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Transmission electron microscope characterisation of molar-incisor-hypomineralisation

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Abstract Molar-incisor-hypomineralisation (MIH), one of the major developmental defects in dental enamel, is presenting challenge to clinicians due, in part, to the limited understanding of microstructural changes in affected teeth. Difficulties in the preparation of site-specific transmission electron microscope (TEM) specimens are partly responsible for this deficit. In this study, a dual-beam field emission scanning electron microscope (FESEM)/focused ion beam (FIB) milling instrument was used to prepare electron transparent specimens of sound and hypomineralised enamel. Microstructural analysis revealed that the hypomineralised areas in enamel were associated with marked changes in microstructure; loosely packed apatite crystals within prisms and wider sheath regions were identified. Microstructural changes appear to occur during enamel maturation and may be responsible for the dramatic reduction in mechanical properties of the affected regions. An enhanced knowledge of the degradation of structural integrity in hypomineralised enamel could shed light on more appropriate management strategies for these developmental defects.

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1 Introduction

Molar-incisor-hypomineralisation (MIH) is the term used to describe a condition in which one or more of the first permanent molar teeth and often at least one incisor tooth is hypomineralised [1] and has been reported to affect up to 19.3% children [2]. MIH differs from enamel hypoplasia, a quantitative enamel defect, since MIH is a qualitative defect identified clinically as demarcated enamel opacities that are also distinguishable from the more diffuse opacities associated with enamel fluorosis. MIH affected enamel is consistently soft and chips away easily under masticatory forces from the time the teeth first erupt into the oral cavity, exposing dentine. The defects significantly increase dental treatment need as the teeth are more susceptible to plaque accumulation and dental caries. Consequently MIH poses a significant problem for both the patients and their clinicians because the affected teeth have significant tissue loss, are often hypersensitive and are difficult to restore.

To date, most of the research in this area has focused on the composition, physical and mechanical properties of the MIH enamel. An increase in carbon content coupled with a decrease in calcium and phosphorous content and a decrease in Ca/P ratio have been reported in the affected teeth [3]. In addition, a 20% volume reduction in mineral content suggests that there is an increase in the organic component in these lesions [4]. Moreover, nanoindentation tests reveal that the mechanical properties, i.e. stiffness and

hardness, of the hypomineralised enamel are significantly inferior to sound enamels. However, the reason for this marked reduction in mechanical properties is currently unclear [5]. Recent research on naturally occurring structural materials, such as deepwater sponge [6] and seashell [7], has revealed that structural hierarchy has a significant influence on mechanical properties of these biological materials. Although hierarchical structure has been identified in sound enamel [8], reports on microstructure of MIH enamel have been very limited. Initial morphological studies with polarised light microscopy demonstrated areas of porosity of varying degrees [9]. Recent microstructural analysis by means of scanning electron microscopy (SEM) identified a less well organised prism structure with voids present, compared to sound enamel whose prism structure is well arranged [5, 10]. Notably, previous histological analyses of enamel, including that affected by MIH, were focused on a length scale greater than 10 μm, and microstructural characteristics at levels of prism and sheath are unclear. A better understanding of enamel microstructure at these levels is therefore essential to clarifying the aetiology of the MIH enamel and its mechanical behaviour during mastication and will also assist in addressing the problems associated with the management of this MIH condition.

Transmission electron microscopy (TEM) has long been used for microstructural examinations of biological calcified tissues [11]. Prerequisite to the success of such TEM analysis is the preparation of high-quality electron transparent specimens. Ultramicrotomy with diamond knives has routinely been used to prepare thin sections of calcified tissues for TEM analysis. Several issues have been identified with this technique; it can be time-consuming, requires multi-stage chemical fixation and embedding and also needs mechanical sectioning with a diamond knife, which may introduce artificial damage. Given the small size of hypomineralised regions, it is also difficult to prepare site-specific TEM specimens using this conventional technique. To overcome the difficulties associated with ultramicrotomy, dual-beam FESEM/FIB instrument has been introduced for TEM preparation of biological calcified tissues in recent years [12, 13]. This technique uses a beam of energetic Ga ions to remove material. Both the specimen surface and the resultant cross-section can be viewed in-situ using the field emission electron column interfaced to this system, enabling site-specific TEM preparation.

In this work, electron transparent specimen of the MIH enamel was prepared using dual-beam FESEM/FIB system and then examined under TEM to identify microstructural change associated with hypomineralisation. Microstructural analysis was focused on the prism and sheath structure of the MIH enamel that would elucidate key structural factors that are responsible for its inferior mechanical properties. This study is also intended to provide insight into the origin of the MIH enamel and advance understanding of treatment problems related to this condition.

2 Materials and methods

Ethical approval and patient informed consent was gained from Western Sydney Area Health Service Ethics Committee for the collection of teeth in this study. To begin with, aesthetic differences between sound and hypomineralised dental enamel have served as criteria for tooth selection in this work. Seven teeth, i.e., three first permanent molars extracted with significant developmental defects in the enamel and four sound controls tooth (first premolars extracted for orthodontic reasons, as it is difficult to access perfectly normal first molar teeth to use as controls), were chosen in this study. There is no evidence that the structure of a premolar has any difference to that of a molar. Moreover, given the little we know about hypomineralisation occurs, it would be appropriate to think that using the same defective tooth might not be a good control because we do not know much about the effect the developmental disruption has on the developing enamel across the whole tooth. As we were only interested in accessing normal healthy enamel, the premolars used here can therefore be regarded as an appropriate control. Upon extraction teeth was placed into Hanks' Solution (SIGMA-ALDRICH CO., St. Louis, USA) with thymol crystals added to inhibit bacterial growth and stored at 4°C.

For the initial preparation of the specimens, each tooth was encased in cold-cured epoxy resin and sectioned with two parallel cuts through the center of the lesion in the mesial-distal axial planes using a water-cooled diamond saw (Isomet, Buehler Ltd., Lake Bluff, Illinois, USA). The cutting directions were selected so that prisms were parallel with the cut surface. The resulting slice, approximately 2 mm thick, was polished on one cut surface with successively finer grade silicon carbide paper and finally with 9 and 1 μm diamond suspensions. Once prepared, specimens were kept fully hydrated in Hanks' Balanced solution with the addition of thymol crystals.

The identical regions within the selected teeth underwent both nanoindentation tests [14, 15] and cross-sectional scanning electron microscope (SEM) examination using a dual beam FESEM/FIB system (Nova Nanolab 200, FEI, USA) [16] prior to TEM to confirm the nature of the affected enamel region in comparison to the sound enamel. Electron transparent sections were prepared both parallel and perpendicular to the direction of the prisms. Four TEM cross sections were prepared for one designated direction on each of the selected teeth to ensure the reliability of microstructural characterisation. The procedure of such operation has been

given in detail in a previous paper [17]. First, a layer of platinum (1 μm thick) was deposited onto a desired surface area to protect the surface from ion-beam damage during the milling processes. A "rough" mill was then performed with a current of 10 nA, in which trenches were cut both sides of the platinum strip to obtain a section ∼3 μm thick. A number of "fine" mills were performed at reduced currents (5–1 nA) and the section thinned to ∼1 μm. Final mills were carried out at further reduced currents (300–100 pA) to reduce the thickness of the already thinned section down to ∼100 nm for electron transparency.

The transfer of the TEM specimen from the sample holder to the copper TEM grid coated with a carbon membrane was performed ex-situ using a high precision micromanipulator (Kleindiek Nanotechnik GmbH, Reutlingen, Germany), which operates by means of an electrostatic force at the tip of a glass needle. The specimen was examined using a field emission electron source transmission electron microscope (Philips CM200, Eindhoven, Netherlands) at 200 keV.

3 Results

TEM specimens parallel to the direction of the prisms were first examined. In order to understand microstructural changes associated with hypomineralised enamel, analysis was performed on both sound and hypomineralised enamel. Cross-sectional TEM images of the sound enamel from the control tooth containing narrow sheath regions are shown in Fig. 1a. The densely packed and well-organised elongated apatite crystalline grains are visible. Within the central region of the enamel prism are elongated apatite crystals running in a direction parallel to the direction of the prisms. Away from the central region the apatite crystals deviate from the prism axis progressively and run at ∼60 degrees to the sheath region. The broken line on the image delineates the closely interconnected nature of the sheath region between the prisms. At higher magnification (Fig. 1b), the sheath region is observed to be extremely narrow and bridged by crystallites. Cross-sectional TEM images of hypomineralised enamel also sectioned parallel to the direction of the prisms demonstrate a well-organised, but less dense structure (Fig. 2a). Most significantly, the hypomineralised enamel was found to have an average sheath width of ∼100 nm. A higher magnification TEM image (Fig. 2b) also reveals that adjacent to the sheath region are low density areas, extending up to hundreds of nanometres into the prisms.

To confirm the observations from the TEM sections parallel to the direction of the prisms, and more importantly, to gain further understanding of microstructure characteristics of hypomineralised enamels, TEM analyses were performed on sections perpendicular to the direction of the prisms. For the sound enamel, the general appearance is of a dense microstructure with poorly defined and discontinuous enamel sheath regions (Fig. 3). The apatite crystals appear to run with their longitudinal axes perpendicular to the direction of the prisms in the tail region. There is a gradual change in the orientation of crystallites in the area between the head (central region) and the tail of the prism. At higher magnifications, the prism structure is observed to consist of tightly packed crystallites (inset (a) of Fig. 3); the sheath appears to contain isolated pores (∼20 nm across) between regions where crystal bridging is extensive (inset (b) of Fig. 3). Observations of the hypomineralised enamel are shown in Fig 4. The basic microstructure of MIH enamel is similar to

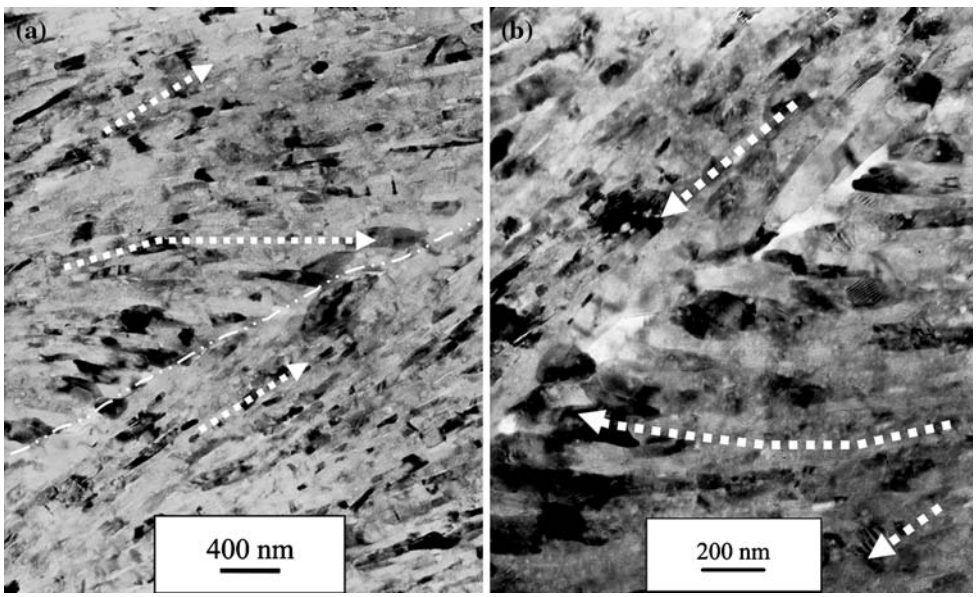

Fig. 1 (**a**) Bright field TEM images of a sound enamel showing tightly packed prism structure and closely interconnected sheath region marked by a dotted and broken line. (**b**) A higher magnification image showing narrow sheath region and adjoining apatite crystallites. The bold broken lines with arrows indicate the crystal orientation. TEM specimens prepared parallel to the direction of the prisms

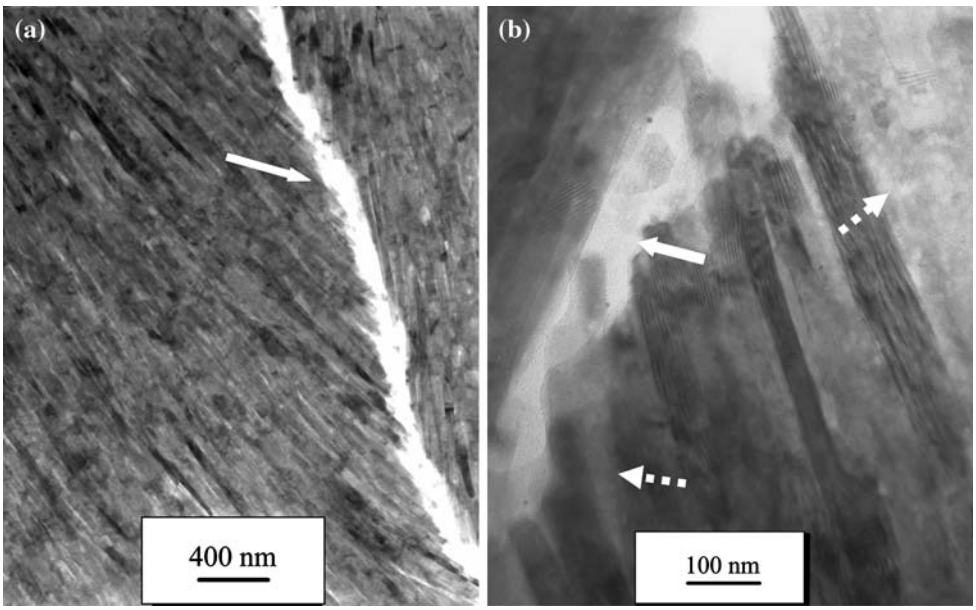

Fig. 2 (**a**) Bright field TEM images of a hypomineralised enamel showing wider sheath region. (**b**) A higher magnification image showing poorly mineralised sheath region and low density areas adjacent to the sheath boundary. Solid arrows indicate sheath regions and broken arrows indicate the low density areas. TEM specimens prepared parallel to the direction of the prisms

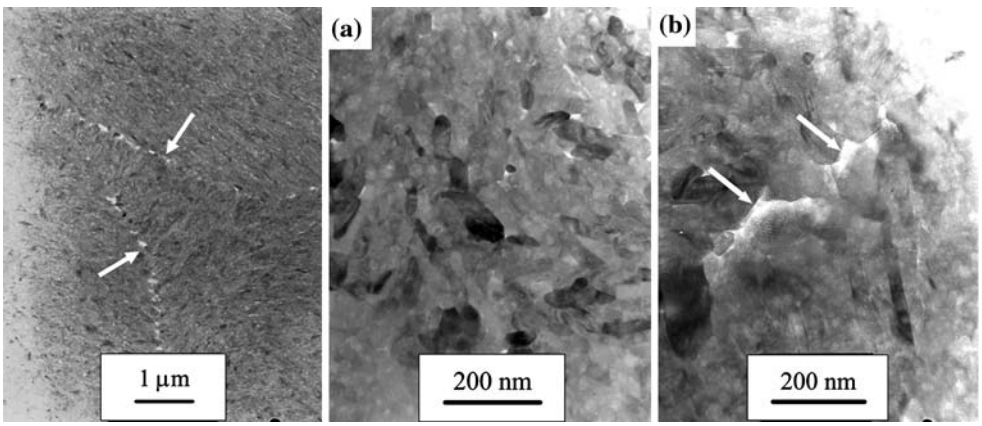

Fig. 3 Bright field TEM images of a sound enamel showing enamel microstructure with poorly defined sheath regions indicated by arrows. Insets showing (**a**) dense prism structure and (**b**) sheath region marked by arrows. TEM specimens prepared perpendicular to the direction of the prisms

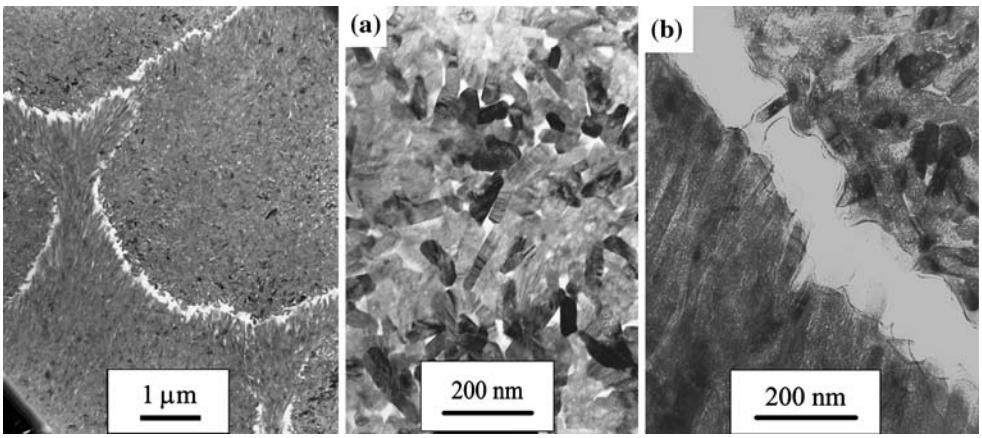

Fig. 4 Bright field TEM images of a hypomineralised enamel showing enamel microstructure. Insets showing (**a**) less dense prism structure and (**b**) sheath region filled with low density substance. TEM specimens prepared perpendicular to the direction of the prisms

the sound enamel in terms of crystal morphology, size and orientation, except that it appears to be less dense compared to the sound enamel. Regions of lower density, typically 20 nm or less across, are clearly evident among crystallites (Inset (a) of Fig 4). The sheath of the hypomineralised enamel is considerably wider (inset (b) of Fig 4), compared to the sound enamel. Partially mineralised crystallites covered with a thick 'coating' can also be seen in the sheath region. Low density substance appears to bridge the neighbouring prisms and lie between more clearly defined crystals adjacent to the sheath boundary. The size of the apatite crystals within the prisms for both types of enamel appears to be comparable.

4 Discussions

In this work, hypomineralised enamel was examined in comparison to sound enamel. This study represents the first site-specific TEM analysis of enamel microstructure, which is important from the perspective of understanding hypomineralisation-related microstructural changes, the structure and property relationship and treatment strategies for affected enamel.

Traditionally, the enamel sheath is considered to be 100–200 nm wide and consist predominantly of proteins [18]. A recent investigation using atomic force microscopy (AFM) has revealed the organic sheath to be 800–1000 nm in width [19]. However, this study suggests that a much thinner, if any, sheath (average thickness < 20 nm) is separating neighbouring prisms in sound enamel, very often interrupted by interconnecting apatite crystals. Such direct physical evidence challenges our current knowledge of the enamel sheath structure. Notably, most previous work has used acid etching to reveal the size of this region. It is speculated that the etching acid probably attacked the mineral at the peripheries of the prisms and extended the size of sheath region. In contrast, the preparation of TEM specimens using the FIB milling technique imparted minimal interference/damage to original enamel microstructure, making subsequent microstructural analysis more reliable. An earlier study had discerned only modest levels of anisotropy in fracture toughness of enamel along prism and across prism [20], which may result from the interconnective nature of adjacent prisms as seen in this study. In-depth study is needed to explore this finding more quantitatively.

Two significant changes in microstructure were found in the hypomineralised enamel;

- A less dense prism structure, i.e. loosely packed apatite crystals filled up with low density substance.
- A wider sheath region filled predominantly with a low density substance. Partially mineralised crystallites with a thick coating of a low density substance were also identified at the sheath boundaries.

Dental enamel development consists of two major stages; the formative secretory phase when the matrix is laid down and the maturation phase when the mineralisation process dominates [21]. The matrix architecture that is created during the secretory stage is probably the most crucial factor that determines crystal orientation and growth [22]. During the maturation stage, the apatite crystallites are understood to increase in both thickness and width, but not in length or in number [18]. Final maturation occurs as most of the proteins are removed from the enamel matrix, the ameloblasts disintegrate and the resulting enamel is fully mineralized with little remaining matrix. Given that the basic microstructure of the hypomineralised enamel is similar to the sound enamel (i.e. organised apatite crystals forming prisms), the resulting defects seen, i.e. the less dense prism structure and wider sheath regions, are most likely to arise from disrupted ameloblastic activity during maturation. It is speculated then that in these hypomineralised teeth, the removal of the residual enamel proteins (amelogenin, enamelin etc) may be incomplete [23, 24]. Further work to identify these proteins is required.

While the biological and physiochemical factors that cause this disruption remain as yet unclear, the consequences of these microstructural changes on mechanical properties of the affected tissue, particularly hardness and modulus, are quite obvious. The wider sheath region, combined with less dense prism structure, reduces the ability of enamel to resist deformation, and is ultimately responsible for the marked reduction in hardness and elastic modulus of the hypomineralised enamel [14]. Moreover, an investigation of the effect of microstructural changes upon the inelastic deformation of enamel has revealed a marked reduction in damage resistance of hypomineralised enamel [25].

This enhanced knowledge concerning the microstructural change in hypomineralised enamel improves our understanding of some of the problems associated with the clinical management of these teeth. In particular, the frequent occurrence of enamel fractures and inadequate retention of adhesive materials both of which are recognized as significant clinical challenges preventing successful restoration of these compromised teeth. It is known that organic matter such as proteins have poor acid solubility. The presence of increased amounts of organic matter in the hypomineralised enamel, specifically within both prism structure and sheath regions as demonstrated by the low density substance in TEM images, may inhibit the creation of an adequate etch profile which in turn compromises the adhesion between resin based restorative materials and the defective enamel [26]. Improved clinical outcomes are likely to depend, at

least in part, on the successful treatment of these proteins prior to any enamel etching or adhesive strategies.

5 Conclusions

Microstructure of sound and hypomineralised enamel was examined using TEM supported by a dual-beam FESEM/FIB system. Two marked changes in microstructure were identified in the affected enamel region; less dense prism structure and wider sheath regions. Such microstructural changes may reduce the ability of enamel to resist deformation, leading to a dramatic reduction in mechanical properties of the affected enamel. The degradation of structural integrity identified in this study is expected to inform more appropriate treatment strategies for restoring MIH affected dentitions.

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