Journal of Oral Biology and Craniofacial Research 13 (2023) 8–12

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Effect of salivary urea, pH and ureolytic microflora on dental calculus formation and its correlation with periodontal status

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ARTICLE INFO

Keywords:
Dental calculus
Urea
pH
Ureolytic bacteria
Microflora
Saliva

ABSTRACT

Context: Dental calculus, formed by mineralization of plaque predisposes to the development of periodontal disease.

Aim: To evaluate the influence of salivary urea and the presence of ureolytic bacteria on dental calculus formation and periodontal status in patients with good, fair and poor oral hygiene.

Material and methods: An observational cross-sectional study was carried out on 135 patients, 18–60 years of age. Based on the simplified calculus index, patients were divided into three groups, good oral hygiene, fair oral hygiene and poor oral hygiene. Clinical parameters such as plaque index, gingival index, pocket probing depth and clinical attachment level and salivary pH were recorded for each subject. Saliva samples were collected to evaluate the urea levels using autoanalyzer method. Supragingival calculus samples were collected and presence and quantification of ureolytic bacteria were done by gram staining and bacterial culture and confirmed by biochemical reaction. For statistical analysis, test like Shapiro-Wilk test, Kruskal Wallis and Spearman's rho were used.

Results: Increase in salivary pH was associated with increased odds of higher calculus index score (odds ratio = 2.785). There was a non-significant weak correlation between salivary urea and ureolytic bacteria in dental calculus in all the three groups ($p > 0.05$). Higher calculus index score was associated with increased odds of presence of ureolytic bacteria (odds ratio>1).

Conclusions: Higher level of ureolytic bacteria with increasing calculus index score may breakdown the salivary urea to ammonia resulting in a ureolytic pH rise that facilitate calcium phosphate saturation leading to more calculus formation.

1. Introduction

Saliva is a complex biological fluid that has a crucial role in oral physiology. Changes in the composition and properties of the saliva are responsible for several oral problems, such as dental calculus formation, caries and periodontal disease. The pH of saliva ranges from 5 to 8. Urea, present in saliva, is an organic nitrogenous substance synthesized from amino acids and carbon dioxide. The normal levels in the saliva range from 12-70 mg/dl (3–10 mmol/l).[1,2] Some oral microbes (ureolytic bacteria) hydrolyse salivary and dietary urea via enzyme urease to produce ammonia and carbon dioxide, thus facilitating the development of an increased alkaline pH. This ureolytic pH response promotes calculus formation by increasing the saturation degree of calcium phosphate in plaque fluid.[3] According to Kleinberg, urea is the only nitrogenous substrate that can produce alkali fast enough to buffer salivary acids and thereby contribute to a rise in pH.[4]

Dental calculus is mineralized dental plaque covered by a layer of unmineralized plaque. It is composed of inorganic components (primarily the calcium phosphate mineral salts) and organic matrix derived from saliva, gingival crevicular fluid and bacterial products. It acts as a retentive surface for plaque, which is the primary causative factor in the initiation and progression of periodontal destruction. Thus, calculus has a secondary role in accentuating the progress of periodontal disease.[5] The porous nature of calculus may provide a favourable environment for oral bacteria, which release their toxic antigenic metabolites and by-products, initiating inflammatory responses in the soft tissues.[6]

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https://doi.org/10.1016/j.jobcr.2022.10.004
Received 15 September 2021; Accepted 10 October 2022
Available online 13 October 2022

A plethora of studies evaluating the bacterial presence and viability in calculus, or studies assessing the influence of salivary urea as well as pH on dental calculus have been reported in the literature.[7–10] To the best of our knowledge, this is the first time that a study has been attempted evaluating the clinical, biochemical and microbiological parameters with the aim to explore the influence of salivary urea, pH and ureolytic bacteria on the formation of dental calculus and periodontal status in patients with good, fair and poor oral hygiene. The objectives were to assess the levels of salivary pH, urea and determine its influence on calculus formation and periodontal status; to identify and quantify the amount of ureolytic bacteria within dental calculus using gram-staining, bacterial culture and biochemical reactions and determine a possible correlation between salivary urea and ureolytic bacteria within dental calculus.

2. Materials and methods

This observational cross-sectional study was conducted in the Department of Periodontics of our institution. The study protocol was approved by the Institutional Ethics Committee and a written informed consent was signed by each patient before commencement of the study. The study included 135 patients in the age group of 18–60 years, with a minimum of 20 functional teeth and presence of dental calculus. Patients with history of any systemic disease, antibiotics, anti-inflammatory or immunosuppressive drug therapy 6 months prior to the study, history of periodontal treatment in the last 6 months, salivary gland diseases, any apparent oral infections (i.e. herpes or candidiasis), history of radiation therapy, pregnant and lactating women, chronic smokers, tobacco users, alcohol users, aggressive periodontitis and denture wearers were excluded from the study.

Based on objective evaluation of simplified oral hygiene calculus index (subgroup of OHI–S index), patients were divided into three groups:

Group A (control group): 45 patients with good oral hygiene (calculus index score 0.0 to 0.6).

Group B (test group 1): 45 patients with fair oral hygiene (calculus index score - 0.7 to 1.8).

Group C (test group 2): 45 patients with poor oral hygiene (calculus index score - 1.9 to 3.0).

All the subjects underwent thorough history-taking followed by evaluation of clinical parameters such as plaque index (PI), gingival index (GI), pocket probing depth (PPD) and clinical attachment level (CAL).

Salivary pH was recorded by using pH indicator strips (Indikrom Papers, India) placed near the opening of the salivary duct in the floor of the mouth.

2.1. Collection of saliva samples

Unstimulated saliva was collected by passive drool technique, between 9 a.m. and 12 noon, after the patients refrained from oral intake and tooth brushing for at least 2 h before saliva collection. The patients were asked to rinse the mouth thoroughly with water twice, before the saliva collection and then instructed to allow saliva to pool in the floor of the mouth, without swallowing. With the head tilted forwards, the patients were asked to drool the saliva in sterile plastic vials for duration of 5 min.[11] The tubes were kept on ice pack and salivary urea levels were estimated with urea reagent kit (Erba Mannheim XL packs, Transasia Bio-Medicals Ltd, India) using an autoanalyzer (Erba Mannheim XL-200, Erba Diagnostics, USA) within 24 h.

2.2. Collection and preparation of calculus samples for cultural analysis

Supragingival calculus samples were collected from sites with greatest amount of calculus. Care was taken to obtain large single pieces (2–5 mm), to maintain the integrity of the calculus samples. These samples were transferred to sterile eppendorf tubes, which were exposed to ultraviolet (UV) light in the class II type B1 biological safety cabinet at 250–260 nm wavelength for 30 min to kill the microorganisms present on the surface of the calculus.

2.3. Cultural evaluation of calculus samples

After UV exposure, the calculus samples were ground to fine particle size using sterile mortar and pestle and then inoculated in sterile tubes containing 1 ml glucose broth. These tubes were vortexed for 30 s. Primary gram staining was done on the vortexed material followed by bacterial culture on Blood agar and MacConkey agar. Both the inoculated plates were incubated aerobically at 37° centigrade for 18 h. Ureolytic microflora was identified by gram staining of colony, growth characteristics on culture media and biochemical reactions.

3. Statistical analysis

Data analysis was carried out using Statistical Package for the Social Sciences (SPSS) 20.0 software. Quantitative data is presented as Median (interquartile range). Normality of the data was checked using Shapiro-Wilk test. Inferential statistics like Kruskal Wallis test was used to compare the mean values of salivary pH, periodontal parameters, salivary urea and presence of ureolytic bacteria in calculus of all the three groups. P value less than 0.05 was considered significant. Spearman's rho correlation coefficient was used to find out the correlation between salivary urea and pH, salivary urea and presence of ureolytic microflora in dental calculus in all the three groups.

4. Results

The study consisted of 135 patients (67 males and 68 females) with a mean age of 39.99 years and standard deviation of 11.66. The median (IQR) urea levels for good, fair and poor oral hygiene groups were 21 (17.5–26), 23 (18–32) and 25 (20–34.5) respectively (Graph 1). There was a statistically significant difference in the distribution of salivary urea ($p = 0.030$) and pH ($p = 0.000$) between the three groups (Table 1). However, a statistically non-significant weak correlation between salivary urea and periodontal parameters was observed in all three groups ($p > 0.05$) (Table 2). Salivary urea did not influence calculus formation with an odds ratio = 1.009 in all three groups ($p = 0.601$) (Table 3).

Gram staining revealed the presence of gram positive bacteria like Staphylococcus and gram negative bacteria like Klebsiella, Proteus, Pseudomonas and Citrobacter. The presence of these bacteria was confirmed by bacterial culture and biochemical reactions of dental calculus. Proteus showed characteristic swarming colonies, Klebsiella showed dome-shaped mucoid colonies, Pseudomonas showed non-lactose fermenting colonies with the production of pigments and Citrobacter showed smooth, low convex, moist, translucent or opaque colonies on MacConkey agar (Fig. 1). Staphylococcus showed haemolytic colonies on Blood agar (Fig. 2). There was a statistically significant difference in the distribution of the ureolytic bacteria among the three groups ($p < 0.005$) except for Citrobacter ($p = 0.243$). Increased colony forming units (CFU) of Klebsiella, Proteus, Pseudomonas, and Citrobacter was found in poor oral hygiene group as compared to fair and good oral hygiene groups. Staphylococci were seen in more number of patients in the fair oral hygiene group in comparison to the other groups. Calculus in patients with good oral hygiene group did not show presence of the Proteus, Pseudomonas and Citrobacter species (Table 1). An increase in the CFU of Klebsiella, Proteus, Pseudomonas, Staphylococci and Citrobacter was associated with an increase in the odds of considering poor oral hygiene (increased calculus score) with odds ratio = 1.954, 1.634, 1.844, 1.437 and 1.61 respectively (Table 3).

Table 1
Intergroup comparison of different parameters.

	Groups	N	Mean	SD	Median (IQR)	Range	Chi square value	P-value
Age	Good	45	39.96	11.66	41(30.5–50)	21–59	0.89	0.957
	Fair	45	39.44	11.26	39 (30–49)	21–60		
	Poor	45	39.6	12.15	39 (29.5–49.5)	19–60		
Salivary urea	Good	45	21.77	6.36	21(17.5–26)	12–38	6.99	0.030*
	Fair	45	27.77	18.05	23(18–32)	6–113		
	Poor	45	29.02	16.11	25(20–34.5)	13–111		
Salivary pH	Good	45	7.5	0.726	7(7–8)	7–10	63.79	0.000**
	Fair	45	8.07	0.688	8(8-8)			
	Poor	45	9.07	0.688	9(9–9.5)	7–10		
Plaque index	Good	45	0.53	0.40	0.46(0.31–0.59)	0.07–2.59	86.56	0.000**
	Fair	45	0.89	0.33	0.87(0.66–1.02)	0.38–1.87		
	Poor	45	1.65	0.41	1.62(1.38–1.84)	0.53–2.65		
Gingival index	Good	45	0.5	1.76	0.2(0.13–0.32)	0–12	94.35	0.000**
	Fair	45	1.23	0.55	1.14(0.7–1.8)	0.28–2.11		
	Poor	45	1.98	0.32	2(1.75–2.23)	1.17–2.6		
Pocket probing depth	Good	45	1.88	0.28	1.9(1.7–2.07)	1.12–2.42	83.38	0.000**
	Fair	45	2.21	0.46	2.2(2.05–2.33)	1.01–4.13		
	Poor	45	2.93	0.52	2.86(2.62–3.31)	2.13–5.17		
Clinical attachment level	Good	45	0.38	0.44	0.28(0–1.34)	0–0.6	40.37	0.000**
	Fair	45	0.55	0.56	0.37(0–0.96)	0–1.96		
	Poor	45	1.59	1.12	1.3(0.87–2.39)	0–4.61		
Klebsiella	Good	45	0.04	0.298	0(0–0)	0–2	20.32	0.000**
	Fair	45	0.29	1.21	0(0–0)	0–7		
	Poor	45	4.98	9.16	0(0–10)	0–39		
Proteus	Good	45	0	0.00	0(0–0)	0–0	11.49	0.003*
	Fair	45	0.64	2.77	0(0–0)	0–18		
	Poor	45	2.62	5.83	0(0–0)	0–22		
Pseudomonas	Good	45	0	0.00	0(0–0)	0–0	14.42	0.001*
	Fair	45	0.6	1.60	0(0–0)	0–8		
	Poor	45	5	9.93	0(0–11)	0–49		
Staphylococci	Good	45	0.09	0.00	0(0–0)	0–4	17.36	0.000**
	Fair	45	2.73	4.25	0(0–6)	0–20		
	Poor	45	2.07	5.07	0(0–0)	0–19		
Citrobacter	Good	45	0	0.000	0(0–0)	0–0	2.90	0.243
	Fair	45	0.31	1.47	0(0–0)	0–8		
	Poor	45	0.62	2.55	0(0–0)	0–12		

N-number of patients, SD - standard deviation, IQR-interquartile range.
<0.001(**) highly significant (HS); $p < 0.05$(*) significant (S); $p > 0.05$ not-significant (NS).

Table 2
Spearman's rho correlation of salivary urea with other parameters in all the three groups.

	Good oral hygiene		Fair oral hygiene		Poor oral hygiene	
	CC	P value	CC	P value	CC	P value
PI	0.201	0.185	0.126	0.410	0.103	0.502
GI	−0.009	0.953	0.090	0.555	0.197	0.195
PPD	−0.006	0.969	−0.059	0.698	0.079	0.605
CAL	**0.344***	0.021	−0.116	0.448	−0.039	0.799
PH	0.084	0.584	−0.211	0.164	0.009	0.956
Klebsiella	0.204	0.179	0.133	0.384	−0.156	0.306
Staphylococci	0.227	0.134	−0.164	0.281	0.070	0.647
Proteus			0.027	0.859	−0.013	0.931
Pseudomonas			0.258	0.087	0.247	0.102
Citrobacter			0.102	0.503	−0.148	0.332

CC-Correlation coefficient; CC=<0.3-weak relationship, CC > 0.3 Moderate relationship, CC > 0.4 strong relationship. P < 0.05- significant, P < 0.001- highly significant.

Table 3
Effect of clinical, biochemical and microbiological parameters on calculus formation (OHIS–CI).

Parameters	Wald Chi square	Df	P-value	0dds ratio (r)	Confidence Interval (CI)	
					Lower	Upper
pH	10.292	1	0.001	2.785	1.49	5.208
Salivary urea	0.273	1	0.601	1.009	0.975	1.044
Klebsiella	12.203	1	0.000	1.954	1.342	2.846
Proteus	12.995	1	0.000	1.634	1.251	2.135
Pseudomonas	14.694	1	0.000	1.844	1.349	2.522
Staphylococci	22.380	1	0.000	1.437	1.237	1.67
Citrobacter	8.643	1	0.003	1.61	1.172	2.212

5. Discussion

Calculus generally predisposes to the development of periodontal disease. Supragingival calculus derives most of its mineral and matrix content from saliva. It acts as a substrate for bacteria and hinders effective plaque control.[5] Salivary urea is one of the prominent factors that play a role in the formation of calculus. The ureolytic pH response due to production of ammonia from urea promotes calculus formation by increasing the degree of saturation of calcium phosphate in plaque.[12] The present study evaluated the levels of salivary urea and pH, presence and quantity of ureolytic microflora in dental calculus and assessed their influence on dental calculus formation, in patients with good, fair and poor oral hygiene.

The results demonstrated that the plaque index, gingival index, probing depth and clinical attachment level values were higher in patients with poor oral hygiene compared to patients with fair and good oral hygiene ($p = 0.000$). Salivary urea levels in fair and poor oral hygiene groups exceeded the normal levels in saliva. However, there was a non-significant, weak correlation between salivary urea and periodontal parameters in patients with fair and poor oral hygiene ($CC < 0.3$). The results could be due to the hydrolysis of urea to ammonia, which

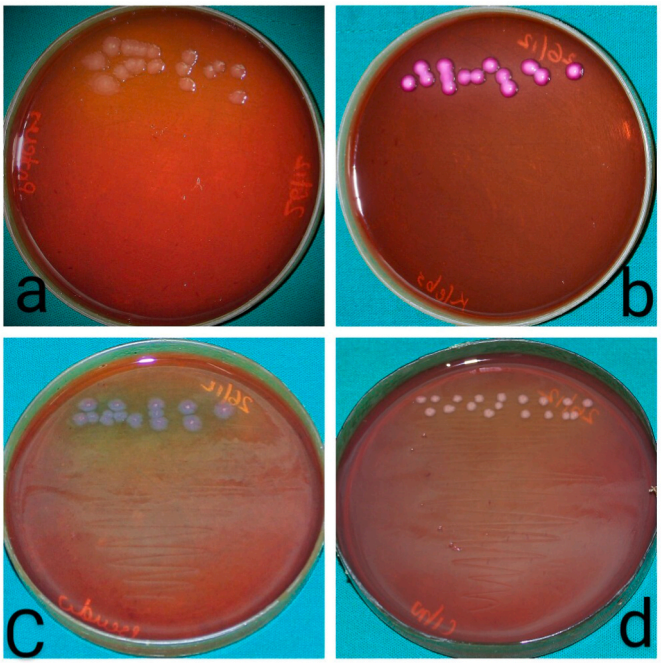


Fig. 1. MacConkey agar showing colonies of:
a) Proteus
b) Klebsiella
c) Pseudomonas
d) Citrobacter

Fig. 2. Blood agar showing colonies of Staphylococci.

increases the permeability of the sulcular epithelium to antigenic substances and is cytotoxic to periodontal tissues, thereby playing a fundamental role in the initiation of periodontal disease.[13]

The results of this study were consistent with research conducted by Junior AB et al.,[8] Hernandez-Castañeda AA et al.,[10] Khozeimeh F et al.,[14] Nasution AH et al.,[15] which demonstrated high concentration of urea in saliva of patients with gingivitis and periodontitis. The urea levels evaluated in the study, as explained by Nakamura et al.,[16] may be correlated with inflammatory agents such as interleukins and growth factors. Variation in salivary urea in different individuals may be related to variation in salivary flow rate, passive diffusion of nitrogenous waste from serum into the saliva, several factors, such as stimulation, circadian rhythm, diet, age and hydrogen (H+) ion concentration.[17]

Every one unit increase in the pH was associated with an increase in the odds of considering poor oral hygiene (increase in the calculus index score) with an odds ratio = 2.785. The results indicate that increase in the salivary pH is associated with increased odds of calculus formation in all three groups. This could be attributed to the increased breakdown of salivary urea to ammonia which results in a concomitant increase in salivary pH that promotes calculus formation. Thus, presence of urea increases the probability of rapid calculus formation.[18]

Our finding of salivary urea not influencing calculus formation is supported to some extent by previous in vitro and in vivo studies. Fure et al. in a double-blind, cross-over study of three months' frequent use of sugar-free chewing gum-with or without urea showed that urea neither promotes nor inhibits calculus formation.[19] Moreover, Belting et al. in an in vitro study demonstrated that urea could interfere with artificial calculus formation by dissolving the mucoproteinaceous material and/or by increasing the solubility of calcium salts in saliva.[20]

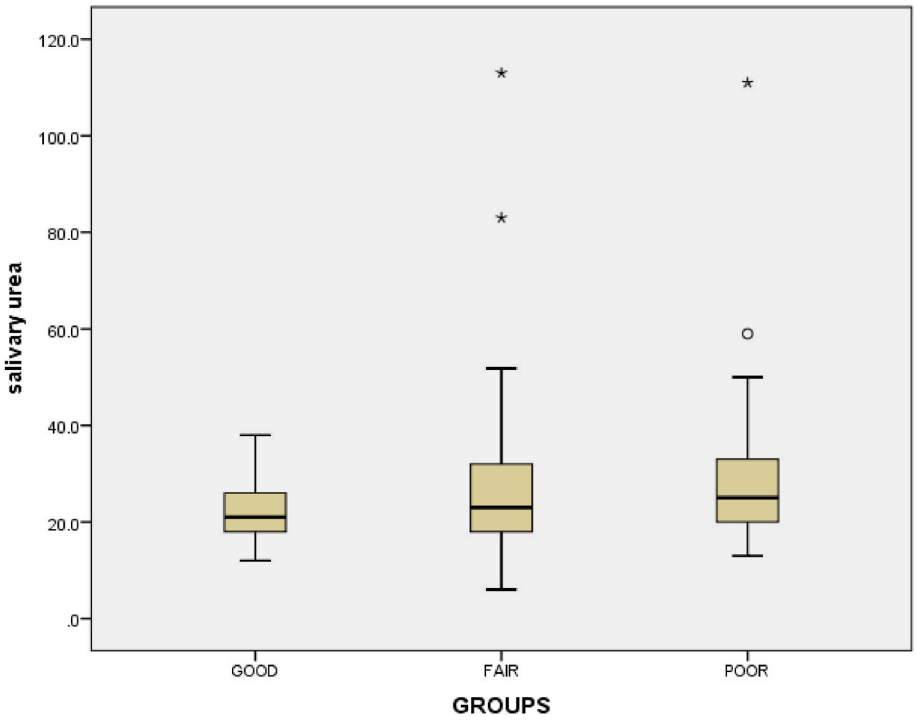
Saliva acts as an alternate route for excretion of urea in compromised renal function state. Increase in blood urea levels results in concomitant increase in the salivary urea levels due to diffusion of nitrogenous waste from serum to saliva. Studies by Epstein SR et al.,[21] Bhatsange A et al.,[22] Kovalcıkova AG et al.[23] have reported that higher salivary urea increases the prevalence of calculus and periodontal disease in chronic kidney disease (CKD) patients.

Transmission electron microscopic and bacterial culture studies on calculus by Sidaway DA,[24] Friskopp J et al.,[25] Tan BT et al.,[7] Moolya NN et al.[9] have proved the presence of intact and viable bacteria within the non-mineralized channels, islands and lacunae in supragingival and subgingival calculus. Thus, calculus may serve as a reservoir of viable microorganisms and play a crucial role in the etiology and recurrence of oral infections even after treatment.

Oral ureolytic activity is ubiquitous, with microorganisms having ureolytic properties being found in soil, water, human and animal bodies.[26,27] Oral ureolytic microflora include S. salivarius, coagulase-negative staphylococci, Actinomyces viscosus/naeslundii, transient Enterobacteriaciae, unknown anaerobes, and Haemophilus species. Normal inhabitants of supragingival plaque with stable ureolytic activity include H. parainfluenzae, A. viscosus, A. naeslundii and coagulase-negative staphylococci.[14,24,28] According to various studies, Eubacteriumsaburreum, Corynebacterium matruchotii, Veillonella parvula, Streptococcus salivarius, Streptococcus sanguis and Streptococus mutans comprise the calcifying species that may predominate in supragingival calculus.[18]

In the present study, gram staining, bacterial culture and biochemical reactions of dental calculus showed the presence of aerobic ureolytic microflora such as Klebsiella, Proteus, Pseudomonas, Staphylococci and Citrobacter. A non-significant weak correlation between salivary urea and ureolytic bacteria in dental calculus (CC < 0.3) was observed. The increase in the CFU of Klebsiella, Proteus, Pseudomonas, Staphylococci and Citrobacter species was associated with an increase in the odds of considering a higher oral hygiene calculus index score with odds ratio of 1.954, 1.634, 1.844, 1.437 and 1.61 respectively indicating that the number of ureolytic bacteria increases with increase in calculus index score.

The presence of ureolytic activity is an important marker of a number of bacterial infections. Urease released by the ureolytic bacteria is an immunogenic protein recognized by antibodies present in human sera. The presence of such antibodies is connected with progress of several long-lasting diseases, like rheumatoid arthritis, atherosclerosis or urinary tract infections.[26] Ureolytic bacteria have also been found to be associated with the formation of renal calculi suggesting a similar mechanism of formation of renal and dental calculus.[29] The ureolytic bacteria may thus be responsible for formation of dental calculus.



Graph 1. Box plot showing comparison of median salivary urea levels between all the groups.

This study had a few shortcomings. Only verbal documentation of the systemic condition of patients was recorded. Patients' dietary habits were not taken into consideration as this could act as a confounding factor. Some bacteria are difficult to culture but can be easily identified by electron microscopy and dark field microscopy due to the presence and motility of filamentous organisms. Further interventional studies with larger sample size, anaerobic bacterial culture may be carried out to better understand and substantiate the observations on calculus formation.

6. Conclusion

The present study suggests that, higher level of ureolytic bacteria with increasing calculus index score may breakdown the salivary urea to ammonia resulting in a ureolytic pH rise that facilitate calcium phosphate saturation leading to more calculus formation. Hence, despite of good oral hygiene, rapid calculus formers may require frequent dental visits. Since calculus plays a key role in maintaining and accentuating periodontal disease, adequate and thorough periodontal as well as prophylactic therapy is necessary to prevent calculus formation.

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