Calc. Tiss. Res. 1 1,222-237 (1973)

Uhrastructural Observations on the Calcification of Human Dental Pulp

J. Appleton and M. J. R. Williams

School of Dental Surgery, University of Liverpool

Received November 19, 1971, accepted May 24, 1972

The calcifications in dental pulp appeared to consist of discrete, smooth-surfaced laminated den~icles and irregularly shaped, non-laminated denticles, together with a diffuse calcification characterized by small loci scattered throughout the fibrous pulp matrix. Calcification appeared to be initiated in relation to the interfibrillar matrix, collagen fibres and connective tissue cells. In each case the inorganic phases showed a distinct morphology; electron diffraction suggested hydroxyapatite. Both the laminated and non-laminated denticles had an organic matrix consisting of collagen fibres together with a background of electron dense material between the fibres. The laminated denticles appeared to grow by the addition of layers of collagen to the surface, leaving an uncalcffied border zone which gradually calcified. The matrix of the non-laminated denticles was formed by the collagen fibres orientated in the long axis of the pulp, and no border zone was present. These denticles grew by the addition of mineral to the adjacent matrix fibres. Some small denticles did not have a collagen fibre matrix, but an electron-dense granular matrix was present. One such denticle was being resorbed by a giant multi-nucleated cell. The non-laminated denticles contained areas devoid of fibrils in which the crystallites were larger but gave a diffraction pattern indicative of hydroxyapatite. Between the matrix fibres in diffuse calcification an electron dense granular material was present.

Key words: Ultrastructure -- Calcification-- Dental Pulp -- Teeth.

Les calcifications de la pulpe dentaire semblaicnt eonstituer par de petits pulpolithes lamellaires, ~ surfaces lisses, par des pulpolithes non lamellaires, s contours irröguliers, ainsi que par une calcification diffuse, constitu6e de petits foyers diss6min6s dans la matrice fibreuse pulpaire. La calcification semble döbuter au niveau de la matrice intcrfibrillaire, du collagbne et des cellules conjonctives. Dans chaque cas, les phases inorganiques ont un aspect morphologique distinct: la diffraction 61ectronique semble indiquer la prösence d'hydroxyleapatite. Les 2 types de pulpolithes ont une matrice organique constituöe de collagöne et d'un matöriel dense aux 61ectrons, situ6 entre les fibres. Les pulpolithes lamellaires semblent s'accroitre par adjonction de couches de collag~ne ~ leur surface, laissant subsister un rebord non calcifi6, qui se caleifie progressivement. La matrice des pulpolithes non lamellaires est form6e de fibres de collagbne orient6es lc long de l'axe de la pulpe et aucun rebord n'est visible. Ces pulpolithes s'accroissent par d6p6t de min6ral aux fibres de la matrice adjacente. Certains petits pulpolithes n'ont pas de matrice eollag6nique, mais une matrice granulaire dense aux 61ectrons. Un de ces pulpolithes est r6sorb6 par une cellule g6ante multinucl66e. Les pulpolithes non-lamellaircs pr6sentent des zones afibrillaircs oh les cristaux sont plus larges et donnent des clich6s de diffraction 61ectronique d'hydroxyleapatite. Entre les fibres de la matrice des calcifications diffuses, un matöriel granulaire dense est visible.

Die Verkalkungen in Zahnpulpa scheinen zu bestehen aus: gctrennten, lamellenf6rmigen Dentikeln mit glatter Oberfl/~che und unregelmaBig geformten, nicht-lamellenfSrmigen Dentikcln, zusammen mit einer diffusen Verkalkung, welche dutch kleine Foci charakterisiert ist, die in der ganzen fibrösen Pulpamatrix verteilt sind. Die Verkalkung schicn dutch die inter-

ior *reprints:* Dr. J. Appleton, School of Dental Surgery, Pembroke Place, P.O. Box 147, Liverpool, L69 3BX, England.

fibrills Matrix, die Kollagenfasem und die Bindegewebszellen hervorgerufen zu werden. Bei jedem Fall zeigten die anorganischen Phasen eine ausgepr/igte Morphologie; die Elektronendiffraktion deutete auf Hydroxyapatit. LamellenfSrmige und nicht-lamellenfSrmige Dentikel besaBen eine organische Matrix, welche aus Kollagenfasem und elektronendichtem Material zwischenden Fasem bestand. Die lamellenfSrmigen Dentikel schienen zu wachsen, indem sie Kollagenschichten auf der Oberfl/icheanfiigten, wobeieine l~andzone zuerst unverkalkt blieb und dann allm-hlich verkalkte. Die Matrix der nicht-lamellenf6rmigen Dentikel wurde durch die Kollagenfasem der L~ngsachse der Pulpa gebildet, und eine Ran@one war nicht vorhanden. Diese Dentikel wuchsen, indem den angrenzenden Matrixfasem Mineral zugcffigt wurde. Einige kleine Dentikel wiesenkeine Kollagenfaser-Matrix auf, aber eine elektronendichte granul~re Matrix wurde festgestellt. Ein solcher Dentikel wurde yon einer vielkemigen Riesenzelle resorbiert. Die nlcht-lamellenfSrmigen Dentikel enthielten Zonen ohne Fibrillen, in welchendie Krist/illchen grSf~er waren, aber ein Diffraktionsmuster zeigten, welches auf Hydroxyapatit hindeutete. Zwischenden Matrixfasem in der diffusen Verkalkung wurde ein elektronendiehtes granuls Material festgestellt.

Introduction

Dental pulp is a typical connective tissue which is normally enclosed by the dentine of the tooth except at the apical foramen where the nerves and blood vessels enter the pulp canal (Selzer and Bender, 1965). Calcifications of the pulpal tissue are common at all ages (Miles, 1961) but caries and other pathological changes affecting the pulp are known to increase their incidence (Hall, 1968).

The calcifications are classified generally into three types; attached stones (true denticles), which are attached to the dentine forming the wall of the pulp chamber, free stones (false denticles), lying freely in the body of the pulp, and diffuse calcifications in which numerous small foci of calcification are scattered throughout the pulp (Selzer and Bender, 1965; Orban, 1966; Kraus *et al.,* 1969). Free and attached stones are spherical or ovoid and may become large enough to occlude the pulp cavity completely. They normally exhibit concentric laminations which indicate incremental growth, and which are most apparent following decalcification and staining with haematoxylin and eosin (Selzer and Bender, 1965; Orban, 1966; Kraus *et al.,* 1969) and in ground sections examined in polarized light (Schmidt and Keil, 1971). Free stones are thought to fuse with the dentine of the pulp cavity as they enlarge and as secondary dentine is formed to become attached stones. Therefore, the stones become surrounded by odontoblast-like cells from the pulp and are often permeated by some of the odontoblast processes of the dentine. On this basis Langeland (1957) and Johnson and Bevelander (1965) maintain that any histological distinction between false and true denticles is invalid and term both types of stones as denticles.

Histological methods have not clearly revealed the nature of the microenvironment at the sites of initiation of these forms of pulpal calcification since the earliest sites are not resolved in the light microscope. It has been suggested, however, that dead or inflamed cells, bacteria, matrix fibres (Johnson and Bevelander, 1956; Selzer and Bender, 1965; Hall, 1968; Kraus *et al.,* 1969) or bloodvessels and nerves (Langcland, 1957; Orban, 1966; Kraus *et al.,* 1969)form loci for calcification. However, it is known that teeth not obviously affected by any histologically-detectable pathological change, for example, unerupted teeth (Langeland, 1957; Orban, 1966; Hall, 1968) and young teeth extracted for orthodontic reasons (James, 1958) often show pulpal calcification.

Since little is known of the detailed structure and composition of pulpal calcifications, apart from brief histoehemical (Burstone, 1953; Johnson and Bevelander, 1956; Okada, 1970), electron microscopical (Harrop and Mackay, 1968) and polarized light investigations (Sehmidt and Keil, 1971) the object of the present study is to describe the formation, structure and composition of denticles and diffuse calcifications.

Materials and Methods

Light Microscopy. Carious and caries-free, single-rooted anterior teeth were obtained fresh from extraction. The teeth were cracked open in a bench vice and the pulps carefully dissected out and fixed in 5% formol saline and examined under a binocular microscope during fixation for visible calcifications. Pulps with visible calcifications, from five carious and five caries free teeth, and pulps without visible calcification from five carious and five caries-free teeth were dentineralized using Brain's (1966) technique before being processed for embedding in paraffin wax. Sections 5 ~ in thickness were stained with haematoxylin and eosin. Araldite embedded sections between 2-5 ix in thickness were also examined, under phase contrast or following staining with 1% toluidine blue in 1% borax, for the purposes of orientation.

Electron Microscopy. Fresh teeth were obtained as above and the pulps were carefully dissected out intact and fixed for 2 h in 6.25% cacodylate buffered glutaraldehyde pH 7.4 (Sahatini *etal.,* 1963). During fixation the pulps were examined in a closed container under a binocular microscope for visible denticles. Pulps with visible calcification front five carious and five caries-free teeth and pulps without visible calcification from five carious and five caries-free teeth were selected and three from each group were decalcified in 5% EDTA for 3 h, the progress of decalcification being followed under a binocular microscope. Following a brief washing in distilled water all the pulps were post-fixed for 1 h in 1% veronal acetate buffered osmium tetroxide pH 7.2 (Palade, 1952), washed briefly in distilled water, dehydrated in methanol and embedded in Araldite. Thin sections were prepared using a glass or diamond knife in a Reichert OMU 2 ultramicrotome. The water bath was maintained at alkaline pH by the addition of sodium hydroxide and the sections were collected immediately in order to minimize the possibility of decalcification and non-specific precipitation (Boothroyd, 1964). Thin sections were mounted unsupported on copper grids, stained with a 25% solution of uranyl acetate in absolute methanol and examined in a Philips EM 300 electron microscope equipped with an anti-contamination device cooled with liquid nitrogen. Selected area diffraction was also utilized using a 30 ~ diffraction aperture.

Results

Light Microscopy. The free denticles were of two types, those with distinct concentric laminations (Fig.]A) and those without distinct laminations (Fig. IB). The laminated type were round or ovoid and often large enough to occlude the pulp canal. They had smooth surfaces and were not usually closely associated with other smaller denticles. In the sections examined no focus of cells or cell debris could be distinguished in these denticles although each denticle had adistinct centre of origin. It appeared that blood vessels and nerves were diverted rather than enclosed b y the denticles as t h e y grew in size (Fig. 1A).

The denticles without laminations (Fig. IB) were also occasionally large enough to occlude the pulp canal but although the smaller denticles were round or ovoid the larger dentieles did not have a definite shape and the surface was not as smooth as in the jaminated type. The large denticles were usually associated wl~h smaller dentieles which were often attached to their surfaces.

Diffuse calcification was distinct (Fig. 1C) and consisted of numerous small loci of calcification scattered throughout the fibrous matrix of the pulp but particularly along its long axis in the pulp canal.

Electron Microscopy. There were numerous foci of calcification of 1 ~ diameter or less in most of the pulps examined in the electron microscope. These foci were either in the form of smooth-surfaced spherical clusters (Fig. 2A), or in a closely packed layer around collagen fibres (Fig. 2B, E), or in the form of intracellu]ar deposits (Fig. 2 C, D).

The smooth surfaced spherical clusters often appeared to push the collagen fibres to one side (Fig. 3A, B), and the crystallites were closely packed, plate-like, diffuse and varied considerably in size (Fig. 3A, B). The broken nature of the lines in electron diffraction patterns obtained from these clusters also indicated the presence of a few crystallites consisting of hydroxyapatite-like material (Fig. 3D).

Following EDTA demineralization of these spherical clusters there was an evenly-dispersed, electron-opaque granular material present often surrounded by an electron dense narrow band in which the granular material was more closely aggregated. Occasionally there was also a more electron dense area present centrally, where there was also a greater concentration of matrix granules (Fig. 3 C).

In some early foci fine needle-like crystallites measuring about 50 x 1000 A were closely associated with the matrix collagen (Fig. 2B, E). The randomly orientated crystallites were enclosed within an electron-opaque granular material which coated the fibres and was most apparent following demineralization with EDTA (Fig. 9).

Examples of the intracellular deposition of crystallites were seen in the fibro-blast-like cells of the pulp of several teeth (Fig. 2 C). In each case the crystallites appeared to be enclosed within the mitochondrial membrane (Fig. 2D). The crystallites were distinctly needle-like, measuring approximately 25 A in width and 500 A in length. Both the crystallites surrounding the fibrils and intracellular crystallites gave a similar diffraction pattern typical of an hydroxyapatite-like material (Fig. 2 E, inset).

The large free denticles, which appeared spherical in outline and had concentric laminations following demineralization (Fig. 1A), also had a distinctive appearance in the electron microscope. Each denticle consisted of an electron dense central zone surrounded by a less dense peripheral zone about 2-3 ~ in width (Fig. 4A). The central zone consisted of closely-packed crystallites approximately 50 X 300 A associated with collagen fibres just visible in the background (Fig. 4A). Following EDTA demineralization, however, collagen fibres about 300 ~ in width were revealed with a distinct periodicity of about 640 A (Fig. 5B). Between the deeper fibres and coating the surface fibres was a layer of electron opaque

granular material between 100-500 ,~_in thickness (Fig. 5B). The collagen fibres were orientated parallel to each other in large bundles. There were round and oval spaces or lacunae present in both the central and peripheral zones. Around the spaces in the central zone fewer crystallites were present and the matrix was less electron dense. There appeared to be no cells or cell debris present in these spaces (Fig. 4A).

At the surface of the denticles, where the fibres were not so densely packed, small ring like dusters of granule-like crystal]ites were seen where, it is suggested, collagen fibres and erystallites, some orientated with their long axes parallel to the fibres, had been cut in transverse section (Fig. 5A). Electron diffraction revealed that the inorganic phase consisted of hydroxyapatite-like material (Fig. 5, inset). The electron opaque coating of granular material around the fibres was most apparent in the surface layer following EDTA demineralization (Fig. 5B).

There were also smaller denticles between 2 ~ and 10 ~ in diameter formed of crystallites measuring 50 X 300 A and with a similar external appearance to the dentieles described above being surrounded by a less dense peripheral zone (Fig. 6). These denticles did not have a matrix of collagen fibres, however, demineralized sections showing an electron opaque granular matrix similar to that surrounding the collagen fibres of the larger dentieles described above.

One of these denticles, which did not have a collagen fibre matrix, was being resorbed by a giant multi-nucleated cell (Fig. 7). A portion of the cell occupied a shallow depression on the denticle at which point its surface was irregular with clusters of detached granule-like crystallites. A brush border was not evident in the plane of section but its presence was indicated by the numerous round and elongated membrane profiles situated between the cell membrane and the border of the denticles. The cell contained numerous mitochondria and smooth vesicles of varying diameter many of which contained clusters of crystallites (Fig. 7, inset).

The non-laminated free denticles showed considerable individual variation in their structure and were irregular in outline by comparison with the laminated denticles (Fig. 8A). The bulk of these dentieles consisted of small granular crystal-lites measuring about 50 x 300 1 deposited on a collagenous matrix similar to that described in the laminated dentic]e. Enclosed exclusively and consistently within these dentieles, however, were less densely mineralized areas, devoid of collagen fibres within which the crystallites were sometimes deposited either in concentric layers (Fig. 8A, B), or in groups consisting of larger crystallites measuring up to 100 • 600 A (Fig. 8C). The enclosed crystallites gave a diffraction pattern indicative of hydroxyapatite-]ikc material (Fig. 8, inset).

Diffuse mineralization was distinctive and consisted of irregularly-shaped areas of mineralization of varying size scattered throughout a fibrous matrix (Fig. 9). Following demineralization a distinct electron dense background was evident coating the collagen fibres (Fig. 9).

Discussion

Normal calcification takes place in a preformed organic matrix produced by specialised secretory cells e.g. odontoblast and osteoblasts. The current view, based principally on the studies of bone, suggests that these matrices calcify by inducing the deposition of mineral from metastable solution by the stereospecificity of matrix collagen (Neuman and Neuman, 1958; Glimcher, 1960; Glimcher and Krane, 1968). It is known, however, that collagen is not unique in inducing the deposition of the inorganic phase and other organic matrices, for example, keratin (Blakey and Lockwood, 1968) and interfibrillar material in cartilage (Cameron, 1963; Bonucci, 1967; Appleton, 1969) have been implicated. It is also apparent from this and other studies that calcification can take place at sites where there is no specialised matrix, for example, in the kidney (Eisenstein *et al.,* 1960). Deposition of calcium in such a tissue which does not normally calcify, therefore, can be termed pathological (Scott and Symons, 1970).

It has been shown that pathological calcification is often preceded by tissue damage which may produce localised metabolic changes thereby promoting calcification (Eisenstein et *al.,* 1960). Histological examination of a large sample of pulps (Hall, 1968), for example, has shown that most pulpal calcifications are clearly associated with pulp pathology, particularly long standing chronic irritation. A small number of pulpal calcifications, however, were found in apparently histologically normal pulps. The question is posed, therefore, as to why and how loci of calcification appear in pulps, particularly those in which there is no detectable pathology.

The calcification that appears to take place within the mitochondria of some ceils of the pulp may be a reflection of a local metabolic change within the cell. The initial step may be the formation of amorphous calcium phosphate which can be accumulated in mitochondrial matrix (Greenwait *et al.,* 1964) in the form of electron-dense, rosette-like granules. However it is also possible that this appearance is an artifact of preparation since mitochondria are known to form crystals readily under the right conditions (Legato *et al.,* 1968).

The small smooth-surfaced loci of mineralization in dental pulp do not appear to have any relationship with a specific matrix component. However, following

EDTA demineralization a floccular, electron-opaque organic phase is visible but the concentration of matrix peripherally is probably a diffusion artifact. This matrix is unlike that seen following the EDTA demineralization of small loci in cartilage which has been described as filamentous (Bonueei, 1967 ; 1969) or granular (Appleton, 1969).

The fine crystallites embedded in an electron dense granular material coating small groups of collagen fibres presents a similar appearance to that seen in the early stages of bone formation (Robinson and Watson, 1955; Fitton-Jackson and Randall, 1956; Fitton-Jackson, 1957; Molnar, 1959; H6hling *et al.,* 1966). It was suggested that this granular material represented amorphous calcium phosphate (Robinson and Watson, 1955; Fitton-Jaekson and Randall, 1956; Molnar, 1959). Following EDTA demineralization of the denticles, however, an electron opaque granular material is still present suggesting it is principally organic in nature. Moreover, electron microscope studies of non-crystalline calcium phosphate show that it consists of distinctive sphere like shells with a hollow inner core (Weber *et al.,* 1967). Numerous small foei associated with collagen fibres are present throughout the pulp in the condition of diffuse calcification.

The smallest spherical dentieles, in which there is no fibrous matrix, appear to develop by the gradual deposition of mineral as evidenced by the narrow even less densely mineralized band at the surface. The large laminated dentieles appear to develop by the deposition of concentric layers of closely-packed collagen fibres which then become mineralized. The initial nidus in both eases could be, for example, a calcified cell or a small smooth surfaced focus of eMeification. No evidence as to the nature of the nidus was discovered in this study, however, but it has been shown in tissue culture that concentric layers of fibrils gradually enveloped degenerating pulp cells to form dentieles (Gerstner, 1971). The less densely mineralized surface zone suggested again that there was a gradual and advancing deposition of mineral. The non-laminated dentieles also had a collagen fibre matrix but grew by the addition of crystallites to the collagen fibres orientated in the long axis of the pulp. These dentieles appeared to gradually envelop the small dentieles which surrounded them and also enclosed areas devoid of fibrils in which the crystallites were able to develop to a larger size.

It would appear that deposition of mineral in the pulp is coincident with the deposition of organic material other than, or in addition to, the collagen fibres already present in the matrix. The presence of glycoprotein, acid polysaceharides and retieulin fibres has been indicated from previous studies (BurstOne, 1953; Johnson and Bevelander, 1956). It is apparent, therefore, that simple fibrous epitaxy as proposed for the calcification of bone does not apply and that other organic phases may be involved. It is possible that local precipitation may occur and once crystallites are formed they would probably become a site for further precipitation. The extracellular factors which could produce such a local rise in the concentration of calcium and phosphate are unknown. However it has been shown in guinea-pig eostoehondral cartilage that Ca ions are bound in increasing concentration, prior to the appearance of crystallites, to small osmiophilie bodies in the matrix (Hall *et al.,* 1971). Such a process would probably be under cellular control and it has been suggested that chondroeytes act as a calcium binding and concentrating system (Matthews *et al.,* 1968) and also that a calcium phospho-

protein complex may be secreted via the Golgi apparatus of osteoblasts (Taves, 1965).

The variation in size and morphology of the crystallites composing the dentieles suggests they may represent different forms of calcium phosphate (Schroeder, 1965). For example, it is generally accepted that hydroxyapatite forms small needle-like erystallites while octaealeium phosphate forms long ribbon like erystallites (Hayek *et al.,* 1960). The electron diffraction pattern of the various crystallites composing the denticles are similar, however, and only indicative of hydroxyapatite-like material. This was expected since it is known, for example, that octaealeium phosphate and brushite can be hydrolysed by the heating effect of the electron beam (Hayek *et al.,* 1960; Saxton, 1968). Any differences in the intensity and character of the lines could be due to differences in statistical distribution, orientation, and numbers of erystallites.

The giant multinuclear resorbing cell present in one of the pulps was quite characteristic of its type in structure in having a brush border, large numbers of mitochondria, vacuoles and several nuclei (Itaneox and Boothroyd, 1963). Resorbing cells of this type are present in pulps in which there is inflammation where they are more usually associated with the resorption of dentine (Selzer and Bender, 1965).