Original Article

Surface quality and endothelial cell viability after femtosecond laser-assisted donor lenticule preparation for endothelial keratoplasty - An *in-vitro* study

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Purpose: To compare surface quality and endothelial cell viability of descemet stripping automated endothelial keratoplasty (DSAEK) donor lenticules prepared with femtosecond laser (FSL) or microkeratome (MK). **Methods:** Experimental ex-vivo evaluation of 15 DSAEK donor lenticules prepared from optical quality donor corneas using 200 KHz FSL (9 eyes) or MK (6 eyes). Surface quality and smoothness of the cut were assessed using atomic force microscopy and endothelial cell viability was assessed using transmission electron microscopy. **Results:** Mean lenticule thickness was 121.89 ± 17.13 µm in FSL group and 112.67 ± 5.89 µm in MK group (P = 0.33). Average roughness of stromal surface (RMSavg) [FSL- 30.51 ± 4.55 nm, MK-22.37 ± 1.83 nm; P = 0.02] and root mean square roughness (RMSrough) [FSL-31.39 ± 5.75 nm, MK-23.08 ± 0.40 nm; P = 0.012] was significantly more in FSL group. Increased granular and linear irregularities were observed in the FSL group. Endothelial cell disruption was more in FSL group (FSL- 29.49 ± 6.91% MK-13.28 ± 3.62%; P < 0.001) with decreased mean nucleus length (FSL-5.56 ± 0.17 µm, MK-7.52 ± 0.65 µm; P < 0.001). **Conclusion:** Automated MKs are still the standard of care for donor lenticule preparation and MK-assisted donor lenticules have smoother surface with less endothelial cell disruption than FSL. Further research is mandatory before FSL platforms can be considered a viable alternative to the MK.

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Endothelial keratoplasty is the preferred procedure for cases with corneal endothelial dysfunction. The increasing popularity of the procedure may be attributed in part to the introduction of automated microkeratomes (MKs) which allow surgeons to create thin reproducible donor lenticules with minimal tissue loss and endothelial cell damage.[1] Ultra-thin descemet stripping automated endothelial keratoplasty (DSAEK) with less than 100 µm thickness is associated with faster visual recovery and better visual quality, with outcomes comparable to that of descemet membrane endothelial keratoplasty.[1,2]

Technological advancements have led to the use of femtosecond lasers (FSLs) in full-thickness and lamellar keratoplasty for donor as well as host preparation, owing to the enhanced precision and predictability of cuts.[3] However, the use of FSLs for the preparation of donor lenticules for DSAEK is not well established. The mechanism of action of FSLs is based on the principle of photodisruption which may result in stromal surface irregularity, cellular inflammation, and apoptosis.[4,5] Various studies have reported good visual acuity and quality in FSL-assisted DSAEK.[6,7] On the contrary, a more irregular interface with poor graft adhesion has also been reported with the use of FSL-donor lenticules.[8,9] The ultrastructural changes in corneal endothelium after FSL application have not been well-characterized.

We herein compared the surface quality and endothelial cell viability of DSAEK donor lenticules prepared with a 200 Kilohertz (KHz) FSL or MK.

Methods

We performed an ex-vivo evaluation of 15 experimental optical quality donor corneal tissues at a tertiary ophthalmic care center. Ethical clearance was obtained from the Institute Review Board and the study adhered to the tenets of Declaration of Helsinki. The donor corneal tissues had medical contraindications for use in keratoplasty.

This was a pilot laboratory study to evaluate the feasibility and safety of using a 200 KHz FSL to prepare donor endothelial lenticules. A formal sample size calculation was not performed, as no similar study using a 200 KHz FSL on human donor corneas has been published in literature.

The donor corneas were mounted on an artificial chamber filled with balanced salt solution and donor lenticules were prepared with a 200 KHz FSL (Alcon Wavelight FS200; Alcon Laboratories Inc, Germany) in nine eyes and an automated MK (Gebauer SLc Microkeratome System, Germany) in six eyes. Preoperative endothelial cell count was assessed with a

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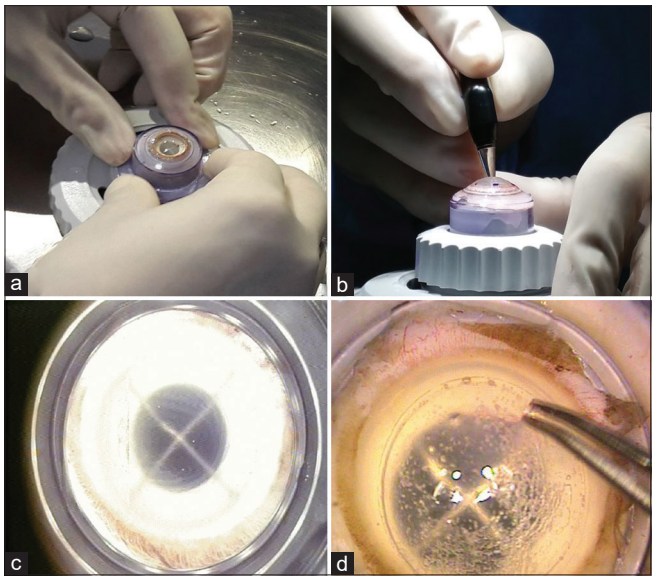

Figure 1: Femtosecond laser-assisted preparation of donor lenticule. (a) Donor cornea mounted epithelial side up on an artificial chamber. (b) Central corneal thickness measured using ultrasonic pachymeter. (c) Femtosecond laser application to create lamellar cut and side cuts. (d) Anterior stromal cap peeled off from the donor lenticule by forceps

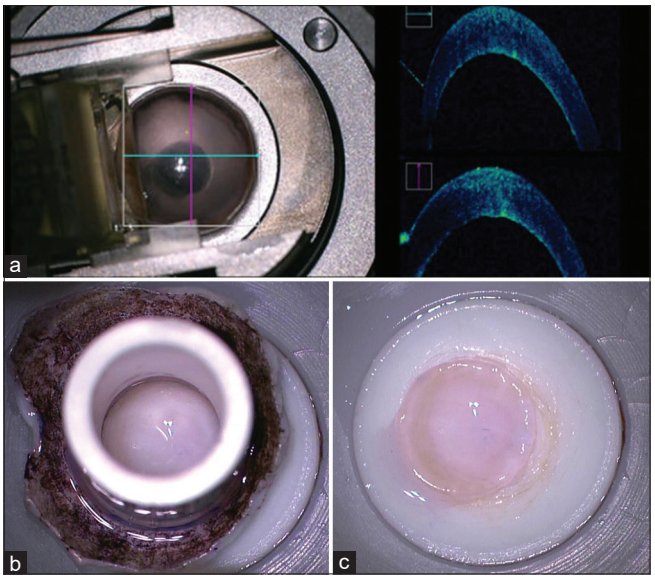

Figure 2: Microkeratome-assisted preparation of donor lenticule. (a) Donor cornea mounted on artificial chamber epithelial side up and lenticule prepared using 500 μm microkeratome head. (b and c) Donor lenticule placed endothelial side up on a Teflon block and trephined with hand-held disposable trephine to achieve a 8 mm diameter donor lenticule

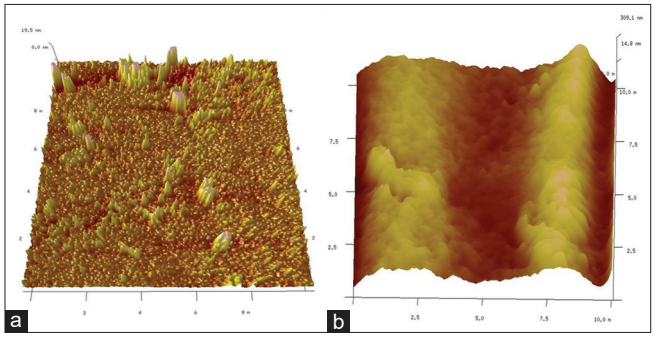

Figure 3: Stromal surface quality as assessed by atomic force microscopy. (a) Increased roughness with granular and linear irregularities in femtosecond laser-assisted donor lenticules. (b) Smooth interface with wave-like irregularities in microkeratome-assisted donor lenticules

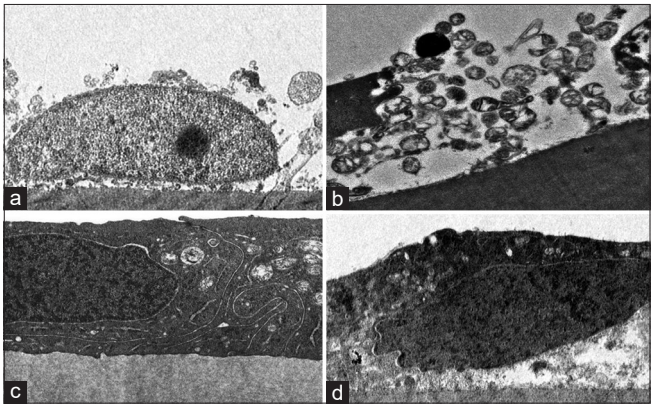

Figure 4: Endothelial cell viability assessed by transmission electron microscopy. (a and b) Decreased endothelial cell viability with disruption of endothelial cell nuclei and discontinuity of plasma membrane in femtosecond laser-assisted donor lenticules. (c and d) Intact endothelial cell nuclei, cell organelles and plasma membrane in microkeratome-assisted donor lenticules

specular microscope (Konan Eye Bank KeratoAnalyzer EKA-10; Konan Medical Group, Hyogo, Japan). The epithelium was debrided using a blunt spatula and the central corneal thickness was measured using ultrasonic pachymetry.

For FSL assisted lenticule preparation, a new disposable applanation cone was used for each cornea without a suction ring [Fig. 1a-d]. The diameter of the FSL stromal bed was kept at 8 mm and the depth of lamellar cut was decided based on the corneal thickness in order to create a donor lenticule with intended thickness of 100 microns. The FSL settings for the bed cut were pulse energy of 0.6 μJ with spot and line separation of 4 μm each. For side cut, 90° angle, pulse energy 1 μJ, spot separation 4 μm, and line separation of 2 μm were selected. The energy parameters were selected based on the manufacturer guidelines for keratoplasty settings of their laser system. We used a pulse energy of 0.6 μJ for the lamellar bed cut, which is the lowest recommended energy setting for keratoplasty lamellar cuts in Wavelight laser system. After laser application, the anterior cap was peeled off with forceps and the donor lenticule was separated. Manual dissection was not required in any case.

For MK-assisted donor lenticule preparation, suitable MK head was chosen to cut the donor corneal tissue to achieve donor lenticule of 100 μm thickness, using a single-pass technique [Fig. 2 a-c]. An 8 mm donor lenticule was trephined from the endothelial side using a hand-held disposable trephine after the MK pass.

Anterior segment optical coherence tomography was performed to assess donor lenticule thickness in all cases. Central 3 mm of donor lenticule was trephined and transferred to a vial containing 2.5% gluteraldehyde and 2% paraformaldehyde for transmission electron microscopy (TEM) to study the endothelial cell viability and peripheral 8 mm ring was transferred onto a glass slide for atomic force microscopy (AFM) to study the surface quality of the cut.

Surface analysis using atomic force microscopy

AFM images were obtained using the Bioscope Catalyst AFM (Bruker Corporation, Billerica, MA) having a Nanoscope V

controller. The sample was prepared by layering the posterior donor lenticule over a freshly peeled mica surface with the stromal surface exposed. The stromal surface was imaged and analyzed using standard ScanAsyst mode in air at room temperature. For imaging, silicon nitride cantilevers having a nominal spring constant of 0.03 to 0.6 N/m were used. A standard scan rate of 0.5 Hz with 512 samples per line was used for imaging each sample. The areas close to the center of the specimen were analyzed to avoid edge artifacts. The imaging of a single area of the cornea was repeated with the same results in order to confirm reproducibility of the results and ensure the absence of artifacts. The images were processed using Nanoscope analysis, v.1.4 and a single third-order flattening of height images with a low pass filter was done followed by section analysis to determine the dimensions in each case. For surface measurements and roughness analysis, ten sections (1 μm^2 each) in each sample were analyzed to obtain the average of the roughness within the given area (RMSavg) and the root mean square value of the roughness within the given area (RMSrough). All data were compared, averaged, and plotted for comparative estimation of surface property of each sample.

Transmission electron microscopy

For electron microscope examination, thin sections of gray-silver color interference (70-80 nm) were observed under a Tecnai G2 20 high-resolution transmission electron microscope (Fei Company, The Netherlands) at an operating voltage 200 kV. Images were digitally acquired at 3000-5000 X magnification by a charge-coupled device (CCD) camera using Digital Micrograph software (Gatan, Inc). The parameters assessed were nuclear length, nuclear width, and percentage of endothelial cells that were disrupted. Endothelial cell disruption was defined as discontinuity of the plasma membrane along with loss of cytoplasm, cellular organelles with or without loss of nucleus. Nuclear dimensions were measured manually with the help of scale provided along with the images and ImageJ software (version 1.5J8) developed by National Institute of Health, USA. The length was measured in the greatest dimension from tip-to-tip. Multiple measurements were taken and an average value was recorded. Nucleus width was measured in a similar manner with three measurements along the entire nucleus and its average was recorded.

Statistical analysis

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS 11.0; SPSS Inc., Chicago, Illinois). Continuous variables were expressed as mean ± standard deviation and compared using the Mann–Whitney U test. *P* value less than 0.05 was considered significant.

Results

The mean age of donors were 50.0 ± 18.1 years in FSL group and 49.5 ± 13.2 years in MK group ($P = 0.86$). The mean death-to-excision time was 7.0 ± 3.8 hours in FSL group and 8.1 ± 3.6 hours in MK group ($P = 0.22$). The donor corneoscleral rim was immediately transferred to preservative solution upon retrieval. The reasons for ineligibility of donor corneas for use in transplantation were positive serology (Hepatitis, HIV) [nine tissues], metastatic malignancy [two tissues], and prolonged ventilator support >72 h [four tissues].

Mean lenticule thickness was 121.89 ± 17.13 μm in FSL group and 112.67 ± 5.89 μm in MK group ($P = 0.33$). Mean pre-cut endothelial cell count was 2171.60 ± 129.6 cells/mm^2 in the FSL group and 2192.50 ± 109.07 cells/mm^2 in the MK group ($P = 0.69$).

Atomic force microscopy analysis

The average roughness of stromal surface (RMSavg) was 30.51 ± 4.55 nm in FSL group and 22.37 ± 1.83 nm in the MK group ($P = 0.02$). Root mean square roughness (RMSrough) was also significantly more in FSL group (FSL-31.39 ± 5.75 nm, MK-23.08 ± 0.40 nm; $P = 0.012$). Increased granular and linear irregularities were observed on the cut surface in the FSL group, in contrast to a relatively smooth surface with wave-like irregularities in the MK group [Fig. 3 a and b].

Transmission electron microscopy analysis

Endothelial cell disruption was more in FSL group (FSL- 29.49 ± 6.91% MK-13.28 ± 3.62%; $P < 0.001$) with significantly decreased mean nucleus length (FSL-5.56 ± 0.17 μm, MK-7.52 ± 0.65 μm; $P < 0.001$) [Fig. 4a-d]. The mean nucleus width was comparable between the two groups (FSL-1.42 ± 0.07 μm, MK-1.61 ± 0.29 μm; $P = 0.14$)

Discussion

FSLs have established their safety and efficacy in various ophthalmological surgical procedures including laser-assisted *in situ* keratomileusis, refractive lenticule extraction, and cataract surgery.[10] Experimental laboratory studies have demonstrated the feasibility of preparing FSL-assisted donor lenticules for DSAEK.[11,12] However, the comparability of FSL and MK-assisted donor lenticules in terms of surface smoothness, endothelial cell viability, and clinical outcomes is a matter of debate.[6-9,11,12]

We evaluated the FSL induced ultrastructural changes in the stromal surface and corneal endothelium of donor endothelial lenticules and compared them with the conventional MK-assisted donor lenticules.

AFM enables high-magnification corneal surface investigation with minimal tissue preparation. It allows a qualitative as well as quantitative assessment of stromal surface regularity and has been used to compare stromal surface smoothness in donor lenticules prepared with MK or FLSs.[11,12] We observed a significantly rougher stromal surface with increased granular and linear irregularities in the femtolaser group. The craters and streaks may be a result of the intersection of cavitation bubbles, whereas granules may represent coagulated collagen fibers. A rougher stromal interface with the use of FSL has been reported in various studies; however, lower energy parameters have been observed to result in a smooth stromal interface of FSL-donor lenticules comparable to MK-donor lenticules.[12-16] We observed a rougher interface in the FSL group despite using low energy parameters and the results were significantly inferior to MK-donor lenticules. Our ultrastructural findings correlate with the clinical observations by Ivarsen *et al*. who reported poor graft adhesion with suboptimal visual acuity and quality with FSL-assisted donor lenticules.[8]

The preparation of FSL-donor lenticules from endothelial side may be associated with a smoother stromal surface.[17] However, increased endothelial cell loss has been reported with this method with poor graft adhesion and a significantly higher re-bubbling rate.[8,18]

We observed significant nuclear shrinkage in the femtolaser group on transmission electron microscopy, which may indicate impending apoptosis. There was an increased proportion of disrupted endothelial cells in the femtolaser group. Transmission electron microscopy allows the assessment of ultrastructural integrity of the corneal endothelium and may be better indicator of cellular level damage during donor lenticule preparation. Vital dye staining with a combination of trypan blue and alizarin red is an accepted method for the assessment of endothelial cell viability.[19] However, apoptotic cells may not be recognized by the stain leading to an overestimation of endothelial cell viability.[20] Moreover, non-contiguous areas of dead cells may not be resolved by standard microscopy photography.[21,22] Previous studies have

observed similar endothelial cell viability after FSL application from the epithelial side as compared with MK, when assessed with vital dye staining or TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assays.[13,16,23,24] Our results may be indicative of subthreshold FSL-induced endothelial cell damage which cannot not be adequately elucidated with conventional vital dye staining at low magnifications. FSL-induced ultrastructural damage may have implications in long-term graft survival and maintenance of endothelial cell function.

A limitation of transmission electron microscopy is that it analyses only small sections of the cornea which may not be representative for the entire graft. We did not compare the results of transmission electron microscopy with vital dye-assisted light microscopy. Further studies may be performed to compare the two methods of endothelial cell analysis.

The post-cut endothelial cell count was not analyzed, as the primary aim of the study was to assess the ultrastructural damage caused to the endothelial cells by FSLs or MK. Moreover, the specimens were processed for electron microscopy making a post-cut specular microscopy infeasible. Post-cut endothelial cell loss has been observed to be comparable between the two methods of donor preparation in previous studies.[16] Post-cut specular microscopy provides an overall assessment of the endothelial cell loss; however, it does not differentiate between healthy and pre-apoptotic cells. The ultrastructural damage observed on electron microscopy may not manifest as an anatomical loss of cells or decrease in cell density but rather as a functional loss and endothelial dysfunction.

Conclusion

We believe our results raise concerns on the safety and feasibility of FSLs for DSAEK donor lenticule preparation. Automated MKs are still the standard of care for donor lenticule preparation and further research is mandatory before FSL platforms can be considered a viable alternative to the MK. To our knowledge, this is the first study comprehensively comparing both endothelial cell viability and stromal surface quality in donor lenticules prepared with 200 KHz FSL or MK. Randomized clinical trials comparing long-term outcomes with MK and FSL- assisted donor lenticules may help to elucidate the functional significance of the ultrastructural changes induced by FSLs.

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Conflicts of interest

There are no conflicts of interest.

References

1. Price FW Jr, Feng MT, Price MO. Evolution of endothelial keratoplasty: Where are we headed? Cornea 2015;34:S41-7.
2. Busin M, Madi S, Santorum P, Scorcia V, Beltz J. Ultrathin descemet's stripping automated endothelial keratoplasty with the microkeratome double-pass technique: Two-year outcomes. Ophthalmology 2013;120:1186-94.
3. Farid M, Steinert RF. Femtosecond laser-assisted corneal surgery. Curr Opin Ophthalmol 2010;21:288-92.
4. de Medeiros FW, Kaur H, Agrawal V, Chaurasia SS, Hammel J, Dupps WJ Jr, *et al*. Effect of femtosecond laser energy level on corneal stromal cell death and inflammation. J Refract Surg 2009;25:869-74.
5. Angunawela RI, Riau A, Chaurasia SS, Tan DT, Mehta JS. Manual suction versus femtosecond laser trephination for penetrating keratoplasty: Intraocular pressure, endothelial cell damage, incision geometry, and wound healing responses. Invest Ophthalmol Vis Sci 2012;53:2571-9.
6. Cheng YY, Schouten JS, Tahzib NG, Wijdh RJ, Pels E, van Cleynenbreugel H, *et al*. Efficacy and safety of femtosecond laser-assisted corneal endothelial keratoplasty: A randomized multicentre clinical trial. Transplantation 2009;88:1294-302.
7. Cheng YYY, van den Berg TJTP, Schouten JS, Pels E, Wijdh RJ, van Cleynenbreugel H, *et al*. Quality of vision after femtosecond laser-assisted descemet stripping endothelial keratoplasty and penetrating keratoplasty: A randomized, multicenter clinical trial. Am J Ophthalmol 2011;152:556-66.e1.
8. Ivarsen A, Hjortdal J. Clinical outcome of Descemet's stripping endothelial keratoplasty with femtosecond laser-prepared grafts. Acta Ophthalmol 2018;96:e655-6.
9. Vetter JM, Butsch C, Faust M, Schmidtmann I, Hoffmann EM, Sekundo W, *et al*. Irregularity of the posterior corneal surface after curved interface femtosecond laser-assisted versus microkeratome-assisted descemet stripping automated endothelial keratoplasty. Cornea 2013;32:118-24.
10. Ozulken K, Cabot F, Yoo SH. Applications of femtosecond lasers in ophthalmic surgery. Expert Rev Med Devices 2013;10:115-24.
11. Serrao S, Lombardo M, De Santo MP, Lombardo G, Schiano Lomoriello D, Ducoli P, *et al*. Femtosecond laser photodisruptive effects on the posterior human corneal stroma investigated with atomic force microscopy. Eur J Ophthalmol 2012;22:S89-97.
12. Lombardo M, De Santo MP, Lombardo G, Schiano Lomoriello D, Desiderio G, Ducoli P, *et al*. Surface quality of femtosecond dissected posterior human corneal stroma investigated with atomic force microscopy. Cornea 2012;31:1369-75.
13. Phillips PM, Phillips LJ, Saad HA, Terry MA, Stolz DB, Stoeger C, *et al*. "Ultrathin" DSAEK tissue prepared with a low-pulse energy, high-frequency femtosecond laser. Cornea 2013;32:81-6.
14. Rousseau A, Bensalem A, Garnier V, Savoldelli M, Saragoussi JJ, Renard G, *et al*. Interface quality of endothelial keratoplasty buttons obtained with optimised femtosecond laser settings. Br J Ophthalmol 2012;96:122-7.
15. Cheng YYY, Kang SJ, Grossniklaus HE, Pels E, Duimel HJ, Frederik PM, *et al*. Histologic evaluation of human posterior lamellar discs for femtosecond laser Descemet's stripping endothelial keratoplasty. Cornea 2009;28:73-9.
16. Jones YJ, Goins KM, Sutphin JE, Mullins R, Skeie JM. Comparison of the femtosecond laser (IntraLase) versus manual microkeratome (Moria ALTK) in dissection of the donor in endothelial keratoplasty: Initial study in eye bank eyes. Cornea 2008;27:88-93.
17. Liu Y-C, Teo EPW, Adnan KB, Yam GH, Peh GS, Tan DT, *et al*. Endothelial approach ultrathin corneal grafts prepared by femtosecond laser for descemet stripping endothelial keratoplasty. Invest Ophthalmol Vis Sci 2014;55:8393-401.
18. Bernard A, He Z, Gauthier AS, Trone MC, Baubeau E, Forest F, *et al*. Femtosecond laser cutting of endothelial grafts: Comparison of endothelial and epithelial applanation. Cornea 2015;34:209-17.
19. Saad HA, Terry MA, Shamie N, Chen ES, Friend DF, Holiman JD, *et al*. An easy and inexpensive method for quantitative analysis of endothelial damage by using vital dye staining and Adobe Photoshop software. Cornea 2008;27:818-24.
20. Gain P, Thuret G, Chiquet C, Dumollard JM, Mosnier JF, Burillon C, *et al*. Value of two mortality assessment techniques for organ cultured corneal endothelium: Trypan blue versus TUNEL technique. Br J Ophthalmol 2002;86:306-10.
21. Sperling S. Early morphological changes in organ cultured human corneal endothelium. Acta Ophthalmol (Copenh) 1978;56:785-92.
22. Bhogal M, Balda MS, Matter K, Allan BD. Global cell-by-cell evaluation of endothelial viability after two methods of graft preparation in Descemet membrane endothelial keratoplasty. Br J Ophthalmol 2016;100:572-8.
23. Feng Y, Qu H-Q, Ren J, Prahs P, Hong J. Corneal endothelial cell loss in femtosecond laser-assisted descemet's stripping automated endothelial keratoplasty: A 12-month follow-up study. Chin Med J (Engl) 2017;130:2927-32.
24. Heinzelmann S, Maier P, Böhringer D, Auw-Hädrich C, Reinhard T. Visual outcome and histological findings following femtosecond laser-assisted versus microkeratome-assisted DSAEK. Graefes Arch Clin Exp Ophthalmol 2013;251:1979-85.