

## ***Ex-vivo* Potential of Cadaveric and Fresh Limbal Tissues to Regenerate Cultured Epithelium**

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**Purpose:** To evaluate and compare the *ex-vivo* growth potential and formation of cultured corneal epithelium from residual corneo-limbal rings obtained from the operating room after penetrating keratoplasty, and fresh limbal tissues from patients undergoing routine cataract surgery.

**Methods:** With the approval of the Institutional Review Board and informed consent from patients, 1-2mm of limbal tissues from 15 patients and 31 tissues from the cadaveric limbal ring preserved in MK medium (16 tissues) and Optisol (15 tissues) were used for the study. Donor data included age, time lapse between death and collection, collection and preservation and preservation and culture. Tiny bits of the limbal tissue were explanted on the de-epithelialised human amniotic membrane prepared following standard guidelines, and cultured using Human Corneal Epithelial cell medium. Radial growth from the explant was observed and measured by phase contrast microscopy over 2-4 weeks. After adequate confluent growth, whole mount preparation of the membrane was made and stained with haematoxylin and eosin. Part of the membrane was fixed in formalin and processed for routine histologic examination. The sections were stained with haematoxylin and eosin.

**Results:** Forty-six tissues were evaluated from 42 eyes (15 from patients, 31 from cadaveric eyes) with a mean age of 55.3 years  $\pm$  21.23 years (range 18 years – 110 years). The growth pattern observed was similar in all the positive cases with clusters of cells budding from the explant over 24- 72 hours, and subsequent formation of a monolayer over the next 2-3 weeks. The stained whole mount preparation showed a radial growth of cells around explants with diameter ranging from 5 to 16mm. Histologic evaluation of the membrane confirmed the growth of 2-3 cell-layered epithelium over the amniotic membrane. Cultivated epithelium around explant cell cultures was observed in 100% (15/15) of limbal tissue obtained from patients, as against 56% (9/16) of MK medium preserved tissues and 46.7% (7/15) of Optisol preserved tissues. This was statistically significant ( $P=0.0131$ ) There was no significant statistical difference in the growth properties, i.e, the mean percentage of fragments showing growth ( $P=0.229$ ) or the mean diameter of growth ( $P=0.479$ ) in the cultures obtained from fresh and preserved tissues. The time lapse at various stages between death and utilisation and donor age had no significant influence on the growth potential of the limbal tissues.

**Conclusion:** The potential for generating cultured corneal epithelium from fresh limbal tissues obtained from living subjects is higher than that observed with preserved tissues. It would also be worthwhile to address the factors that could further enhance the proliferative potential of the cadaveric tissues obtained from eye banks.

**Key Words:** Limbal stem cells, human amniotic membrane, cadaveric limbal tissue, cultured corneal epithelium, explant tissue cultures, histopathology

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Ever since the role of limbal stem cells in maintaining the integrity of the ocular surface was established,<sup>1-5</sup> various approaches have been adopted to treat limbal stem cell deficiencies and other ocular surface disorders. These treatment modalities include transplantation surgeries like autologous limbal transplantation,<sup>4-5</sup> allogenic limbal transplantation from cadaveric tissues, living related or unrelated donors,<sup>5-9</sup> the use of human amniotic membranes,<sup>10,11</sup> and use of cultured limbal epithelium.<sup>12-18</sup> Various investigators have reported highly successful results of autologous limbal transplantation (direct or as cultured cells) in unilateral limbal stem cell deficiency.<sup>4,5</sup>

The outcome of allografted limbal tissue, however, has varied, depending on the type of tissue (fresh Vs cadaveric), use of HLA-matched or unmatched tissues, and the use of immunosuppressants. The debate arising in the context of allografts relates to the source of limbal tissue: (cadaveric Vs living-related individuals), and the utility of HLA matching. In our clinical experience (unpublished observation), we found that the conjunctival-limbal allografts from live-related individuals survived better than cadaveric allografts, both treated with immunosuppression. We hypothesised that the viability, survival and growth of progenitor cells in fresh limbal/conjunctival tissues is possibly better than that in the preserved tissues obtained from the cadaveric sources. To test our study hypothesis, we evaluated and compared the generating potential of limbal epithelium *ex-vivo* from limbal cadaveric tissues obtained from residual corneoscleral rings after completion of keratoplasty, with limbal tissues from a control group of patients.

## Material and Methods

### Limbal tissue

The study protocol was approved by the Institute's Review Board. After obtaining informed consent from patients undergoing routine cataract surgery, 1-2 mm of limbal tissue was taken randomly from superior or inferior quadrants. The conjunctiva was incised at the 12 to 1 o' clock position, 3 mm behind the limbus, avoiding the Tenon's capsule. The dissection was continued using # 15 blade, towards the limbus into clear cornea, following the plane of limbal insertion of conjunctiva. About 2x2 mm strip of limbal tissue was excised and submitted for culture. The cadaveric limbal tissue was obtained from the residual corneo-scleral rings after keratoplasty. About 2x2 mm of limbal tissue was harvested approximately 1mm on either side of the corneo-conjunctival junction. After making a conjunctival flap the dissection was carried from the conjunctival surface towards limbus and cornea, following the plane of limbal insertion of conjunctiva. This facilitates mostly a full-thickness biopsy of epithelium with minimal amount of stroma.

Corneo-scleral rings from Optisol as well as McCaurney Kauffman preserved tissues were included in the study. Donor data noted in all cases included the age of the donor, and the various time intervals including that between death and retrieval, duration of preservation in the eye bank, and between keratoplasty and tissue culture. Growth from any one fragment of the explanted tissues was taken as positive growth. The results of the growth from the fresh and preserved limbal tissue was compared using the Student's t test, while the growth between two types of preserved tissue was compared using Fischer's exact test, and the Wilcoxon rank sum test.

### Human amniotic membrane preparation

The human amniotic membrane was prepared using the standard protocol reported by Kim.<sup>10</sup> In brief, amniotic membrane was taken from placenta obtained from Caesarian section deliveries, after testing the donor for HIV,

HBS Ag and VDRL. This placenta was washed with Ringer's solution containing antibiotics and the amnion was separated from the chorion. The amniotic membrane thus separated with epithelial side up was spread on to the nitrocellulose paper. Then the nitrocellulose paper along with the amniotic membrane was cut into 2.5 x 4 cm pieces and stored in DMEM vials at -70°C. Just before use, the amniotic membrane was thawed at 37° C for half an hour, peeled from the nitrocellulose paper and spread over the cut glass slide with the epithelial surface up. The amniotic epithelium was digested using 0.25% trypsin in 0.2% EDTA at 37° C for half an hour, and then scraped with the help of a glass slide for 1-2 minutes. After the membrane was repeatedly washed with phosphate buffered saline, it was microscopically examined under sterile conditions for complete removal of cells. Some membranes were stained by haematoxylin and eosin to confirm de-epithelialisation. The de-epithelialised membrane was then spread on a glass slide measuring 2.5 x 3cm (used as culture inserts) in a petri plate, tucking the edges for a uniform surface. The whole process was carried out in the tissue culture room, under a laminar flow hood with filters and UV light.

### Culture media

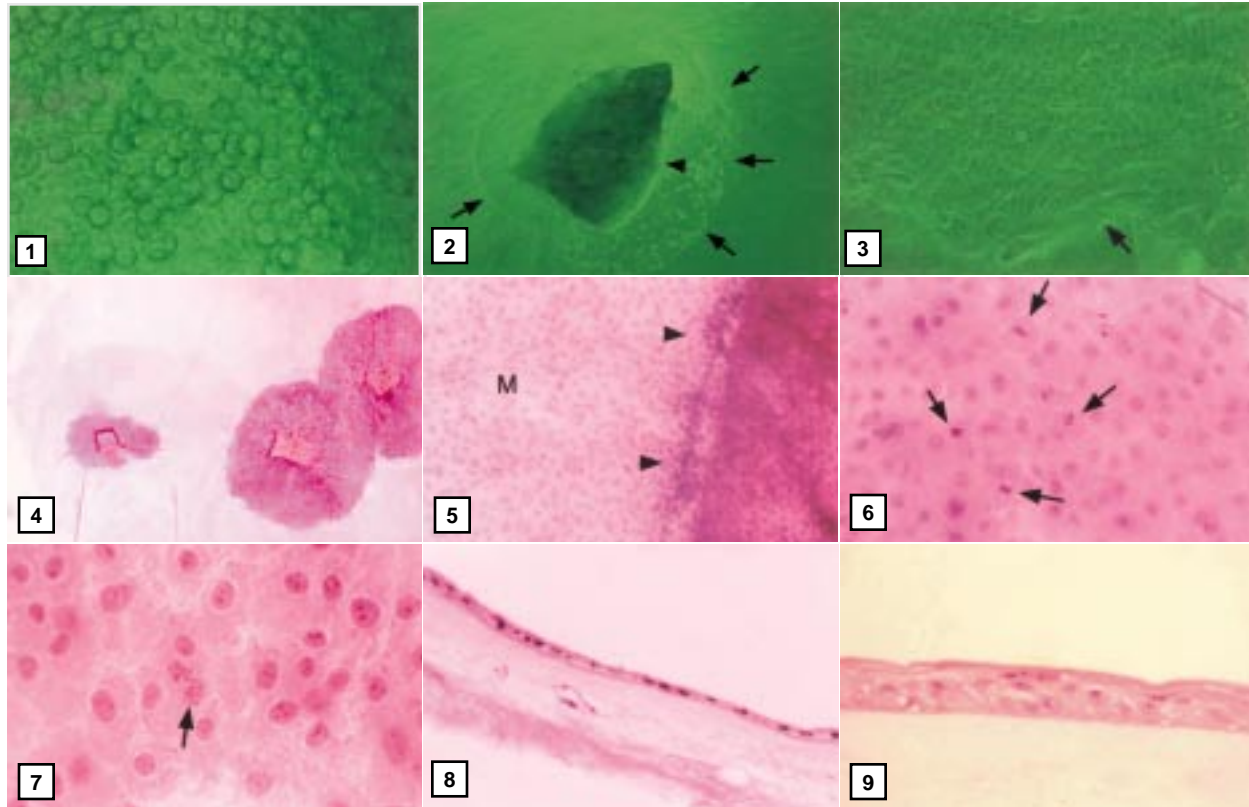
The medium was similar to that used to culture the human corneal epithelial cells.<sup>19</sup> In brief, it was prepared using 3.9g/1 Modified Eagle medium with addition of 6.66g/1 Ham's F12 serum, 0.01mg/1 epidermal growth factor, 5mg/1 insulin, 0.1mg/1 cholera toxin, and 4mg/1 of gentamicin. This was supplemented with 10% foetal calf serum (FCS) at the time of use. The pH of the medium was adjusted to 7 and was filtered using Millipore vacuum (0.22micron) filter. [Source of reagents: Sigma -Aldrich Chemie (Steinheim, Germany) and Sigma Chemical Co (St.Louis, USA). 0.22 filters were from Millipore Corporation (Bedford, MA, USA).]

### Explant culture technique

Each limbal tissue from both sources was cut into 4-6 tiny fragments and separately placed on to the de-epithelialised membranes. After allowing the tissues to settle on the membrane for half an hour, the membrane was flooded with culture medium and the plate was left overnight in an incubator at 37° C with 5% CO<sub>2</sub>. The following day, 2-2.5ml of human corneal epithelium (HCE) medium with 10% FCS was added to the plates and they were incubated for 15-21 days, changing the medium on alternate days.

### Whole mount preparation

After confirming confluent growth from the explanted tissue over 1-2 weeks under an inverted microscope, the growth was terminated by replacing the media with 10% buffered formalin. The amniotic membrane with the cultured cells was used to make the whole mount preparation stained with haematoxylin and eosin. The mounted preparation of the explants was examined for the ring of cultured cells. The diameter of the visible outgrowth from each fragment of the explanted tissue and the degree of growth (number of fragments showing growth) were measured from the whole mount



**Figure 1.** The microscopic picture of explant culture from fresh tissue on day 2 with clusters of round cells growing from the edge of the explanted limbal tissue. (x 400); **Figure 2.** The explant cultures on subsequent days showed a circular growth (arrow) arising from the edge of the explant (arrow head)(x 40); **Figure 3.** On day 7, there is formation of a monolayer of polygonal epithelial cells which are closely packed. (x200); **Figure 4.** The hematoxylin and eosin stained whole mount preparation of amniotic membrane with cultured cells around the fresh limbal tissues. (x200); **Figure 5.** The microscopic picture of the stained whole mount reveals the cultured cells around the edge of the explant (arrow). The cells are seen to form a monolayer over the amniotic membrane. (hematoxylin and eosin, x 250); **Figure 6.** At higher magnification, the cells show a vesicular nucleus with prominent nucleoli. Frequent mitotic figures were noted (arrow). (hematoxylin and eosin, x 500); **Figure 7.** A few cultured cells show nuclear condensation and fragmentation suggestive of apoptotic cell death (arrow). (Hematoxylin and eosin, x 500); **Figure 8 & 9.** (8) The section of the human amniotic membrane with the native cuboidal epithelium. (hematoxylin and eosin, x 250) (9) The section of the human amniotic membrane (HAM) with the cultured epithelial cells (C. Epi) forming 1-2 cells of squamous cells (Haematoxylin and eosin, x 500).

preparations in all cases. The growth was qualified *good* (diameter of the cultured cells around the explanted  $\geq 12$ mm) *average* (diameter of cultured cells 8-11mm), and *poor* (diameter of cultures cells < 7mm.) The culture potential of fresh and preserved tissues was compared using statistical methods. In three cases, part of the amniotic membrane with the explant culture was processed for routine histopathologic examination, to confirm the nature of the cultured cells.

#### Histopathology of cultured cells

In three cases, part of the membrane with the cultured limbal tissue and the cultured cells was fixed in formalin and processed for routine histopathology with paraffin embedding. The sections were cut at 4-5 microns and after deparaffinisation, stained by haematoxylin and eosin stain.

Normal amniotic membrane without trypsinisation was also studied in 3 cases by routine histology.

#### Results

In this study, a total of 46 limbal tissues were evaluated for the growth potential of the limbal cells. Of these 15 were fresh tissues (from patients); 31 were from cadaveric corneo-scleral rims - 15 from MK media preserved tissues and 16 from Optisol preserved tissues. The tissues were obtained from 42 subjects including 24 males, 13 females; and in 5 subjects, the gender information was not available. In 4 cases, tissues were obtained from both corneo-scleral rims of the same donor.

The mean age of live donors was  $37.8 \pm 18.8$  (range 18-65) years and the cadaveric donors was  $63.7 \pm 16.8$

(median 65 years, range 25-110) years with a majority of samples (29/31, 92%) from patients above 50 years. There was no difference in the growth potential of tissues obtained from male (18 of 24, 75%) and female (9 of 14, 69%) donors in each group.

### Explant cultures of fresh tissues

Successful limbal explant cultures were noted in all (15/15) fresh limbal tissues obtained from patients undergoing routine cataract surgery. The pattern of growth was the appearance of clusters of rounded cells at the periphery of the explant by day 2-3 (Figure 1). By day 3-5, there was a monolayer of polygonal to angulated cells in the background (Figure 2&3), that expanded to cover the entire amniotic membrane in 2-3 weeks.

The whole mount preparations on naked eye examination revealed a ring of pink darkly stained areas around the explanted tissue (Figure 4). The ring was absent in explants with no growth, which showed only the original explanted stained tissue. The mean percentage of growth in all fragments in positive explants as observed in the stained mounts was  $85.5\% \pm 23.4\%$ . The average diameter of the explant was  $13.8\text{mm} \pm 6.4\text{mm}$  (range 8 mm to 21 mm).

The stained whole mount preparation of the cultured explants when observed under the microscope revealed a monolayer of polygonal cells, originating from the edge of the explanted tissue (Figure 5). The nuclei of the epithelial cells were vesicular with 2-3 nucleoli in the cells. Frequent mitotic figures were noted (Figure 6). Some of the cells showed nuclear fragmentation, morphologically suggestive of apoptosis (Figure 7).

### Explant cultures of preserved tissues

The average duration from death to utilisation of these preserved tissues was 52.7 hours for MK medium preserved tissues and 382.52 hours for the Optisol preserved tissues. The details of various time intervals in each media is given in Table 1. Explant growth from the preserved tissues was noted in 51.6% (16/31) of all cases; 56% (9/16) in MK preserved tissues and 46.7% (7/15) in Optisol preserved tissues. The negative cultures were reconfirmed after terminating the growth. None of negative cultures revealed any cells around the explants in the stained whole mount preparation, confirming the total lack of growth. The mean percentage of growth in explanted tissue bits was  $76\% \pm 19.2\%$ .

### Histopathology

The histopathology of the normal human amniotic membrane revealed a single layer of cuboidal epithelium (Figure 8) whereas the de-epithelialised membrane had no lining cells. In the three cases where the explant culture was studied by histology, there were 1-2 layers of cuboidal cells (Figure 9). Focal areas of stratification were noted with pigmentation of cells near the edge of the explants.

### Comparison between live and cadaveric tissues:

The differences in the growth properties of fresh and preserved tissues are given in Table 2. The growth potential of live-donor tissues was significantly better than that seen in the preserved tissues (100% vs 52%);  $P=0.0131$ ). Once the growth was initiated, there was no difference in the growth properties of both tissues. The mean diameter of explant cultures of fresh and preserved tissues ( $13.8$  vs  $12.37\text{mm}$ ) was not statistically

**Table 1. The time lapse between death to enucleation, enucleation to receiving of consignment, preservation to utilization and the total time lapse for the MK preserved tissues and Optisol preserved tissues**

Medium	Number	Min. hours	Max. hours	Mean duration	Std deviation
MK					
Enu-D	16	0.50	8.60	3.1750	1.8036
Enu- rec	16	0.50	17.00	3.4063	5.3431
Prep-Util	16	12.00	96.00	45.8938	31.4445
Total time	16	17.50	102.00	52.7500	32.4861
Optisol					
Enuc-D	15	1.20	7.10	3.8200	1.5138
Enu-Rec	15	121.00	227.50	171.6800	36.3766
Pre-Uti	15	134.70	265.00	209.1067	39.2716
Total time	15	267.00	488.60	382.5267	65.0426

MK- McCarney Kauffman medium; Enuc-D- Enucleation to death; Enu- Rec- Enucleation to Receiving of consignment; Pre-Uti: Preservation to Utilization time

**Table 2. Comparison of growth potential of fresh and preserved limbal tissues obtained from cadaveric eyes.**

Growth pattern	Fresh tissues	Preserved tissues	Significance
Successful	15/15(100%)	16/31(51.6%)	P=0.0131
Mean diameter *	13.8mm	12.37mm	P=0.479
Mean Degree of growth**	85.5%	76%	P=0.229
Mean Age of donor	37.8	63.7	P<0.0001

\*Mean diameter of cultured epithelium in all cases of that group.

\*\* Mean Percentage of fragments showing growth around explants of all cases, in that group.

significant (P=0.479) as shown in Table 3. Similarly, there was no significant difference (P=0.229) in the percentage of fragments showing growth in live and preserved tissues (85.5 vs 76%).

### Influence of preservative media and donor age on cultures

There was no significant difference (P=0.724) in the growth potential of tissues preserved in MK media, and in Optisol medium (56.3 vs 46.7%). Though the time interval between enucleation and utilisation of tissues for explant cultures was significant (P=0.0001) in MK medium and Optisol preserved tissues (Table 1), the growth characteristics of tissues obtained from both media did not differ. The difference in the mean diameter of growth (13.5mm vs 10.8 mm) and percentage of fragments showing growth (77.3 vs 74.5) in MK and Optisol preserved tissues was not significant (P=0.252 and P=0.0918 respectively).

In view of the lower mean donor age of live donors, further attempts were made to evaluate the effect of different age groups on the proliferative potential of the tissues. The donor age of the cadaveric tissues was divided into 3 categories, (Table 4) (<40years; 40-60 years and >60 years) but was found to have no significant influence on the growth potential in these preserved tissues (P=0.716). The various time intervals between death and enucleation, enucleation to preservation, preservation to utilization or the total duration between death and utilisation of the tissues did not make any difference in the growth potential of preserved tissues.

### Discussion

Studies on corneal cell cultures were attempted long before the limbal stem cells concept was understood.<sup>20-22</sup> With increasing knowledge of limbal stem cells, various groups have successfully generated corneal epithelium from limbal tissues and used it for reconstruction of the damaged ocular surface in limbal stem cell deficiency. Pelligrini et al<sup>12</sup> were the first to report the successful clinical application of the cultured epithelium in two

patients with severe chemical injuries, followed by Tsai, Schwab, Koizumi, and Shimazaki et al.<sup>13-18</sup>

The techniques of culturing limbal epithelium developed by various workers differ in the nature of cultures- direct explant culture or subculture from the trypsinised cells, with or without the use of feeder cells, type of media used, attempt to multilayering the cultured cells, and other features. At our centre, we established an explant culture technique of generating epithelium from the limbal tissue, a presumed source of stem cells. Though the limbal epithelium has been cultured by many groups, the evidence of “stem cells” in the cultured epithelium and the promise of permanent cure to limbal stem cell deficiency in clinical cases has been documented only recently by Rama et al.<sup>23</sup> There is evidence that amniotic membrane when used as a substrate to culture the limbal cells, could propagate the stemness of the limbal stem cells.<sup>24</sup> What is interesting is that despite the lack of evidence of stem cells in the cultured epithelium in other studies, the cultured limbal epithelium was successfully transplanted. The methods of documenting the stem cells in the cultured epithelium include clonal assay, p63 positivity and the keratin profile.<sup>25</sup> We have characterised the cultured cells by morphological features, whole mount preparation of cultures, and histopathology. During the initial period of culturing corneal epithelium from limbal tissues of cadaveric eyes, we noted that the limbal tissues from freshly obtained cadaveric eyes yielded better results than those obtained from the preserved eyes, similar to the results of James et al.<sup>26</sup> We speculated that fresh tissues possibly have a better potential of proliferation as compared to the preserved tissues. In patients suffering from severe bilateral limbal stem cell deficiency, it is important to decide on the choice of allogenic limbal tissue for limbal transplantation procedures, i.e, whether it is to be taken from a living related donor or from the cadaveric tissues. In the belief that our study would help in this decision making, we aimed to evaluate the *ex-vivo* growth potential of limbal cells from fresh and preserved limbal tissues.



**Table 3. Diameter of explant culture noted in fresh and preserved limbal tissues.**

Size of Explant	3-7 mm	8-12 mm	12-21 mm	Total Number
Fresh tissue	4 (26.7%)	2 (13.3%)	9 (60.0%)	15 (100.0%)
MK tissue		5 (55.6%)	4 (44.4%)	9 (100.0%)
Optisol tissue	2 (28.6%)	3 (42.9%)	2 (28.6%)	7 (100.0%)
Total	6 (19.4%)	10 (32.3%)	15 (48.4%)	31(100.0%)

In this study, the *ex-vivo* growth potential of fresh tissues was excellent (100%) compared to 51% in preserved tissues. In a similar kind of study by James et al,<sup>26</sup> the growth potential of limbal tissue from discarded corneo-scleral rings obtained from the operating room was 66.6% (14/21), as compared to 100% in tissues obtained from freshly enucleated eyeballs. Extrapolating these results to predict the success of the limbal allografts from cadaveric and living related subjects, it appears that the allografts from the latter should have a better outcome when compared to the cadaveric tissues, with adequate immunosuppression in both groups. However, the *in-vivo* behavior of limbal cells could be further influenced by factors like survival of the transplanted cultured epithelium, percentage of limbal stem cells in cultured epithelium, the persistence of these cells in the new environment, homing of the donor cells into the recipient limbal niche, establishment of the communication between the limbal stem cells (internal factors) and the surrounding stroma (niche or external factors).

Tsubota et al,<sup>6</sup> have reported the technique of confirming the viability of limbal cells in 35 cadaveric limbal tissues (obtained from the eye bank) by culture techniques. They documented successful growth in all cases after 15 days of culturing the limbal tissue. This was evidenced by an increase in the proliferating cell nuclear antigen (PCNA) as documented by immunofluorescence in all the 35 samples. Though the aim of that study was to report the outcome with the limbal allografts, the observations of the preliminary testing are striking as well as encouraging. This difference could possibly be due to the method of cultures or the limited parameters of assessing the growth potential. Further studies like clonality of the cultured cells, identifying the stem cell marker, comparing the growth potential of limbal cells from different quadrants of the limbus would shed more light on these findings.

The other interesting issue related to donor factors was that no added harm is caused by the minor delays at various stages of tissue harvesting. Furthermore, once the culture is initiated from the explanted tissues of cadaveric origin, it is as good as that derived from live donor for transplantation purposes except for the point of allogenicity. Since living donors are not always

available, the potential of cadaveric corneo-scleral rings should be explored further by harvesting the tissues from fresh globes and utilising them in the appropriate manner.<sup>27,28</sup>

In a study by James et al<sup>26</sup>, a decreasing trend in growth potential of limbal tissues was observed with increasing age. However in this study we could not support or confirm the same findings. The mean age of patients donating the fresh tissues was lower than the age of the cadaveric donors. Therefore to evaluate the influence of age on the growth potential of the cadaveric tissues, the donors were categorised into three groups (<40, 40-60, >60 years). The effect of age on growth potential in the three age groups of cadaveric tissues was not significant ( $p=0.219$ ). Since the growth was 100% in the fresh tissues, effect of age on the preserved tissues could not be evaluated. This can be explained by the fact that we generally do not get donor tissue from foetal, neonatal or young adults. However, a prospective age-matched study, we believe, would strengthen the observations made in this pilot study.

The cultured limbal cells possibly undergo differentiation and termination with time. The stratification of cultured cells noted by us without the use of an air-water interphase possibly represent this phenomenon. By having an air-water interphase in their cultures. The cultured cells possibly undergo a programmed cell death as evidenced by cells showing

**Table 4. Effect of donor age on the growth potential of preserved tissues**

Age of donor	Growth in MK media	Optisol media	Significance <60 Vs >60 years
<40 years	1/2 (50%)	1/1 (100%)	P=0.558
40-60 years	1/3 (33%)	3/4 (75%)	
>60 years	7/12 (58%)	3/8 (38%)	P=0.265

morphological features of apoptosis in the whole mount preparation and also in histology sections. Human amniotic membrane is shown to have anti-inflammatory and anti-apoptotic influence on the corneal epithelial cells as well on keratocytes.<sup>29-31</sup> However, more detailed studies are required to comment on the cell kinetics, mode of differentiation, termination and cell death of the cultured cells, specific to the cells cultured over human amniotic membranes.

None of the control patients in our study had any complication related to the donor site, following limbal biopsy, as a very tiny fragment of tissue was excised. Similar observations have been made in clinical studies

wherein limbal tissues were harvested for limbal transplantation from the patient's own healthy eye and from living-related donors.<sup>4-9</sup>

In summary, fresh limbal tissue taken from living subjects have a better *ex-vivo* growth potential to generate cultured limbal epithelium as compared to the preserved limbal tissues obtained from the eye bank. We speculate this might have an impact on the success of the allogenic transplantation using cultured cells, and therefore warrants further clinical studies. It would also be worthwhile to address the factors that could enhance the proliferative potential of the cadaveric tissues obtained from eye banks.

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