

Viability of Anaerobic Bacteria in Dental Sub-Gingival Calculus and Its Minerlization – An Observational Microbiological Study

Dr. Richa Bhosale^{1*}, Dr. Nikesh N. Moolya², Dr. Nilima Rajhans³, Dr. Nilima Daule², Dr. Asawari Lawande⁴, Dr. Sayli Ujgare¹, Dr. Ashlesha Arekar¹, Dr. Yogita Landge¹

¹Post Graduate Student, Department of Periodontics and Oral Implantology, YC.M.M & R.D.F's Dental College & Hospital, Ahmednagar, Maharashtra, India

²Professor, Department of Periodontics and Oral Implantology, YC.M.M & R.D.F's Dental College & Hospital, Ahmednagar, Maharashtra, India

³Professor and Head of the Department, Department of Periodontics and Oral Implantology, YC.M.M & R.D.F's Dental College & Hospital, Ahmednagar, Maharashtra, India

⁴Lecturer, Department of Periodontics and Oral Implantology, YC.M.M & R.D.F's Dental College & Hospital, Ahmednagar, Maharashtra, India

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*Corresponding author: Dr. Richa Bhosale

Post Graduate Student, Department of Periodontics and Oral Implantology, YC.M.M & R.D.F's Dental College & Hospital, Ahmednagar, Maharashtra, India

Abstract

Original Research Article

Background: Dental calculus, a mineralized product of plaque, remains ignored and is considered as a fossilized remnant of minor significance. Dental plaque is still considered as the main culprit in etiopathogenesis of periodontal disease. Several studies suggest that calculus has a porous and spongy nature because of which it may act as a reservoir for endotoxins and bacterial antibodies. However, the viability of bacteria in sub gingival calculus and its role in the pathogenesis and disease progression still remains a mystery. The present study aims to investigate the viability of bacteria within sub-gingival calculus and to assess the degree of mineralization of dental calculus and the variation of viable micro flora corresponding to the extent of mineralization. **Materials and Methods:** Eighteen samples of sub gingival calculus were harvested from patients with chronic inflammatory periodontal disease. The samples were divided into two groups, Group 1 (non-irradiated) and Group 2 (samples exposed to UV radiation) and Group 3 (samples assessed for mineralization). Group 1 and 2 samples were utilized for anaerobic culture to detect the presence of anaerobic bacteria. Group 3 samples were crushed in a Universal testing machine and mineralization was assessed according to the force required for crushing. **Results:** All study specimens showed positive bacterial growth under anaerobic conditions. The bacterial cultures revealed decrease in the bacterial count in Group 2 i.e. Irradiated group (I) when compared with Group 1 i.e. Non-irradiated group (NI). The results obtained from computerized Universal Testing Machine showcased varying values required to crush the sub-gingival calculus samples suggesting variation in mineralization of each sample. The correlation between mineralization and viable anaerobic bacterial count revealed an inverse relation. **Conclusion:** Sub-gingival calculus plays a key role in harbouring pathogenic anaerobic bacteria, owing to its porous nature. Thereby, increasing the severity of the periodontal disease by providing the microorganisms a suitable environment to flourish, leading to various pathological changes. The Inverse relationship between viability of bacteria and mineralization of calculus has also been observed.

Keywords: Viability, subgingival calculus, anaerobic bacteria, calculus mineralization.

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INTRODUCTION

Dental calculus is a mineralized oral plaque biofilm that preserves biomolecules such as DNA, protein and bacterial colonies over long periods of time. Most oral microbiological studies focus on dental plaque as it represents an active biofilm and is directly responsible for progression of periodontal disease.

Comparatively, less is known about the structure and formation of dental calculus.

Although calculus forms from dental plaque, microbial profile differences have been noted between calculus and dental plaque [1]. The initial gingival damage occurs due to immunologic and/or enzymatic

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effects caused by the microorganisms of the plaque which result in pocket formation, chronic inflammation, and further promotes sub-gingival calculus formation [2]. The mineralization of sub-gingival calculus results from the interaction of sub-gingival plaque with the influx of mineral salts that is part of the serum transudate and inflammatory exudate. It is not clear to what extent the presence of mineralized deposit enhances gingival inflammation. Hence, assuming that sub-gingival calculus, at a minimum may expand the radius of plaque induced periodontal injury is still the chronology. However, it should not be the basis for relegating calculus as mere ash heap [3].

It is thought that the role of calculus is associated principally with its physical character, in that it is plaque retentive and may impede natural and mechanical oral hygiene activities. However, there is evidence that calculus is not a solid mineralized mass but has a porous, spongy nature as established by histological studies, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) studies. These studies suggest that dental calculus may act as a reservoir for irritating substances such as endotoxins and bacterial antibodies. The unmineralized channels and lacunae within the calculus allows an environment which is able to support viable bacterial communities through molecular diffusion of nutrients through channels [4]. Hence, "The presence of microorganisms must be regarded as one of the characteristic criteria for dental calculus. However, it is not yet known whether bacteria are active or passive in the formation of the dental calculus matrix" [2].

The role of microorganisms in mineralization of calculus is already established and there is change in microflora as the transition from plaque to calculus occurs. It is also a well-known fact that rate of calculus formation and its brittleness can vary among individuals [5].

The aim of the present microbiological study was to investigate the viability of anaerobic, bacteria within sub-gingival calculus and to assess the degree of mineralization of dental calculus and the variation of viable micro flora corresponding to the extent of mineralization.

MATERIALS AND METHODS

The study was approved by the Institutional Ethics Committee and the Institutional Research Committee-Late Shri Yashwantrao Chavan Memorial Medical & Rural Development Foundation's Dental College & Hospital, Ahmednagar, Maharashtra, India, 04 / 01 / 2023, (YCDC/IEC-IRC/2022-2023/122). All the participants provided written informed consent for the participation in the study. All procedures performed in the study were conducted in accordance with the ethical standards given in 1964 Declaration of Helsinki, as revised in 2013.

Subjects with chronic inflammatory periodontal disease within 18-55 years of age group were included in the study after obtaining informed consent. Exclusion criteria for patients was presence of any systemic disease, any salivary gland disease and/or xerostomia, antimicrobial therapy since past six months, patients who underwent oral prophylaxis for at least six months prior to harvesting the samples, pregnant and lactating women.

Eighteen samples of sub-gingival calculus from subjects having clinical evidence of chronic inflammatory periodontal disease and presence of substantial calculus deposits were procured. Care was taken to obtain large pieces of calculus samples and to maintain the integrity of the calculus samples. The obtained samples were divided into three groups, Group 1 (Non- Irradiated group), Group 2 (Irradiated group) which were subjected to anaerobic microbial culture, and Group 3, where the samples were assessed for mineralization by subjecting them to (UTM) Universal Testing Machine (Indian Technologies, Coimbatore, India) Group 1 (non-irradiated, NI): (Six samples) were immediately placed in vials with Reduced Transport Fluid (RTF) obtained from Central Research Laboratory. The superficial plaque layer present on Group 1 samples was kept intact. Group 2 (irradiated, I): (Six samples) were exposed to Ultraviolet light in a UV chamber for 30 minutes in order to render the superficial surface of sub-gingival calculus free of microorganisms. This procedure was mainly done in order to kill the viable bacteria on the surface of calculus to eliminate contamination due to the overlying plaque layer. The samples were turned over intermittently to ensure complete irradiation of all the surfaces. After irradiation, the samples were placed in RTF. All the samples of Group 1 and Group 2 were then transported to Research Laboratory for anaerobic bacterial culture within 72 hours (Figure 1 & 2).

Bacterial culture: All the vials containing samples from Group 1 and Group 2 were exposed to UV light in Laminar Air Flow Station, (Labline Biological Safety Cabinet) (Figure. 3) to prevent any contamination of samples prior to handling. The vials were then opened and under aseptic conditions, the samples were crushed with sterile forceps within the vial. A portion of 10 μ l of these samples was vortexed with 490 μ l of Thioglycolate broth using digital vortex mixer (Talboys digital vortex, Troemner, USA) (Figure 4). It is a multi-purpose, enrichment, differential medium which consumes oxygen and permits the growth of anaerobes. The portion from these samples was inoculated on the surface of Laked Brucella Blood Agar (LBA) and Fusobacterium Selective Agar (FSA) (Figure 5, 6) using an Auto-pipette and incubated at 37°C in 10% H₂, 10% CO₂ and 80% N₂ for 5 days in the anaerobic gas chamber and digital incubator (Figure 7).

Analysis of Mineralization: Samples from Group 3 were used to crush under computerized UTM (Indian Technologies, Coimbatore, India) (Figure 8) to

assess the mineralization of calculus samples by determining the force required in Newton (N) to crush the samples so that samples of sub-gingival calculus can be categorized as less mineralized, moderately mineralized and highly Mineralized based on the Newton Units recorded. Samples were classified as less mineralized, if less than 30 N force was required to crush the calculus samples. If 31-60 N force was required to crush the calculus samples, they were termed as moderately mineralized and calculus samples which required more than 61 N force was required to be crushed were categorized as highly mineralized.

All the findings obtained from this study were recorded at each step and were tabulated systematically. These findings were then subjected to statistical analysis using SPSS 24 software.

RESULTS

The results for the observational microbiological study were as follows:

Bacterial culture: All study specimens showed positive bacterial growth under anaerobic conditions.(Figure 9) Sample 2 which belonged to the non-irradiated group (NI) showed highest number of Colony Forming Units (CFU) 224 colonies whereas sample 12 which belonged to the irradiated (I) group showed minimum of 02 CFU on Laked Brucella Blood Agar. Maximum number of CFU, 296 colonies were seen in sample 4 which belonged to the (NI) group while minimum CFU, 30 colonies were seen in sample 7 on Fusobacterium Selective Agar, which belonged to irradiated (I) group. The bacterial cultures revealed decrease in the

bacterial count of organisms in Group 2 i.e. Irradiated group (I) when compared with Group 1 i.e. Non-irradiated group (NI) (Figure 10). The results of total number of counts of bacterial colonies cultured from the sub-gingival calculus samples are detailed in Table 1.

Assessment of Mineralization: The results obtained from computerized UTM showcased varying values required to crush the sub-gingival calculus samples suggesting variation in mineralization of each sample. Sample 5 required maximum amount of force, 88.89 N/mm² to crush making it a highly mineralized calculus sample while sample 2 (11.11 N/mm²) and sample 4 (12.08 N/mm²) required minimum force to crush making them less mineralized samples of calculus. The rest of the samples required moderate amount of force ranging from 35.88 N/mm² to 69.02 N/mm² as seen in Table 2. These values provide a scope for classification of calculus samples on the basis mineralization by categorizing the samples based on force required in N/mm² to crush the particular calculus sample. Thereby classifying calculus as shown in Table 3

Correlation of mineralization with viable bacterial count: The correlation between mineralization and viable anaerobic bacterial count revealed an inverse relation. Highly mineralized sub-gingival calculus samples i.e. sample 5 and sample 3 showed presence of less number of anaerobic bacterial count. On the other hand less mineralized samples i.e. sample 4 and sample 2 showed maximum number of bacterial counts. Thus, confirming the inverse relation between mineralization of calculus and viability of bacteria.

Table 1: Total number of Colony Forming Units (CFU) on Laked Brucella Blood Agar (LBA) and Fusobacterium Selective Agar (FSA) from Sub-gingival calculus samples

Sl. No	Groups	LBA (CFU)	FSA (CFU)
01	NI 1	120	120
02	NI 2	224	242
03	NI 3	84	249
04	NI 4	212	296
05	NI 5	40	180
06	NI 6	164	197
07	I 1	12	30
08	I 2	132	120
09	I 3	60	147
10	I 4	72	234
11	I 5	15	40
12	I 6	02	32

Note: CFU: Colony Forming Units, LBA: Laked Brucella Blood Agar, FSA: Fusobacterium Selective Agar

Table 2: Force required in N/mm² to crush the sub-lingual calculus sample

Sr. No	Force required in N/mm ²
Sample 1	43.05
Sample 2	11.11
Sample 3	69.02
Sample 4	12.08
Sample 5	88.89
Sample 6	35.88

Table 3: Classification of calculus samples based on mineralization by categorizing the samples based on force required in N/mm² to crush the calculus sample. (*The above classification is based on similar grounds as that of the classification given by Gupta *et al.*, in 2016) [5]

Mineralization	Force Required in N/mm ²
Less mineralized	01- 30 N/mm ²
Moderately mineralized	31- 60 N/mm ²
Highly mineralized	61 N/mm ² and above



Figure 1:



Figure 2:



Figure 3:



Figure 4:



Figure 5:



Figure 6:

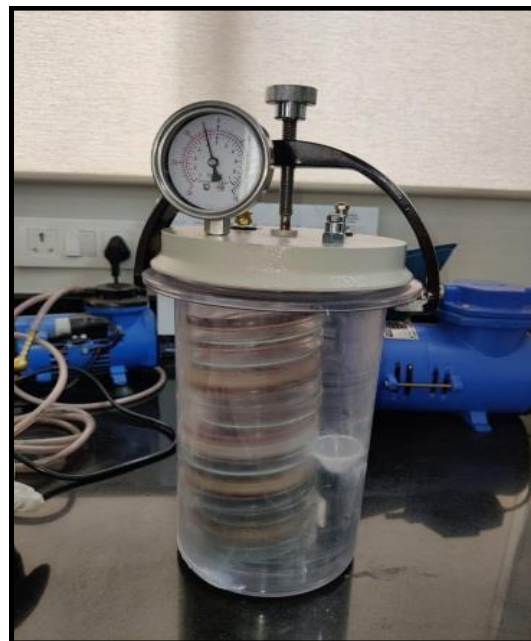


Figure 7:



Figure 8:



Figure 9:

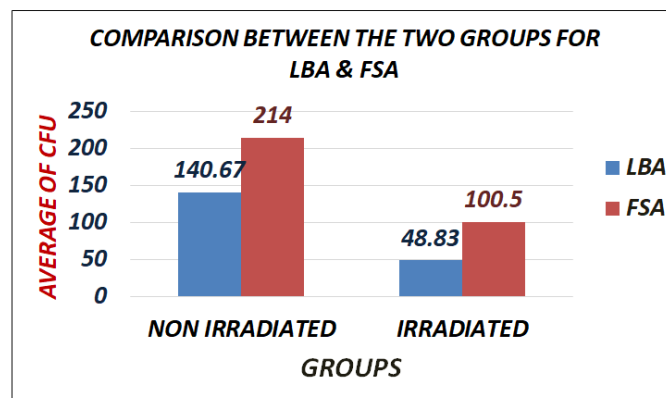


Figure 10:

DISCUSSION

The current study was undertaken to shed light on the unexplored actions of sub-gingival calculus, by assessing the viability of anaerobic microbial flora in the sub-gingival calculus and correlating it with mineralization of calculus by categorizing it on the basis of hardness. The study aimed at confirming the presence of viable bacteria within dental sub-gingival calculus along with its identification and the extent of mineralization of the same. It tried to assess the relationship between the degree of mineralization and viability of the micro-flora. This comparison will help to understand the relationship between viable microflora within the calculus and how passage of time dictates the pathogenicity of sub-gingival calculus.

The complexity of the subgingival microbiota has been recognized since the 1st microscopic examination of this ecosystem by Van Leeuwenhoek in 1683 [6]. Subgingival plaque was frequently characterized by a zone of gram negative and/or motile species located adjacent to the epithelial lining of the pocket while gram positive rods and cocci appeared to be forming a tightly adherent band of organisms on the enamel or root surface (Listgarten 1976, Listgarten 1994) [7, 8]. Subgingival calculus is a porous substrate and can adsorb a variety of substances. Hence, “The permeability of calculus makes it a reservoir for irritating substances from microbial plaque which can permeate and diffuse

out again to irritate the periodontal tissues [9]”. The age group selected for this study was 18-55 years. This was primarily done to involve patients having maximum amount of calculus deposition. A longitudinal study by Anerud *et al.*, (1991) suggests that sub-gingival calculus formation starts in early 20’s and also noted presence of calculus deposition since a minimum of 14 years of age [10]. Another study by Hassan *et al.*, (2005) stated that salivary calcium concentration was significantly high in younger individuals, and it plays major role in the formation of supra or subgingival calculus [11]. This study tried to identify the presence of anaerobic bacteria in subgingival calculus and showed their presence in varying numbers.

A study by Sidaway *et al.*, in 1978 found presence of anaerobic organisms *F. nucleatum*, *Veillonella alcalescens*, and *A. naeslundii* in subgingival calculus samples [12]. This finding correlates with the results of samples 1, 2, 3, 4, 5, 6 since the superficial plaque layer had been incorporated for both studies during examination. Tan *et al.*, in 2004 confirmed presence of anaerobic organisms but within supra-gingival calculus [4], also they did not identify the type of anaerobic organism. Similar results were obtained by Gupta *et al.*, 2016; Moolya *et al.*, 2010; and Kaur *et al.*, 2013; respectively [5, 13, 14].

In this study, anaerobic microbial culture was also obtained in sub-gingival calculus samples devoid of plaque layer. Similar method was used by Tan *et al.*, Gupta *et al.*, and Moolya *et al.*, respectively [4, 5, 13]. The samples were exposed to UV light to render the superficial surface of calculus free of microorganisms and to eliminate contamination due to the overlying plaque layer. Although the microbial count was drastically reduced, it is known that obtaining successful bacterial culture is an important sign of bacterial viability. Similar results were found in studies by Gupta *et al.*, who confirmed presence of positive aerobic growth in irradiated, supra-gingival calculus samples [5], and Moolya *et al.*, who revealed aerobic bacterial culture growth in all irradiated samples of supra as well as sub-gingival calculus [14] but results from study by Tan *et al.*, are in contradiction since bacterial culture was not obtained in irradiated calculus samples [4]. No study has been conducted till date which has obtained positive anaerobic culture in dental sub-gingival calculus devoid of superficial plaque layer. Therefore, all these findings lead us to one common road that, calculus is not just a dead organic material or a mere fossilized remnant but a reservoir of viable organisms.

Reduced Transport Fluid (RTF) was used as a transport medium and buffer to maintain the viability of organisms within the calculus samples. RTF is a dithiothreitol poised balanced mineral salt solution which has proven to show better results in aiding survival of anaerobic species within the samples [15]. The present study also investigated the possible correlation between mineralization of calculus and variation of viable micro flora corresponding to the extent of mineralization.

The results of this study revealed presence of more number of bacterial colonies in samples which required less amount of force to crush representing the less mineralized calculus sample while samples that required greater amount of force to crush, forming the highly mineralized calculus revealed lesser number of microbial colonies thus, presenting an inverse relation between mineralization of calculus and viability of bacteria within it. For this purpose the Universal Testing Machine (UTM) was used, which is a versatile equipment that can perform numerous standard tensile and compression tests on materials. It has a computer interface to chart and analyse the readings obtained. To the best of our knowledge, no study has been conducted till date making use of such equipment to obtain precise measurements. A similar study was conducted by Gupta *et al.*, to classify calculus on the basis mineralization but no significant relevance was noted between mineralization and viability of bacteria [5]. The present study also puts forward a classification based on mineralization of calculus on the basis of force required to crush the calculus sample. This classification was made by taking into consideration a classification given by Gupta *et al.*, [5].

According to the results of this study mineralization of calculus forms an important aspect to determine the pathogenicity of calculus. It suggests that even if the viable bacterial count decreases at a later stage of mineralization, the porous nature of the calculus will still aid the microorganisms to thrive till the end by continuous supply of necessary nutrients. A similar observation was made by Baumhammers *et al.*, and Shirato *et al.*, Friskopp *et al.*, in their respective studies [9, 16, 17]. Thus, supporting the statement by Mandel and Gaffar that, calculus indeed should be considered a “toxic waste dump site” and in a sense a “slow release device” delivering pathogenic products [3].

This helps us to understand the pathogenic effect of subgingival calculus due to its assistance in maintaining the viability of bacteria leading to progression of periodontal disease and the importance of its removal to prevent this progression. It also describes that how, regardless of the mineralization, calculus still acts as a reservoir of pathogens and it is not merely a plaque retentive substrate.

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CONCLUSION

This study strongly confirmed the presence of viable anaerobic bacteria within dental sub-gingival calculus and describes an inverse relationship between viability of bacteria and mineralization of calculus. It also gives a classification of calculus based on mineralization. This study supports the fact that how thorough complete removal of sub-gingival calculus is necessary for achieving optimum periodontal health, and explains its active participation in periodontal disease progression.

Clinical significance: Sub-gingival calculus thus plays an active role in etiology of periodontal disease rather than just serving as a nidus for accumulation of plaque.

Future considerations: Since this study had a limited sample size, future studies should be aimed at performing bacterial cultures on larger sample size including criteria such as age, gender, oral hygiene practices of individuals, impact of tobacco, and identification of different types of bacteria using highly sensitive and definitive techniques like PCR.

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