J Clin Periodontol 2004; 31: 364–369 doi: 10.1111/j.1600-051X.2004.00484.x
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Journal of Clinical Periodontology

A preliminary investigation into the ultrastructure of dental calculus and associated bacteria

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Tan B, Gillam DG, Mordan NJ, Galgut PN. A preliminary investigation into the ultrastructure of dental calculus and associated bacteria. J Clin Periodontol 2004; 31: 364–369 doi: 10.1111/j.1600-051X.2004.00484.x. © Blackwell Munksgaard, 2004.

Abstract

Introduction: Though dental calculus is generally recognised as comprising mineralised bacteria, areas of non-mineralised bacteria may be present.

Aim: To investigate the ultrastructure of non-decalcified young and mature supragingival calculus and subgingival calculus, and the possible presence of internal viable bacteria.

Materials and methods: Supragingival calculus was harvested from five patients, 9–10 weeks after scaling and root debridement. Five samples of mature supragingival and subgingival calculus were taken from patients presenting with adult periodontitis. Specimens were fixed and embedded for transmission electron microscopy.

Results: The ultrastructure of young and mature supragingival calculus was similar with various large and small crystal types. Non-mineralised channels were observed extending into the calculus, often joining extensive lacunae, both containing intact non-mineralised coccoid and rod-shaped microorganisms. Subgingival calculus possessed more uniform mineralisation without non-mineralised channels and lacunae.

Conclusion: Supragingival calculus contains non-mineralised areas which contain bacteria and other debris. The viability of the bacteria, and their identification could not be determined in this preliminary investigation. As viable bacteria within these lacunae may provide a source of re-infection, further work needs to be done to identify the bacteria in the lacunae, and to determine their viability.

Key words: bacteria; calculus; plaque; subgingival; supragingival.

Accepted for publication 19 June 2003

Dental calculus is defined as mineralised dental plaque that is permeated with crystals of various calcium phosphates (Schroeder 1965, 1969). X-ray diffraction studies have revealed the presence of four main crystal structures; hydroxyapatite (HA), whitlockite (WH), octacalcium phosphate (OCP) and dicalcium phosphate dihydrate (DCPD). Crystallographic aspects of dental calculus were described on a systematic basis by Jensen & Danø (1954), Jensen & Rowles (1957), Grøn et al. (1967) and more recently by Sundberg & Friskopp (1985). HA and OCP were found to be most abundant in supragingival calculus, and WH to be most abundant in subgingival calculus by these workers.

While traditionally regarded as an aetiologic agent of periodontal disease (Weinberger 1948), the importance placed on calculus changed with the advent of studies on dental plaque. However, the evidence for the role of calculus in the initiation and progression of periodontal diseases is inconclusive. Earlier epidemiologic studies (Ramjford 1961, Lilienthal et al. 1965) showed a stronger correlation between calculus and disease than plaque and disease. These studies unfortunately, could not provide significant information of causality because they, as well as their antecedents, employed indices attempting to correlate mean values for deposits and disease, while in fact the nature of periodontal disease is site specific (Schroeder 1969). Furthermore, the studies did not include plaque adherent to dental calculus in the evaluation (Schroeder 1969). The current view is that dental calculus is not in itself harmful and that the major reason for preventing its formation or removing it once it has formed is because it is always covered by a layer of unmineralised, viable and metabolically active bacteria (Newman 1994).

Supragingival calculus has been shown to contain non-mineralised areas and, by nature of its porosity (Friskopp & Hammarstrom 1980, Friskopp 1983), it has been proposed that it may act as a reservoir for irritating substances such

as endotoxins, which can affect the chronicity and progression of periodontal disease (Mandel & Gaffar 1986). Furthermore, the mineralisation of calculus has been shown to be highly variable, containing a variety of different crystalline forms, which seem to predominate depending on the age of the calculus. Although some mineralisation may occur within a few days of professional prophylaxis (Theilade 1964), and in some individuals may be clinically evident as soon as 2 weeks (Galgut 1996), it appears clinically to be relatively chalk-like. With time it becomes more condensed and crystalline (Schroeder & Baumbauer 1966). Various terms for the development of calculus with time are found in the literature. Terms such as young, mature, crystalline or old calculus are found in the literature, but are not defined. In this paper, young calculus is defined as calculus that had reformed within 12 weeks after thorough professional prophylaxis, as opposed to mature calculus, which is defined as calculus harvested from subjects requiring periodontal treatment who had not received professional prophylaxis for at least 6 months prior to harvesting the samples.

The aim of the present study is to investigate the ultrastructure of non-decalcified young and mature supragingival calculus and subgingival calculus and the possible presence of viable bacteria within them.

Materials and Methods

Recently formed supragingival calculus was harvested from five patients, 9–10 weeks after thorough scaling and root debridement. Mature supragingival calculus was taken from five patients presenting with moderate to advanced adult periodontitis as part of their periodontal treatment. Subgingival calculus was harvested from three patients undergoing surgical therapy for moderate to advanced adult periodontitis and two patients who had their teeth extracted due to advanced adult periodontitis. Care was taken to obtain large, single pieces of calculus and to maintain the cross-sectional integrity of the structure from the tooth surface through to the external surface.

The harvested calculus was immediately placed in a fixative solution of 3% glutaraldehyde in 0.1 M cacodylate buffer, for a minimum of 3 h at 4°C. The fixed calculus specimens were post-fixed in 1% osmium tetroxide for 2 h at 4°C. After the fixation procedures all specimens were dehydrated in a graded series of ethanols (20%, 50%, 70%, 90% and 3 × 100%) at room temperature, and then infiltrated with 100% propylene oxide. All the specimens were embedded in Araldite CY212 resin (Agar Scientific Ltd, Stansted, UK).

Semi-thin sections of 0.5–1 μm were cut on an ultramicrotome (Reichert, Leica, UK) with a glass knife and then stained with toluidine blue for light microscopic examination. The specimens were viewed under a standard light microscope (Carl Zeiss, Oberkochen, Germany) and the images captured with a colour video camera (JVC TK-870E, Victor Company, Tokyo, Japan) and digitised (Image Pro Plus v 3.01, DataCell, Wokingham, UK).

For transmission electron microscopy (TEM), ultra-thin sections of 90–100 nm were cut on an ultramicrotome (Reichert, Leica, UK) with a diamond knife (Diatome, Bienne, Switzerland). These sections were mounted onto carbon-formvar coated 200 mesh copper grids, stained and viewed with a JEOL 100CXII transmission electron microscope (JEOL, Oberkochen, Germany) operating at 80 kV.

Results

Light microscopy

Young and mature supragingival calculus

In toluidine blue stained sections, the organic material was stained blue, mineralised areas remained unstained and appeared grey and white areas were artefacts due to the loss of mineralised material during sectioning.

The light microscope pictures presented of young supragingival calculus (Fig. 1a) and mature supragingival calculus (Fig. 1b) reflect the variation observed in these specimens, though generally the mature supragingival calculus specimens were larger. The interface with the tooth surface was fairly smooth and slightly curved following the shape of the tooth whereas the external mineralised surface was generally irregular and covered by a non-mineralised plaque layer of variable thickness. Specimens ranged from containing many (Fig. 1a) to fewer (Fig. 1b) non-mineralised lacunae and, in some sections, the lacunae formed a continuous connection with the external bacterial plaque, and extended to the calculus/tooth interface (Fig. 1a, arrows).

Subgingival calculus

Semi-thin sections of subgingival calculus (Fig. 1c) demonstrated that the mineralised component was uniform. Unlike supragingival calculus, lacunae of stained organic material were not seen within the body of subgingival calculus. The calculus surface previously in contact with the tooth was usually flat and mineralised whilst the external/oral surface was fairly regular and covered by a non-mineralised plaque layer of variable thickness.

Transmission Electron Microscopy

Young and mature supragingival calculus

The ultrastructure of young and mature supragingival calculus was similar. The mineralised intermicrobial areas of the body of the calculus contained predominantly small, randomly orientated needle-shaped/platelet-shaped crystals (Fig. 2a). Areas containing crystals of larger columnar and roof-tile shapes were also observed (Fig. 2b). Individual microorganisms present within the mineralised matrix showed varying degrees of calcification, in particular, individual non-mineralised coccoid and rod/filamentous bacteria were observed within fully mineralised matrix (Fig. 2c). Some of these bacteria showed vacuolation but appeared otherwise normal and possessed intact cell walls, with good ultrastructural preservation evident in the cell walls between two filamentous bacteria (Fig. 2d).

Large thin crystals, not associated with the microorganisms, appeared to be growing from the previously formed calculus (Fig. 2e). These crystals were found both at the calculus/plaque interface where there was no overlying established plaque, and in apparent splits in the mineralised areas. Such splits differed from the non-mineralised channels in that they contained few bacteria.

Channels of organic matrix containing non-mineralised bacteria were often observed extending into the calculus from the calculus/plaque interface (Fig. 3a). The width of these channels ranged from a single bacterial cell to many cells although not all channels contained bacteria. Channels were observed to join extensive non-mineralised lacunae within

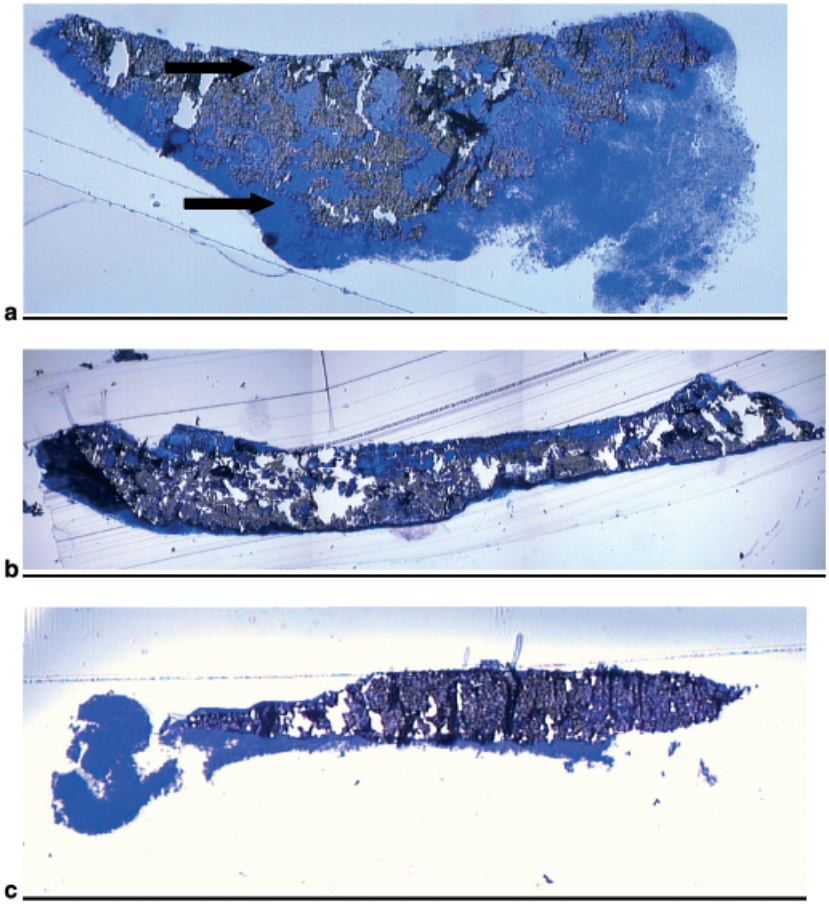

Fig. 1. 1 μm light microscope sections of supra- and subgingival calculus. The bacteria are stained blue and the mineralised areas appear grey. White areas correlate to missing pieces of mineralised calculus. (a) 1 μm section of young supragingival calculus stained with toluidine blue. There are large areas within the calculus that are unmineralised. (See arrow.) (Original magnification $\times$100.) (b) 1 μm section of mature supragingival calculus stained with toluidine blue which illustrates the continued presence of non-mineralised areas. (Original magnification $\times$40.) (c) 1 μm section of subgingival calculus stained with toluidine blue showing the lack of non-mineralised regions. (Original magnification $\times$40.)

the calculus, many of which were found to enclose large Gram +ve cells, which were apparently intact (Fig. 3b). Other lacunae contained within them large and small coccoid and rod-shaped micro-organisms (Fig. 3c), many of which appeared to possess intact cell walls and normal cell structure, though there was a varying degree of vacuolation (Fig. 3d). Individual mineralised bacteria could also be observed within these generally non-mineralised areas (Fig. 3b and d).

Subgingival calculus

The calcification within the body of subgingival calculus was more homogeneous than supragingival calculus and consisted of small randomly orientated needle- and platelet-shaped crystals (Fig. 4a). Mineralised bacteria were randomly arranged and appeared to have mineralised to the same extent as the surrounding matrix, rendering them difficult to identify (Fig. 4a). There were also areas with flat ''bulk-shaped'' crystals, as described by Sundberg & Friskopp (1985), within which were fewer bacterial cell structures (Fig. 4b). Throughout the body of the calculus there were non-mineralised areas resembling the shapes of single coccoid- or rod-shaped bacterial cells (Fig. 4c), however, no intact bacteria were found within these structures. Non-mineralised islands resembling those seen in supragingival calculus were not observed in subgingival calculus.

Discussion

There is good evidence that DCPD and OCP are formed in the calculus before HA. Thermodynamic solubility studies showed that upon local supersaturation, DCPD and OCP can precipitate very quickly and that other calcium phosphates use them as precursor crystals so that further mineralisation can take place (Driessens & Verbeeck 1989). Furthermore, X-ray diffraction studies have shown that young dental calculus contained a higher amount of DCPD and OCP than mature dental calculus (Kani et al. 1983, Sundberg & Friskopp 1985). For these reasons, it has been suggested that DCPD and OCP are formed as precursor minerals during the initial stage of mineralisation of dental plaque and that they are gradually hydrolysed and transformed into HA (Driessens & Verbeeck 1989), or in the presence of magnesium, to WH (Newesely 1968).

This ultrastructural investigation of non-decalcified young and mature supragingival calculus has observed the same small needle- and platelet-shaped crystals in both groups, which appeared to form the bulk of the calculus with groups of much larger columnar, ribbon and roof-tile crystals, which generally occurred at the plaque-free surface or in splits in the calculus. However, the identification of mineral by the crystal shape is circumstantial and inconclusive (Schroeder 1969) and more recent studies have found needle-shaped crystals forming alone in newly mineralising calculus (Hayashi 1993a, b). Therefore, theoretically, the similarity between the young and mature calculus could be partially accounted for if, in areas of microrganisms, DCPD and OCP precipitate out as small crystals which are transformed to HA, whereas in cracks and areas of low concentrations of bacteria, the DCPD and OCP can readily form much larger crystals which may also subsequently slowly transform to HA.

The presence of areas of non-mineralised matrix was a frequent finding at all levels within the body of supragingival calculus (Fig. 1), and such areas have been observed in other studies of supragingival calculus (Lustmann et al. 1976, Friskopp 1983). Two possible explanations have been suggested (Friskopp 1983):

(1) Filamentous bacteria that are predominant in supragingival plaque could have properties that inhibit mineralisation, resulting in non-mineralised regions within the calculus.

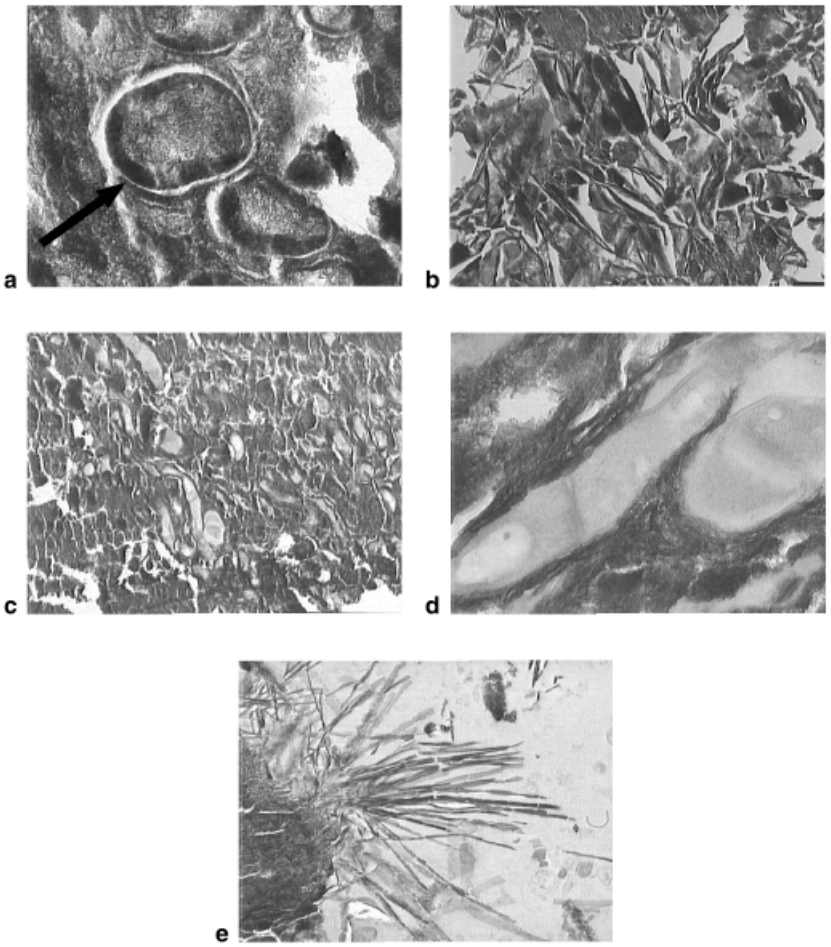

Fig. 2. Micrographs showing the ultrastructure of mature supragingival calculus. (a) Micrograph showing the small, randomly oriented, needle-shaped crystals found throughout the mineralised areas. There are mineralised microorganisms in the field with the characteristic unmineralised perimeter. (Original magnification $\times 27{,}000$.) (b) An area showing the larger columnar and roof-tile shaped crystals. (Original magnification $\times 5000$.) (c) Section showing the several non-mineralised microorganisms, which appear pale, within the fully mineralised matrix. (Original magnification $\times 5000$.) (d) Higher magnification image of enclosed non-mineralised bacteria, which appear to have a normal ultrastructure. (Original magnification $\times 27{,}000$.) (e) Micrograph showing long thin crystals originating from a mineralised region but not associated with microorganisms. (Original magnification $\times 4000$.)

(2) Differences in calcification capability between different bacterial colonies located in different parts of supragingival plaque could lead to calcification of superficial parts of the dental plaque with consequent interruption of the supply of fluid salts necessary for calcification to occur in deeper layers.

This investigation has shown that these islands may in fact not be isolated entrapments within the supragingival calculus, but may be connected to the external environment and to each other by non-mineralised channels. The channels may range from a single bacterial cell to many cells in width, and non-mineralised islands or bacteria that appear isolated in the two-dimensional representation of a single section or micrograph may potentially be interconnected in three dimensions. This study however, found few filamentous microorganisms within the non-mineralised areas, or in the vicinity of the calculus/plaque interface of supragingival calculus, perhaps lending weight to the second of the theories for the formation of non-mineralised areas. Indeed many of the channels and islands appeared to contain Gram +ve cocci with similar appearance to Staphylococcus species, which remained non-mineralised or were only slowly becoming mineralised. Thus the occurrence of, sometimes extensive, lacunae in supragingival as opposed to subgingival plaque may be explained by the presence of large numbers of slow mineralising aerobic species mostly associated with supragingival plaque.

WH has been found to be abundant in subgingival calculus (Jensen & Rowles 1957) and has been described as ''bulk-shaped'' crystals in a TEM study (Sundberg & Friskopp 1985). The present investigation found the presence of small roof-tile shaped crystals that resembled the ''bulk-shaped'' crystals described by Sundberg & Friskopp (1985) in subgingival calculus (Fig. 2b). These crystals were not observed in either young or mature supragingival calculus in this study.

The results of the ultrastructural investigation of subgingival calculus in this study were in agreement to that of Friskopp (1983). The finding that calcification was homogeneous would suggest that the combination of environment and bacteria found in subgingival plaque were more readily calcified (Friskopp 1983). The TEM investigations found few unmineralised bacteria and no apparently viable bacteria within subgingival calculus. This result would be consistent with recent controlled animal (Nyman et al. 1986) and clinical (Nyman et al. 1988, Mombelli et al. 1995) studies which showed that the removal of subgingival plaque covering subgingival calculus without complete root planning resulted in healing of periodontal lesions provided that good oral hygiene was maintained and supragingival deposits removed on a regular basis.

In a susceptible host, the presence of a periodontopathogenic community of bacteria in sufficient numbers, and in an environment that is conducive to the expression of virulence factors by the pathogens, will lead to the initiation and progression of periodontal disease (Socransky & Haffajee 1997). In the light of this understanding it is the bacterial component within plaque that causes disease, the plaque matrix itself merely serves as a carrier of pathogens in a conducive environment. The network of non-mineralised islands and channels within supragingival calculus may serve as such an environment for the expression of virulence factors by possible periodontopathogens within the calculus. This study has demonstrated that

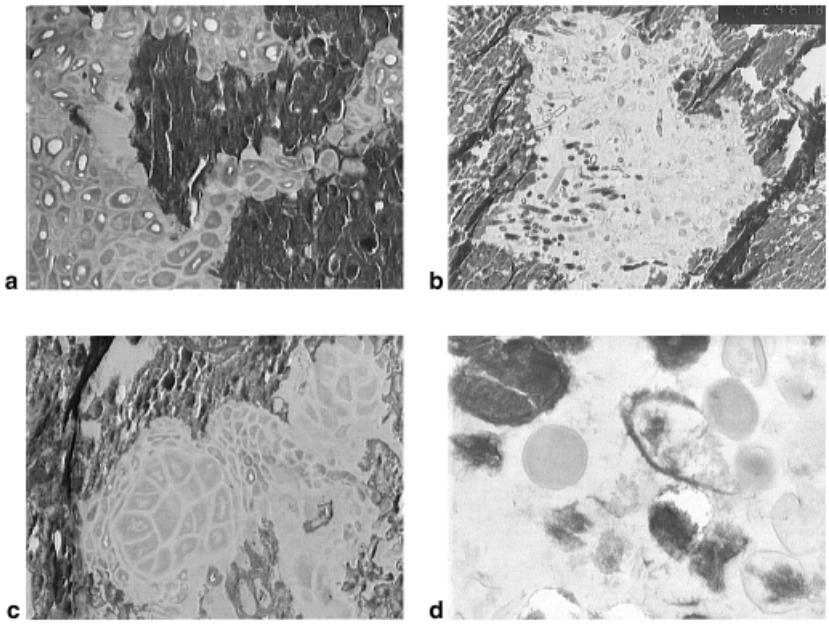

Fig. 3. Ultrastructure of the large non-mineralised areas within the mineralised calculus. (a) Micrograph showing a channel containing non-mineralised bacteria extending from the calculus/plaque interface into the body of the calculus. (Original mag $\times 5000$) (b) A non-mineralised lacuna within the calculus containing many varied non-mineralised rod and coccoid organisms. (Original magnification $\times 2700$) (c) Image showing a non-mineralised lacuna that appeared to contain a colony of one type of organism. Such organisms were often encountered. (Original magnification $\times 4000$) (d) Individual, apparently normal microorganisms located within a non-mineralised lacuna. (Original magnification $\times 20{,}000$).

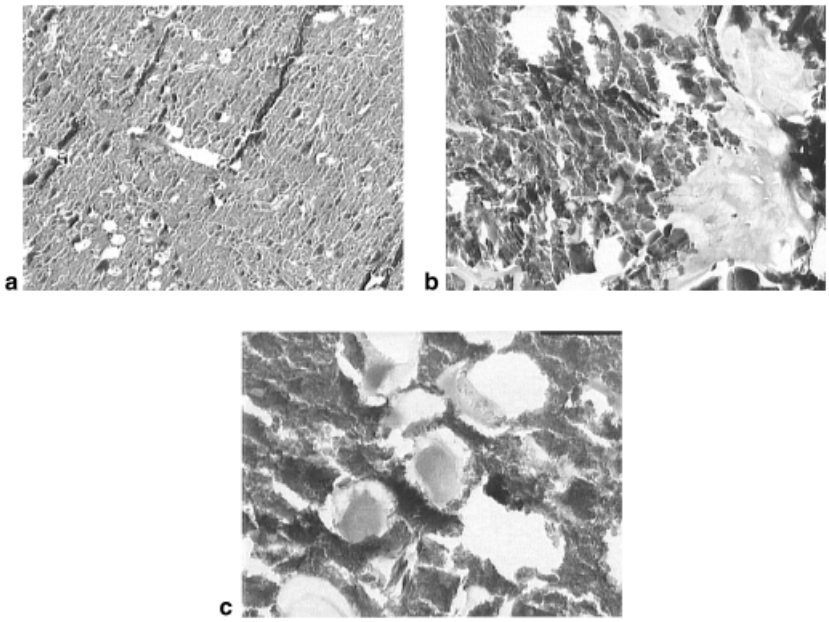

Fig. 4. Ultrastructure of subgingival calculus. (a) Micrograph showing the more homogenous nature of the mineralisation of subgingival calculus, comprising mainly small needle and small plate shaped crystals. (Original mag $\times 2700$) (b) Some small non-mineralised areas were found within the calculus but none larger than a few cells across. (Original magnification $\times 8000$) (c) Areas resembling single, non-mineralised organisms observed throughout the calculus, did not contain intact, apparently normal bacteria. (Original magnification $\times 20{,}000$).

supragingival calculus is a mineralised structure containing non-mineralised islands. These islands are present throughout the whole cross-section of the calculus and may be in communication with the external environment and with each other through a network of channels. Investigation of these islands showed that they contained microorganisms, some of which were degenerating and partially mineralised, while others possessed intact cell walls and appeared to be viable. Subgingival calculus, on the other hand, is highly mineralised and does not demonstrate the non-mineralised islands seen in supragingival calculus.

Observation of intact bacterial cells under the TEM does not necessarily imply that these bacteria are viable and, though they appear to be structurally normal, studies need to be undertaken to ascertain whether they are actually viable or not.

There are compelling arguments for ensuring thorough calculus removal, and maintenance of a calculus free environment in those individuals who have a propensity for rapid reformation calculus, if supragingival calculus is shown to contain pools of viable, periodonto-pathogenic bacteria.

References

Driessens, F. C. M. & Verbeeck, R. M. H. (1989) Possible pathways of mineralisation of dental plaque. In *Recent Advances in the Study of Dental Calculus*, ed. Cate, T., pp. 7–17. Oxford: IRL Press at Oxford University Press.

Friskopp, J. (1983) Ultrastructure of nondecalcified supragingival and subgingival calculus. *Journal of Periodontology* **54**, 542–550.

Friskopp, J. & Hammarstrom, L. (1980) A comparative scanning electron microscopic study of supragingival and subgingival calculus. *Journal of Periodontology* **51**, 553–562.

Galgut, P. N. (1996) Supragingival calculus formation in a group of young adults. *Quintessence International* **27**, 817–820.

Grøn, P., van Campen, G. J. & Lindstrom, I. (1967) Human dental calculus. Inorganic chemical and crystallographic composition. *Archives of Oral Biology* **12**, 829–837.

Hayashi, Y. (1993a) High resolution electron microscopy of the initial mineral deposition on enamel surface. *Journal of Electron Microscopy* **42**, 342–345.

Hayashi, Y. (1993b) High resolution electron microscopy of the junction between enamel and dental calculus. *Scanning Microscopy* **7**, 973–978.

Jensen, A. T. & Danø, M. (1954) Crystallography of dental calculus and the precipitation of certain calcium salts. *Journal of Dental Research* **33**, 741–750.

Jensen, A. T. & Rowles, S. L. (1957) Magnesium Whitlockite. A major constituent of dental calculus. *Acta Odontologica Scandinavica* **15**, 121–139.

Kani, T., Kani, M., Moriwaki, Y. & Doi, Y. (1983) Microbeam x-ray diffraction analysis of dental calculus. *Journal of Dental Research* **62**, 92–95.

Lilienthal, B., Amerena, A. & Gregory, G. (1965) An epidemiological study of chronic periodontal disease. *Archives of Oral Biology* **10**, 553–566.

Lustmann, J., Lewis, E. J. & Shteyer, A. (1976) Scanning electron microscopy of dental calculus. *Calcified Tissue Research* **21**, 47–55.

Mandel, I. D. & Gaffar, A. (1986) Calculus revisited. *Journal of Clinical Periodontology* **13**, 249–257.

Mombelli, A., Nyma, S., Bragger, N., Wennstrom, J. & Lang, N. P. (1995) Clinical and microbiological changes associated with an altered subgingival environment induced by periodontal pocket reduction. *Journal of Clinical Periodontology* **22**, 780–787.

Newesely, H. (1968) Calcifying processes within superficial layers of the dental plaque. *Caries Research* **2**, 19–26.

Newman, H. N. (1994) The development of dental plaque. In *Primary Preventive Dentistry*, eds. Harris, N. O. & Christen, A. G., pp. 19–58. East Norwalk, CT: Appleton & Lange.

Nyman, S., Sarhed, G., Ericsson, L., Gottlow, J. & Karring, T. (1986) Role of ''diseased'' root cementum in healing following periodontal disease. An experimental study in the dog. *Journal of Periodontal Research* **21**, 496–503.

Nyman, S., Westfelt, E., Sarhed, G. & Karring, T. (1988) Role of ''diseased'' root cementum in healing following periodontal disease. A clinical study. *Journal of Clinical Periodontology* **15**, 464–468.

Ramfjord, S. P. (1961) The periodontal status of boys 11–17 years old in Bombay, India. *Journal of Periodontology* **32**, 45–53.

Schroeder, H. E. (1965) Crystal morphology and gross structures of mineralising plaque and of calculus. *Helvetica Odonologica Acta* **9**, 73–86.

Schroeder, H. E. (1969) *Formation and Inhibition of Dental Calculus*. Berne: Hans Huber Publishers.

Schroeder, H. E. & Baumbauer, H. U. (1966) Stages of calcium phosphate crystallization during calculus formation. *Archives of Oral Biology* **11**, 1–14.

Socransky, S. S. & Hafferjee, A. D. (1997) Microbiology of periodontal disease. In *Clinical Peridontology and Implant Dentistry*, eds. Lindhe, J., Karring, T. & Lang, N. P., pp. 138–188. Munksgaard.

Sundberg, M. & Friskopp, J. (1985) Crystallography of supragingival and subgingival human dental calculus. *Scandinavian Journal of Dental Research* **93**, 30–38.

Theilade, J. (1964) Electron microscopic study of calculus attachment to smooth surfaces. *Acta Odontologica Scandinavia* **22**, 379–387.

Weinberger, B. W. (1948) *An Introduction to the History of Dentistry, Vol 1*, pp. 203–207. St Louis: Mosby.

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