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Original Article

The injection of DisCoVisc into the anterior chamber improved corneal preservation and transplantation for cornea blind patients

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Abstract: Purpose: In this study, we aimed to provide a new method of corneal preservation by injecting DisCoVisc into the anterior chamber of eyeballs and evaluate its efficiency for corneal transplantation. Methods: Three pairs of eyeballs (n=6) were preserved by DisCoVisc viscoelastic agent, and the corneas were stored for 1 to 6 days. Then, the structure and morphology of cornea were analyzed by hematoxylin-eosin (H&E) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and transmission electron microscopy (TEM). The corneas preserved by this method were transplanted into 15 patients with corneal disease and the efficacy was assessed. Results: Epithelial cells and endothelial cells were intact and a normal morphology was preserved in both fresh corneas and corneas stored for 1-6 days by DisCoVisc viscoelastic agent. Corneal endothelial cells did not appear apoptosis in fresh corneas and corneas stored for 1-3 days, whereas a few apoptosis positive cells were shown on the 4th day. The results of TEM showed that all corneas had active corneal endothelial cells with normal nuclei and homogenous nucleoplasm. Desmosomes and hemidesmosomes were closely connected. Mild nuclear pyknosis and autophagic cell death were only found from the 6th day, and mitochondria appeared a little bubble from the 5th day. Visual acuity in 11 of the 15 patients receiving transplantation of the preserved corneas was improved by more than 0.5. Average corneal endothelial cell counts, areas of corneal endothelial cells, and CV% of average area were not affected during the 6-month follow-up. Compared to the values obtained one-month postoperatively, the values of corneal thickness were significantly reduced in the three-month and six-month periods. Conclusions: Corneal preservation technology with the injection of DisCoVisc viscoelastic agent may effectively extend the preservation time of corneas for five days, which could be used for patients as penetrating keratoplasty surgery.

Keywords: DisCoVisc, transmission electron microscopy, endothelial cells, corneal preservation, corneal transplantation.

Introduction

There are currently about 1,233,000 visual disabilities in China. Among them, 4 million people's disabilities are caused by corneal blindness and followed by corneal disorder [1]. Technologies to preserve cornea include short-term preservation, mid-term preservation, and long-term preservation. Corneas are mainly stored in wet room in China, and the preservation time is short (about 24-48 h) because cell viability of corneal endothelial cells decreases 50% at 48 hours later [2, 3]. Thus, corneal transplant surgery in China is always thought to be an emergency surgery. It is crucial to cut down the cost of corneal preservation, prolong the preservation time, and improve the preservation approach.

DisCoVisc is a mixture of chondroitin sulfate (CS) and sodium hyaluronate (SH) [4]. CS belongs to sulfated glycosaminoglycan (GAG), which is one of the main components of the stroma of corneal endothelial cells [5]. In the 1960s, CS was originally used to preserve corneas [6]. It has been confirmed that corneal preservation solution containing CS had a remarkable protection in corneal endothelial

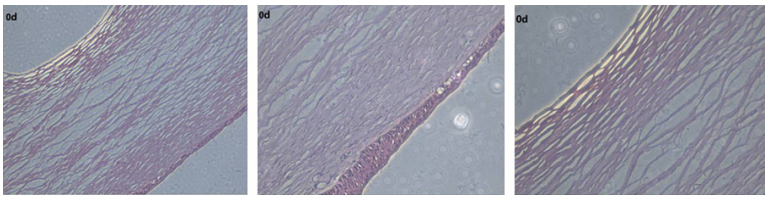

Figure 1. Microstructure of fresh cornea. Left panel (magnification: 100 x): clear corneal edema, epithelial cell and endothelial cell layer, and slightly loose matrix layer; middle panel (magnification: 200 x): corneal epithelial cells were normal in morphology, and the nuclei were homogeneous; Right panel (magnification: 200 x): corneal endothelial cells without shedding and edema.

cells [7, 8]. Additionally, CS is a diffuse viscoelastic agent with a small molecular weight, which can be uniformly coated on the corneal endothelium. SH is a cohesive viscoelastic agent and has a high surface tension accumulation, which can maintain the anterior chamber of the eye shape. Therefore, the mixtures of CS and SH are likely to keep the shape of endothelium in the cornea preservation process.

In the current study, we sought to explore the effect of DisCoVisc on corneal preservation and determine the safety and preservation time of this method.

Materials and methods

Patient description

This study included 15 patients who had received penetrating keratoplasty surgery at the Department of Ophthalmology, Shanghai Tenth People's Hospital, between December 2013 and July 2015. Inclusion criteria were as following: age ranged from 20 to 60 years; no acute infectious or ocular disease; no allergic history to fluoroquinolone or aminoglycoside. Patients unable to understand the characteristics and objectives of the study or with acute conjunctivitis, blepharitis, or dacryocystitis were excluded from the study. General information, including age, sex, systemic diseases, and eye symptoms, was recorded.

All donors were derived from Shanghai Branch of Red Cross Society of China, who had signed agreement to donate their corneas for corneal transplantation or scientific research purposes. This prospective clinical quality-control trial was performed according to the World Medical Association (WMA) Declaration of Helsinki under the Policy of "Ethical Principles for Medical Research Involving Human Subjects". All patients signed informed consent and the protocols were under the approval of Ethics Commission of the Institutional Review Board, Shanghai Tenth People's Hospital.

Preparation of corneas and injection of DisCoVisc

The eyeballs were removed within 2 h after the death of the donors, and 1/4 of the cornea was removed immediately after disinfection (fresh cornea control group). The water was taken out and viscoelastic agent was injected into the anterior chamber. In addition, the anterior chamber puncture was performed under the aseptic condition without damaging the corneal endothelia. Sterile adhesive was injected at the puncture site and coated on the corneal epithelium. Corneas were preserved in the wet room thereafter for 1-6 days and the corresponding part of cornea was removed for indicated experiments. The tissues were cut into three pieces for hematoxylin-eosin (H&E) staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and transmission electron microscopy (TEM). TEM was applied to observe the activity of the nuclei and organelles of corneal endothelial cells. DisCoVisc sterile ophthalmic viscoelastic agent (Alcon) included 1.6% hyaluronic acid (also found in connective tissues) and 4% chondroitin sulfate (also found in cartilage).

H&E staining

The tissues were fixed in 4% paraformaldehyde. The pathological changes of corneal tissue were observed by H&E staining following paraffin embedding and sectioning.

TUNEL

TUNEL staining was performed in 30-μm slices using the ApopTag In Situ Apoptosis Detection Kit (C1089, Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instruction. After staining, the sections were imaged using FV1000 Olympus Confocal Laser Scanning Microscope (Olympus, Japan).

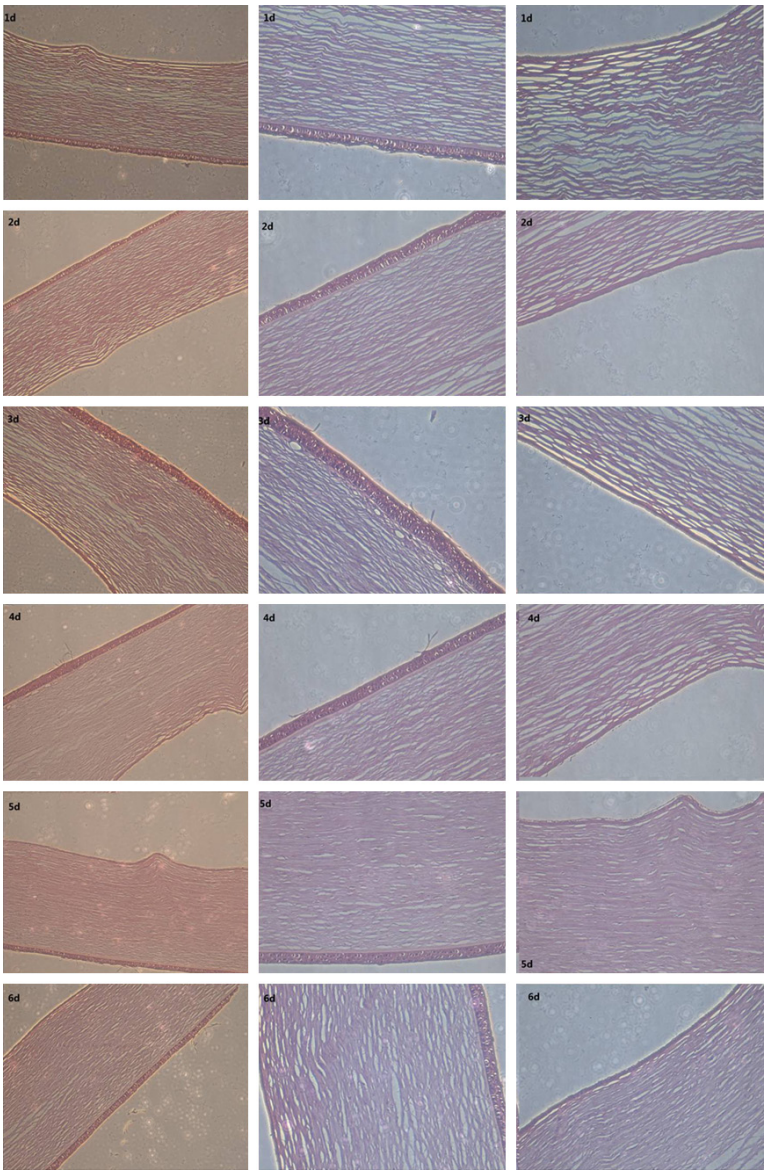

Figure 2. Microstructure of corneas preserved by DisCoVisc for 1-6 days. Left panel (magnification: 100 x) showed that there was no edema observed in the whole cornea. Structure of epithelial cell and endothelial cell layer were clear without cell shedding. Endothelial cell layer showed slightly wrinkled with clear matrix layer; Middle panel (magnification: 200 x) showed that corneal epithelial cells were normal in morphology, and the nuclei were homogeneous. Right panel (magnification: 200 x) showed that there was no edema observed in the cornea and endothelial cells were normal.

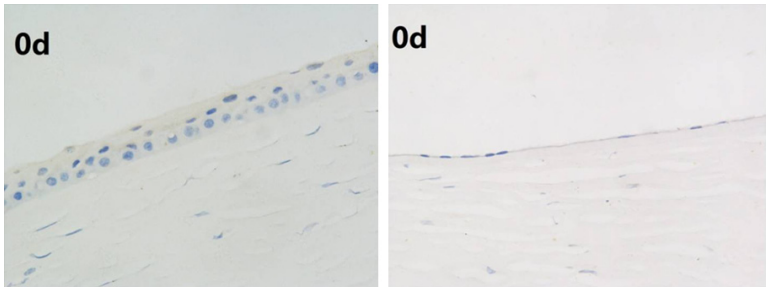

Figure 3. TUNEL staining of fresh cornea. There was no TUNEL-positive staining in the fresh cornea.

TEM

The tissue was fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4) for at least 2 h to overnight. After dehydration in 8% (0.2 M) sucrose overnight, the tissue was post-fixed in 1% osmium tetroxide and in 0.1 M PB for 1 h. Then, the tissue was sectioned into 1-μm slices. The images were taken under the electric microscope (Hitachi, Japan).

Clinical application of the preserved corneas

DisCoVisc was used to preserve the eyeball for 3 days. All penetrating keratoplasty surgeries were performed by the same experienced surgeon under topical anesthesia. The patients included corneal white spots and keratoconus without other eye diseases. Postoperatively, the patients were treated with Tobramycin and Dexamethasone Eye Drops (three times per day) and 0.5% Levofloxacin (twice per day), and Tropicamide Phenylephrine Eye Drops (once per day). The patients were examined at 24 h, 48 h, 7 days, 1 month, 3 months, and 6 months postoperatively. Corneal endothelial cell count and best-corrected visual Snellen acuity (BCVA) were measured at one and six months postoperatively.

Statistical analysis

Statistical analyses were performed using SPSS using the unpaired *t*-test and one-way ANOVA. The *P* value less than 0.05 was considered as significant difference.

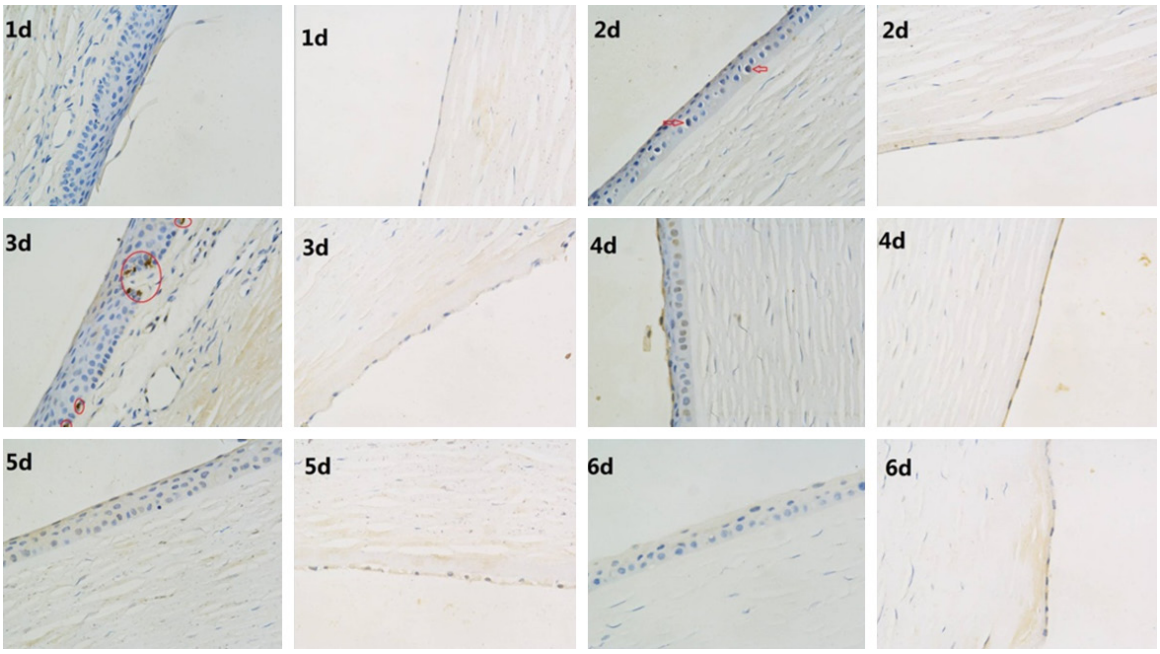

Figure 4. TUNEL staining of corneas preserved by DisCoVisc for 1-6 days. Compared to the fresh cornea, corneal endothelial cells did not show obvious apoptosis, and the corneal epithelial cells appeared positive staining from the fourth day.

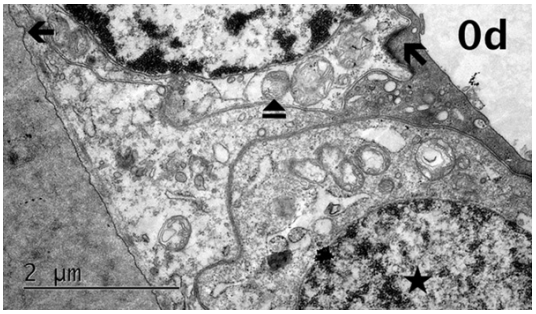

Figure 5. The structure of corneal endothelial cells from fresh cornea by transmission electron microscopy. Triangle showed normal mitochondria without swelling degeneration. Arrow indicated the normal hemidesmosome connection between the basement membrane and endothelial cells, and normal desmosomes between corneal endothelial cells. Five-pointed star showed the normal nuclei, chromatin uniformity, and no nuclear pyknosis.

Results

Pathological changes

H&E staining was used to detect the structure of each layer of the cornea. The results showed that both fresh corneas (**Figure 1**) and corneas preserved by DisCoVisc for 1-6 days had normal microstructure (**Figure 2**). Corneal epithelial cells and endothelial cells were normal in morphology, and the corneal structure of stromal layer was regular. Up to the sixth day, there was a slight wrinkle, but no cell shedding, in corneal endothelia.

There were only moderate numbers of TUNEL-positive cells in cornea preserved by DisCoVisc, with typical morphological changes of apoptosis, such as small, round, and nuclear pyknosis (**Figure 4**), whereas cells in control group were not stained by TUNEL method (**Figure 3**).

The cells in corneas from control (**Figure 5**) and 1-6-day preservation groups (**Figure 6**) had normal corneal endothelial cells, and active nuclei, homogenous nucleoplasm. Intercellular desmosomes and hemidesmosomes were closely connected. There was mild nuclear pyknosis in the preservation group on the sixth day, a few bubbles in mitochondria from the 5th days, and a small amount of lysosomal autophagy from the 6th day.

Clinical efficacy

For the transplantation, a total of 15 patients were included in the study. Visual acuity of eleven patients was improved by more than 0.5. As shown in **Table 1**, mean corneal endothelial cell counts were not significantly changed at 6

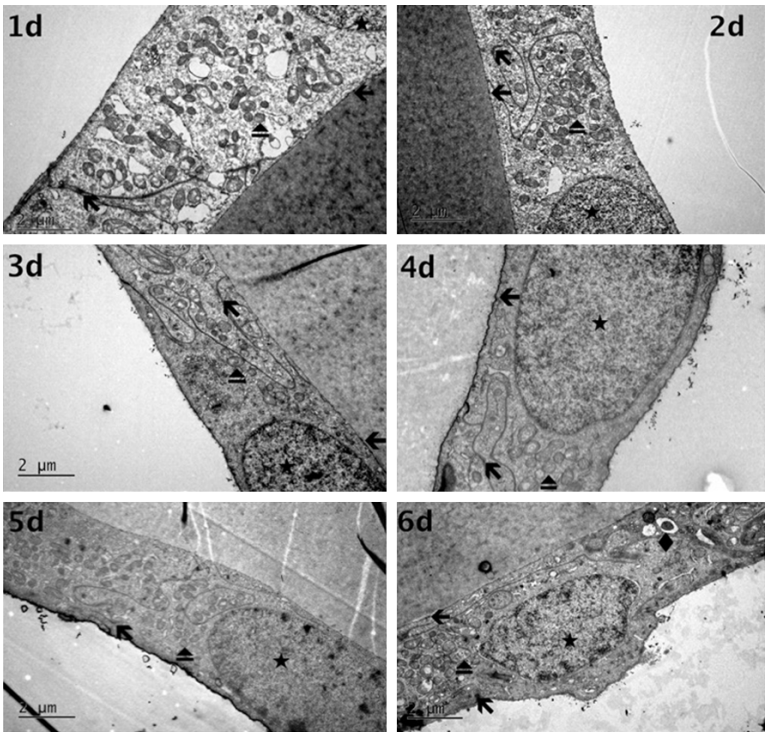

Figure 6. The structure of corneal endothelial cells preserved by DisCoVisc for 1-6 days by transmission electron microscopy. 1-5 d: Triangle showed normal mitochondria without swelling degeneration. Arrow showed the normal hemidesmosome connection between the basement membrane and endothelial cells, and normal desmosomes between corneal endothelial cells. Five-pointed star showed normal nuclei, chromatin uniformity and no nuclear pyknosis. 6 d: Triangle showed mitochondrial color fades, mild swelling. Arrow showed the normal hemidesmosome connection between the basement membrane and endothelial cells, and normal desmosomes between corneal endothelial cells. Five-pointed star showed abnormality of nuclei, mild pyknosis. Diamond showed autophagic bodies.

months postoperatively. Average areas of corneal endothelial cells and CV% of average area were also not affected during the 6-month follow up. Compared to the value in one-month postoperatively, the value of corneal thickness significantly decreased in 3-month and 6-month periods.

Discussion

CS is a penetrating agent and has a lot of negative charges, which can accumulate a layer of chondroitin sulfate film on the surface of corneal endothelial layer. It is resistant to free radical in the tissue and cells [9, 10], and protects the cells against cytotoxin. As a membrane stabilizer, CS has mechanical drag-reducing effect and can also fight against physical damage caused by mechanical vibration during transport process [11]. It maintains the activity of corneal endothelial cells in a long-term [12] and reduces the endothelial cells from histolysis [13]. Optisol corneal preservation solution usually contains CS and low molecular dextran to keep translucent state of the cornea [8, 14, 15]. Similar to CS, SH has the function of penetration and corneal protection [16]. In the eye surgery, the mixture has high viscosity and good coating effect, which maintain the anterior chamber and protect cornea endothelial cells.

This method of corneal preservation is an improvement in comparison with the preservation in wet room. Corneal dystrophy preservation is one useful mid-term corneal preservation method, which can obviously prolong the time of corneal preservation and maintain the activity of endothelial cells. In China, donated corneas are limited and mid-term corneal preservation is limited in most of the hospital due to the expensive cornea preservation fluid and easier contamination. In order to facilitate the operation of central sampling drilling, we employed a syringe to extract the aqueous humor of the anterior chamber and then inject DisCoVisc agent into the anterior chamber, and coat them on the surface of the cornea. Hyaluronic acid is a physiological substance of the eye, whereas chondroitin sulfate is one of the main components of tissue culture liquid. Both have neurotrophic effects on corneal epithelial cells and endothelial cells to ensure cell activity in the process of corneal preservation. Some studies had shown that the use of DisCoVisc in cataract surgery significantly reduced the rate of corneal endothelial cell loss [17, 18]. In this present study, the preserved corneas were detected by electron microscopy, HE staining, and TUNEL staining. We reported that the corneal structure and endothelial cells were well preserved within 5 days. Satisfactory results were obtained in clinical trials. Most of the patients who underwent

Table 1. Corneal endothelial cell count, area of endothelial cells and corneal thickness before and after surgery (n=15)

	1 week	1 m	3 m	6 m
Corneal count	2633.19±141.65	2713.01±161.34	2502.80±124.00	2438.94±161.78
Average size	-	424.67±46.37	406.42±20.44	456.09±50.41
CV%	-	40.76±1.72	40.69±1.53	39.34±1.88
Corneal thickness	-	538.64±17.49	485.71±17.02*	478.20±12.78**

*p<0.05, **p<0.01 compared to one month.

corneal transplant surgery had an excellent corrected visual acuity and a stable number of corneal endothelial cells.

Overall, our study is an attempt to use DisCoVisc viscoelastic agent to explore the methods for the preservation of corneas. This method can extend the time of preservation and reduce the cost of the mid-term preservation of cornea. Whole eye preservation method can prolong the original preservation from 48 h to 5 days. Our data provided a detailed explanation on corneal pathological changes with clinical efficacy that warrants further corneal transplantation in clinical medicine. Therefore, cornea blind patients in the remote areas will potentially benefit from this new method.

Acknowledgements

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Disclosure of conflict of interest

None.

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