocols (SRP in control group and SRP plus SP in the test group). When comparing both groups, a greater decrease in *Pg* levels was seen in the test group, which may be attributable to the benefits achieved by the adjunctive use of SP. This is the first in vivo trial investigating the effects of SP on subgingival microbiota, which is a dynamic environment unlike those in in-vitro studies. In an invitro study utilizing the methanolic SP extract (200 mg/ml) it was observed that its antibacterial activity against tested oral pathogenic bacterial isolates was comparable to that seen with standard antibiotic (ampicillin).[41]

While all subjects in this clinical trial had confirmed diagnosis of chronic periodontitis, not all showed detectable levels of *Pg* at baseline. In the presence of standardized plaque sample collection methods and optimized PCR protocols along with the high sensitivity and specificity of PCR, this finding points at the fact that not all cases of CP have *Pg* as part of the subgingival microbiota. Other studies detecting *Pg* in subgingival samples of CP showed varying prevalence amongst different populations: 60% [42], 60% [43], 53% [44], 75.8% [45], 85.7% [46], while a study conducted in Pakistan showed that 83% of subjects with chronic periodontitis showed detectable *Pg* levels.[47] This variation in prevalence amongst different populations corroborates with the finding that

marked differences exists in the microbial profiles of subgingival biofilm amongst subjects from different countries.[48]

An essential finding related to the *Pg* levels was that despite thorough and complete manual debridement and satisfactory mechanical plaque control practices followed by the subjects, at 6 weeks follow-up some level of *Pg* was seen in the plaque and its complete eradication was not observed, even in the test group where subgingival SP gel was placed. This observation could be hypothesized to be the result of the presence of fimbriae and strong virulence factors of *Pg* which allow it to invade the sulcular tissues and multiply, presenting again in the gingival sulcus.[49]

Thus, within the limitations of the present study, the authors concluded that a statistically significant decrease in the counts of *Pg* was observed with adjunctive use of subgingival SP gel, indicating an anti-microbial role against periodontopathogenic bacteria.

Ethical Disclosure/Approval

Ethical Review Committee of Ziauddin University (0860319THOM)

Source of Funding

This research project has been partially funded by Board of Advanced Studies and Research (BASR), as part of MDS training.

Author Contribution

1. **T.H:** Data collection, contribution data tools, performed the analysis, wrote the manuscript
2. **S.Q:** Conceived and designed the analysis
3. **R.K:** Conceived and designed the analysis, performed PCR

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