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Improved Ultrastructural Preservation of Retinal Cells in *Drosophila melanogaster*

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초고압동결장치를 이용한 초파리 레티나 세포의 향상된 미세구조

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ABSTRACT

The *Drosophila* retinal cell is widely used to study cell development and cell signaling processes. In the past decades, conventional chemical fixation had been used to study the structure of retinal cells in *Drosophila*. Rapid freezing methods are superior to chemical fixation methods due to their fixation speed. Some *Drosophila* tissues, such as the eyes, should not be freezed due to their surrounding cuticle layer. Therefore, in the case of the *Drosophila* retina, the benefits of high pressure freezing and freeze substitution (HPF-FS) had not been verified. In this study, a retinal cell from *Drosophila melanogaster* had been studied by using the HPF-FS method. Compared to chemical fixation, the preservation of the cytoplasm in the HPF-FS sample was improved on the whole. The HPF-FS cell membranes were smoother than that of chemical fixation. In addition, HPF-FS preserved the mitochondria structures very well. These results of the present study suggest that HPF-FS is superior to other fixation methods for the preservation of the retinal cell structure.

Keywords : *Drosophila melanogaster*, High pressure freezing-freeze substitution, Retinal cell

INTRODUCTION

The cryo-fixation technique, which emerged in around 1980, has generally been proven to be a more effective preparation method than conventional chemical fixation (CF) for the preservation of cell structures (Dempsey & Bullivant, 1976; Ornberg & Reese, 1981; Ikeda et al., 1984; Steinbrecht & Zierold, 1984; Nagano & Kamimura, 1987; Studer et al., 1989; Monaghan et

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al., 1998; Murata et al., 2002; Muller-Reichert et al., 2003; Nitta & Kaneko, 2004). Freezing is a much better technique for preserving cell structures due to its speed of fixation. Fast freezing also freezes every cell component regardless of its chemistry (McDonald & Morphew, 1993; McDonald, 1994; McDonald, 1999). In contrast, glutaraldehyde, paraformaldehyde, osmium, and other chemical fixatives are selective in their cross-linking (Hayat, 2000). The high pressure freezing and freeze substitution (HPF-FS) method, which involves the formation of vitreous ice down to a depth of approximately 700 nm through quick freezing at a temperature of 251 K and a pressure of 2.1 kbar, is a type of cryo-fixation technique that is currently being exploited (Studer et al., 1989; Studer et al., 1995). Freezing under high pressure is capable of solidifying water rapidly as vitreous ice, an amorphous state of water, which physically preserves tissues.

The *Drosophila* retina had been used to study a broad range of topics, such as signal transduction, cell fate specification, morphogenesis and cell death. Over the past 40 years, extensive research investigating the ultrastructures of the *Drosophila melanogaster* retina has been thoroughly conducted (Perry, 1968; Ready et al., 1976; Tomlinson, 1988; Wolff and Ready, 1993; Kumar and Ready, 1995; Longley and Ready, 1995; Alone et al., 2005). However, the research into the ultrastructure has primarily focused on pre-fixation by glutaraldehyde, post-fixation with osmium tetroxide and dehydration with alcohol or acetone, etc. In other words, all such research had been conducted using CF methods at room temperature or at 4°C.

The HPF method can be expected to yield better preservation than the CF method, as already shown for *Drosophila* eggs, embryos and pupae (Schulte et al., 2003; Grosshans et al., 2005; Moussian et al., 2005). However, the facet eyes of adult *Drosophila* are difficult to freeze without damaging the cells, which may be due to the fluids surrounding the cells and cuticle layer (McDonald, 1994). Therefore, some question remains as to whether HPF-FS is truly effective with regard to the preservation of the cell organelles present in the retinal cells of *Drosophila melanogaster*. Therefore, we compared the preservation efficiency of CF and HPF-FS in *Drosophila* retinal cells in the present study.

MATERIALS AND METHODS

1. *Drosophila melanogaster* culture

Drosophila melanogaster strains (Oregon-R standard strain) were maintained on a standard diet [4.16% (w/v) corn powder; 4.16% (w/v) dextrose, 10% (w/v) yeast extract, 1.7% (w/v) agar] at 25°C with a 12-h/12-h light/dark cycle.

2. Conventional chemical fixation

The cuticle was peeled off from the retinas of adult flies 2~3 days after eclosion. The retinas were then fixed with 2.5% glutaraldehyde in 1% tannic acid (0.1 M cacodylate buffer) (PH 7.0) for 3 hours, and then in 2% osmium tetroxide for 2 hours, at 4°C (Walz and Baumann, 1989). Then, we stained *en bloc* with 2% uranyl acetate, followed by dehydration with a graded acetone series, and embedding into Spurr medium (Electron Microscopy System).

3. High pressure freezing and freeze-substitution

For the high-pressure freezing-freeze substitution (HPF-FS) method, we added one drop of 20% dextran onto each sample in order to fill any air cavities. We then carefully loaded the samples into a flat specimen holder. After the loading step, the samples were cryo-immobilized in HPF (EM PACT, Leica Microsystems, Vienna, Austria). The FS was conducted in dry acetone containing 2% osmium tetroxide. The substitution proceeded as follows: 8 hours at −90°C, heating at a rate of 5°C / hr to −60°C, 8 hours at −60°C, another heating

step at a rate of 5°C to −30°C, 8 hours at −30°C, followed by the subsequent transfer of the samples to room temperature at a rate of 5°C / hr (Harvey, 1982; Muller-Reichert et al., 2003). The samples were then embedded with a Spurr kit.

4. Electron microscopy

The samples were sectioned (60 nm and 250 nm) with an ultra-microtome (RMC MTXL) and double stained with uranyl acetate and lead citrate. The sections were viewed under a Tecnai 12 electron microscope (Philips, Netherlands) at 120 kV and under a high-voltage electron microscope (HVEM) at 1250 kV (Jeol JEM-ARM 1300S, Japan) and 1000 kV (Hitachi 1250M, Japan).

5. Statistical analysis (Quantification of changes in mitochondria)

To quantitatively examine the changes in mitochondria, the cristae surface area per mitochondria, and the mitochondrial surface area per cytoplasm were measured in 6 HPF (n=36) and 6 CF (n=36) retinas taken by HVEM. The HPF and CF mitochondria were traced and calculated using IMOD software. The IMOD software is the program for image processing, modeling and display developed by the Boulder Laboratory at the University of Colorado.

RESULTS

1. Overall structure of retinal cell

Drosophila retinas were peeled off from the cuticle layer and chemically fixed with aldehyde, tannic acid, and osmium acid. The structure was compared with those of retinas fixed using HPF-FS. In the chemical fixation method, membrane-bound organelles were rarely found and the cytoplasm was usually disrupted. On the other hand, these structures were well preserved in the HPF-FS sample. In addition, the variable elec-

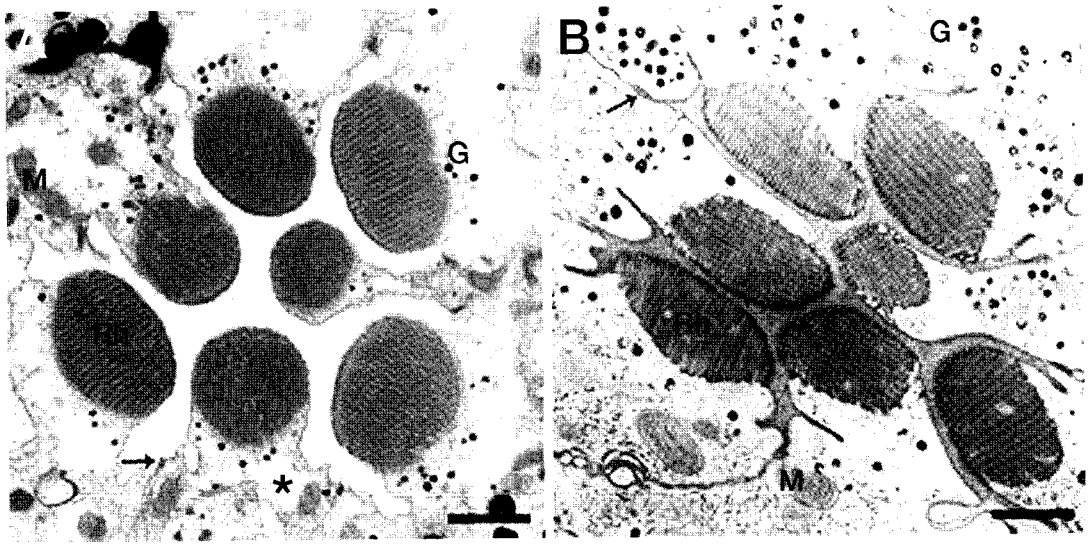

Fig. 1. Morphology of retinal cells in *Drosophila melanogaster* processed by CF (A), HPF-FS (B). Size Bar=1,000 nm, CF: Chemical Fixation, HPF-FS: High Pressure Freezer-Freeze Substitution. These images are tangential sections through a single adult ommatidium. There are seven cells in this section of ommatidium, and the cells were connected with adherens junction (arrow). Cells exposed to both mechanical stress and chemical solution (CF) were changed in their morphology (A, asterisk). The preservation of variable electron-dense structures, including the granule (G) and organelles such as the mitochondria (M), was improved in the HPF-FS cells (B). Rh-Rhabdomere.

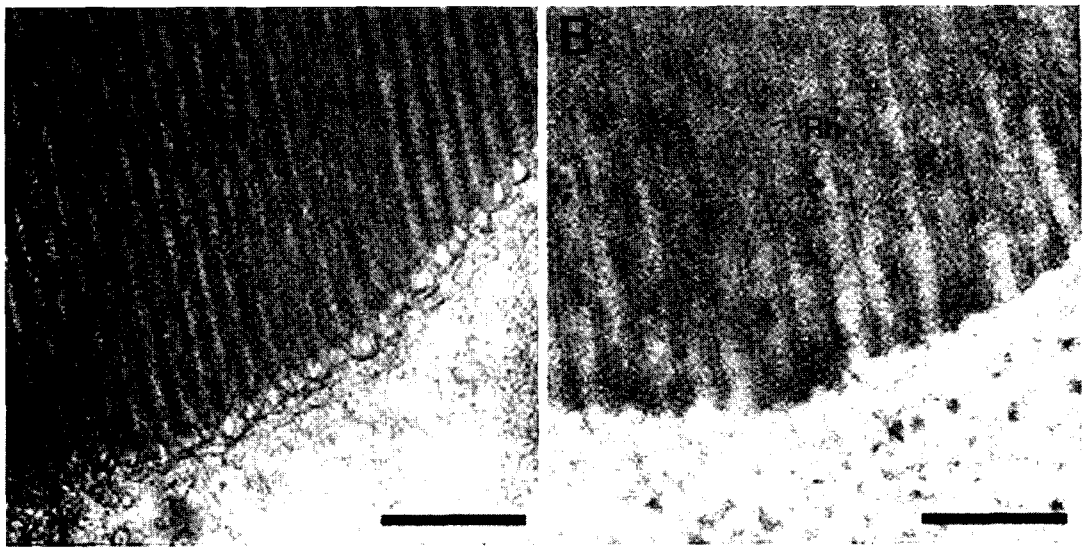

Fig. 2. The comparison of chemically fixed and HPF-FS fixed photoreceptors. These are longitudinally sectioned microvill. CF (A), HPF-FS (B). Size Bar=500 nm
The microvillus loop is more regular and wider than the chemically fixed microvillus.

tron-dense structures, including the granule and ribosome, were better preserved in HPF-FS than in CF. HPF-FS resulted in the preservation of a larger amount of ribosome than was observed when the CF method was used (Fig. 1 A, B).

2. Morphology of microvilli

The photoreceptor plasma membrane area, known as the rhabdomere, is an array of densely-packed microvilli (Wolff & Ready, 1993). We observed that the microvilli of rapidly frozen samples were distinctly different from those seen in the chemically-fixed cells. Figure 2 shows the longitudinally sectioned microvilli in the CF- and HPF-FS-fixed cells. In a chemically fixed retina, each microvillus is a cylindrical projection, approximately 0.05 um in diameter. However, the diameter of each microvillus in the HPF-FS fixed sample was about 0.07 um. Furthermore, the terminal loop was more regular in the HPF-FS-fixed microvilli than in the chemically fixed microvilli (Fig. 2. A, B).

3. Morphology of intercellular membrane

The HPF-FS freezing method employed in these experiments ultimately generated structures that were qualitatively different from those seen with CF. The HPF cell membranes appeared to be smoother than the CF cell membranes (Fig. 3. A, B). The thickness of this intact bio-membrane structure also appeared to vary from region to region. In addition, the structure of the junction was different depending on the fixation method employed.

4. Morphology of mitochondria

The mitochondria observed in the samples fixed with CF were smaller than those in samples fixed via HPF-FS (Fig. 4. A, B). The average areas of the individual mitochondria per cytoplasm in the CF, and HPF-FS fixed samples were $0.20 \pm 0.05\ \mu m^2$, and $0.26 \pm 0.08\ \mu m^2$, respectively. The mitochondrial surface area in the samples fixed via CF was determined to have shrunk by 20% more than the mitochondria of samples fixed using HPF-FS. In addition, the cryo-fixed mitochondria exhi-

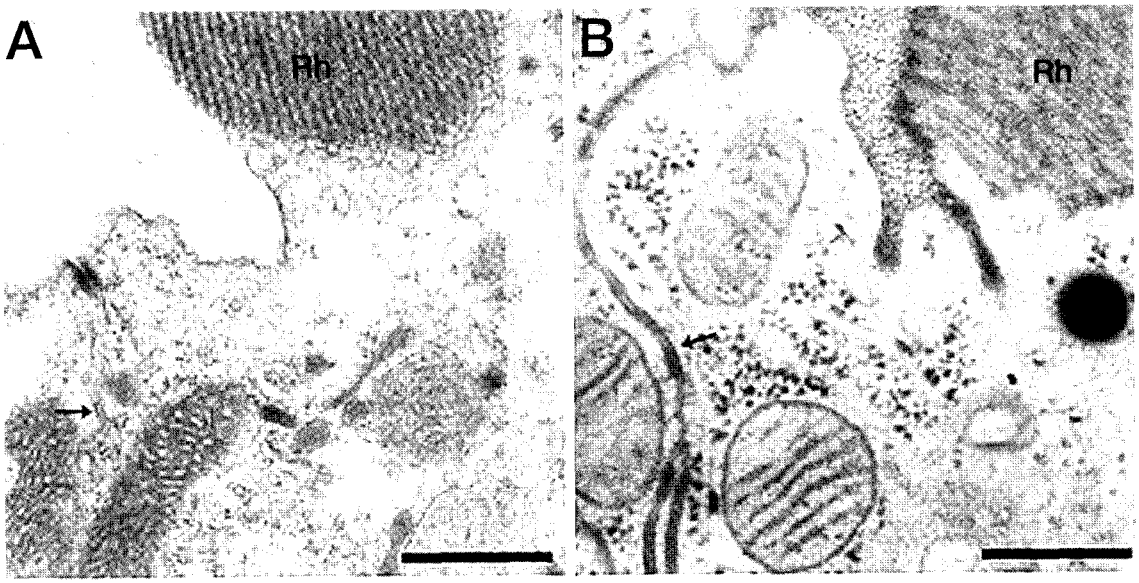

Fig. 3. Membrane comparison of CF and HPF-FS samples. The HPF cell membranes appeared to be smoother than the CF cell membranes. Membrane structures of various thicknesses appeared in HPF-FS samples (arrows). CF (A), HPF-FS (B). Size Bar=500 nm

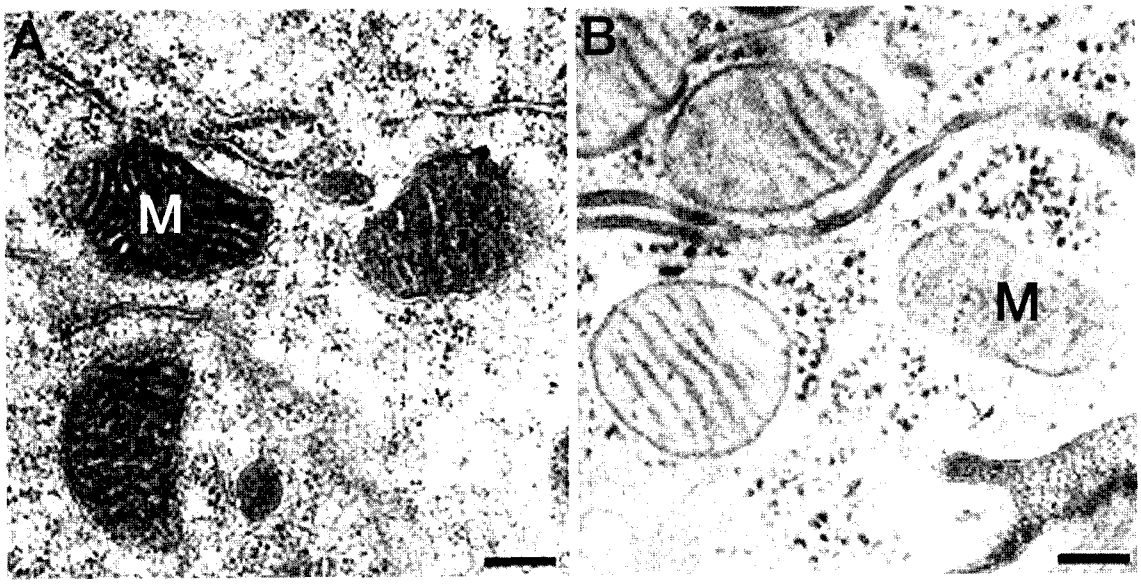

Fig. 4. The difference in mitochondria morphology between the samples according to the fixation method used, CF (A) and HPF-FS (B). Size Bar=250 nm. Mitochondrial surface area in CF sample was shrunken by 20% in comparison to that of the HPF-FS sample.

bited the same cristae morphology as the stacked lamellar cristae connected to the inner membrane. The outer and inner membranes were also closer together in the cryo-fixed mitochondria.

DISCUSSION

This paper provides the first detailed structural analysis of the retinal cell by cryo-fixation in *Drosophila*. Compared to CF, the HPF-FS method appears to be more effective in the preservation of the structure of the *Drosophila* retinal cell. Variable electron-dense structures, including the ribosome and lysosome, were well preserved in the sample fixed using HPF-FS. The higher number ribosomes observed in the frozen samples is thought to reflect a constant interaction and communication talking place between the membranes. This suggests that conventional fixatives may not penetrate rapidly enough to preserve the sub-cellular organelles.

Rapidly frozen microvilli show some differences in comparison to those seen in chemically fixed cells. The rapidly frozen microvillar loop is more regular and its diameter is wider in the rapidly frozen microvillus than in the chemically fixed microvillus. The rhabdomere is the photoreceptor light-sensing organelle that includes most substances that receive light and those that are involved in signal transduction. Susuki and co-workers reported that discrepancies in rhodopsin concentration seem to be primarily due to changes in the structure of the membrane (Suzuki, 1993). It has been said that the photoreceptive microvillus is a labile structure, accessible to the damaging effects of chemical fixatives. A similar result was reported in the squid (Walrond, 1992).

The HPF-FS cells showed smooth and rounded cell membranes, which are indicative of excellent preservation. In contrast, the same cell membrane was more wrinkled and damaged when preserved by the CF method. The solutions typically used for chemical fixation are hyper-osmotic, which can exacerbate tissue contraction and membrane ruffling. However, the high-pressure freezing method arrests tissues in a vitrified state.

Membranes of variable thickness were observed in the HPF-FS sample. The plasma membranes of various thicknesses that are enriched in steriol-sphingolopids are called lipid rafts. Lipid rafts range in diameter from 50 ~ 700 nm (Simons & Vaz, 2004).

We confirmed the possibility of the lipid raft-like characteristic of these structures by evaluating flotillin expression (Mun JY, unpublished). Flotillin, known as a family of lipid raft components (Perkins et al., 2003; Simons and Vaz, 2004), forms a stable structure in the plasma membrane and triggers signaling cascades (Sargiacomo et al., 1993). Recently, the dysregulation of flottilin has been closely related to the immunoreactivity of interommatidial cells and to the mass of cone cells or primary pigment cells (Bared et al., 2004). In *Drosophila*, as invertebrates, the lipid raft can be involved in the phagocytotic process and the internalization of the trans-membrane receptor-ligand complex (Harder et al., 1998; Hoehne et al., 2005). Using HPF-FS, membranes of various thicknesses resembling lipid rafts as an inter-photoreceptor signaling site can be easily detected.

Mitochondria are cytoplasmic organelles that are crucial players in numerous cellular processes, including bioenergetics, the metabolism of amino acids, lipids and iron, as well as in programmed cell death (apoptosis), differentiation, and aging. Mitochondria are dynamic organelles that constantly fuse and divide. These mitochondrial dynamics play a role in mitochondrial and cellular functions. Mitochondrial functions are affected by changes in mitochondrial membrane topology. Mitochondria are abundant in photoreceptors, and it has been reported that mitochondrial damage is a factor in photoreceptor degeneration. Mitochondrial defects, such as deletions in mtDNA (mitochondrial DNA), are abnormally frequent in the degenerating retina (Moraes et al., 1991). Recent studies have shown that somatically acquired mutations, such as the deletion of mt-

DNA, are caused by oxygen damage or UV irradiation during the life of the individual. The accumulation of these somatic mutations in postmitotic cells, such as neurons, causes bioenergetic deficiency leading to age-associated dysfunction of cells and organs (Bravo-Nuevo et al., 2003). An accelerated accumulation of mt-DNA fragmentation leads to premature aging and/or degenerative retinal disease. These diseases include kerns-sayer, MEALS and PEO. Diseases that occur due to the deletion or mutation of mitochondrial DNA usually show structural abnormalities of mitochondria, which may be a useful clue for the detection of these diseases. We compared the ultrastructure of mitochondria in photoreceptor cells prepared by cryofixation, followed by freeze substitution with those in cells prepared conventionally, and we found important similarities and significant differences between the two groups. Considering that cells may shrink by approximately 20～70% during dehydration together with the infiltration of the embedding media (Lee, 1984), our result suggests that CF has a more profound effect on cell deformation than the other method. While CF might result in the constriction of mitochondrial membranes or the distortion of cristae, HPF-FS appears to preserve the structure of mitochondria very well. A similar phenomenon has been reported in quick-frozen frog photoreceptor cells (Willson 1998). Therefore, the HPF-FS method must be used to correctly diagnose mitochondrial diseases.

In conclusion, the HPF-FS technique was determined to be an excellent method for the preservation of the photoreceptor structures, including the membranes, cell organelles inside the cytoplasm, inclusions, granules and intercellular membranes.

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<국문초록>

정확한 세포의 구조 분석을 위해서는 조직을 가능한 한 자연 상태 그대로 보존하는 것이 무엇보다 중요하다. 그러나 지금까지 이용되고 있는 화학적인 고정방법은 조직의 변형을 유도하는 것으로 알려져 그 해결방법에 관한 연구가 활발히 진행되고 있다. 그 연구의 결과로 현재 급속 동결법이 제시되었고, 그 중 초고압동결법(high-pressure freezing method)는 가용 두께가 200 μm로서 10~15 μm정도인 침윤동결법(plunging method) 혹은 접촉동결법(slamming method)보다 우수한 방법으로 보고되고 있다.본 연구팀에는 노랑초파리(*Drosophila melanogaster*)의 레티나를 화학고정법과 고압동결법으로 고정하여 미세구조를 비교하였다. 먼저 120 kV 전자현미경을 이

용하여 각 세포 소기관을 비교하였고, 그 중 미토콘드리아의 형태변화를 좀 더 자세히 비교하기 위하여 초고압 전자현미경을 이용하였다. 그 결과 급속동결 세포의 세포막과 미토콘드리아의 고정에서 특히 차이가 있음을 알 수 있었고, 이는 주로 탈수에 의한 구조변형 때문인 것으로 추측된다.