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Rat Odontoblasts as Revealed by High-Pressure Freezing Followed by Freeze-Substitution

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Abstract *It is difficult to slam freeze odontoblasts that are surrounded by enamel organ and dental papilla, because they must first be mechanically exposed using a razor blade, which damage the cells. Furthermore, because the area optimally cryo-preserved is less than 10 μm below the freezing surface, it is difficult to obtain specimens without mechanical or ice crystal induced damages. In this study, high-pressure freezing, which permits cryo-fixation of tissues up to 0.5 mm thick, was used to cryo-preserve rat odontoblasts. This method permits optimal cryo-preservation without ice and mechanical cell damage to the entire odontoblast layer. This method produced marked qualitative differences with regard to the mitochondria and various membrane-bounded structures.*

Key words : High-pressure freezing, odontoblasts, mitochondria

INTRODUCTION

There are differences in the ultrastructure or localization of certain proteins and ions in specimens fixed by conventional chemical fixation and cryo-fixation[1～3]. Although fast-freezing/freeze-substitution best preserves the living state of tissues and/or cells, problems occurr during cryo-preservation of ameloblasts and/or odontoblasts. Slam and/or plunge freezing methods, which preserve tissue to a relatively shallow depth (10 μm), have been used to cryo-preserve these tall cells[4,5]. Because the cells must be mechanically exposed prior to cryo-fixation, however, mechanical damage is unavoidable with these freezing techniques[6]. Mechanical damage such as rupture of the cell membrane causes severe cell injury, with resulting ultrastructural changes of various organelles.

High-pressure freezing was recently used to immobilize cell and tissue components in their native state[7～15]. This freezing method permits optimal cryo-preservation to a depth of 0.5 mm and is able to vitrify a variety of large biologic specimens[7,8,13～15]. The present study investigated the rat odontoblast ultrastructure using this cryo-technique.

MATERIALS AND METHODS

Tooth germs of rat newborn upper and lower incisors were dissected and sandwiched between two dome-shaped golden sample holders and cryo-immobilized with a high pressure freezing machine (HPM010, Balzers). These samples were then freeze-substituted in anhydrous acetone containing 1% osmium tetroxide and/or 0.5% tannic acid at −80℃ for 48h. The temperature was then gradually allowed to rise to −30℃ over a period of 3 h and placed under a fume hood in a −30℃ acetone bath, which gradually warmed to room temperature over a period of 5h. The tissues were then washed in pure acetone and embedded in Taab 812 Resin.

RESULTS AND DISCUSSION

High-pressure freezing permitted optimal cryo-preservation without ice crystal damage to the whole layer of odontoblasts (Figs. 1～5). Although the ultrastructure of the various cell organelles was

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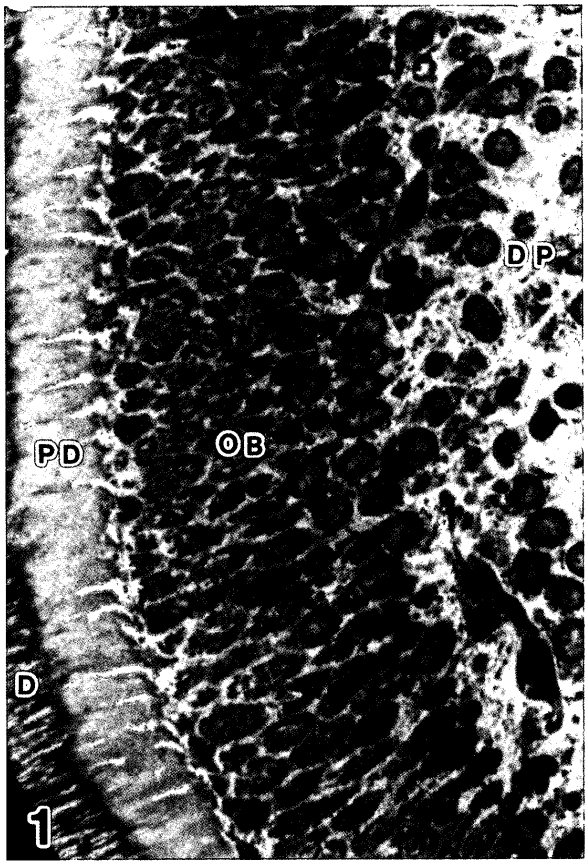

Fig. 1. Light micrograph of odontoblast layer (OB) processed by high pressure freezing/freeze substitution. DP : dental papilla PD : predentine D : dentine ×400

very similar to that in conventionally fixed specimens, there were pronounced qualitative differences with regard to the mitochondria and various types of membrane-bounded structures.

Mitochondrial ultrastructure was considerably different in unfrozen and frozen specimens. Characteristic features of chemically-fixed mitochondria indicated an open peripheral membrane space, open intracrystal space, and to a variable degree, angulation of the cristae (Fig. 6b). In contrast, mitochondria processed by high-pressure freezing were characterized by a smooth contour, electron-dense matrix, well-defined cristae, very narrowed intracrystal and peripheral membrane spaces (Figs. 3, 6a). The intracrystal spaces were usually observed as an electron-dense line (Fig. 6a). No mitochondrial granules were observed in mitochondria of the intact odontoblasts (Figs. 3, 6a), but many electron-dense granules appeared in mitochondria of mechanically damaged odontoblasts (Fig. 9).

Transport vesicles budding from the endoplasmic reticulum (ER) were often observed (Fig. 8b). The ER-derived vesicles fused with each other and formed vesicular tubular clusters (Fig. 8b). In the distal portion of the odontoblasts and the proximal parts of Tomes' processes, there were many large and small coated vesicles, dumb-bell shaped and/or roundish dense bodies, multivesicular bodies (electron dense and lucent), tubulo-vesicular structures, and various types of secretory granules (round, oval, cigar-shape, or fusiform) (Figs. 7a, b, 8a). Some granules contained a homogeneous electron-dense materials in which electron lucent structures comparable to the collagen fibrils in predentine were observed (Fig. 7b). Some granules included homogeneous materials of moderate opacity as well as intensely electron-dense particles (Fig. 7a). There were also granules containing filamentous or membranous structures (Fig. 7a).

Mitochondrial ultrastructure is proposed to provide a sensitive indicator of the quality of cryopreservation. Dalen et al. (1992)[16] have reported that examples of severe cryodistortions are manifested by large-ice crystal cavities in the cytoplasm and mitochondria with angular and complete collapse of the peripheral membrane and intracrystal spaces. In

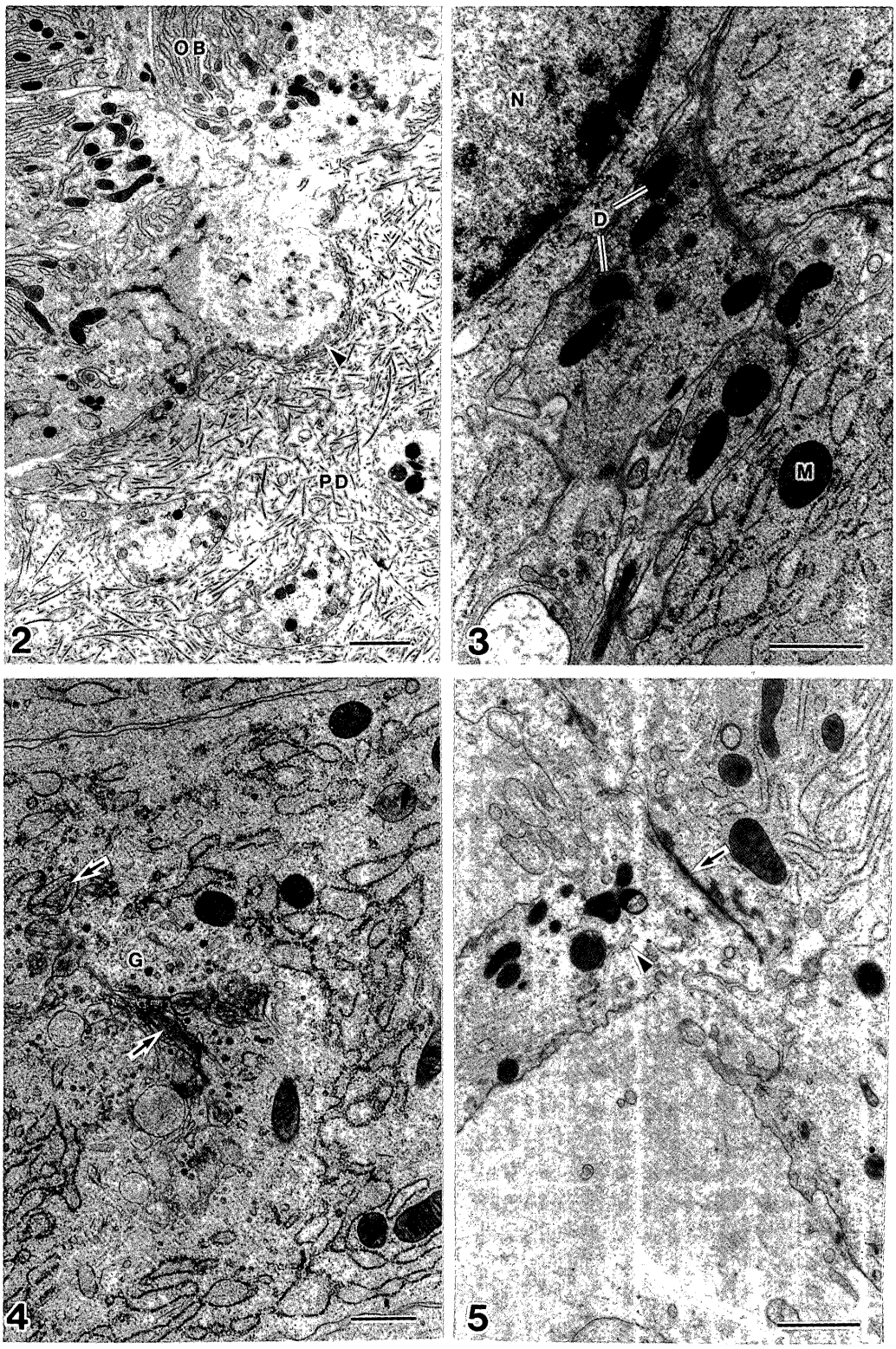

Fig. 2. Apical region of odontoblasts (OB) and predentine (PD). Various types of granules can be seen in the odontoblast processes. Note that there are many tubulo-vesicular structures adjacent to the plasmalemma (arrowhead). Bar=2 μm

Fig. 3. Proximal portion of odontoblasts. Electron dense mitochondria (M) and dense bodies (DB) can be observed. No reticulation is observed in the nucleus (N). Bar=500 nm

Fig. 4. Golgi region (G) in the supranuclear cytoplasm of the odontoblast. Golgi vacuoles contain filamentous structures (arrows). Bar=500 nm

Fig. 5. In the odontoblast process, multi-vesicular bodies, various forms of dense bodies and tubulo-vesicular structures (arrowhead) can be observed. The terminal web (arrow) is taken as the boundary between cytoplasm and odontoblast process. Bar= 1 μm

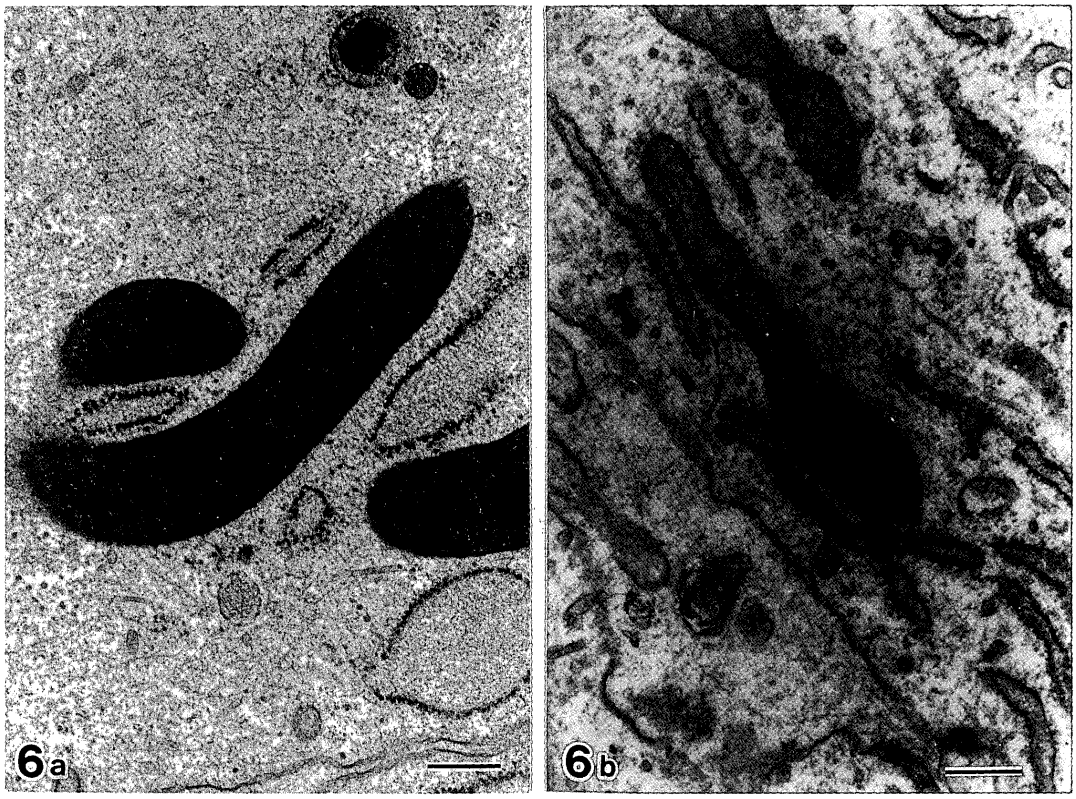

Fig. 6. a. A higher magnification view of mitochondria. Note that the mitochondria matrix has high electron density and that intracrystal and peripheral membrane spaces are very narrow. Bar=200 nm
b. Mitochondria processed by conventional chemical-fixation. Note that intracrystal spaces appear open. Bar=200 nm

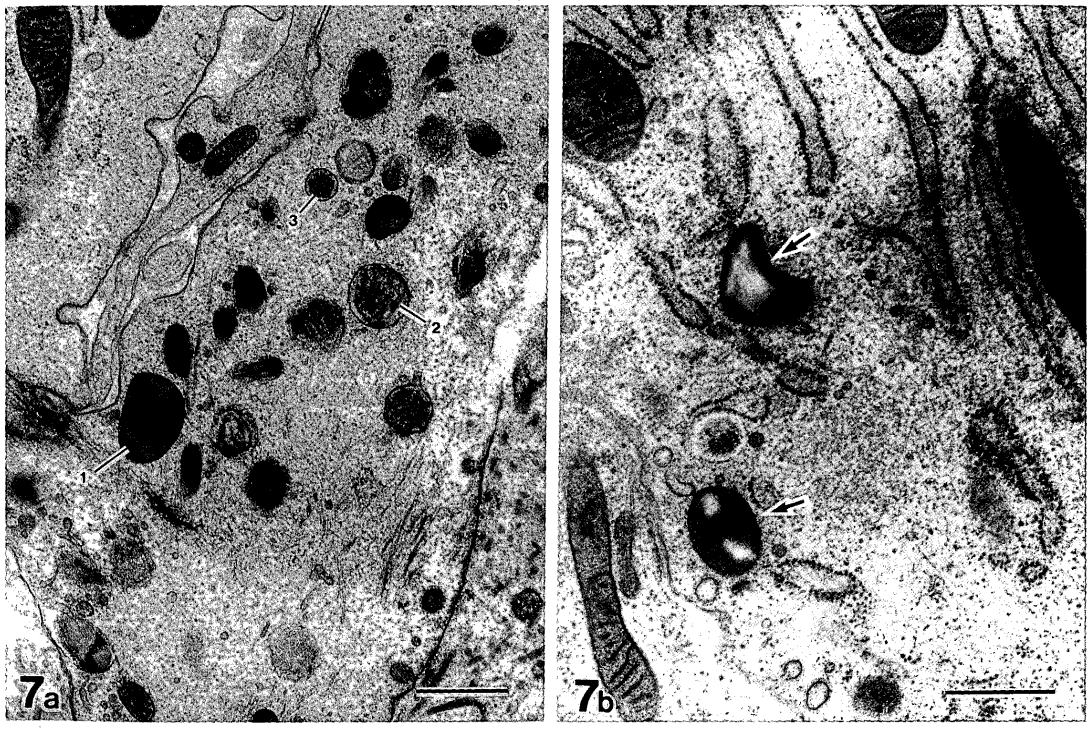

Fig. 7. Various types of membrane-bounded structures can be observed in the distal portion of odontoblasts.
a. There are at least three different types of granules, which contain almost homogeneous materials and some electron dense particles (type 1), filamentous structures (type 2), and membranous structures (type 3), respectively. Bar=500 nm
b. These granules contain a homogeneous electron dense material in which electron lucent structures are included. Bar=500 nm

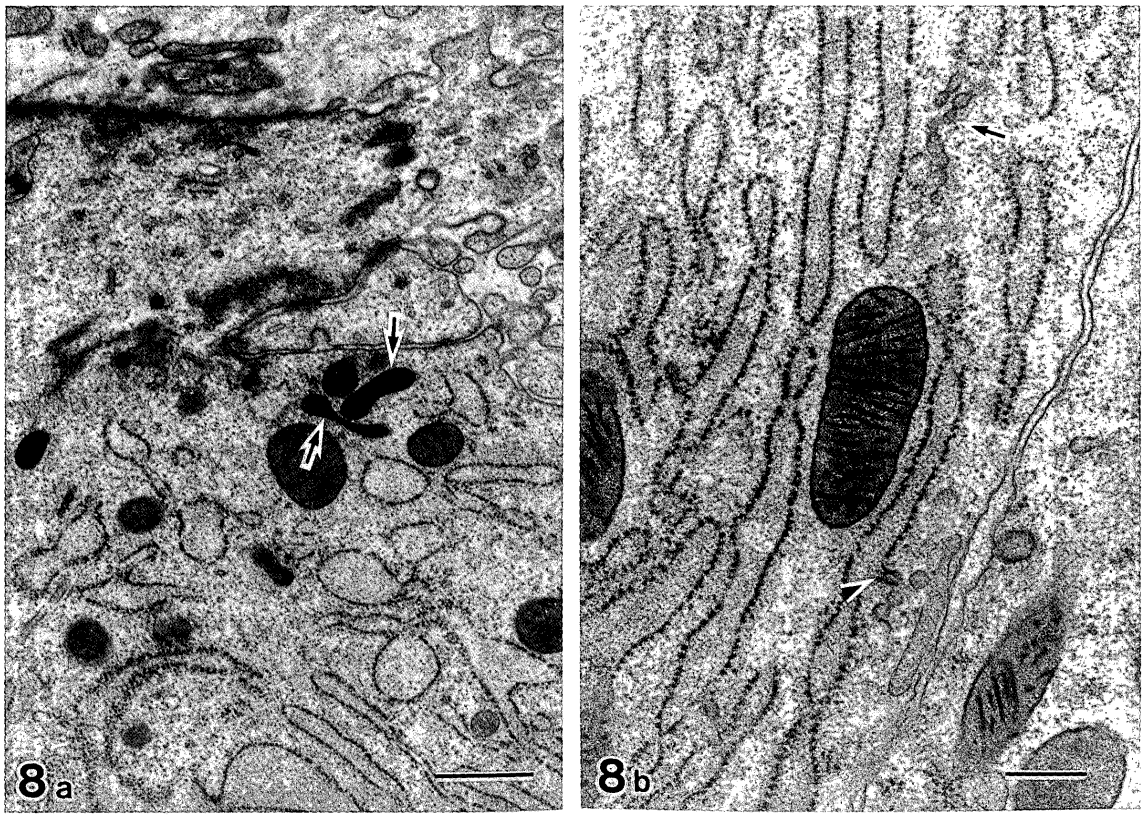

Fig. 8. a. Arrows indicate dumb-bell shaped dense bodies. Bar=500 nm
b. Transport vesicles budding from an ER exit site (arrowhead). Note that some vesicles fuse with each other (arrow). Bar= 200 nm

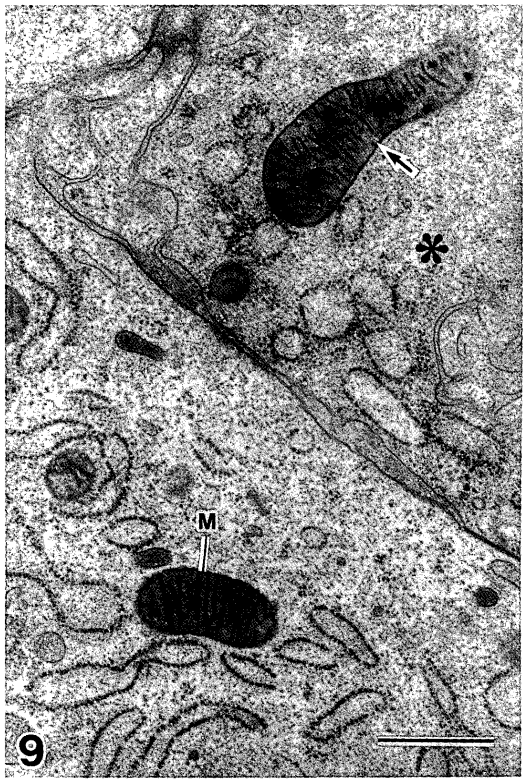

Fig. 9. Note that in mitochondria (arrow) of mechanically-damaged odontoblasts (asterisk) many mitochondrial granules, but that in mitochondria (M) of intact odontoblasts, granules are not seen. Bar=500 nm

specimens that were well cryo-preserved by slam and/or plunge freezing, mitochondria with open peripheral membrane and intracristal spaces are usually observed[17]. In contrast, mitochondria processed by high pressure freezing have very narrow peripheral membrane and intracrystal spaces[16]. The morphology of the phospholipids used to model membranes are drastically changed when frozen under high-pressure conditions[18]. In this study, characteristic features of the high-pressure frozen mitochondria were smooth mitochondrial contours, and narrow peripheral membrane and intracrystal spaces. Although the smoothness of the mitochondrial contours indicates that the cells were successfully cryo-preserved, it remains unclear whether the narrowness of the peripheral membrane and intracrystal spaces is an artifact caused by high pressure.

Mitochondrial granules are observed in various types of cells processed by chemical- and/or cryo-fixation[19~22]. Further, the presence of calcium-rich mitochondrial granules might be associated with mechanical cell damag[23]. In fact, the number of electron-dense mitochondrial granules increases by loading with divalent cations prior to fixation and/or by mechanical damage of cells[20,24]. In the present study, no

electron-dense mitochondrial granules were observed in intact odontoblasts, but they appeared in the mechanically-damaged odontoblasts. Although at physiologic levels, the intercellular calcium concentration is less than 10 mM, damage to the cell membrane causes an increase in intracellular calcium concentration. Kogaya et al. (2001)[22] reported that mitochondrial granules of various types of cells include sulfated substances and that the electron dense mitochondrial granules might be caused by an accumulation of large amounts of calcium ions that influx into mechanically-damaged cells.

Type I collagen fibrils are secreted by odontoblasts into the predentine matrix at the proximal portion of the odontoblast process, which is also a site of endocytosis of the organic matrix breakdown product[25]. Secretory granules transport not only collagen precursors but also phosphophoryn and proteoglycans. For secretory granules to release their contents by exocytosis, the membranes of the granules must be incorporated into the plasma membrane of the odontoblasts process. The excess membrane is disposed of large bristle-coated vesicles frequently observed adjacent to the plasmalemma of the process. In the distal regions of the odontoblasts, including their processes, various types of membrane-bounded structures, cigar-shaped or round secretory granules, dumb-bell shaped, or round lysosome-like dense bodies, are observed, thus representing the diversity of secretory substances contained in odontoblasts.

In conclusion, we used a high-pressure freezing technique to study the ultrastructure of odontoblasts, because the mechanical damage to the cells during tissue preparation is unavoidable with the slam and/or plunge freezing methods. The present study demonstrated that (1) high-pressure freezing permits optimal cryofixation without mechanical or ice crystal damage to the entire odontoblast layer, (2) high-pressure frozen mitochondria are characterized by a smooth contour, electron-dense matrix, well-defined cristae, and very narrowed intracrystal and peripheral membrane spaces, (3) electron dense mitochondrial granules are not observed in mitochondria of intact odontoblasts but many granules appear in the mitochondria of mechanically damaged odontoblasts, and (4) transport vesicles budding from endoplasmic reticulum are often observed.

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高圧急速凍結・凍結置換固定したラット象牙芽細胞

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キーワード：高圧急速凍結，象牙芽細胞，ミトコンドリア

抄録　エナメル器と歯乳頭に囲まれている象牙芽細胞を圧着法で凍結固定するためには，カミソリ刃を用いて細胞を露出しなければならない．この操作で象牙芽細胞は機械的な損傷を受けることになる．さらに適正に凍結固定される領域は圧着面から10μm以下であるので，機械的および氷結晶による損傷のない試料を得ることは極めて困難である．本研究では，0.5mm厚の組織の凍結固定が可能な高圧急速凍結法を用いてラット象牙芽細胞の微細構造を検索した．象牙芽細胞層全域で氷結晶や機械的損傷の無い凍結固定像が得られ，殊にミトコンドリアや膜周囲性構造物の微細構造に質的な違いが認められた．