Original Article

Comparison of structural integrity and functional status of corneal endothelium stored in Cornisol and Optisol-GS

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Purpose: To compare the structural integrity and functional status of the donor corneas stored in Cornisol and Optisol-GS. **Methods:** Fifteen optical grade corneal donor buttons (6 pairs; 3 individual) obtained from Rotary Aravind International Eye Bank were used for the study. The left eye of the paired sample was preserved in Cornisol and the right in Optisol-GS. The three individual buttons were used for the baseline data. The corneas were assessed with slit lamp and specular microscope before and after storage time (7, 10, or 14 days). They were then immunostained for markers of structural integrity (ZO-1, Phalloidin) and functionality (Na^+/K^+ ATPase). The images were acquired using confocal microscope and analyzed using ImageJ software. **Results:** There was no difference in the clinical evaluation of the corneal layers between the two media. No marked variation was observed in the immunostaining data with reference to the storage period. Intact cellular integrity was identified in 91% (51%, 98%) [Median (min, max)] of cells in Cornisol and 94% (38%, 98%) cells in Optisol based on ZO-1 staining, comparable to the baseline data [87% (76%, 97%)]. Stress fibers were detected in 42.5% (1%, 88%) cells in Cornisol stored corneas and in 55% (11%, 94%) in Optisol when stained for actin cytoskeleton, which correlated with the presence of epithelial defect before storage and vacuolated endothelial cells after storage. No difference was observed between the two media based on the staining pattern for Na^+/K^+ ATPase. **Conclusion:** Cornisol and Optisol-GS are equivalent in maintaining the structural integrity and functionality of the donor corneas.

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Corneal blindness is the fourth (5.1%) leading cause of blindness after cataract, glaucoma and age-related macular degeneration in world.[1] Corneal transplantation stands to be the only option for restoration of vision in these patients. Due to the acute shortage of donor eyes most of the cases go untreated. According to WHO, approximately 4 million people are blind due to corneal pathology.[2]

For better utilization, the healthy corneas procured from deceased donor are stored in a biochemically defined tissue culture medium. McCarey-Kaufman (MK) medium is short-term storage medium which can preserve the corneal tissue only up to 4 days,[3-5] while Eurobio Cornea Cold and organ culture medium has been used successfully for long-term storage in Europe and Australasia but not globally.[6] One of the most widely used intermediate term storage medium is Optisol-GS[7] and is traditionally considered as a gold standard intermediate one.

The corneal storage media available so far have been validated for preserving the cornea for successful transplantation based on (i) specular microscopic analysis which assess the endothelial cell count, variability in cell size,[8-10] (ii) slit lamp microscopic analysis to validate the tissue quality,[11,12] (iii) electron microscopic analysis to elucidate the changes at cellular level,[13,14] (iv) endothelial cell viability[15] and (v) corneal deturgescence activity. Recently, a new intermediate corneal storage medium Cornisol has been introduced and is the most widely used storage medium in India. Preliminary studies have established Cornisol to be equivalent to Optisol-GS by analyzing the endothelial cell count and viability after storage.[15,16] The corneas that have been stored in Cornisol are now regularly used for clinical application. For further validation, the current study was carried out to compare the functional status of the corneal endothelium by immunostaining of corneas stored in Cornisol and in Optisol-GS using a specific marker Na^+/K^+ ATPase which plays a significant role in maintaining the corneal homeostasis. In addition, the expression of markers for structural integrity like tight junction protein, ZO-1, and actin were also analyzed.

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Methods

Sample collection

The donor corneas for this study were obtained from Rotary Aravind International Eye Bank, Aravind Eye Hospital, Madurai. The inclusion criteria for the selection of donor corneas were: (1) Corneas received within 24 hours of death, (2) Optical grade tissues not suitable for transplantation due to septicemia (3 pairs), seropositivity to Hepatitis B (2 pairs), and non-availability of blood sample (1 pair and three single globes), (3) Corneal endothelium with a good specular count (Median (min, max): 2793 (2217, 3225) cells/mm^2) and (4) no age limit (47 to 84 years). The research adhered to the tenets of the Declaration of Helsinki, and the study was approved by the Institutional Ethics Committee of Aravind Eye Care System.

Assessment of corneal parameters

Before storage

The whole globes were collected and the corneoscleral buttons were harvested according to the guidelines of the National Programme for Control of Blindness.[17] The dissected paired corneoscleral buttons were stored in Cornisol (left eye) and Optisol-GS (right eye). The following parameters were assessed by a single clinician (GGG) to determine the quality of the corneal tissue. (1) The corneas were evaluated in slit lamp (Topcon, USA) for epithelial defect, stromal edema, Descemet's membrane (DM) folds, endothelial defects and vacuolated cells, and were graded based on SightLife guidelines.[18] Corneas which showed no abnormalities in the slit lamp evaluation were graded as zero. They were scored in increasing negative grades (from -1 to -3) corresponding to the abnormalities of the structure [Table 1]. Score of all layers were added and total score was given for tissue before and after storage [Table 2]. (2) The endothelial cell density was examined by specular microscopic (Konan, Japan) analysis. Though the optimal storage of corneas is 14 days in the intermediate storage medium, the tissues are normally utilized within 7 to 10 days of storage due to high demand. Hence in this study, the corneas (2 pair each) were stored for 7, 10, and 14 days, respectively at 4°C. For baseline data, 3 unstored donor tissues were used within 24 hours of death.

After storage

After the storage period, the media vials containing the corneal tissue were placed at room temperature for 1 hour. The above-mentioned corneal parameters were re-examined after storage. Additionally, the pH of the medium was determined. The anterior surface of the corneas was then trimmed using microkeratome (Moria, France) with 350 micron head to reduce corneal thickness for proper mounting after immunostaining.

Since the number of tissues used for each time period was small the average of endothelial cell count and pH for the three time points were analyzed.

Immunohistochemistry

The corneoscleral buttons were immunostained as described earlier[19] with minor modification. Briefly, the tissues were washed with 1X PBS and fixed in 1% paraformaldehyde (Sigma Aldrich, Saint Louis, MO) for 2 minutes. The corneal buttons were cut into 4 equal quadrants (1 quadrant for control and 3 quadrants for 3 markers). This was followed by ice cold acetone fixation for 5 minutes. The quadrants were then washed thrice with 5% dextran (Sigma Aldrich, Saint Louis, MO) and 1% DMSO (Sigma Aldrich, Saint Louis, MO) dissolved in 1X PBS. After treating the tissues with avidin biotin blocking system (Dako, Glostrup, Denmark), the quadrants were stained using anti- ZO-1 (Invitrogen, Grand Island, NY), phalloidin (Invitrogen, Grand Island, NY) and anti- sodium potassium ATPase (Merck Millipore, Billerica, MA) at a dilution of 1:100, 1:40, and 1:200 in 1% BSA, respectively. After overnight incubation at 25°C, biotinylated secondary antibody (anti-mouse IgG, Dako, Glostrup, Denmark) was added to the quadrants at a dilution of 1:200 in 5% BSA. Visualization was carried out with streptavidin fluorescein isothiocyanate (FITC, BD Pharmingen, SanDiego, CA). Between the steps, the tissues were washed thrice in 1 X PBS. The quadrants were mounted in cavity slides (Blue Star, Mumbai, India) with vectasheild fluorescent mounting media containing nuclear counterstain DAPI (Burlingame, CA). The control quadrant was stained similarly but without the addition of any primary antibody.

Confocal microscopy

Acquisition of confocal images were carried out as described earlier by Arpitha *et al*. (2005).[20] Briefly, fluorescence Z stack images in five different regions of corneal endothelium were captured with a laser scanning microscope (Leica SP8 confocal microscope, Germany) using 40X objective. The emission (band width) for FITC ranged from 496 nm to 535 nm when excited using 488 nm argon laser and 350 nm to 470 nm for DAPI using He-Ne laser.

Analysis

Using confocal microscopic images, the number of corneal endothelial nuclei was counted based on DAPI staining and the number of cells expressing specific markers (FITC staining) in five different fields using ImageJ software, and the average values were calculated. The percentage of positivity was calculated by counting the total number of cells and the number

Table 1: Grading of donor corneas based on Sight life guidelines[25]

Corneal tissue	Scoring of corneal tissues based on abnormalities using Sight life guidelines			
Epithelium	No Epithelial defect	10 mm² epithelial defect	11-50 mm² epithelial defect	More than 50 mm² epithelial defect
Stroma	Crystal clear stroma	Light cloudiness	Moderate Cloudiness	Severe cloudiness
DM	No folds	Few peripheral light folds	Numerous but shallow folds	Numerous deep central folds
Endothelium	No defects No vacuolated cells	Few vacuolated cells (none within central 8 mm cornea)	Central vacuolated cells	Endothelial defects and vacuolated cells
Score	0	-1	-2	-3

Table 2: Clinical scoring of the corneal buttons based on the slit lamp analysis

Media	Pair	Slit lamp analysis									
		Before storage					After storage				
		Epithelium	Stroma	Descemets membrane	Endothelium	Score	Epithelium	Stroma	Descemets membrane	Endothelium	Score
					7 days storage						
Cornisol	1	2×2 mm^2 epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-3	5×2 mm^2 epithelial defect	Light Cloudiness	Numerous but Shallow folds	No Defects No vacuolated cells	-4
Optisol		No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2	1×2mm^2 epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No Defects No vacuolated cells	-3
Cornisol	2	No Epithelial defect	Crystal clear stroma	Few peripheral light folds	No defects No vacuolated cells	-1	No Epithelial defect	Crystal clear stroma	No folds	Few vacuolated cells (none within central 8mm cornea)	-1
Optisol		No Epithelial defect	Crystal clear stroma	Few peripheral light folds	No defects No vacuolated cells	-1	NO Epithelial defect	Crystal clear stroma	No folds	Few vacuolated cells (none within central 8mm cornea)	-1
					10 Days storage						
Cornisol	1	No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2	No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2
Optisol		No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2	No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2
Cornisol	2	No Epithelial defect	Crystal clear stroma	Numerous deep central folds	No defects No vacuolated cells	-3	3×2mm^2 epithelial defect	Light Cloudiness	Numerous deep central folds	No defects No vacuolated cells	-5
Optisol		No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2	No Epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-2
					14 Days storage						
Cornisol	1	2×1 mm^2 epithelial defect	Crystal clear stroma	No folds	No defects No vacuolated cells	-1	4×1 mm^2 epithelial defect	Crystal clear stroma	Numerous deep central folds	No defects No vacuolated cells	-4
Optisol		No Epithelial defect	Crystal clear stroma	No folds	No defects No vacuolated cells	0	3×3 mm^2 epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-3
Cornisol	2	3×2 mm^2 epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-3	4×2 mm^2 epithelial defect	moderate Cloudiness	Numerous deep central folds	Endothelial defects and vacuolated cells	-9
Optisol		4×2 mm^2 epithelial defect	Crystal clear stroma	Numerous but Shallow folds	No defects No vacuolated cells	-3	5×2 mm^2 epithelial defect	Light Cloudiness	Numerous but Shallow folds	Endothelial defects and vacuolated cells	-7

The corneas were evaluated for epithelial defect, stromal edema, Descemet's membrane folds, endothelial defects and vacuolated cells before and after storage and were graded

of cells positive for each marker in five different fields in the corneal endothelium (0.085 mm²/field).

Statistical analysis

Mann-Whitney U test was performed to compare the difference between the two storage media, while Wilcoxon sign rank test was used to find the difference between before and after storage. Kruskal Wallis test was done to find out the difference between storage days in Cornisol and Optisol-GS. A *P* value < than 0.05 was considered as statistically significant.

Results

Effect of storage on clinical features of cornea

The median corneal endothelial cell density was 2816 cells/mm² (min, max: 2624,3225) before storage and 2320 cells/mm² (2082, 3165) after storage in Cornisol (p = 0.14). In Optisol-GS, the density was 2544 cells/mm² (2217, 3086) before and 2564 cells/mm² (2214, 3294) after storage (p = 0.5). No significant variations were found in endothelial cell count between the media and also the storage period. Compared to baseline pH of 7.4, pH after storage was 7.19 (min, max: 6.43, 7.3) and 7.28 (min, max: 6.46, 7.33) in Cornisol and Optisol-GS, respectively. Acidic pH was noted in 2 pairs- after 10 days (pair 2, Cornisol- 6.43 and Optisol-GS- 6.46) and 14 days (pair 2, Cornisol- 6.89 and Optisol-GS- 6.99) of storage, equally in both media. After 7 days of storage, slit lamp analysis revealed deterioration of -1 in one pair (pair 1) in both the media. Upon 10 days, Cornisol stored tissue of the pair 2 had a deterioration of -2 due to the epithelial defect. Highest deterioration was observed after 14 days of storage and a -3 change in clinical grading was observed in both the pair 1 tissues. The presence of moderate stromal cloudiness and deep central folds in Cornisol stored cornea of pair 2, resulted in an additional variation of -2 compared to Optisol stored tissue. In spite of all the above variations, the difference between clinical score before and after storage was not significant (p = 0.61) and the endothelium behaved similarly in both the media in all time intervals [Table 2].

Structural integrity of corneas after storage

Analysis of confocal microscopic images of unstored corneal tissues without storage revealed that a median 87% (min, max: 76%, 97%) of the endothelial cells to be positive for the marker ZO-1 which expressed apical pericellular staining pattern with Y junction gaps [Fig. 1a]; 12% (min, max: 3%, 13%) to have fragmented positivity due to ruptures in the staining pattern [Fig. 1c] and remaining cells to be negative [Figs. 1b and 2a]. After storage in Cornisol and Optisol-GS, the median percentage of ZO-1 positive cells were 91% (min, max: 51%, 98%) and 94% (min, max: 38%, 98%), respectively. This difference between Cornisol and Optisol-GS stored corneas were not significant (p = 0.7). The median percentage of Fragmented positivity was observed in 8.5% (min, max: 2%, 47%) and 5.5% (min, max: 2%, 50%) of the cells (p = 0.8) and the remaining cells were negative for the tight junction protein [Fig. 2b] No variations were observed in the staining pattern at different time periods.

Similarly, analysis of phalloidin stained baseline tissue identified that a median of 97% (min, max: 93%, 98%) cells to be positive for the cytoskeletal protein actin expressing double cortical dense peripheral bands [Fig. 1d], 2% (min, max: 2%, 3%) to have stress fibers characterized by the presence of radial cytoplasmic fibers [Fig. 1f] and the remaining to be negative [Figs. 1e and 2a]. After storage, the median percentage of cells having the double cortical staining pattern reduced to 42.5% (min, max: 1%, 88%) in Cornisol and 55% (min, max: 11%, 94%) in Optisol-GS. This difference between Cornisol and Optisol-GS stored corneas were not significant (p = 0.38). The presence of stress fibers increased to 55.5% (min, max: 10%, 93%) and 15.5% (min, max: 6%, 87%) respectively (p = 0.23) [Fig. 2c].

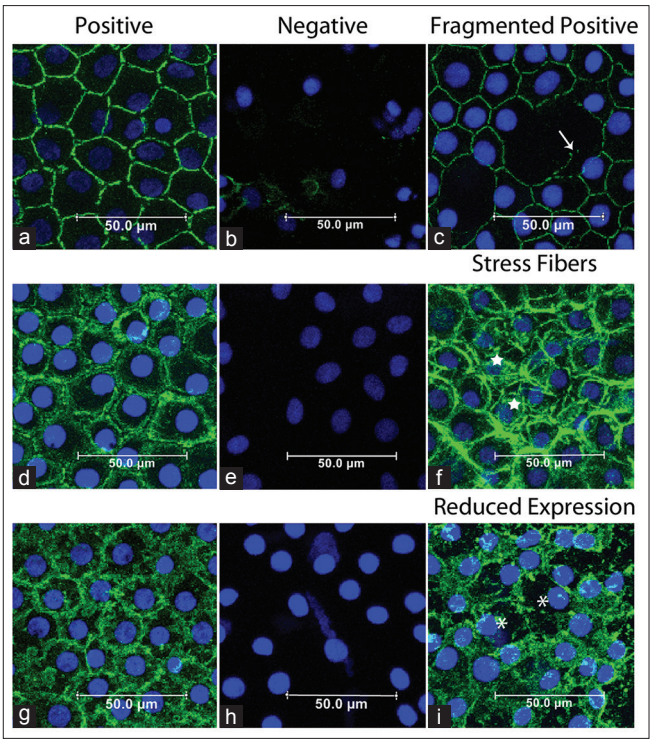

Figure 1: Representative confocal microscopic images of stored corneal endothelium stained for the structural and functional markers. (a-c) Expression pattern of ZO-1, (a) apical pericellular staining pattern with Y junction gaps, (b) Negative (c) fragmentation in the staining (arrow); (d-f) Expression pattern of Actin cytoskeleton, (d) double cortical dense peripheral bands, (e) Negative (f) cells with radial cytoplasmic stress fibers (star); (g-i) Expression pattern Na^+/K^+ ATPase, (g) ubiquitous staining pattern throughout the cell surface (h) Negative (i) indicates the reduced expression of Na^+/K^+ ATPase (asterisks)

Functional status of corneas after storage

Immunostaining for Na^+/K^+ ATPase in unstored donor tissues identified that all endothelial cells (100%) to be positive expressing ubiquitous staining pattern throughout the cell surface [Figs. 1g and 2a]. In one pair (10 days, pair 1 in Table 2) all endothelial cells were negative for Na^+/K^+ ATPase after storage [Fig. 1h]. Excluding this pair, the percentage of cells positive for the marker was 100% in both Cornisol and Optisol-GS [Fig. 2d]. Among these positive cells, a median of 1% (min, max: 1%, 1%) of cells in unstored corneal tissue, 0% in Cornisol (min, max: 0%, 100%) stored tissue and 0% (min, max: 0%, 20%) in Optisol-GS stored had a reduced staining in certain regions on the periphery of the cell giving the appearance of fragmented positivity [Fig. 1i].

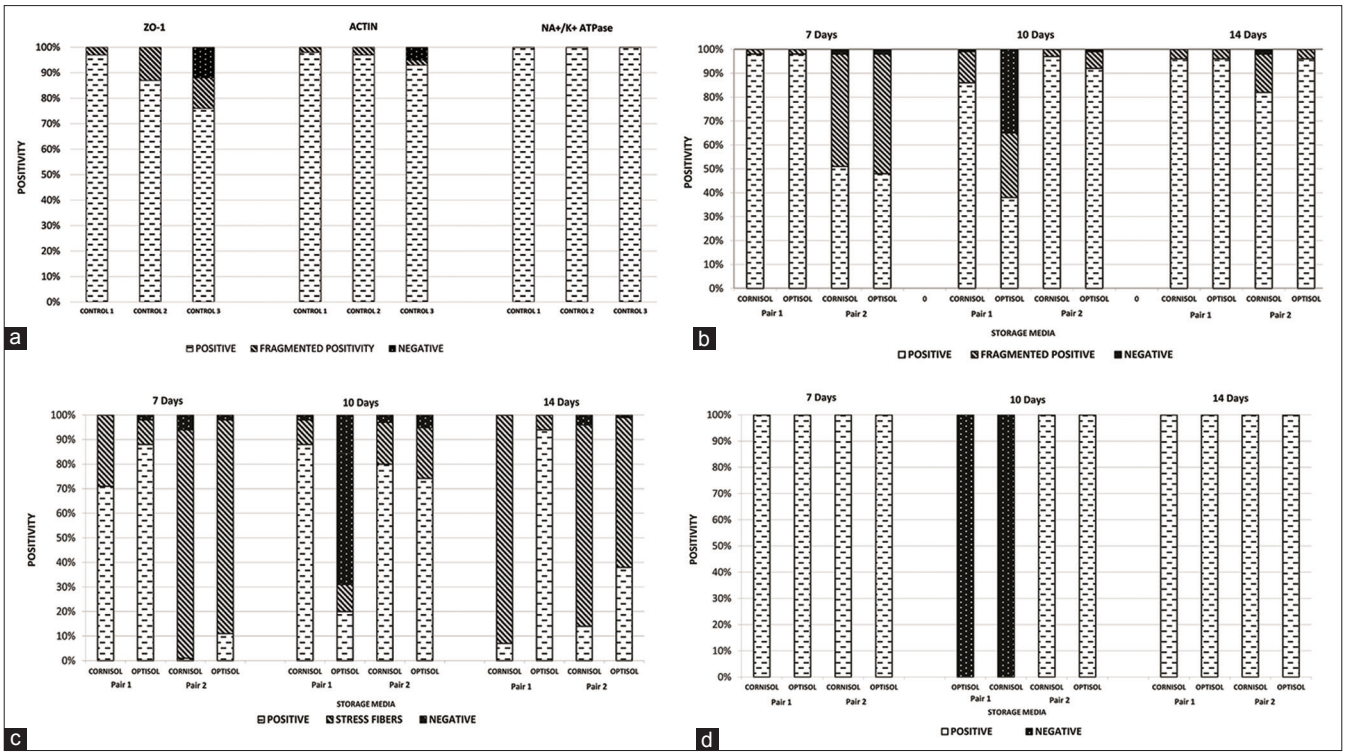

Figure 2: Graphical representation of the percentage of marker positivity in Baseline, Cornisol and Optisol-GS stored corneas. (a) Baseline expression of markers for structural and functional integrity in corneal endothelium. The graph represents the quantitative staining pattern to ZO-1, phalloidin and Na^+/K^+ ATPase in unstored donor tissues. Comparison of the percentage of cells expressing (b) ZO-1, (c) Actin cytoskeleton and (d) Na^+/K^+ ATPase after 7, 10, and 14 days of storage. Based on the staining pattern, the cells were graded as positive, fragmented positive/stress fibers, and negative

Discussion

Cornisol is now used as an intermediate term corneal storage media widely in India and also in other developing countries like Nepal and Kenya. Based on the endothelial cell count and cell viability, Cornisol is proved equivalent to Optisol-GS.[15,16] Analysis of stored corneas (i) by slit lamp microscopy for corneal parameters and (ii) for specific marker expression by immunostaining to elucidate the structural and functional integrity of the endothelium in this study further strengthens its equivalence to Optisol-GS.

Slit lamp analysis revealed the donor cornea quality before storage to be 0 to -3 which upon storage deteriorated to -1 to -9 in Cornisol and -1 to -7 in Optisol-GS [Table 2]. The highest level of changes were observed in corneas stored for 14 days (pair 2). Changes observed in endothelium were comparable between the pairs for each time interval. Similar to the clinical grading, the average endothelial cell counts decreased equally in Cornisol and Optisol-GS compared to baseline. Further validation was based on immunostaining using specific markers which have been employed for the structural and functional assessment of cultured corneal endothelial cells.[19,21] The barrier integrity of the corneal endothelium is conferred by its tight junction protein ZO-1 that blocks the passage of water and water soluble substance. In addition, the establishment and maintenance of these tight junctions are influenced by actin cytoskeleton.[22,23] Analysis of these markers for the structural integrity in Cornisol and Optisol-GS stored corneas indicated a similar level of changes. Compared to baseline, a higher percentage of fragmented ZO-1 positivity was observed in both corneas of pair 2 after 7 days of storage [Fig. 2b]. This fragmented positivity was clinically associated with the presence of few vacuolated cells after storage [Table 2]. The presence of stress fibers in this pair as well as in 14-day stored tissues (Pair 1 and 2) correlated with epithelial and/or endothelial defect due to storage. Thus, these changes in the corneal parameter and staining pattern for these specific markers were either similar between the individual pairs or were associated with changes in the corneal epithelium/stroma/Descemet's membrane.

Functionally, the Na^+/K^+ ATPase present on the basolateral membrane of the corneal endothelium plays a significant role in the maintenance of stromal deturgescence by pumping the excess fluid out of stroma through ionic pumps.[24,25] The reduced expression in a proportion of cells was associated with the presence of vacuolated cells as per slit lamp microscopic examination. The significance of this reduced expression for Na^+/K^+ ATPase and its influence in corneal endothelial functions remains unclear. Among the six pairs, one pair of cornea (Pair 2, 10 day storage) was an exception wherein the functional marker was observed to be 100% negative and the structural integrity was also altered more in Optisol-GS than Cornisol stored tissue. This feature is contradictory to the slit lamp observation wherein no changes were observed due to storage. In spite of inter-donor variation, no significant difference in the clinical grading or in the immunostaining pattern was observed after storage at the endothelial level.

One limiting factor of the study was the small sample size due to the high demand for optical grade corneas and low availability of tissues not suitable for transplantation. In addition, the functional status of the stored corneas needs to be confirmed by other analysis like deturgescence assay or transendothelial resistance measurement by Ussing chamber.[25] An effective and an affordable storage medium is an integral part for a successful corneal transplantation program. It is imperative that a wider choice of affordable options are available to the international ophthalmological community in pursuit of establishing cost effective corneal transplantation care. Cornisol is half expensive than the most subsidized version of Optisol-GS.

Conclusion

This study confirms the equivalence of Cornisol to Optisol-GS with regard to structural and functional integrity of the cornea.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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