Very early endothelial cell loss after penetrating keratoplasty with organ-cultured corneas

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ABSTRACT

Aims After keratoplasty, postoperative endothelial cell loss is calculated between the eye bank endothelial cell density (ebECD) and the postoperative specular microscopy (SM). To elucidate the very early cell loss, always described after penetrating keratoplasty (PK), we designed two complementary studies.

Methods (1) Clinical prospective study of 90 consecutive PKs (keratoconus, Fuchs' corneal dystrophy, lattice dystrophy, bullous keratopathy) with organ-cultured corneas and postoperative follow-up by SM at day 5 (D5), D15, month 1 (M1) and M3. This series provided a quantification of the difference between ebECD performed 2 days before graft and very early postoperative ECD. (2) Ten pairs of corneas with comparable ebECD in both corneas and same organ-culture (OC) duration were randomised: one cornea was grafted, and, at the same time, the viable ECD (vECD) of the other was measured after labelling with Hoechst/ethidium/calcein-AM. The relationship between vECD and very early postoperative ECD was studied.

Results vECD at the time of graft did not differ from ECD 5 days after PK, with a difference of 39 (−356; 355) cells/mm^2 (median (10°; 90° percentile, p=0.799)), whereas a significant difference of 755 (359; 1146) cells/mm^2, corresponding to 28% (95% CI 26 to 30) of cells, was measured between ebECD and ECD 5 days after PK (p<0.001).

Conclusions In OC, ebECD provided to surgeons significantly overestimate the number of viable ECs grafted to patients. The absence of difference between the vECD at D0 and ECD at D5 indicates that the very early endothelial cell loss is almost negligible in recipients.

INTRODUCTION

Penetrating keratoplasty (PK) always entails a postoperative decrease in endothelial cell density (ECD).[1] This decrease is calculated by subtracting the postoperative ECD determined in recipients by specular microscopy (SM) to the eye bank ECD (ebECD) determined a few days before grafting. The reasons for ECD decrease after corneal graft remain unclear, and possible factors are surgical trauma, centrifuge cell migration and acute and subclinical chronic immune reactions. Postoperative ECD decrease is not linear. Several decay models have been elaborated during the last years from clinical series.[1–3] They all use different mathematical methods and results essentially differ by their capacity to model the medium and long-term decrease. They all comprise an initial very early important EC loss. From a biological point of view, the chronic slow cell loss is supposed to be triggered mainly by a subclinical innate immune reaction, but the early decrease (during the first month) has not been fully explained. The trauma caused by the trephination itself is very limited,[4–6] and no obvious acute immune reaction after corneal graft (no clinical sign of inflammation) is typically observed, so the large difference between ebECD and the very early postoperative cell counts is unexplained. Understanding this initial ECD decrease could allow interventions liable to extend graft survival.

We recently demonstrated, using an in vitro destructive test involving triple labelling with Hoechst/ethidium/calcein-AM applied to the whole endothelium followed by image analysis, that, during organ-culture (OC), ebECD overestimates the pool of living ECs carried by the cornea and thus grafted in patients.[7] As ebECD is the only reference available to calculate postoperative ECD decrease, we postulate that the very early rapid decrease universally observed in all types of keratoplasty involving ECs may be mostly artifactual, due to this overestimation. Study of this phenomenon is limited by the fact that very early ECD determination by SM is often difficult because of the residual corneal oedema and Descemet folds, which last several weeks. Most postoperative ECD values are reported only at 3 or 6 months, when SM images become easy to obtain. Nevertheless, our team reported a prospective series of 25 PKs where SM was reliably performed as early as 5 days postgraft in 75% of cases.[8] In addition, the usual animal corneal models (rabbit, rat) are not suitable for this study since no satisfying long-term storage in OC has ever been described.

Recently, we reported a case of bilateral simultaneous PK comprising an allograft with an OC cornea and an autograft, showing that almost no very early EC loss occurred after autograft, for which all ECs were by definition viable.[9] In this cornea, preoperative and postoperative cell counts were reliably determined using the same SM. This case suggested that the surgical trauma is indeed minimal.

To further study the gap between preoperative and very early postoperative ECD, we designed two complementary clinical and experimental studies with very early postoperative ECD monitoring.

MATERIALS AND METHODS
Study design and ethics statement

This work comprised one observational clinical study and one experimental study (figure 1). All procedures conformed to the tenets of the Declaration of Helsinki for biomedical research involving human subjects. For the two studies, a total of 100 consecutive patients who underwent PK in our department were enrolled during year 2013. We previously determined the variance of the distribution of ebECD in our European donor population (n=1591 consecutive donors) using the same methods of ebECD counting.[10] We calculated that a sample of 100 patients would allow obtaining 95% confident intervals with a ±5% width, which we consider as a reasonable accuracy. Patients received the usual care except for non-contact SM, which was systematically performed at day 5 (D5) postoperatively (discharge from hospital). A preliminary study (data not shown) had confirmed that SM images could be obtained by D4 in <10% of cases. The rest of the follow-up was performed as per the routine protocol in our department. Given this minor change to the usual postoperative procedure, the study belongs to a category of research on routine care, requiring that patients be informed of the research goal and protocol but not their signed consent (Ethics Committee ruled that approval was not required for this study).
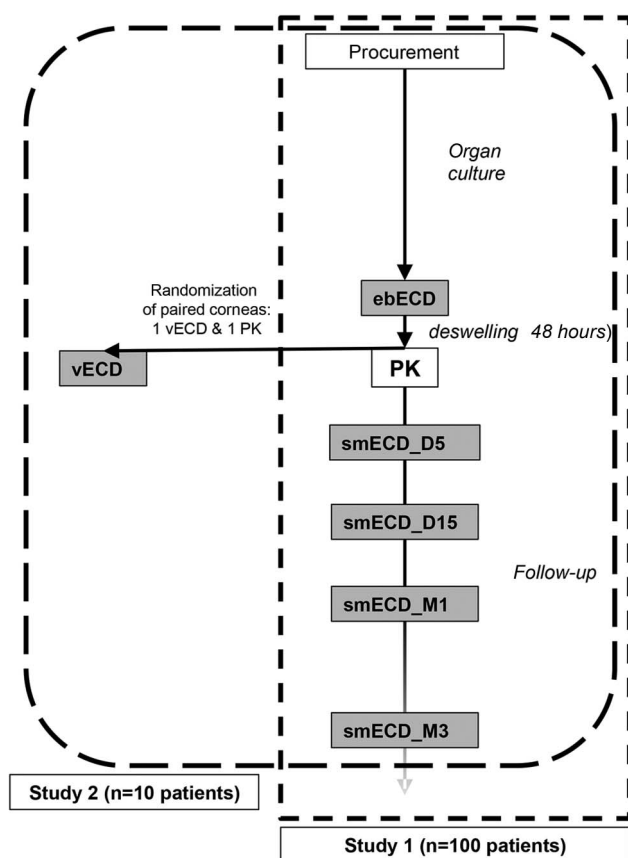

Figure 1 Study design. In study 1 (observational), very early endothelial cell density (ECD) was determined in 100 patients after penetrating keratoplasty (PK); in study 2 (experimental), 10 paired corneas with similar ECD were randomised. One cornea was grafted and the recipients were monitored (subgroup of study 1); the same day, the other cornea was sent to our laboratory for determination of its viable ECD (vECD). ebECD, eye bank ECD; smECD, specular microscopy ECD; D, day; M, month.

Patients

All PKs were performed under general anaesthesia by a senior surgeon (PG, more than 1500 PKs). Briefly, the cornea was trephined at 8.00 mm using a Vacuum trephine (Barron Radial Vacuum Trephine, Katena, Denville, New Jersey, USA) and replaced by an OC cornea, trephined at 8.25 mm with a vacuum punch on its endothelial side (Barron Vacuum Donor Cornea Punch, Katena) and sutured with intracameral viscoelastic protection (Viscoat, Alcon, Rueil Malmaison, France) by a single running suture with a 10/0 nylon monofilament. At the end of surgery, dexamethasone was injected subconjunctivally. Postoperative treatment was eye drops consisting of a fixed association of tobramycin and dexamethasone, and of indometacin, three times a day for the first month. Dexamethasone was then prescribed and was tapered over the following 8 months.

Methods of ECD determination

Three methods were used at different times to measure ECD.

Transmitted light microscopy in the eye bank

All corneas were stored at 31°C in 100 mL of commercial OC medium containing 2% of foetal calf serum (CorneaMax, Eurobio, Les Ulis, France). Preoperative ECD was determined using the standard process of our eye bank as described elsewhere,[11 12] during transfer to the final deswelling step in dextran-containing medium (CorneaJet, Eurobio), that is, 48 hours before surgery. This storage and endothelial assessment sequence respected European eye-banking guidelines. Briefly, the cornea was handled under a laminar flow hood in a clean room, rinsed with balanced salt solution (BSS, Alcon) and the endothelial side was exposed during 1 min to 0.4% trypan blue (Eurobio). After gentle rinsing with BSS, the endothelial side was incubated at room temperature with 0.9% sodium chloride (Aguettant, Lyon, France) renewed every minute, to trigger an optimal intercellular space dilation that make cells visible under the light microscope. Finally, the cornea was placed in a sterile hermetic Petri dish and observed under a direct transmitted light microscope (Leica, Leitz Laborlux, Wetzlar, Germany) equipped with a digital camera (DXC-390P, Sony, Tokyo, Japan). Ten microscopic fields of 768×576 µm were acquired in the 8 mm central area. ECs were counted in three non-overlapping fields selected at random, using the Sambacornea software (TribVn, Chatillon, France), after calibration with a certified Leitz micrometre. Cell boundaries of more than 300 cells were automatically detected, and carefully verified by a skilled technician (more than 5000 cell counts) who performed all necessary corrections. Accuracy of the analyser, based on cell boundary recognition in a variable frame, had been validated and we showed that it halves the variability of cell counts compared with manual counts done with the conventional fixed-frame technique.[11–14] We also showed that it had very high accuracy.[15] This ECD is hereafter referred to as ebECD.

Fluorescence microscopy in the laboratory

For a subset of 10 pairs of corneas with similar ebECD (intra-pair difference <5%), a centralised randomisation was performed. One cornea was grafted as usual. For the other, viable ECD (vECD) was determined on the same day to obtain a reliable estimation of the number of living ECs grafted in the patients. vECD was measured using a method that we recently reported, called HEC staining (for Hoechst/ethidium/calcein), and improved by the use of dedicated image analysis software (CorneaJ, PlugIn for imageJ). Briefly, corneas, placed endothelial

side up in a sterile Petri dish, were incubated for 45 min at room temperature with 150 mL of Hoechst 33 342 (10 mM), ethidium homodimer-1 (4 mM) and calcein-AM (2 mM) in phosphate-buffered saline, then were gently rinsed in phosphate-buffered saline. The 8.25 mm central buttons were flat-mounted under a coverslip to avoid parallax error due to corneal curvature, the whole flat endothelium was observed with an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan) and ECD was determined with CorneaJ, a specific plugin for the freeware ImageJ,[16] after proper calibration using the same certified micrometre.

Postoperative SM of the patients in the ophthalmology department

All images were acquired using the same non-contact SM (SP2000P, Topcon, Tokyo, Japan). The central endothelium was observed at D5, D15, D30, D60 and D90 postoperatively. Central ECD was subsequently determined using the cell analysis module of the Imagenet software (Topcon). Cell boundaries were drawn manually on the widest area permitted by the software. In all cases, the reliability of boundary drawing was verified by two independent experts (ASG and GT) on a screen copy of the processed pictures, and corrections were made if necessary until cell recognition was considered perfect by both experts. ECD in patients is hereafter referred to as SM ECD (hereafter referred as smECD).

Statistics

Data distribution normality was tested using Shapiro-Wilk and Kolmogorov-Smirnov tests with a threshold for non-normality set at $p < 0.05$. Non-normal data were described using their median (10°; 90° percentiles). Normal data were described using the mean and its 95% CI. Analysis was performed using IBM SPSS Statistics V.23 (IBM, Armonk, New York, USA).

RESULTS

Donors' characteristics

One hundred corneas of 74 donors (40 males) were studied. Donor age was 71 (48; 87) (1h00–24h30) (1–24h30) hours. OC lasted 21 (14; 27) days. At the end of storage, ebECD, measured 2 days before surgery, was 2614 (2181; 3180) cells/mm² and was determined by counting 329 (310; 389) cells.

Recipients' characteristics

The 100 recipients were grafted for late endothelial failure on a previous PK (37%), pseudophakic bullous keratopathy (27%), stromal dystrophy (mostly, and unusually, lattice in our central French region of Auvergne Loire) (11%), keratoconus (10%), Fuchs' endothelial corneal dystrophy (FECD) (7%), postinfectious keratitis scars (6%), corneal perforation (2%). They were grouped according to their preoperative endothelial status as low (63%) or normal (30%), excluding FECD (7%) because of the frequently large peripheral endothelial reserve that may partly explain the good prognosis of PK in this subpopulation. All surgery and postoperative periods were uneventful. No rejection episode was observed during follow-up. For 87% of the patients, the first SM was possible at the fifth postoperative day. It was possible at D15 for seven more patients, at month 1 (M1) for five more patients, and only at M3 for one patient. The number of ECs able to be counted, an indicator of image quality, logically increased significantly over time: 40 (24; 59) at D5, 62 (40; 97) at D15, 71 (46; 112) at M1 and 80 (50; 107) at M3 ($p < 0.001$).

Overall postoperative ECD (study 1)

The decrease in smECD was represented in figure 2. Calculated as a percentage of ebECD, as in all papers studying postoperative cell loss, the decrease in ECD was 28% (95% CI 26 to 30) at D5, 33% (95% CI 31 to 36) at D15, 37% (95% CI 35 to 40) at M1 and 42% (95% CI 40 to 44) at M3 ($p < 0.001$, calculated over the whole series). Cell loss followed a Gaussian distribution. Between D5 and M3, ECD and postoperative time were inversely correlated with Pearson's $r = -0.305$ ($p < 0.001$), and D5 to M3 cell loss was 19% (95% CI 16 to 22) (calculated on paired data). None of the postoperative cell losses differed between recipients irrespective of endothelial status (normal, low, or FECD) (data not shown).

Direct comparison between ebECD, vECD and smECD (study 2)

For the experimental study of 10 pairs of corneas, the ebECDs were 2466 (2160; 3113) cells/mm² for the grafted corneas and did not differ from the paired corneas intended for vECD determination, with 2446 (2168; 2747) cells/mm² ($p = 0.139$, Wilcoxon non-parametric rank test). The 10 corneas were grafted after 17 (14; 29) days of OC, a duration not different from the whole series ($p = 0.587$, Mann-Whitney non-parametric test). Surgical indications did not differ ($p = 0.559$, χ^2 test). vECD was 1642 (1168; 1950) cells/mm², that is, 33% (22; 48) lower than ebECD ($p < 0.001$, Wilcoxon non-parametric rank test). vECD was comparable with smECD at D5 with 1577 (1327; 2060) cells/mm² ($p = 0.799$), and with smECD at D15 with 1490 (1128; 1847) cells/mm² ($p = 0.203$) (figure 2). The difference between endothelial image types at each time point is illustrated in figure 3.

DISCUSSION

Using a large prospective series, we show that ECD measured 5 days after PK with organ-cultured corneas in CorneaMax is approximately 30% below the ECD determined by an eye bank 2 days before surgery using an accurate endothelial analyser. Furthermore, we show that this number of cells, measured days after PK, is very similar to the number of living cells counted immediately before surgery in paired corneas having undergone exactly the same storage process. In consequence, in OC, ebECD substantially overestimate the number of living ECs.

Reliability (accuracy and reproducibility) of smECD 5 days after graft is of course lower than smECD determined later because residual stromal oedema and endothelial folds limit the number of ECs clearly visible. Nevertheless, cell counts repeated at D15, M1, M2 and M3 are in total coherence with D5 (figure 2).

Assessment of ECD in the very first days after PK has already been reported in a series of 32 grafts performed with 4°C stored corneas, with ebECD determined by SM.[17] Four days after PK, ECD, determined by contact confocal microscopy, ranged from 1666 to 2548 cells/mm² (mean±SD, 2125±283 cells/mm²) corresponding to the difference with the ebECD, which varied from 0% to 29% (mean, 12%). The gap between ebECD and post-PK ECD, which seems smaller than in our study, is likely explained by the significant differences between 4°C storage and OC. Generally speaking, three characteristics of 4°C storage may have a particular impact: (1) limited stromal swelling due to the presence of dextran and chondroitin sulfate throughout storage and consequently few endothelial folds; (2) absence of the final deswelling step causing osmotic stress and (3) counting
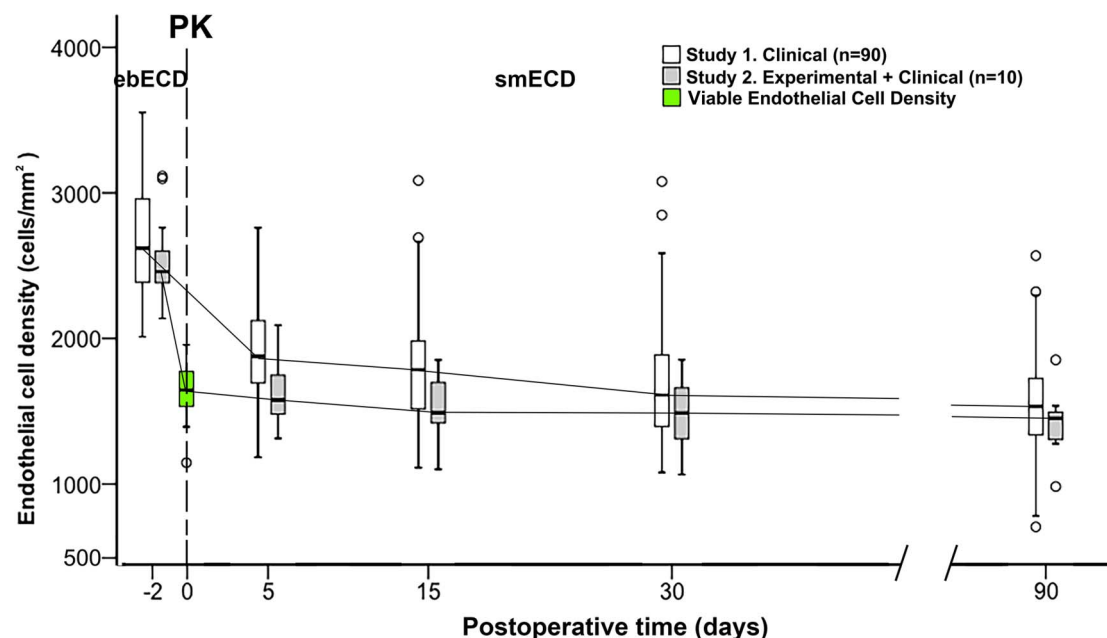
Figure 2 Box plots of endothelial cell density (ECD) over time. Study 1 was the follow-up of 90 penetrating keratoplasties (PKs), and study 2 of 10 pairs of corneas: one grafted as usual, the other with laboratory determination of its viable ECD at day 0, highlighted in green. Thick horizontal lines show the distribution median; boxes, the IQR and individual circles, the outliers. Whiskers mark the highest and lowest non-outlying values. A circle is between 1.5 and 3 times the IQR. The vertical dashed line indicates time of surgery. ebECD, eye bank ECD on grafts; smECD, specular microscopy ECD in recipients.
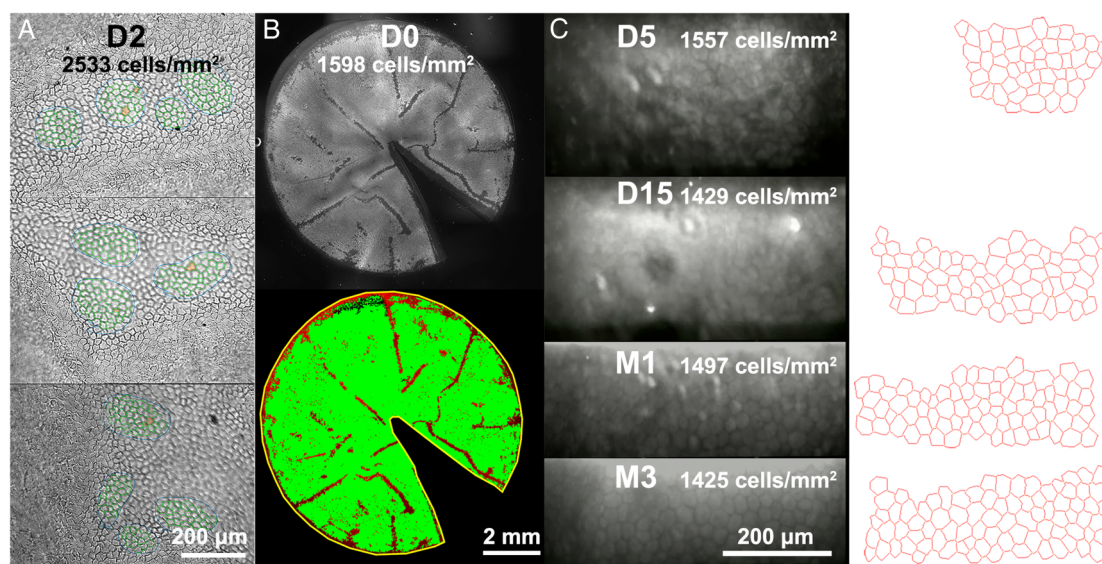

Figure 3 Representative example of the three methods of endothelial cell counting used in the study. (A) Eye bank endothelial cell density (ebECD) of the grafted cornea determined 2 days before penetrating keratoplasty with image analysis on 3 images and 325 cells counted. (B) Viable ECD (vECD) determined on the paired corneas on the day of surgery, with laboratory triple Hoechst/ethidium/calcein-AM labelling. The top image is calcein-AM staining of the 8.25 mm diameter graft after flat mounting with a radial incision. The black area revealed non-viable cells or area without cells, such as folds. The bottom image shows image analysis with the CorneaJ plugin for ImageJ, necessary to measure areas covered by living cells. (C) Postoperative sequence of specular microscopy showing the raw image and corresponding manual cell contours in red with, respectively, 52, 69, 76 and 81 cells counted. vECD was 37% lower than ebECD, but in noticeable agreement with postoperative smECD. D, day; M, month.

of ECs with similar specular microscopes in the eye bank and in patients.

The limitations of measuring ebECD in OC were recently discussed.[9] The reasons for ebECD overestimation can be summarised as follows: (1) failure to take account of the areas deprived of ECs and of dying cells, especially in endothelial folds (also called stress lines);[7] (2) failure to take account of supplementary ECs dying between counting and graft, especially during deswelling time in OC;[18–21] (3) considering too small a sample (50–100 cells is valid for SM in living patients, but probably not for stored endothelium, which is not homogeneous) and (4) failure to take account of the three-dimensional aspect

of the stored corneas, especially of folds where cell counting results in overestimation caused by parallax error. In addition, a degree of corneal shrinkage, due to absence of intraocular pressure and to tissue swelling, could also contribute to overestimating ECD (same number of ECs on a smaller area) and could also be true for 4°C stored corneas. There is thus sufficient evidence that even the most accurate ebECD overestimates vECD. This study quantifies this overestimation for PK. The rather large variation that we observed in the series of 10 corneas (median 33, with 10° and 90° percentiles, respectively, of 22% and 48%) may also reflect variation between corneas. Taking all these explanations together, the hitherto-unknown difference between standard ebECD and the vECD actually grafted in patients may be the main explanation for the absence of a relationship, noted in two studies concerning PK, between ebECD and either graft survival[22] or postoperative ECD.[23] In addition, similar findings were recently described for Descemet stripping endothelial keratoplasty for which the 10-year postoperative ECD correlated with the 6-month postoperative ECD but not with the ebECD.[24] Although mechanisms of postoperative EC loss in PK and endothelial keratoplasty may substantially differ, this new result constitutes further argument for the limitation of ebECD value. Improving the reliability of ebECD requires a radical change of concept. The ideal non-toxic stain able to reveal functioning ECs has yet to be discovered. Meanwhile, there is clearly still room for improving storage methods to improve endothelial survival and homogeneity and thus to count cells on a small sample more representative of the whole population. We already suggested replacing dextran by less toxic molecules such as poloxamers[20] or poloxamines[21] to reduce the negative impact of the deswelling step.

The dominant factor in medium- and long-term survival and in EC loss after PK is graft indication.[25] The main factor therein is the endothelial reserve, located at the periphery of the recipient cornea (50% of the whole endothelial area for an 8.00 mm graft).[26] Redistribution of ECs from high-ECD areas to lower ECD areas seems to be the mechanism involved in the excellent survival of graft in keratoconus, which has a normal periphery; good survival in Fuchs' corneal endothelial dystrophy, where there is usually a near-normal reserve of healthy cells at the periphery and, conversely, poor prognosis in bullous keratopathy, which has no normal peripheral ECs. The dynamic of this phenomenon is relatively slow as, for instance, it takes 5 years for 40% of grafts to fail from late endothelial failure in bullous keratopathy[25 27] and 15 years to reach 50%.[27] This study demonstrates that the very short-term behaviour of the central ECD of the graft is not influenced by the recipient's endothelial status. Significant redistribution of ECs between graft and the recipient bed might take weeks, which is probably related to the wound-healing process in the trephination area.[28]

Our study of 10 paired corneas, showing noticeable stability between vECD and very early postoperative ECD, demonstrates that the perioperative period does not trigger a high rate of EC death. The role of surgical trauma, particularly trephination, may only be a minor factor in this very early EC loss. The endothelial destruction triggered by trephination from the endothelial side (the most widespread technique) was characterised more than 35 years ago, and corresponds to a ring of 0.05–0.28 mm width[4] (ie, a near 10% reduction in endothelium area for a standard 8.25 mm diameter keratoplasty), which is partly offset by oversizing the graft. The total number of viable EC may therefore remain the same as if trephination did not kill EC on a smaller graft. In addition, as stated above, in the very short-term, the speed of EC redistribution is not rapid enough for the central ECD to be influenced by EC death in the trephination area.

Surprisingly, we found −28% of ECs at D5, −33% at D15, reaching −42% at M3, percentages that are rather in the range of the typically accepted cell loss at 6 months (−28.8% for Bertelmann et al)[29] or even 1 year in normal-risk PK after OC (−39.4% for Harper et al,[30] or −29.7% for Borderie et al).[2] Looking ECD, instead of cell loss, of different series of PK performed with OC corneas, they were substantially and systematically lower for our patients. Table 1 summarises these comparisons and show that we reach in 1 month, ECD that are usually reported only after 1 year. Despite series are not fully comparable and ECD never reported as early as in our series,

Table 1 Earliest postpenetrating keratoplasty ECD reported in the literature compared with the present series

Author(s), journal, year	Storage method and medium	Donor age (year)	ebECD	ECD and postoperative time	Number of patients and indications
This study	OC CorneaMax/CorneaJet	71 (48; 87)	2614 (2386–2958) 2 days before graft	1857 (1620–2091) at 5 days and 1594 (1392–1865) at 1 month	100 (all comers)
Hagenah et al, Ophthalmologe, 1997[31]	OC at 31°C Homemade	46±18	2643±420	1549±410 at 1 year	30 (6 keratoconus, 10 stromal dystrophy, 5 Fuchs, 5 bullous, 4 others)
Frueh and Böhnke, Archives of Ophthalmology, 2000[32]	OC at 36°C Likely homemade*	50±14	2617±395 (at retrieval)	2327±341 at 1 month	12 (8 keratoconus, 2 Fuchs, 1 Herpes, 1 bullous keratopathy)
Borderie et al, Ophthalmology, 2009[2]	OC at 31°C Commercial media (Likely Inosol, Opsia, Labège France† and CorneaMax)	70±14	2264±295	1604 at 1 year	1062 (all comers)
Patel et al, American Journal of Ophthalmology, 2005[27]	CS in McCarey-Kaufman CS in Optisol-GS OC medium at 34°C	NA	2973±550	2467±675 at 2 months	394 (all comers)
Lass et al; Cornea Donor Study, Ophthalmology, 2008[33]	CS in Optisol-GS	Subgroup of 66–75 (n=108)	2585 (2445–2792)	2350 (2200–2800) at 6 months	60 Fuchs dystrophy and bullous keratopathy

Data are provided as media (IQR) or mean±SD depending on the original data in each article.
*Data interpreted from the corresponding European Eye Bank Association directory.
†Now discontinued.
CS, cold storage; ebECD, eye bank ECD in cells/mm²; ECD, endothelial cell density; NA, not available; OC, organ culture.

data suggest that differences in donor age between series may not be involved because when donors were younger, ebECD were similar. In addition, donor age has very little influence on postoperative ECD.[31] Populations of recipients are comparable, except for the Cornea Donor Study in which only endothelial deficiencies were recruited. The only parameter that substantially differs between series is the storage medium. In consequence, we cannot rule out that EC survival in the commercial medium we used is worse than in other media. Further comparative study would be necessary to confirm these suppositions but in practice, the present medium is the only authorised in France.

In conclusion, we demonstrated that, in OC with CorneaMax/CorneaJet, ebECD determined 2 days before graft overestimates by nearly 30% the number of ECs that are viable at the time of graft, and that surgeons therefore graft fewer living cells than they think. Until we have a non-toxic method to determine vECD in eye banks, calculation and modelling of postoperative EC loss after PK should take as its reference not ebECD but smECD, measured in the recipient as soon as possible. Consequently, models of postoperative ECD decrease should be revisited because there is no high cell loss immediately after PK, and therefore no specific biological background.

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