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Ultrastructural Changes in the Rat Corneal Endothelium Preserved at Low Temperature

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The ultrastructural differences in rat corneal endothelia preserved at 0˚C and 4˚C were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Some corneas were preserved as whole globes in an eye preservation medium (EP-II) either at 4˚C or at 0˚C for 1, 2 and 7 days. The specimens were prepared for SEM and TEM according to routine methods. For all storage times, the endothelium preserved at 0˚C showed less irregularity of the cell surface, swollen mitochondria or vacuoles than that at 4˚C. A significant difference in structural changes between the endothelium preserved at 4˚C and 0˚C was seen after 7 days' storage. The former showed destruction of the surface cell membrane and degenerated cell organella, while the latter maintained the structure of the surface cell membrane and cell organella. These findings suggest that the lifetime of a rat endothelium preserved at 0˚C is longer than that at 4˚C.

Key words: corneal endothelium; low temperature preservation (0˚C); scanning electron microscopy; transmission electron microscopy

Since Filatov (1935) succeeded in transplanting stored human corneas, corneal preservation techniques have markedly improved (Doughman, 1988; Wilson and Bourne, 1989). From the 1930s to the 1970s, the corneas from whole eye balls were stored in moist chambers for 24 h at 4˚C (Bourne, 1991).

Although a lot of effort was made to develop an effective storage medium, less attention has been paid to the temperature at which the corneas are kept. Cryopreservation techniques, by which the corneas are preserved at –196˚C, were introduced for prolonged storage (Capella et al., 1965), but they are not widely used today because of the requirement of special expensive equipment and trained technicians (McCarey and Kaufman, 1974).

It is well known that distilled water freezes at 0˚C. However, the freezing-points of cells and tissues are below 0˚C because they contain proteins, sugars and lipids. Judging from the freezing temperature of other organs (Wicomb and Cooper, 1984; Storey and Storey, 1990; Yoshida et al., 1999), it is obvious that the cornea does not freeze at 0˚C. Accordingly, we tried to preserve rat corneas at 0˚C and examined the ultrastructural changes in the endothelium.

Today, most eye banks in the United States store isolated corneas at 4˚C (Bourne, 1991), but in Japan there are still many eye banks which preserve corneas as whole globes in a storage medium (EP-II) (Manabe et al., 1984) at 4˚C and their storage periods are limited to within 48 h.

In this study, we tried to preserve rat corneas as whole globes with storage medium at 0˚C to prolong the storage period. We compared the ultrastructural preservation between corneal endothelia stored at 4˚C and those stored at 0˚C using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy

Materials and Methods

Fourteen adult female rats, weighing 145 to 330 g, were used in this study. All experiments conformed to the Association for Research in Vision and Ophthalmology Resolution on the Humane Use of Animals in Research. After the animals were anesthetized by an intraperitoneal injection with sodium pentobarbital (50 mg/kg body weight), they were sacrificed by removal of blood from the right auricle. The eye globes were immediately enucleated and were stored in glass vials containing 15 mL of a storage medium (EP-II, Kaken Pharmaceutical Co., Tokyo, Japan). The vials were stored either at 4˚C or 0˚C in a special refrigerator regulated by thermomodules (NH-60, Ninomiya Co., Ltd., Chiba, Japan). After preservation for 1, 2 and 7 days, the corneas were excised with a 1- to 2-mm scleral rim to make corneal buttons. After fixation with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 2 h, the cornea was cut into 2 pieces: one for SEM and the other for TEM. A freshly excised cornea was used as a control.

For SEM, the specimens were washed in a buffer and postfixed in buffered 1% osmium tetroxide for 2 h. After washing again in the buffer, they were conductive-stained with 1% tannic acid and 1% osmium tetroxide (Murakami, 1974). They were then dehydrated in a graded series of ethanol, substituted with *t*-butyl alcohol, and finally freeze-dried (Inoué and Osatake, 1988). The dried specimens were sputter-coated with platinum and observed with a scanning electron microscope (HFS-2ST, Hitachi Ltd., Tokyo) operated at 8 to 25 kV.

For TEM, the specimens were rinsed overnight in a buffer containing 7.5% sucrose, and postfixed in buffered 1% osmium tetroxide for 2 h. They were washed again in the buffer, dehydrated in a graded series of ethanol, and finally embedded in epoxy resin. Ultrathin sections were cut using an ultramicrotome (Ultracut UCT, Leica, Wien, Austria), stained with tannic acid, uranyl acetate and lead citrate (Kajikawa et al., 1975), and examined in a transmission electron microscope (100 CX II, JEOL Ltd., Tokyo) at 100 kV.

Results

Normal corneal endothelium

The corneal endothelium, comprising a single layer of hexagonal cells of 15 to 20 µm wide and 2 to 3 µm thick, was lined with a dense basal lamina, referred to as Descemet's membrane, of approximately 4 µm thick (Figs. 1a and b). The free surface was generally smooth except for cell boundaries, scattered microvilli and single cilia. The cell boundaries were slightly elevated forming hexagonal ridges. The cells included the attenuated nucleus and spherical mitochondria (Fig. 1c). On the free surface of the cell membrane, pinocytotic vesicles were frequently visible. The intercellular space was deeply invaginated between adjacent endothelial cells, and the zonula occludens was usually visible at the apical portion.

Corneal endothelium preserved at 4˚C

After 1 day's storage, the cell boundaries of the corneal endothelium slightly protruded and became somewhat indistinct (Fig. 2a). Most of the mitochondria were swollen and their cristae were indistinct. The zonula occludens was intact. The density of the cytoplasmic matrix was almost the same as normal endothelial cells, but had slightly decreased in the basal region of the cells (Fig. 2b).

After 2 days' storage, the endothelial surface showed prominent irregularity and the cell boundaries became indistinguishable (Fig. 3a). The mitochondria showed marked swelling accompanied by complete disruption of the cristae. The zonula occludens was intact. The cytoplasmic matrix showed a low-density compared with normal endothelial cells (Fig. 3b).

After 7 days' storage, the corneal endothelium showed marked structural damage. The cell surface became very irregular and destruction of the cell membrane was evident (Fig. 4a). Severe damage to intracellular structures was clarified by TEM (Fig. 4b): intracellular structures were unidentifiable except for the nucleus.

The surface cell membrane was disrupted and fragments of clumped chromatin and some other cell organellae protruded out of the cell.

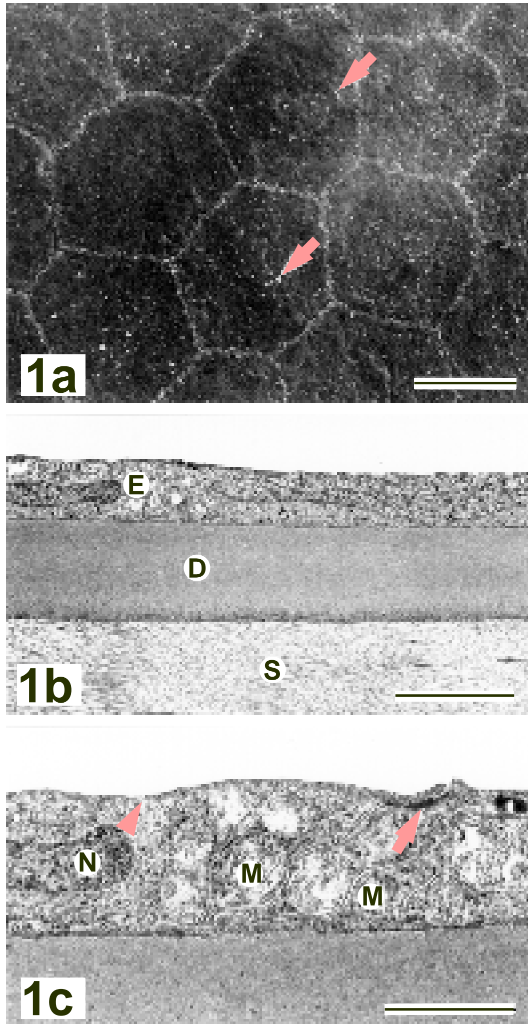

Fig. 1. a: Scanning electron micrograph showing the surface of a normal rat corneal endothelium. The cell surface is generally smooth, except for elevation of the hexagonal cell boundaries. Arrows: single cilia (bar = 10 µm). **b:** Low-magnification transmission electron micrograph of a normal rat corneal endothelium. D, Descemet's membrane; E, corneal endothelium; S, stroma (bar = 5 µm). **c:** High-magnification transmission electron micrograph of a normal rat corneal endothelium. Note the slightly elevated cell boundaries and zonula occludens at the apical side of the intercellular space (arrow). M, mitochondria; N, nucleus; arrowhead, pinocytotic vesicle (bar = 2 µm).

Corneal endothelium preserved at 0˚C

After 1 day's storage, the surface of the endothelium was almost the same as that stored at 4˚C. The cell boundaries appeared more natural than those preserved at 4˚C (Fig. 5a). Although the mitochondria showed slight swelling, other intracellular organellae were relatively well preserved. The cytoplasmic density was almost the same as the normal endothelium (Fig. 5b). The zonula occuludens was intact.

After 2 days' storage, the surface of the endothelium became irregular, as observed in the specimen preserved at 4˚C. The cell boundaries became less distinct than those preserved for 1 day. Some microvilli were elongated (Fig. 6a). The mitochondria became swollen, but their cristae were partly preserved. The rough-surfaced endoplasmic reticulum was relatively well-preserved and the zonula occludens was also intact. The cytoplasmic density was almost the same as the normal endothelium (Fig. 6b).

After 7 days' storage, the surface cell membrane of the endothelium was almost intact, but the microvilli on the peripheral margin of the endothelium were markedly elongated (Fig. 7a). The mitochondria were vacuolated and no cristae were visible. The rough-surfaced endoplasmic reticulum showed swelling. The density of the cytoplasmic matrix decreased in the basal region, but no significant changes were noted in the apical region of the cells (Fig. 7b). The zonula occludens was well preserved.

Discussion

Since Filatov (1935) preserved whole eyeballs in an ice chest, hypothermic preservation of corneas at 4˚C has provided the basis of tissue storage for clinical use. Preservation at reduced temperatures prolongs in vitro survival by slowing metabolism, reducing the demand for oxygen and other metabolites, and by conserving chemical energy (Taylor and Hunt, 1989a). Although cryopreservation at

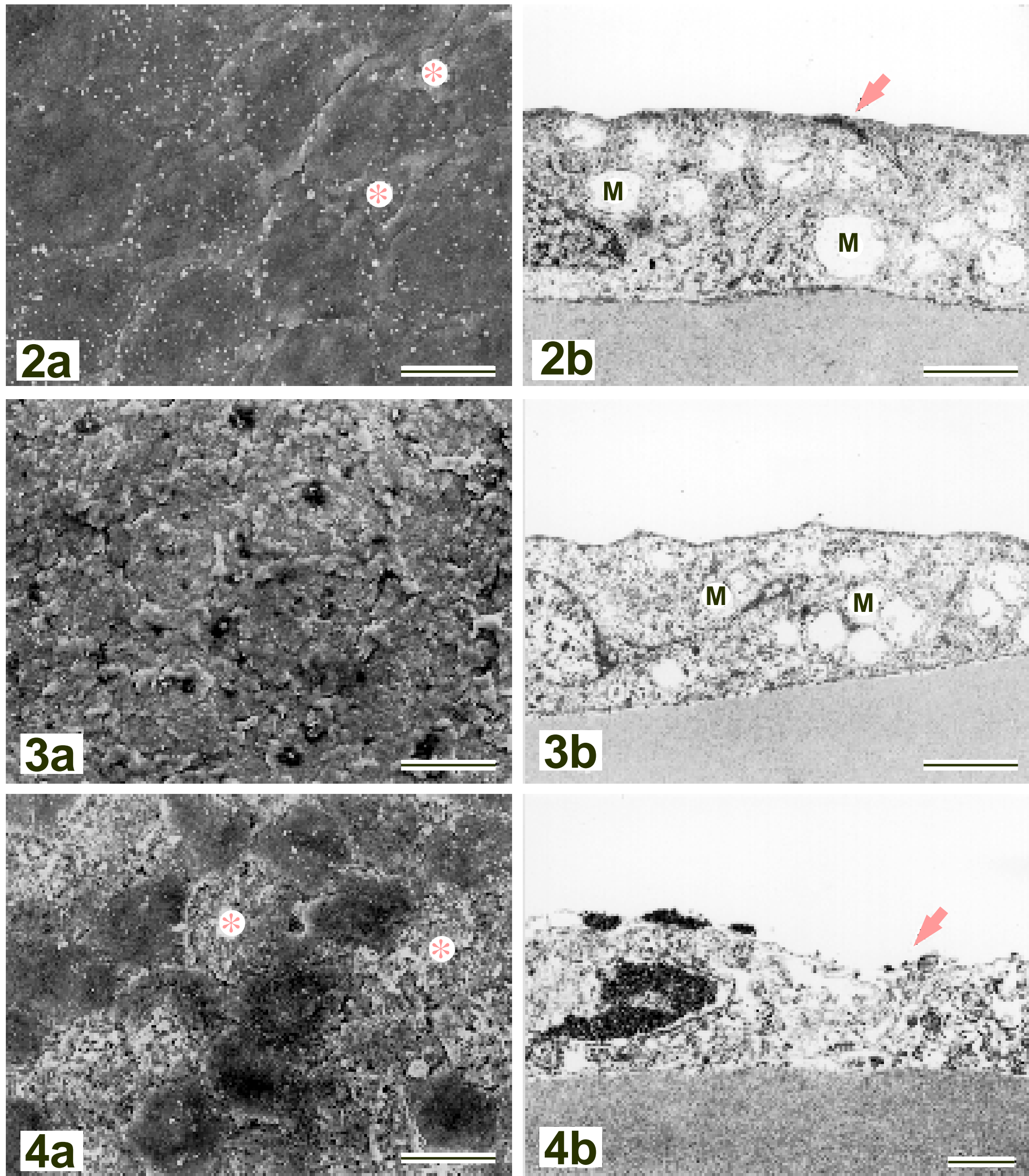

Fig. 2. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 4˚C for 1 day.
a: The cell boundaries protrude slightly (*) and become somewhat indistinct (bar = 10 µm).
b: Most of the mitochondria (M) are swollen and their cristae become indistinct. The zonula occludens (arrow) is intact. The density of the cytoplasmic matrix slightly decreases in the basal region of the cells (bar = 1 µm).

Fig. 3. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 4˚C for 2 days.
a: The cell surface shows prominent irregularity and the cell boundaries cannot be distinguished (bar = 10 µm).
b: Mitochondria (M) are markedly swollen and the cristae are completely disrupted (bar = 2 µm).

Fig. 4. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 4˚C for 7 days.
a: The cell surface shows high irregularity and the surface cell membrane is destroyed (*) (bar = 10 µm).
b: The surface cell membrane is broken and some cell organellae protrude out of the cell (arrow). Note the marked degeneration of the nucleus and cell organella (bar = 1 µm).

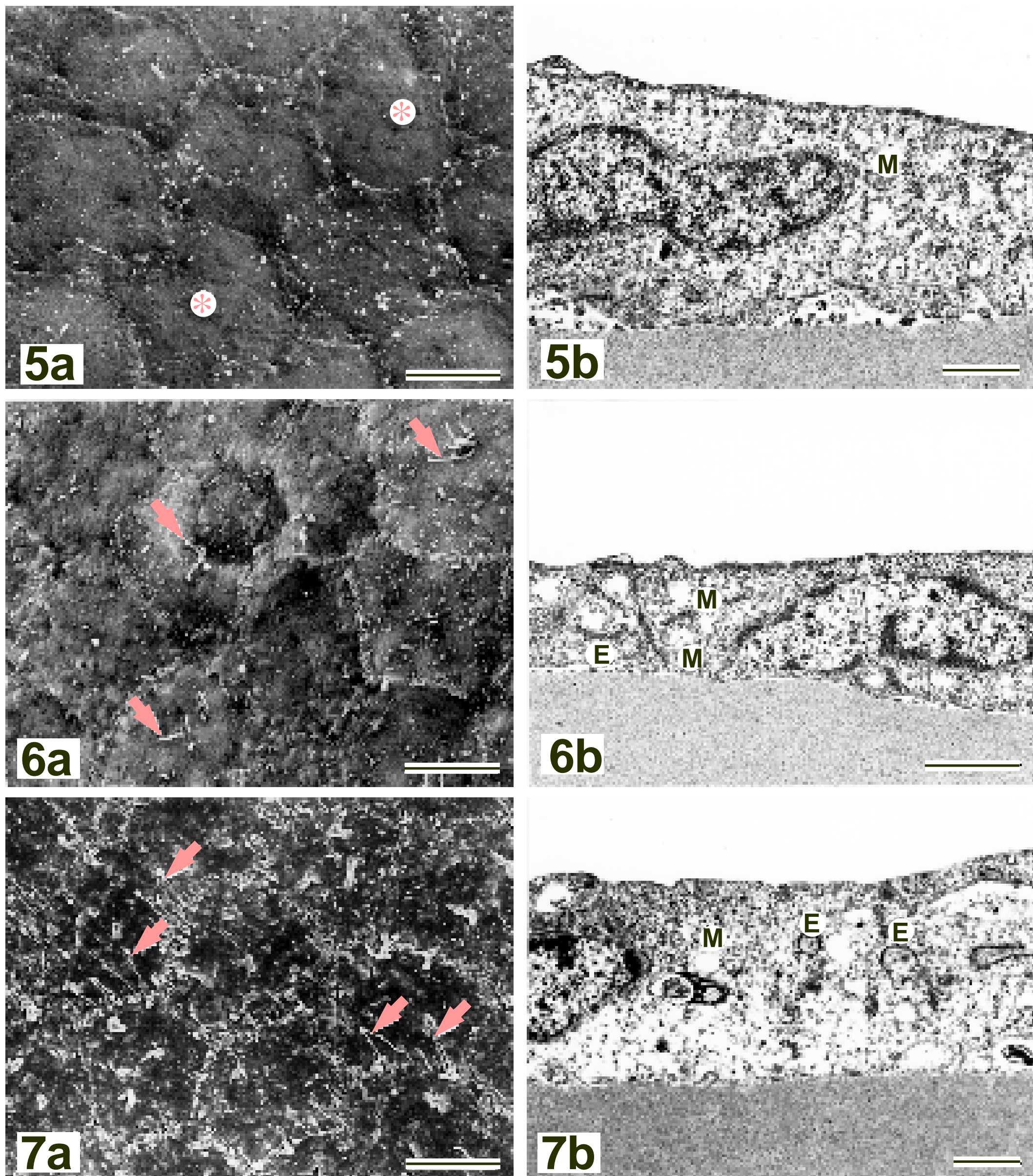

Fig. 5. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 0˚C for 1 day.
a: The cell surface protrudes slightly (*) and the cell boundaries are clearly visible (bar = 10 µm).
b: Mitochondria show slight swelling (M) and other intracellular organellae are relatively well preserved (bar = 1 µm).

Fig. 6. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 0˚C for 2 days.
a: The cell surface is irregular and the cell boundaries are just recognizable. Some elongated microvilli (arrows) are observed (bar = 10 µm).
b: The mitochondria (M) become swollen, but their cristae are partly preserved. The rough-surfaced endoplasmic reticulum (E) is relatively well preserved (bar = 2 µm).

Fig. 7. Scanning (**a**) and transmission (**b**) electron micrographs of a rat cornea preserved at 0˚C for 7 days.
a: The cell surface is almost intact. The microvilli on the peripheral margin of the endothelium are markedly elongated (arrows) (bar = 10 µm).
b: The mitochondria are vacuolated and no cristae are visible (M). The rough-surfaced endoplasmic reticulum is swollen (E). The density of the cytoplasmic matrix decreases in the basal region of the cells (bar = 1 µm).

–196˚C made it possible to prolong storage periods for up to a year or longer, freezing and thawing cause structural damage to the endothelium (Capella et al., 1965; Van Horn et al., 1972; Doughman, 1988). Thus, it is logically assumed that organs should be stored at the lowest temperature possible without freezing (Yoshida et al., 1999).

All organic matter has its own specific subzero freezing temperature: –1.0˚C in the rat liver (Yoshida et al., 1999), –0.6˚C in the rat heart (Wicomb and Cooper, 1984) and –0.8˚C in human plasma (Storey and Storey, 1990). Since the rat corneal endothelium is very thin, it is impossible to measure its freezing point with a thermometer. In this study, we tried to preserve corneas at 0˚C, assuming that corneas do not freeze at this temperature.

Previously, Taylor et al. (1989a,1989b) stored rabbit isolated corneas at 0˚C. They reported that the endothelial ultrastructure was maintained during storage for 3 and 5 days in a hyperkalemic solution, CPTES*. According to their transmission electron microscopic findings, the mitochondria showed moderate swelling with a diffuse pallid matrix, as well as condensed and beaded forms. The smooth endoplasmic reticulum was swollen and vesiculated. Their ultrastructural findings were almost identical to the present study. According to their method, polypropylene vials containing a preservation medium were placed in an evacuated Dewar flask containing ice in a 4˚C refrigerator. In this study, we used a special refrigerator that can precisely control the temperature from room temperature to –5.0˚C at 0.1˚C intervals (Yoshida et al., 1999).

Nowadays, Optisol (Steinemann et al., 1993) and Dexsol (Skelnik et al., 1988; Lass et al., 1990) (Chiron Co., Irvine, CA) are used by most eye banks in the United States as solutions for preserving isolated corneas at 4˚C. These solutions have enabled their preservation for up to 1 week (Bourne, 1991). Although the corneoscleral preservation has increased in Japan, it has not been widely used all over the world (Shimazaki et al., 1993). In this study, we preserved rat whole globes at 0˚C in EP-II, which has been generally used for whole globe preservation in Japan.

Fluid movement in the corneal endothelium is thought to be related to a pump-leak transport mechanism in which fluid from the anterior chamber leaks into the stroma across a leaky apical junction, while fluid is actively pumped from the stroma into the anterior chamber (Barry et al., 1995). The Na^+-K^+ ATPase pump of the corneal endothelium has been described by many investigators: the pump is located in the lateral membrane and actively transports sodium and bicarbonate ions into the anterior chamber; along this osmotic gradient, water moves from the stroma to anterior chamber (Klyce and Beuerman, 1988; Dohlman, 1994; Edelhauser et al., 1994; Barry et al., 1995). Hodson (1971) demonstrated that perfusion with a bicarbonate free medium caused reversible stromal swelling. Thus, it was presumed that the pump was partly inactivated by the removal of bicarbonate (Hodson, 1971). Since EP-II does not contain bicarbonate ions, it extends the lifetime of the corneal endothelium by controlling pump function consumption.

The present scanning electron microscopic study showed no significant ultrastructural differences in the endothelial surfaces of corneas preserved at either 4˚C or 0˚C for up to 2 days. After 7 days' storage at 4˚C, the cells showed marked destruction (Fig. 4a), but were fairly-well preserved at 0˚C. Furthermore, transmission electron microscopic findings indicated that the corneal endothelium preserved at 4˚C showed more distinct mitochondrial swelling than that preserved at 0˚C. These changes became evident after 7 days' storage (Figs. 4b and 7b). Ultrastructural changes were also noted in the corneas preserved at 0˚C: the presence of vacuoles, condensation of the mitochondrial matrix, a swollen rough-surfaced endoplasmic reticulum and a decrease in the cytoplasmic density in the basal region. However, most of the surface cell membrane and cell organellae were retained (Fig. 7b). The density of the cytoplasmic matrix decreased in the basal re-

*CPTES: corneal-potassium-TES, a potassium-rich balanced salt solution containing the impermeant biological buffer compound N-Tris(hydroxymethyl)methyl-2-amino ethane sulphonate.

gion of the endothelia preserved for 1 and 2 days at 4˚C or 7 days at 0˚C (Figs. 2b, 3b and 7b). This may have been due to the leakage of fluid into the paracellular space across the leaky apical junction.

There were fewer ultrastructural changes in the corneal endothelia preserved at 0˚C than those at 4˚C, which are thought to be reversible. Since the endothelial metabolism below 0˚C is reduced in comparison with that at 4˚C, consumption of ATP and activity of the Na^+-K^+ ATPase pump are minimized in an endothelium preserved at 0˚C. Recently, Yoshida (1999) reported that the concentration of ATP in the rat liver preserved at –0.8˚C was higher than that at 4˚C. Thus, the lifetime of the endothelial cells is extended if they are preserved at 0˚C.

The utilization of corneas preserved as whole globes at 4˚C is restricted to within 48 h, because the endothelium is exposed to stagnant aqueous humor which has metabolic waste products and tissue necrosis (Bito and Salvador, 1970; McCarey and Kaufman, 1974). The present findings indicate the possibility for longer preservation of whole globes if they are stored at 0˚C.

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