A PROSPECTIVE, IN VITRO, RANDOMIZED STUDY TO COMPARE AN INDIGENOUS INTERMEDIATE-TERM CORNEAL STORAGE MEDIUM WITH OPTISOL-GS

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CERTIFICATE

This to certify that this dissertation entitled **"A PROSPECTIVE, IN VITRO, RANDOMIZED STUDY TO COMPARE AN INDIGENOUS INTERMEDIATE-TERM CORNEAL STORAGE MEDIUM WITH OPTISOL-GS"** is a bonafide work done by **Dr.Soham Basak** under our guidance and supervision in the Cornea Department of Aravind Eye Hospitals and Post Graduate Institute of Ophthalmology, Madurai during the period of his post graduate training in Ophthalmology for May 2013-April 2016.

DR.N.VENKATESH PRAJNA
Guide,
Head of the Department,
Cornea,
Aravind Eye Hospital,
Madurai.

DR.S.ARAVIND
Head of the Department,
Aravind Eye Hospital,
Madurai.

DR.M.SRINIVASAN
Director,
Aravind Eye hospital,
Madurai.

DECLARATION

I, **Dr.Soham Basak** hereby declare that this dissertation entitled, **‘“A PROSPECTIVE, IN VITRO, RANDOMIZED STUDY TO COMPARE AN INDIGENOUS INTERMEDIATE-TERM CORNEAL STORAGE MEDIUM WITH OPTISOL-GS”** is being submitted in partial fulfilment for the award of M.S. in Ophthalmology Degree by the Tamilnadu Dr.MGR Medical university in the examination to be held in April 2016.

I declare that this dissertation is my original work and has not formed the basis for the award of any other degree or diploma awarded to me previously.

Dr.Soham Basak,
Aravind Eye Hospital,
Madurai.

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CONTENTS

1.1: INTRODUCTION

Corneal blindness is one of the main causes of preventable blindness and is included in the Vision 2020 program. According to 1999-2000 NPCB data, corneal blindness accounts for 0.90% of the total blindness in India(1). Gupta et al did a meta-analysis in 2013 and estimated corneal blindness to be 0.45%(2). Oliva et al in their publication 'Turning the tide on Corneal Blindness', included Trachoma, vitamin A deficiency disease in the corneal blindness and found it to be responsible for 14% of the 8 million blind people in India(3). Of this 14%, 3.9% was from corneal opacities, 1.5% from childhood causes (including VADD), 1.4% due to trachoma and 7.3% undetermined.

Keratoplasty is a time tested surgical treatment for corneal scars, decompensation, dystrophies and ectasias. It is also a globe saving procedure in cases of severe corneal ulcer. In India annual corneal donations are estimated to be between 50600 (EBAI data, 2014-15) to 57944 (NPCB data, 2014). The utilisation however is less than 50% and is estimated to be around 24300 (EBAI data, 2014-15). As per government projections, we need to perform 100,000 corneal transplantation surgeries to reduce the back log and address the incidence every year.

Since 1932 when Filatov gave the concept of moist chamber storage, there has been major advances in corneal storage media which allow superior preservation of endothelium and prolonged storage time of up to 5 weeks in organ culture. In spite of these new technology, the most common storage method in the developing countries is still the moist chamber and MK Medium.

In the USA most tissues are stored in intermediate term hypothermic liquid storage media (most commonly Optisol-GS) and in many European nations, organ culture is preferred. The advantage of an intermediate storage medium is that it allows easy storage for up 14 days.

The main function of the corneal storage medium is to maintain the viability and functionality of the endothelial cells from collection to utilisation. Intermediate chondroitin sulphate media further offer the advantage of extended storage for up to 14 days and the stored tissues are thinner too. This has several clinical implications – more storage time allows more flexible scheduling of surgeries, the surgeon has time to call suitable recipients from far away and not worry about the tissue getting spoiled. Thinner tissue allows better evaluation pre-operatively, intra-operative handling is better and also post-operative visual rehabilitation is faster. Another advantage of intermediate storage is that it allows transportation of tissues.

The National Cornea Distribution System (CDS) was started in India in 2012 through the EBAI-SightLife network(4). Through this system, around 1200 tissues were transported in 2014 (source – personal communication with EBAI). This is being done using Optisol-GS as the intermediate term media.

There are a few disadvantages of using Optisol-GS – firstly, it is expensive (about Rs.2700 per vial) and since it is imported from the USA, the availability is limited. The expenses are ultimately borne by the recipient family as part of tissue processing charges.

Cornisol is an indigenous intermediate storage media being manufactured by Aurolab, Madurai, India and was introduced in 2012. Compared to Optisol-GS it is less expensive (MRP Rs.1300) and being manufactured in the country, it is more easily available. According to EBAI data this storage medium is being used by over 50 eye banks in the country. There has been a positive feedback from the surgeons using the media who say that the tissue stored is thinner than in MK medium.

Till date there has been no in vitro or clinical trial to evaluate the performance of Cornisol or compare it with Optisol-GS. A multi-center trial was done during development of the product but the data was not published (source – personal communication with Aurolab). This is the first study

being done to see how Cornisol compares to Optisol-GS as a hypothermic intermediate storage medium.

1.2: HISTORY OF CORNEAL PRESERVATION

Eduard Zirm performed the first successful human corneal transplantation in 1906. The donor was a living 11 year old boy who underwent enucleation for penetrating trauma. Freshly enucleated eyes was the source of donor tissues for the next three decades. In 1935 V.P.Filatov of USSR reported the use of corneas taken from cadaver donors for the first time.(5) The globes were enucleated within few hours of death, cleaned by washing in brilliant green solution and then stored in tightly closed glass bottles with the cornea positioned upwards. He also gave the concept of storing them in an icebox and reported eyes which were stored for 20-56 hours before surgery. He concluded that freshly harvested cadaver corneas were as good as living donors. This method revolutionised corneal transplantation and Filatov is considered to be the father of eye banking.

This is how the moist chamber method of storage came to be. The storage time was limited to 48 hours. Various methods were attempted to increase the storage time like drying, freezing, freeze-drying, adding paraffin or formalin.

When Stocker published the importance of the endothelium in his landmark paper in 1953(6), the focus of shifted to developing storage

medium to preserve the anatomical and physiological integrity of the endothelium.

Using cryopreserved tissues for surgery was first given by Eastcott in 1954.(7) Other investigators modified and refined the method. In spite of being successful clinically, the method was very complex and the method did not gain much popularity.

Then came the landmark moment in 1974 when McCarey and Kaufman developed the M-K medium. It was composed of tissue culture medium 199 (TC-199), dextran and antibiotics. It allowed corneal storage for up to 96 hours at 4^{o}C.(8) This changed the keratoplasty surgery entirely. It was no longer an emergency procedure. It became a planned surgery allowing the best tissue to be transplanted to the ideal candidate.

The organ culture method was introduced by Doughman in late 70s.(9) This method used Eagle's minimum essential medium, calf serum, anti-bacterials and anti-fungals and could store cornea at 37^{o}C for about a month. It has been refined over the years and a non-bovine medium is used nowadays, chondroitin sulphate and other nutrients are added to preserve endothelium more efficiently.

In the mid-80s the chondroitin sulphate based media started being developed. It started with Chondroitin Sulphate Corneal Storage Medium

(CSM)(10) which was followed by K-Sol(11) and DexSol(12) which was made by adding 1% dextran to CSM. Finally Optisol(13) was introduced in the early-90s. Since then Optisol has been the most commonly used intermediate duration storage medium.

The exact mechanism by which chondroitin sulphate prolongs preservation is not clearly understood but various probable ways have been put forth. CS is thought act as an antioxidant and help in maintenance of endothelial cell integrity by preventing lipid peroxidation which damages cell wall by altering their structure(14). It has been shown that higher concentrations of chondroitin sulphate between 2% and 5% are better at corneal preservation(15). CS has a role in formation of extracellular matrix and maintains physiochemical environment for endothelial function. It may also act as a cation-exchange resin and regulate the passage of ions across the membrane b forming chelation complexes(16).

There have been newer media with varying popularity. Chen medium, approved in 2000, is an isotonic medium with β-hydroxybutyrate instead of glucose to reduce lactate formation.(17) There are other newer media coming up like Eusol-C(18), Life4^{o}C(19) and Cornea Cold(20).

1.3: CLASSIFICATION OF CORNEAL STORAGE MEDIUM

Based on the duration of storage, the corneal storage media are classified as:

Short Term	**Intermediate term**	**Long Term**
Moist chamber	K-Sol	Cryopreservation
MK Medium	Chondroitin sulphate corneal storage medium	Organ culture
	DexSol	
	Optisol-GS	
	Eusol-C	
	Chen Medium	
	Life4^{o}C	
	Cornisol	

Table 1.1: Different types of corneal storage media

1.4: VARIOUS METHODS OF CORNEAL STORAGE

1.4.1: Moist Chamber

This is the simplest method of corneal storage and still one of the leading methods of storage in our country. Whole globe enucleation is done and is kept in an air-tight glass bottle. It can store cornea for up to 48 hours at 4^{o}C but shorter the storage time, better the surgical outcome.

Advantages: Simple and inexpensive. Useful in developing countries without access to liquid storage media.

Disadvantage: Short storage time of 48 hours. For the whole duration of storage, the endothelium is exposed to post-mortem changes in the aqueous like accumulation of waste metabolites, change in pH and ion concentration. This 'aqueous sewer' as described by McCarey and Kaufman(8) can affect the surgical outcome.

1.4.2: M-K Medium

The first liquid hypothermic storage medium. It is used for storing excised corneo-scleral rim at 2-8^{o}C for 96 hours maximum.

MK Medium consists of Tissue Culture Medium-199 (TC-199) as base, with 5% dextran, HEPES (N 1,2 hydroxyethylpiperazine-N-ethane sulphonic acid) and sodium bicarbonate as buffer, phenol red as pH

indicator and mixture of streptomycin and penicillin (100 units/ml) as antibiotic.(8)

In MK Medium, TC-199 provides the necessary nutrients, dextran being an osmotic agent prevents the swelling up of the tissue, the buffers maintain a suitable pH and osmolarity of 7.4 and 290 mOsm respectively, and the antibiotics prevent microbial contamination.

1.4.3: Optisol-GS

Introduced in 1991 by Chiron Ophthalmics, Optisol is the most popular chondroitin sulphate based intermediate duration media. It is a hybrid of K-Sol and DexSol in composition and can store corneal button for a maximum of 14 days. Now it is marketed by Bausch and Lomb.

Optisol contains Tissue Culture Medium-199 (TC-199), Earle's Balanced Salt Solution and Minimum Essential Medium as the base component. 2.5% Chondroitin sulphate(CDS), 1% dextran-40(14). The high concentration of CDS and dextran act together to give greater deturgescence to the stored tissue. Like MK Medium HEPES and bicarbonate act as buffering agents. Optisol contains additional components to increase endothelial viability. ATP precursors like adenine, adenosine and inosine are added to supplement the limited stored ATP. Cobalamine is added as enzymatic co-factor and ascorbic acid acts as antioxidant(12). Other

micronutrients like amino acids, sodium pyruvate, L-glutamine, 2-mercaptoethanol are also present.

Constituent	**Optisol**	**DexSol**	**K-Sol**
Base medium	TC-199 and MEM	MEM	TC-199
Chondroitin Sulphate	2.5%	1.35%	2.5%
Dextran 40	1%	1%	0
HEPES buffer	Yes	Yes	Yes
Gentamicin sulphate	Yes	Yes	Yes
Non-essential amino acids (mmol/L)	0.1	0.1	0
Sodium bicarbonate	Yes	Yes	Yes
Sodium pyruvate (mmol/L)	1	1	0
Antioxidants	Yes	Yes	No

Table 1.2: Comparison of CDS based intermediate media. Optisol was developed as a hybrid of K-Sol and DexSol. [From Kaufman et al (14)]

Initially Optisol had only gentamicin sulphate 100mg/l as the antibiotic. But it provided limited prophylaxis against gram-positive organisms. A study reported the most common organism for post-keratoplasty endophthalmitis to be gram-positive cocci(21). To address this streptomycin 200mg/l was added to Optisol in 1993 and to denote the presence of two antibiotics, the name changed to Optisol-GS (22). Streptomycin was chosen because of its previous use in MK medium which

proved its success and clinical safety. Also, it was much more stable than vancomycin (97% vs. 37%) as solution for up to one year.

The only limitation of Optisol-GS is its inability to preserve epithelium efficiently beyond 1 week(23).

1.4.4: Eusol-C

This is an entirely synthetic storage medium which can store tissues at $4^{o}C$ for 14 days(24).There is no chondroitin sulphate; only dextran is used as osmotic agent. It contains only gentamicin as the antibiotic and phenol red pH indicator. Being synthetic, it can be stored in room temperature before use and can tolerate relatively higher temperature during transportation(23).

1.4.5: Life4^{o}C

It is a new FDA approved intermediate storage medium which is supposedly better than Optisol-GS for corneal preservation. Its composition is similar to that of Optisol-GS. Extra components include L-glutamine, recombinant human insulin which helps in better nutrient uptake by the cells and reduced glutathione as a powerful antioxidant. There are also additional membrane stabilizers, antioxidants and micronutrients(23)(25). In an internal study by the manufacturer Numedis, Life4^{o}C was statistically significant in maintaining endothelial cell density and keratocyte

survival(26). Also it had greater tissue deturgescence and stored tissues were significantly thinner than in Optisol-GS. Unlike other storage media it comes in 30ml vials and there is a specialized transport-cum-viewing chamber available. This increased volume is able to supply more nutrients to the endothelium and also offers better dilution of toxic metabolites(25).

1.4.6: Cryopreservation

It is the only method which can theoretically store cornea indefinitely. Though this method was described in the 50s, it is not very popular because of the technical complexity and mainly because of freezing injury to the cells. There are reports on higher endothelial cell loss and primary graft failures with cryopreserved tissues than liquid hypothermic media(27). Refinements are being made like storing tissues in dimethyl sulphoxide (DMSO) which is a cryo-protectant or using vitrification which is an ice-free method(28).

1.4.7: Organ Culture

This method was first described by Doughman in 1972 where he demonstrated storage of tissue for 5 weeks at 34^{o}C with good endothelial function. The longer storage period allows screening for prion diseases and quarantining tissues for microbial contamination. However due to technical difficulty and high maintenance, it is used only in certain European nations

especially in the Netherlands. There are two methods currently in use – the Minnesota method and the Dutch method(16). Eurosol (Bausch and Lomb) is a serum-free organ culture medium approved for use in Europe(23).

1.5: SPECULAR MICROSCOPY

The specular microscope (SPM) is a specialized microscope which is used to visualize and record the corneal endothelium. And using in-built computer programs we can quantify various parameters of the endothelial structure.

Vogt in 1918 first visualised the endothelial layer with a slit lamp using the method of specular reflection and described it as ‘a graceful honeycomb., Later in 1968 David Maurice made the first specular microscope.(29) Over the years it has evolved from contact, wide field type to non-contact, narrow field and higher resolution type.

1.5.1: Principle of Specular Microscopy:

In normal microscopy we look at light transmitted through the specimen but in specular we are more interested in the light that is reflected. When light strikes a surface, it can be reflected, transmitted (refracted) or absorbed. Specular reflection or mirror-like reflection happens when the angle of incidence is equal to the angle of reflection. This image is captured by the SPM.(29)

Light striking the normal cornea is mostly transmitted but a small fraction is reflected at interfaces of different optical density (or refractive index) e.g. tear-epithelial interface, endothelium-aqueous interface. At the

endothelium-aqueous interface about 0.022% of the light is reflected back.(30) The greater the difference between the refractive indices, the more is the amount of reflected rays. The area of the specular reflex also depends upon the radius of curvature of the reflecting surface. Also it is important to know if the area being images is representative of the entire cornea. For example, one can image an area of focal injury in an otherwise normal cornea and the abnormal counts will get extrapolated to the whole area.

There are certain criteria to obtain the ideal specular image. The endothelium must be at the accurate distance for the focus of objective lens. The part of the endothelium being imaged must be perpendicular to the optic axis of the objective lens.

The quality of the image depends on the numerical aperture of the objective lens, how clean the lenses are. Another important factor is the presence of glass or plastic and storage media between the lens and tissue. Although transparent, these can lead to considerable distortions(31). A viewing chamber can reduce this problem to some extent.

1.5.2: Types of Specular Microscopes:

1: Contact and non-contact depending on the nature of the objective lens. The image resolution depends on the numerical aperture of the objective lens. This is a bit higher with contact objective lenses thus giving a sharper

image(31). They also reduce the ocular movement so the image has less motion artifacts. Contact SPMs can be used to image the epithelium and stroma too and gives accurate pachymetry values. But being more practical and easy to use, the non-contact ones are used more commonly.

2: Clinical and Eye-bank specular microscopes.

1.5.3: Eye Bank Specular

The eye bank SPM is little different from the clinical variety. It allows in-vitro analysis of corneas through the vial of the storage media. Unlike in patients, the cornea is viewed directly from the endothelial side. The stage has a holder for the vial which can be moved in X-Y axis and also can be tilted to allow maximum specular reflection(32).

Older contact varieties could be used to image the endothelium with a whole globe but modern ones are non-contact to allow imaging of cornea in stored media.

It is a must to warm the cornea to room temperature before doing specular imaging. At storage temperature of 2-8^{o}C the endothelial pump is relatively inactive leading to tissue oedema and there is loss of normal smoothness. Rewarming time may vary from 45 minutes to 2 hours(31).

1.5.4: Specular Analysis

Specular microscopic images can be interpret in two ways:

1. Qualitative

2. Quantitative

Qualitative analysis: Here we look at the appearance of cell borders, cell shape, intra-endothelial and inter-endothelial abnormalities.

A normal specular image will show endothelial cells as bright hexagons with dark cell borders. Cells are of uniform size and shape with no abnormal bright or dark areas. The cell density is between 2000-3000 cells/mm^2 with more than 60-70% hexagonal cells.

The light falling on the cell borders are reflected in such a way that they are not received by the SPM and are seen as dark, thin lines whereas the rays falling on the surface of the cell undergo orderly reflection forming a bright area. Normally the borders form a hexagon with three lines meeting at 120^o. In pathological conditions there may be rounding of the borders secondary to oedema, trauma or aging.(33)

Cell shape variation is a part of normal aging. With decreasing numbers the cells increase in size and associated shape alterations take place. Abnormal cells can appear as stretched, scalloped, rounded, square or

triangular.(33) Injured endothelium can show signs of healing like enlargement of cells to fill-up the damaged areas or cell coalescence where the cell borders between two cells disappears slowly as they merge together.(31)

Guttae are excrescences of the Descemet's Membrane. They appear as dark with central bright spot with abnormally shaped surrounding cells. These excrescences and coalesce and hinder visualisation of the endothelium. Hassall-Henle warts are also excrescences seen in the peripheral cornea. These are usually dome shaped with normal endothelial cells. Bacteria and inflammatory cells appear as small bright twinkling objects with changing shape and position.

Quantitative analysis: Here we use in-built computer programs to automatically or semi-automatically calculate various endothelial cell features. The various parameters which are looked at are:

1. Endothelial Cell Density (ECD) – number of cells per square millimetre (cells/mm^2)

2. Mean or Average Cell Area – measured as um^2

3. Coefficient of Variation (CV) – Variations in the areas of different cells

4. Percentage Hexagonality (6A) – percentage of cells that are perfectly hexagonal.

1. Endothelial Cell Density: This can be counted in a number of ways.

a. Fixed frame analysis – A box of known area is overlayed on the specular image and number of cells inside are counted. Cells that are partially in the given area are counted as half. The total is then multiplied by number depending on area selected. This method is very error-prone because of the 'border cells' or cut-off cells and is rarely used nowadays.

b. Variable frame analysis – A variable shape of contiguous cells first outlined, then cells in this defined area are counted. The selected area is determined by computer planimetry. Once the area is known, the ECD is calculated. This is superior to fixed frame method as only complete cells are counted and there is no confusion regarding partially included cells. This is the most accurate and reliable semi-automatic method of counting.(31) However, this method does not provide very accurate data about the cell size distribution as doing it for a large number of cells is very tedious.

c. Center method – A newer method where only the center of the cells are marked. The computer identifies adjacent cells and performs the calculations. It is important to mark at least a hundred cells because cells without adjacent ones maybe discarded while computing.

Center-flex method is a hybrid of variable frame analysis and center method where the user outlines the borders of cells and marks their centers.

d. Corner method – Each cell is marked by identifying the six corners. The software connects these dots and determines the area and ECD. This method was used initially.

e. Comparison to fixed pattern – The image is compared to various specular images of known ECD values and closest match is considered as the count. It gives a very rough estimate.

2. Coefficient of Variation: CV is a numerical representation of polymegathism. Polymegathism refers to the variations in cell sizes.

$CV = SD_{\text{cell area}}/\text{Mean cell area in } \mu m^2$

3. Percentage Hexagonality (6A): Pleomorphism or Polymorphism refers to the variations in cell shapes. 6A is a numerical way to represent that. Polymorphic cells have less or more than six sides and are therefore less hexagonal. Also, CV and 6A are inversely related. Change in cell size is almost always associated with change in shape. So more CV would mean less hexagonality.

In quantitative analysis, the ECD is not the only parameter to look at. Polymorphism and polymegathism are believed to be sensitive indicators of endothelium under stress. A high CV and low 6A indicates unusable relationship between neighbouring cells which can lead to loss of pump and barrier function.

Modern SPMs have completely automated counters which outline and count cells automatically in a given area and generate the morphometric reports. The intelligent program is able to identify and exclude areas of poor or unfocussed image quality. The number of cells counted is much more than manual method thus giving better statistical results. It is also able to give additional data like a cell density histogram, give overlays showing abnormal cell sizes in different colours. And all that is done in less than a minute(31).

Accuracy of the quantitative analysis depends on – image quality, how well the sampled area represents the whole cornea, technician's ability to identify cells and borders and mark accurately. Common errors are missing cells, double counting and not being able to identify the borders correctly. Automated counters should always be supervised by a trained personnel with knowledge of the cornea so that the above errors can be minimized. For good statistical output, there should be only a single dot marking the cells placed exactly in the center of the cells.

The age of the cornea is of less importance than the appearance of the cell in determining the health and functional reserve of the tissue. So instead of determining suitability based on age alone, it is very important to do a SPM evaluation of old donor corneas.

1.5.5: Application of Specular Microscopy

1. Donor cornea evaluation in eye bank and selecting tissues for optical or endothelial surgeries.
2. Penetrating Keratoplasty – cell loss happens more than normal but more-or-less at a steady rate
3. Endothelial Keratoplasty – Initial high loss of cells at 6-12 months but thereafter less than that of penetrating keratoplasty.
4. Fuchs' endothelial corneal dystrophy- documenting progression
5. Cataract surgery – pre and post-op images to study the cells loss during various surgical procedures
6. ICE (irido-corneal-endothelial) syndrome shows a reversal appearance with black borders and white central areas.
7. PPCD (posterior polymorphous corneal dystrophy) – differentiating from ICE
8. Documenting corneal guttae
9. Physiological ECD loss with aging at about 0.5 to 1% per year
10. Keratoconus – shows elongated cells with long axis towards the direction of apex of cone.
11. Document endothelial damage following intraocular inflammation, trauma, vitreous touch, in contact lens users, in diabetics etc.

1.6: GROSS AND SLIT-LAMP EXAMINATION OF THE DONOR EYE

The functioning of the eye bank can be broadly grouped as potential donor screening, procurement, processing and preservation, evaluation and distribution.

The aim of detailed evaluation of donor tissue is to identify which tissues are suitable or unsuitable for surgery and to further group the suitable tissues for different types of keratoplasty technique. This is to ensure that this precious donation is utilised fully by the ideal recipient.

1.6.1: Donor screening and general examination

Donors are of two types - voluntary donors where the donor family contacts the eye bank and donors whose families are counselled through a hospital corneal retrieval program (HCRP).

Before going ahead with the actual procedure it is important to do the following. Identify the donor and confirm the death. Take written informed consent from the family members. Eye donation pledge cards are not a substitute for consent. Document the cause of death. Take detailed ocular and medical and surgical history especially about intraocular and refractive surgeries, contact lens use, medical comorbidities, malignancy, history of ventilator support, blood transfusion etc. A general examination of the

whole body should be ideally performed looking for signs of high-risk behaviour.

1.6.2: In situ examination of the eye

The eye and adnexa should be examined carefully before collection preferably with a pen torch. Examination should focus on looking at signs of infection, details of intraocular surgery especially lens status, icterus of the sclera. Unlike in whole globe enucleation, a detailed slit lamp examination cannot be done with in situ excisions. Therefore the onsite gross examination becomes even more important. Also it is important to note corneal folds and oedema before placing in storage medium which can reverse these findings(34).

Collection of the eye can be done by the followed techniques:

1. In situ corneoscleral rim (CS rim) excision with immediate transfer to storage media (short or intermediate-term)
2. Whole globe enucleation with moist chamber storage
3. Whole globe enucleation followed by CS rim preparation in the eye bank and storage in
    a. Liquid hypothermic media – short or intermediate storage
    b. Organ culture
    c. Cryopreservation

Whole globe enucleation and CS rim preparation have their advantages and disadvantages. In situ technique is gaining popularity recently.

Advantages of in situ excision:

1. More cosmetically acceptable. This sometimes helps in winning over the donor family while counselling.
2. Death to storage time in moist chamber is decreased as tissues are immediately kept in storage media.
3. The contact time of the endothelium with the toxic aqueous is decreased.

Disadvantages:

1. In most cases only a pen-torch examination is done at the site. If the tissue is unsuitable based on slit-lamp examination alone, then the cost of tissue preservation, disposables can be saved which is not possible with in situ collection.
2. Whole globes not suitable for surgery can be used for research and surgical training more widely than CS rims.
3. Inexperienced technicians can cause more damage to the tissue while doing an in situ excision.

1.6.3: Gross examination

On gross in situ examination with pen torch the following findings should be noted:

1. Epithelial oedema and epithelial defects from exposure or trauma
2. Stromal opacities – arcus senilis, central scars, infiltrates
3. Stromal oedema – clarity of cornea and number and severity of deep folds
4. Abnormal corneal shape – keratoconus, micro or megalocornea
5. Status of the anterior chamber – formed, shallow or flat; anatomical abnormality; turbidity of the aqueous humour
6. Evidence of surgery on the cornea, phakic or aphakic, evidence of iris laser
7. Condition of the globe – firm, soft or collapsed

With the whole globe the posterior part of the eyeball can also be examined. Traumatic corneal abrasions, collapsed globe, scleral perforation are evidence of improper enucleation technique.

1.6.4: Slit lamp examination

Slit lamp examination of the whole globe or CS rim is the most important step of quality control in an eye bank. A viewing chamber allows much better optics and easier evaluation. Most storage vials are transparent

glass or plastic and allow examination through the bottom, albeit a bit challenging. A vial holder with mirror attachment on the slit lamp is recommended. Whole globe examination is done in the moist chamber jar with lid open and the tissue brought forward. A viewing brace is used to clamp the vial in position.

First use low magnification with diffuse or wide slit beam at 45^{o} to examine. Then use high magnification with slit beam to systematically study all the layers of the cornea from anterior to posterior. And to complete the examination use coaxial position and retro-illuminate the tissue. This can sometimes reveal defects or opacities which may have been missed on oblique slit(34).

Corneal epithelium: Look for microcystic oedema, abrasions, epithelial defects and foreign bodies. In areas of epithelial defect rule out Bowman's or stromal injury.

Corneal stroma: Stromal opacities are examined in detail to define the extent, depth, location and appearance of the opacity and dystrophies are excluded. It is vitally important to differentiate between opacities and infiltrates. Infiltrates appear as slate grey without adjacent oedema whereas infiltrates have a yellowish white look with surrounding stromal oedema. Deep stromal folds can be seen along with stromal oedema.

Corneal endothelium: Before doing a SPM imaging slit-lamp examination is a fast and efficient method to evaluate the endothelium. A meticulous examination should be done to look for intactness of the endothelial layer and DM, guttata, Descemet's tears, stress lines and fractures and endothelial vesicles.

Anterior chamber: A crystalline appearing aqueous indicates that the globe had been frozen and such tissues ideally should not be used. Examine the iris for signs of trauma, surgery, tumours, and lasers. Note the lens status.

Excised corneoscleral rim: Make sure the rim is at least 2 mm all around so that it fits well on an artificial anterior chamber. A wide area of sclera can be used for scleral grafts.

Storage media: It is also important to examine the state of the media. Clouding and colour change are indicative of infection or major metabolic changes. In case there is a lot of debris, the tissue should be transferred to a new vial.

1.6.5: Specular microscopy

This is the most important investigation to be done before distributing tissues. The technician should be well trained in techniques of counting cells. Tissue should be allowed to rewarm adequately before imaging.

Features of abnormal endothelium on specular microscopy:

a. Cell density of <1500 cells/mm2
b. Severe polymegathism or pleomorphism
c. Corneal guttata
d. Abnormally shaped cells
e. Abnormal single-cell defects
f. Large areas of oedema
g. Presence of bacteria or inflammatory cells
h. Presence of ghost vessels in stroma

The eye bank donor proforma consists of details regarding the time and cause of death, death to enucleation time, refrigeration time if any, transport time and time in moist chamber. Details of slit lamp and specular microscopy are attached. The tissue is then graded as per the eye bank protocol.

1.7: METHODS OF ASSESSING ENDOTHELIAL VIABILITY

A living endothelium is absolutely necessary for normal corneal function. Therefore it is important to assess the ability of the storage method to preserve endothelial viability. Here are few methods which are used to directly or indirectly assess endothelial viability.(35)

1. Staining methods:

a. Trypan blue alone or with Alizarin Red – stains nuclei of cells with damaged membrane while alizarin stains the intercellular membranes

b. Nitro-blue Tetrazolium – Enters and stains cells with damaged membrane.

c. Acridine orange/ ethidium bromide – arcidine orange enters viable cells and gives green fluorescence whereas non-viable ones stain with ethidium bromide and has red fluorescence.

Other methods include lissamine green, Evans blue, indocyanine green and rose bengal(36).

2. Temperature Reversal: The change in corneal thickness is noted as tissue is rewarmed from cold storage. Reactivation of the endothelial pump makes the tissue thinner thus indicating viable function.

3. Bicarbonate Flux: The bicarbonate flux across the endothelium is related to the outward flow by active endothelial pump action and inward flow limited by the barrier function. This is measured using radio-isotope labelled bicarbonate.

4. Electron Microscopy

a. Transmission Electron Microscopy: To study the cellular organelles.

b. Scanning Electron Microscopy: To study the integrity of the cell membrane

5. Specular Microscopy: Allows both qualitative and quantitative analysis of the endothelium. The morphometric analysis can be done at various stages to note change over time.

6. Enzyme release: Enzymes released give an indirect idea about the metabolic activity. Lactate dehydrogenase and acid phosphatase levels in the media indicate about the endothelial metabolism.

7. Nuclear Magnetic Resonance: Uses NMR to detect high and low energy phosphates which indirectly point at energy consumption and metabolism.

8. Redox Fluorometry: This uses the principle of measuring metabolism through the levels of relative concentration of reduced pyridine nucleotides and oxidized flavoproteins.

9. Intracellular glutathione oxidation-reduction status

10. Measuring the uptake of radio-labelled metabolites

11. Measuring oxygen consumption

1.8: ENDOTHELIAL VIABILITY BY HISTOPATHOLOGY

Specular microscopy gives only a picture of the endothelial honeycomb. While it is sufficient to assess the endothelium by looking at the ECD and the variations in cell shape and size, we need something more if we want to assess the vitality of these cells. Histopathological analysis is one such method.

Trypan blue was used as a vital stain to determine areas of endothelial damage since early 1970s. Vital staining of the corneal endothelium by using dual stains was first described by Spence and Peyman in 1976.(37) They used a combination of 0.25% trypan blue and 0.2% alizarin red-S to stain rabbit corneal endothelium. This method combined the vital staining property of trypan blue with alizarin red's property to stain the intercellular membrane.

Later in 1977 Sperling published his findings using similar method.(38) Taylor and Hunt in 1984 in their article titled "Dual staining of corneal endothelium with trypan blue and alizarin red S: importance of pH for the dye-lake reaction" showed that adjusting the pH of alizarin red to 4.2 gave best staining of the intercellular membranes and using glutaraldehyde as fixative preserves the stained tissues.(39) Over the years there has been further refinements to this method in regards to the dye concentration and

duration of staining. Singh et al concluded that 0.3% trypan blue for 1 minute followed by 0.2% alizarin red for ½ to 1 minute is ideal in 1986(40) and recently in 2012 Park et al demonstrated 0.4% trypan blue and 0.5% alizarin red for best staining outcomes.(41)

Principle of Staining

Trypan blue was initially used as the vital stain for looking at areas of endothelial damage. It stains the cells in which the permeability of the cell membrane is increased. Healthy cells are not outlined and only dead or partially damaged cells are stained. Grid counting to quantify is therefore not possible.(37)

This is where dual staining is advantageous. Both stains are impermeable to healthy cells with intact cell membrane. Alizarin red stains the intercellular borders and trypan blue enters cells with damaged membrane and stains the cytoplasm and nuclei.

After staining, the following patterns are seen. A healthy cell appears as a clear area with the hexagonal borders stained with alizarin. Damaged cells which are attached to the Descemet's membrane have outlines stained with alizarin along with nuclei stained dark with trypan blue. Whereas, damaged cells which are dislodged from the Descemet's membrane show

areas of drop out with bare DM stained diffusely with both alizarin and trypan blue giving a pink-red colour.(37)

Due to the natural curve of the cornea, it is difficult to sharply focus the entire area under the microscope. While viewing, some areas will be out of focus. The dense staining tends to fade and decrease in contrast within an hour. To prevent that, one can fix the tissues with glutaraldehyde. Fixation also helps flatten the cornea allowing a larger to be focussed.

This is a superior method than staining with trypan blue only. Since all cells, healthy, dead or damaged and areas of drop out are outlined clearly, a grid count or computer program can be used to accurately quantify the damage.

1.9: REVIEW OF LITERATURE

Review of literature was done with PubMed search. Keywords used were Optisol and Optisol-GS, Cornisol, corneal endothelial viability and articles relevant to this study were considered. Cornisol keyword did not return any results.

Kaufman et al in the article Optisol Corneal Storage Medium(14) compared Optisol with DexSol in an in vitro method. 15 pairs of human corneal tissues were compared in three groups – storage at 4^{o}C for 2 weeks, room temperature storage at 26^{o}C for 1 to 4 days and hypothermic storage with temperature reversal analysis. Finally tissue thickness and scanning electron microscopy was done for all tissues and graded as per a morphological scoring system. They found that Optisol stored tissues stored at 4^{o}C for 2 weeks showed better morphology than DexSol stored tissues and were significantly thinner. However, for tissues stored at 26^{o}C there was no difference between the two media. The investigators concluded that Optisol was superior to DexSol for intermediate storage at 4^{o}C which would allow more flexible surgical scheduling and more appropriate tissue for the patient and reduce tissue wastage.

Lass et al in their article A Randomized, Prospective, Double-Masked Clinical Trial of Optisol vs. DexSol Corneal Storage Media(12) also

compared Optisol with DexSol in a clinical trial. 31 pairs of human corneal tissues were stored at $4^{o}C$, one in Optisol and its paired tissue in DexSol for a period of 24 to 134 hours. Preoperative specular microscopy was done. They were then transplanted to patients, each pair operated by the same surgeon using same technique within 8 hours of each other. The patients were meticulously matched for age, gender, underlying corneal pathology, statues of cornea and lens status in 42% to 82% of cases. Intra-operatively the corneal thickness was measured with ultrasound pachymetry. Lysosomal enzyme release assay was done with the cut portion of the corneal button. Post-operative slit lamp, specular microscopy, pachymetry and applanation tonometry was done at 3, 6 and 12 months. There was one graft failure in the Optisol group, rest all grafts were clear in both groups at one year. 57% patients in Optisol group and 38% patients in DexSol group had CDVA of 20/40 or better. Keratometric and refractive measures did not reveal any significant difference between the two groups. Intraoperative tissue thickness was significantly lower in the Optisol group ($p=0.0001$) but did not vary post-operatively. Serial specular microscopy did not show any significant differences in endothelial cell loss or morphometric parameters between the two media. They hypothesized that Optisol stored tissues are thinner probably because of chondroitin sulphate, better post-storage tissue deturgescence and due to a greater loss of endogenous glycosaminoglycans.

Both groups had rebound swelling in immediate post-operative period probably due to penetration of the media in the stroma which attracts water. The lysosomal enzyme release which is directly related to cell viability was found to be significantly lower in the Optisol group. Therefore they concluded that the advantage of Optisol over DexSol in only apparent during storage and surgery. There is less autolysis during storage and the thinner tissue allows better tissue evaluation pre-operatively with slit lamp and specular microscopy. Also, thinner tissue is easier to handle intra-operatively and gives earlier visual rehabilitation post-operatively.

Lindstrom et al in the article Optisol Corneal Storage Medium(13) compared Optisol with DexSol, K-Sol and McCarey-Kaufman medium. This was an extensive study which combined both in-vitro studies and clinical trial using human and rabbit corneas. Three phases of studies were done – in vitro examination of endothelial preservation in terms of metabolism, structural changes and thickness; in vivo evaluation of epithelial toxicity; and clinical outcome. For the in vitro analysis tissue cultured human endothelium was stored in Optisol or DexSol and the mitotic activity measured with radiolabeled thymidine. Rabbit cornea tissues were randomly divided and stored in MK-medium, Optisol and DexSol. Specular microscopy and ultrasound pachymetry were done. Additionally, cell membrane potential was measured with microelectrodes and redox

fluorometry was done to measure metabolic function. Two human corneas each were stored for 2 weeks at $4^{o}C$ in DexSol or four variants of Optisol with different concentrations of chondroitin sulphate. Corneal thickness was measured at intervals and finally endothelial cell viability was assessed histologically with Alizarin red-S and trypan blue staining. Human corneas not suitable for surgery were stored in Optisol and DexSol and both scanning and transmission electron microscopy done. In vivo studies included checking for corneal toxicity by instilling Optisol drops in rabbit corneas and histopathological analysis done. Finally an open-label clinical trial was done where 51 tissues stored in Optisol were transplanted, intra and post-operative pachymetry was done along with post-operative specular microscopy. They found that Optisol incubated tissues had significantly increased mitotic activity which was found to be due to adenosine component in the media. Rabbit tissues stored in Optisol were significantly thinner, the specular images showed lesser swollen endothelial cells with fewer cytoplasmic vacuoles. The membrane potential was stable across all the media. Human tissues stored in 2.5% chondroitin sulphate was found to be thinnest than other concentrations. Histological examination showed intact endothelium in all the media with Descemet's staining along areas of stromal folds or stress lines. Electron microscopy was comparable till day 7 but by day 14 DexSol sored tissues had lost all cellular integrity. Rabbit

eyes instilled with Optisol did not show any histological abnormality. From the clinical trial, 93% patients had clear graft at three months, endothelial cell loss was about 5% (± 18.4%) at three and 11.5% (± 14.4%) at six months. This study, like the previous ones, concluded that the main advantage of Optisol is thinner tissues during storage and surgery. While the exact mechanism is not known it is due to better endothelial cell function and high concentration chondroitin sulphate.

There are a few clinical studies comparing Optisol-GS with Chen medium which is a non-lactate containing, non-chondroitin sulphate medium.

Nelson et al did an in vitro comparison between the two and reported the results in the article In Vitro Comparison of Chen Medium and Optisol-GS Medium for Human Corneal Storage(17). They found that there was no significant difference between the tissues stored in the media in terms of endothelial cell density, coefficient of variation and percentage hexagonality. Electron microscopy showed intact endothelium for all tissues. There was no difference in percentage of apoptotic keratocytes and TUNEL-positive cells were not correlated with storage time. They concluded that both the media were equally effective for intermediate term corneal storage.

In another study comparing Chen media with Optisol by Bourne et al titled Comparison of Chen Medium and Optisol-GS for Human Corneal Preservation at $4^{o}C$ a clinical study was undertaken(42). 32 surgeries were done with 15 tissues stored in Chen Medium and 17 in Optisol-GS. Specular images were taken pre and post-operatively. Corneal thickness and specular morphometric parameters were compared between the two groups serially over one year follow-up. They found no statistical significance in endothelial cell loss, coefficient of variation and hexagonality. Endothelial cell loss at 2 months post-op was significantly correlated with storage time in both the groups. In conclusion, tissues stored in Chen Medium was not different from Optisol-GS over 1 year follow-up.

Kanavi et al has published a recent study titled Comparing quantitative and qualitative indices of the donated corneas maintained in Optisol-GS with those kept in Eusol-C(18). The authors compared 90 pairs of corneal tissue in an in vitro method. Serial slit lamp examination and specular microscopy was done. Tissues were graded as per eye bank guidelines. There was no significant difference in slit lamp features and specular parameters between the tissues stored in either groups. The authors concluded that there is no superiority of Optisol-GS over Chen medium in preservation of corneas in terms of corneal rating, stromal oedema, Descemet's folds and endothelial cell indices.

Pham et al published an in vitro comparison of Optisol-GS and Life4^{o}C where they looked at the specular image quality of tissues stored in either media. They studied 25 pairs of tissues with serial specular images taken during thawing from 2-8^{o}C to room temperature. The images were graded to a classification system. They found that for good quality specular images Optisol-GS tissues required significantly longer thawing time than Life4^{o}C tissues to get good quality specular images 2.5 hours vs. 2 hours. Though the thawing time was longer, the image quality was significantly better in the Optisol-GS group.

Price et al recently published a clinical study comparing the outcomes of DSAEK and DMEK with tissues stored in Optisol-GS with Life4^{o}C(19). 32 age matched recipients with Fuchs dystrophy were enrolled in each group. Baseline specular microscopy data was compared to post-op data and slit-lamp examination was done. At 6 months follow up all recipient corneas were clear. There was no significant difference between the endothelial cell loss of central cornea (18 ± 18% in Life4oC vs. 20 ± 20% in Optisol-GS group, p=0.55), the endothelial cell density and central corneal thickness was also comparable between the two groups

Spence and Peyman described the technique of double staining of the corneal endothelium to assess the viability in the article A new technique for the vital staining of the corneal endothelium(37). This involved staining

with 0.25% trypan blue followed by 0.2% Alizarin red-s. This was an improvement over the existing method of staining with trypan blue alone which stained the cells with damaged membrane alone. With the dual stain, the normal cells were seen as clear hexagons with the intercellular borders stained with alizarin, the permeable cells had their nuclei stained with trypan blue and areas of bare Descemet's membrane was seen as diffuse red staining. This method allowed visualization of normal and abnormal cells.

Taylor and Hunt in their article on vital staining further refined this method(39). They also showed the importance of maintaining the pH between 4.1 to 4.3 for ideal dye-lake reaction and better staining outcomes.

2.1: AIMS AND OBJECTIVES

The aim of this study was to determine if Cornisol is comparable to Optisol-GS for storing donor corneal tissues over a period of 14 days.

The objective was to compare paired human corneas stored in Cornisol and Optisol-GS for 14 days and look at the endothelial cell loss (ECL) and also the endothelial cell vitality (ECV) histologically.

2.1.1: PRIMARY OUTCOME MEASURE:

The primary outcome measure of this study was endothelial cell loss (ECL) of donor tissue measured as percentage of baseline which were stored for 14 days at 2-8^{o}C. Coefficient of Variance (CV) and percentage hexagonality (6A) was also documented and compared between the groups.

2.1.2: SECONDARY OUTCOME MEASURE:

The secondary outcome measure was to look at endothelial cell viability at the end of 14 days by histopathological staining and to quantify it by analysing photos from the microscope camera with a special software.

2.2: MATERIALS AND METHODS

Human cornea were used for the study from cadaveric donors. Only tissues which were considered not suitable for surgery were taken for the study. Tissues were obtained from Rotary Aravind Eye Bank, Madurai, Aravind IOB Eye Bank, Coimbatore and Prova Eye Bank, Barrackpore.

The storage media was obtained from the respective manufacturers. Cornisol was supplied by Aurolab (Madurai, India) for the study and Optisol-GS was obtained from Bausch and Lomb (Rochester, USA) via Sight Life, India.

2.2.1: Study Design:

This is a **prospective and in-vitro** study conducted at a **single centre**.

The study was **two-armed** wherein both Cornisol and Optisol-GS were **studied in parallel using paired samples**. For each pair **randomization** was done to determine which side when in which media. The observers were **masked**.

Since the paired samples were studied simultaneously under similar conditions, the Optisol-GS tissue served as the control for that paired tissue in Cornisol.

2.2.2: Sample size:

Sample size calculation was done based on the primary outcome of endothelial cell loss in a parallel study. Since there are no prior studies, a 15% difference between the two media was assumed based on clinical experience from various institutes and in-house studies from the manufacturer. Calculations were done using 80% power and 0.05 level of significance. The sample size required was found to be 32 donor pairs (64 total).

2.2.3: Inclusion Criteria:

1. Tissues which are not suitable for surgery
2. Paired tissues of similar optical clarity
3. Medical reasons for not using the tissue like:
    a. Sepsis
    b. Malignancy
    c. Serology positive for HIV, HBV, HCV and Syphilis
    d. Inadequate or no blood sample
4. Very old donors
5. Pseudophakic donors
6. Specular picture available at baseline examination.

2.2.4: Exclusion Criteria:

1. Gross Descemet's membrane folds
2. Excessive damage to the endothelium
3. Scars interfering with specular view
4. One eyed
5. Gross difference between the right and left eye

2.2.5: Tissue Collection and Transport:

Tissues were collected from voluntary donors or through Hospital Cornea Retrieval Program (HCRP) by doctors and trained technicians.

Donor demographics were documented. Time and cause of death; medical and ocular surgical history were also noted.

Some tissues were collected as whole globe enucleation and stored temporarily in moist chamber and later transferred to the liquid storage media after the corneal button was prepared. While other tissues were collected as in situ corneoscleral buttons and stored in the media immediately after collection. Tissues which were transported were kept in ice-lined thermocol boxes to maintain temperature at 2-8^{o}C. Adequate steps were taken to ensure that the cold chain is maintained throughout the process.

5ml donor blood was collected from the carotid or subclavian vessels for serology.

All methodology was performed as the per the eye bank protocol and was done by trained technicians of similar working experience.

2.2.6: Serology:

At the eye bank, serology was done to screen out unsuitable tissues. All samples were tested for the following:

a. HIV 1 and 2 – using a tridot kit (HIV Tri-Dot test, Diagnostic Enterprises, Parwanoo, India)
b. HBV – using an immunochromatograohic test (SD BioLine HBsAg, SD Bio Standard Diagnostics, Gurgaon, India)
c. HCV – using an immunochromatographic test (SD BioLine HCV, SD Bio Standard Diagnostics, Gurgaon, India)
d. VDRL – Treponema Hemagglutination test (Immutrep TPHA, Omega Diagnostics, Scotland, UK)

2.2.7: Whole Globe evaluation:

1. The enucleated globes were examined at the slit lamp (Model SL-1E, Topcon Corp, Japan) by consultants or senior fellows at the Cornea Clinic.

2. They were graded as per the eye bank evaluation proforma (see annexure).
3. If at this point tissues met the inclusion criteria, they were included in the study after being authorised by the evaluating doctor.

2.2.8: Corneo-Scleral Button Preparation:

1. Corneo-scleral button was prepared from these included tissues as per the eye bank protocols by trained technicians with similar work experience.
2. The entire process was done under a laminar flow hood under strict sterile environment.
3. It was then immersed in povidone iodine 5% for 3 minutes.
4. Then it was immersed in a solution of normal saline and amikacin (for injection)
5. Finally it is rinsed with normal saline.
6. The rim was then cut with a number 15 blade and corneal scissors leaving about 2-3mm scleral rim around the periphery.
7. Once the rims were prepared, moxifloxacin (0.5%) eye drop was instilled and the right and left side tissues were transferred to either Cornisol or Optisol-GS. Both were transferred at the same time to ensure the time they stay in the media is same.

8. For tissues collected by in-situ method, they were randomized and transferred to Optisol-GS or Cornisol immediately after collection.

2.2.9: Tissue evaluation:

1. The corneoscleral button was examined under slit-lamp (Model SL-1E, Topcon Corp, Japan) while being stored in the media vial by trained technicians.
2. A vial-holder with a mirror was attached to the slit lamp. The vial was kept upright in the slot and the tissue examined from the bottom of the glass vial with the help of the mirror kept at an angle. This is a simple method and does not require transferring tissues to a specialized viewing chamber thus reducing chances of contamination.
3. The button was examined layer by layer and graded according to the tissue evaluation form (see annexure).

2.2.10: Specular Microscopy:

1. Specular microscopy was done using the Konan Eye Bank specular (Keratoanalyzer EKA-98, Konan Medicals, Japan) by the technicians in a masked fashion. Only which eye and tissue id was mentioned
2. Each tissue was thawed for 45 to 60 minutes and brought to room temperature before imaging. The endothelium was focussed and area with the clearest view taken for analysis.

3. The center method was used to mark individual endothelial cells. A minimum of 100 cells were counted to ensure that the analysis is accurate and representative of the whole cornea. The morphometric analysis was then performed by the in-built software. Data of endothelial cell density, co-efficient of variance and percentage hexagonality was taken. In images where it was not possible to count a 100 cells, at least 50 cells were counted to have a fair accuracy(43).
4. After taking the specular picture, the storage vials were replaced in the fridge and maintained at 2-8^{o}C.
5. A total of five specular readings were taken for each tissue on day 1, 3, 7, 10 and 14. Each time tissue was thawed adequately before imaging and were replaced in the fridge after.
6. Each pair of tissue was always handled together. This was done to ensure that they are exposed to similar conditions for the same amount of time. According to published reports, there has been no observed adverse effects of cooling warming and re-cooling donor corneas multiple times even when done daily over seven days(44).
7. In case it was not possible to focus the endothelium clearly due to tissue oedema or turbid media, the particular reading was skipped. But the tissue was not excluded or dropped out of the study. Observation protocol was maintained till the 14th day.

8. All specular readings were taken by one of three trained eye bank technicians with similar working experience. As per the eye bank reports, there was very minimal inter and intra-observer differences in specular microscopy evaluation.
9. If it was not possible to take the specular picture on a specific day due to Sunday or other holidays, it was taken on the previous day.

2.2.11: Histology:

Histology was performed on the 14th day of storage after the fifth specular count. The thawed tissues were taken to the pathology lab and processed by the lab technician. Microscopy was done by the institute pathologist and the investigator. All was done in a masked way.

Making the solutions:

1. 0.2% Alizarin Red-S solution was made by mixing 0.2gm of Alizarin Red-S powder (SD Fine Chemicals, Chennai, India) in 70 ml 0.9% saline and 30 ml of 0.1% ammonium hydroxide. The final pH was between 4 and 5 (measured approximately with pH strips).
2. 0.25% Trypan Blue solution was made by mixing 0.25gm of Trypan Blue powder (HiMedia, Mumbai, India) in 100ml 0.9% saline.
3. During the period of the study, solutions were made three times at two month intervals.

Staining procedure:

1. The corneal tissues were transferred from the storage media vial to a Teflon corneal cup.
2. Trypan Blue 0.25% was added drop by drop till it covered the endothelium completely. It was allowed to stand for 90 seconds.
3. The tissue was then rinsed twice with normal saline to wash off the stain.
4. Alizarin Red-S 0.2% was added drop by drop in a similar way to cover the endothelial surface and allowed to stand for 90 seconds.
5. Once again the tissue was rinsed twice with normal saline.
6. Using a corneal trephine of 7.5mm diameter, a corneal button was punched from the center of the tissue.
7. The button was lifted gently with forceps and placed on a glass slide. A drop of saline was put on the top and then fitted with a cover slip.

Microscopy:

1. The stained tissues were observed using a Leica clinical microscope (Leica DM-LS2, Leica Microsystems, Germany).
2. Tissues were examined under 100x magnification to get an overall view and then at 200x to see the details of the endothelium clearly.

3. Uniformity of staining pattern, areas of endothelial damage, Descemet's baring and cell dropouts were noted. Polymegathism and pleomorphism was also noted by not quantified.
4. Histological pictures were taking using the mounted camera (Leica DFC-400) and using Leica Application Suite software (LAS version 4.2, Leica Microsystems, Germany).

Image analysis:

1. The images were analysed using ImageJ software (version 1.49v, Wayne Rasband, NIH, USA) which is an open-source program developed by the National Institute of Health as a versatile biomedical analysis tool(45). It has been used in many studies and is a validated program.
2. Cells were counted using this program using a method similar to variable frame analysis.
3. The image photographed with 200x was used for evaluation and the central area with sharpest focus was analysed.
4. Between 140 to 150 contiguous intact cells were counted per image(46). The marked cells were then outlined to delineate the variable frame. After calibrating the software to the microscope magnification settings, the surface area of the outlined was calculated by the program.
5. Endothelial cell density was counted by dividing the cells counted by the area in square millimetres (ECD = Cells (140-150)/ area in mm^2).

6. Additionally, abnormally stained cells and areas of bare Descemet's in the variable frame were also noted.

2.2.12: Data Collection:

Data collection was divided in to four parts and tabulated in a Microsoft Excel spreadsheet.

1. **Donor profile:** Data collected from eye bank donor sheet
    a. Age
    b. Gender
    c. Cause of death
    d. Death to preservation time – This was calculated as death to enucleation time plus the time kept in moist chamber for whole globe enucleation
    e. Reason why the tissue was not suitable for surgery.
2. **Slit lamp evaluation:** Baseline data collected from the eye bank evaluation forms.
    a. Epithelium – graded as:
        i. Clear and intact
        ii. Mild epithelial defect
        iii. Moderate epithelial defect.
    b. Stroma – graded as:

i. Clear and compact

ii. Mild stromal oedema

iii. Moderate stroma oedema

c. Descemet's membrane – graded as:

i. No folds

ii. Few folds

iii. Moderate folds

d. Endothelium - graded as:

i. No stress lines, guttae or drop out areas

ii. Few stress lines, guttae or drop out areas

iii. Moderate stress lines, guttae or drop out areas

e. Lens status:

i. Phakic

ii. Pseudophakic

iii. Aphakic

3. **Specular microscopy:** Collected from the printed specular microscopy images. Five sets of data collected per tissue on day 1, 3, 7, 10 and 14.

a. Endothelial cell density

b. Coefficient of variance

c. Percentage hexagonality

4. **Microscopy:** Data collected from analysis of histology pictures with ImageJ software.
    a. Number of cells counted
    b. Area of the counted cells
    c. Endothelial cell density
    d. Abnormally stained cells
    e. Areas of Descemet's membrane baring

2.2.13: Statistics:

Statistical analysis was performed using Microsoft Excel (Microsoft Office Professional Plus 2013, Washington, USA) and using Stata statistical software (version 13, StataCorp, Texas, USA).

Percentages were calculated and represented as tables and graphs. Chi square test was done to compare categorical data. For inter-group analysis two-tailed student t-test was done. Pearson coefficient of correlation was calculated between specular and histology data. P-value of <0.05 was considered statistically significant. For analysing the specular microscopy data at various days, the sample size was changed to the total number of clear images for that day and group.

2.3: RESULTS

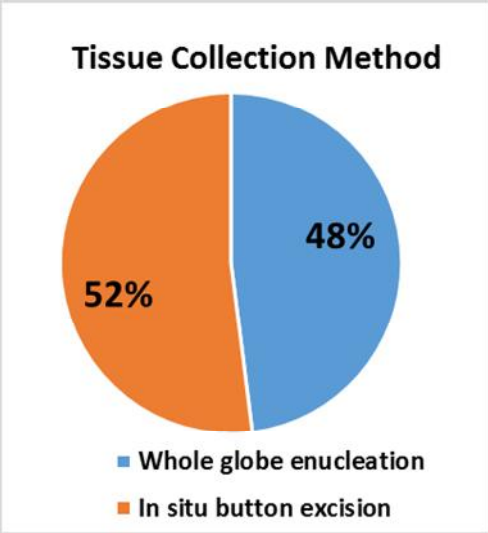
The study period was from February 2015 to August 2015. Over the period of seven months 26 pairs of tissues were included in the study.

One pair of tissue (sample 4) showed turbidity of the media on day 5 and 6 for the left and right eye respectively. Subsequently specular microscopy was not possible. The tissue was observed till day 14 and histology done. Staining showed grossly damaged endothelium and large areas of Descemet's baring. No specific staining patterns could be made out. This sample was excluded from the study. The reason for the turbidity and the tissue getting spoilt was probably due to microbial contamination.

Finally the data and results were compiled using 25 pairs of tissues. The results will be discussed under the following headings – donor profile, slit lamp findings, specular data and histological analysis.

2.3.1: Donor profile:

Of the 25 samples included in the study, 13 (52%) were collected as in-situ corneoscleral buttons and remaining 12 (48%) were collected as whole globe enucleation.



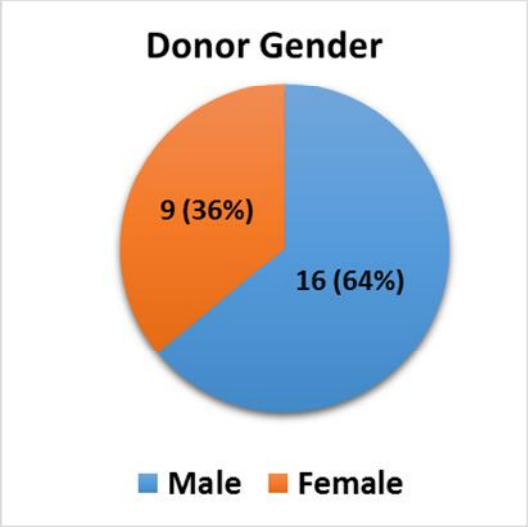


Figure 2.1 and 2.2: Pie charts showing the Tissue collection method and donor gender distribution.

Among the donors, 16 (64%) were male and 9 (36%) were female. The mean donor age was 64.8 ± 18.9 years (range 35 to 95 years). When grouped according to age 5 (20%) were ≤50 years, 11 (44%) were between 51 to 70 years, 8 (32%) were between 71 to 90 years and one donor (4%) was above 90 years of age. The following table shows a more detailed distribution of the age and gender distribution.

Age (years)	**≤50**	**51-60**	**61-70**	**71-80**	**81-90**	**>90**	**Mean Age**	**SD**
Male	3	1	5	3	4	0	60.7	20.7
Female	2	2	3	1	0	1	67.2	18.1
Total	5	3	8	4	4	1	64.8	18.9

Table 2.1 Age and gender distribution of the donors.

The cause of death for the donors were natural deaths in 5 (20%), septicaemia in 9 (36%), malignancy in 5 (20%), cerebrovascular accident in 3 (12%), cardio-respiratory arrest in 2 (8%) and renal failure in 1 (4%).

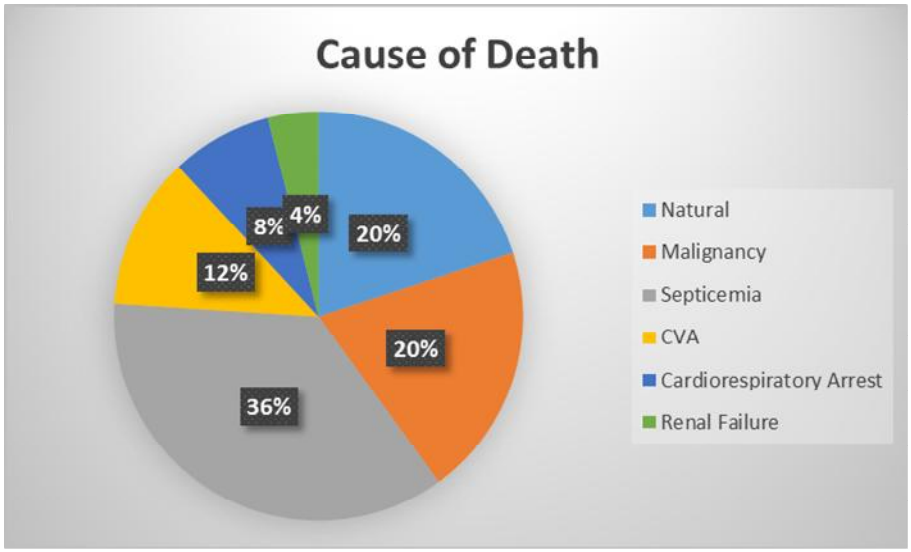


Fig 2.3: Distribution of causes of death

The reasons for considering these tissues unsuitable for transplantation were sepsis in 12 (48%) donors, history of malignancy in 6 (24%) donors, found to be seropositive in 2 (8%) cases (1 for HBV and the other for HIV) and 5 (20%) pairs were deemed unsuitable because of anatomical reasons like dense arcus senilis, old donors with pseudophakic eyes.

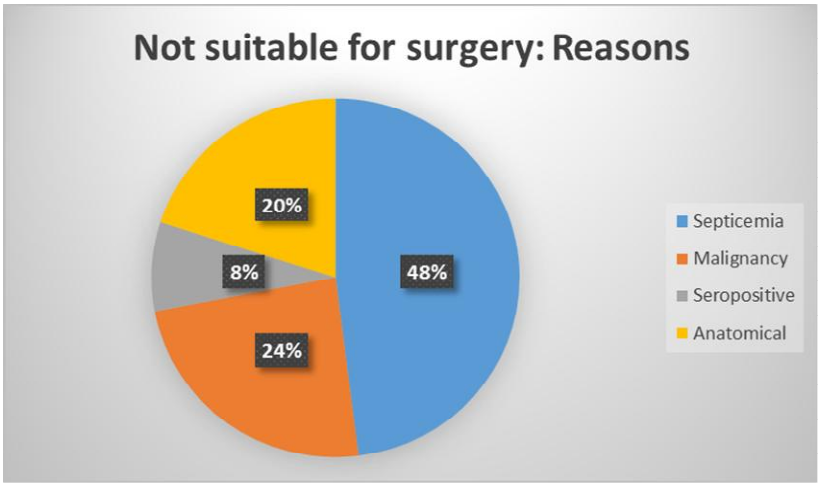


Figure 2.4: Pie chart showing why tissues were not used for surgery

The mean death to preservation time was found to be 393 ± 252 minutes (range 115 to 870). Because of the paired nature of the study, there was no difference in death to preservation time and storage time between the groups. Looking at the in-situ collection group separately, the death to preservation time was 222 ± 59 minutes. In the whole globe collection group, the death to enucleation time was similar at 189 ± 64 minutes. But due to long storage duration in moist chamber of 390 ± 233 minutes, the combined death to preservation was prolonged to 579 ± 250 minutes.

Whole globe collection:	579 ± 250 mins
Death to enucleation	189 ± 64 mins
Moist chamber storage	390 ± 233 mins
In situ collection:	222 ± 59 mins
Total mean:	393 ± 252 mins

Table 2.2: Table showing Death to Preservation time for tissues

After the tissues were included in the study, the decision to put which eye in which media was randomly picked based on a random number generator (www.random.org). If even the right eye tissue was stored in Optisol-GS and if odd it was stored in Cornisol. 15 (60%) right eye tissues and 10 (40%) left eye tissues were stored in Optisol-GS and their mate tissues were simultaneously kept in Cornisol.

2.3.2: Slit lamp findings:

Epithelium examination showed a clear and intact epithelium in 9 (36%) in the Cornisol group and 8 (32%) tissues in the Optisol-GS group, mild epithelial defect in 12 (48%) tissues in both groups and moderate epithelial defect in 4 (16%) tissues and 5 (20%) tissues in the Cornisol and Optisol-GS group respectively (p value = 0.919).

The findings of the stroma showed equal distribution of grades of clarity in the two groups. 19 tissues (76%) were clear and compact, 5 (20%) showed mild stromal oedema and 1 (4%) in each group showed moderate stromal oedema (p-value = 1).

No Descemet's folds were seen in 8 (32%) tissues in the Cornisol group and 7 (28%) tissues in the Optisol-GS. 14 (56%) of the Cornisol stored tissues and 16 (64%) of the Optisol-GS stored tissues had few Descemet's folds. Moderate number of folds were seen in 3 (12%) tissues in

the Cornisol group and 2 (8%) tissues in Optisol-GS group (p-value = 0.819)

Endothelial findings showed a clear endothelium in 6 (24%) tissues stored in Cornisol versus 7 (28%) tissues stored in Optisol-GS. There was mild drop-outs and endothelial stress lines in 19 (76%) tissue of the Cornisol group and in 18 (72%) of the Optisol-GS stored tissues (p=0.747).

The lens status showed 1 (4%) aphakic, 15 (60%) phakic and 9 (36%) pseudophakic tissues in the Cornisol group. Whereas the Optisol-GS group had 1 (4%) aphakic, 16 (64%) phakic and 8 (32%) pseudophakic tissues (p-value = 0.955)

There was no significant difference between the various corneal structures as seen on slit-lamp between the two groups.

Structure	Cornisol group n (%)	Optisol-GS group n (%)	p-value Chi sq. test
Epithelium			
Clear and intact	9 (36%)	8 (32%)	
Mild ED	12 (48%)	12 (48%)	Chi.sq – 0.17
Moderate ED	4 (16%)	5 (20%)	p = 0.919
Stroma			
Clear and compact	19 (76%)	19 (76%)	
Mild oedema	5 (20%)	5 (20%)	Chi.sq = 0
Moderate oedema	1 (4%)	1 (4%)	P = 1
Descemet's membrane			
No folds	8 (32%)	7 (28%)	
Few folds	14 (56%)	16 (64%)	Chi.sq = 0.4
Moderate folds	3 (12%)	2 (8%)	P= 0.819
Endothelium			
Clear	6 (24%)	7 (28%)	Chi.sq = 0.104
Mild dropouts	19 (76%)	18 (72%)	P = 0.747
Lens status			
Aphakic	1 (4%)	1 (4%)	
Phakic	15 (60%)	16 (64%)	Chi.sq = 0.091
Pseudophakic	9 (36%)	8 (32%)	P = 0.955

Table 2.3: Slit Lamp Findings (total n=25 in each group)

2.3.3: Specular microscopy findings:

In the Cornisol group we were able to obtain clear specular images were obtained for all 25 tissues on day 1 and 3, 24 tissues on day 7, 20 (80%) on day 10 and 14 (56%) tissues on day 14. Where as in the Optisol-GS group good specular pictures were taken for all 25 tissues on day 1 and 3, 24 (96%) tissues on day 7, 21 (84%) tissues on day 10 and 15 (60%) tissues on day 14. This served as the sample size for calculations on those particular days.

For few tissues the subsequent specular reading was not possible on the specified date if it fell on a Sunday or hospital holiday. In those cases, the reading was taken on the day before (e.g. day 6 instead of day 7).

As expected, the endothelial cell count showed a decrease in count with each serial imaging. In the Cornisol group the ECD decreased from 2667 ± 334 (range 2016 to 3311; n=25) on day 1 to 2308 ± 329 cells/mm^2 (range 1824 to 2967; n=14) on day 14, whereas in the Optisol-GS this parameter changed from a baseline count of 2828 ± 270 (range 2309 to 3472; n=25) on day 1 to 2431 ± 415 cells/mm^2 (range 1558 to 2881; n=15) on day 14. The following table gives the ECD counts for each observed day and the corresponding t-test result.

	Cornisol (mean ± SD)	Optisol-GS (mean ± SD)	p-value
Baseline (day 1)	2828 ± 270 (n=25)	2667 ± 334 (n=25)	0.076 (n=25)
Day 3	2609 ± 335 (n=25)	2602 ± 319 (n=25)	0.917 (n=25)
Day 7	2580 ± 329 (n=24)	2444 ± 337 (n=24)	0.183 (n=23)
Day 10	2499 ± 303 (n=21)	2370 ± 418 (n=20)	0.053 (n=20)
Day 14	2431 ± 415 (n=15)	2308 ± 329 (n=14)	0.192 (n=12)

Table 2.4: Mean (±SD) ECD at various days in the two groups

For some tissues, SPM image was available for only one eye on certain days. Because a paired two-tailed t-test was done to test the level of significance, the number of samples used to calculate was less than the total images. As seen from the above table, the p-values were not significant and only on day 10 it was close to 0.05.

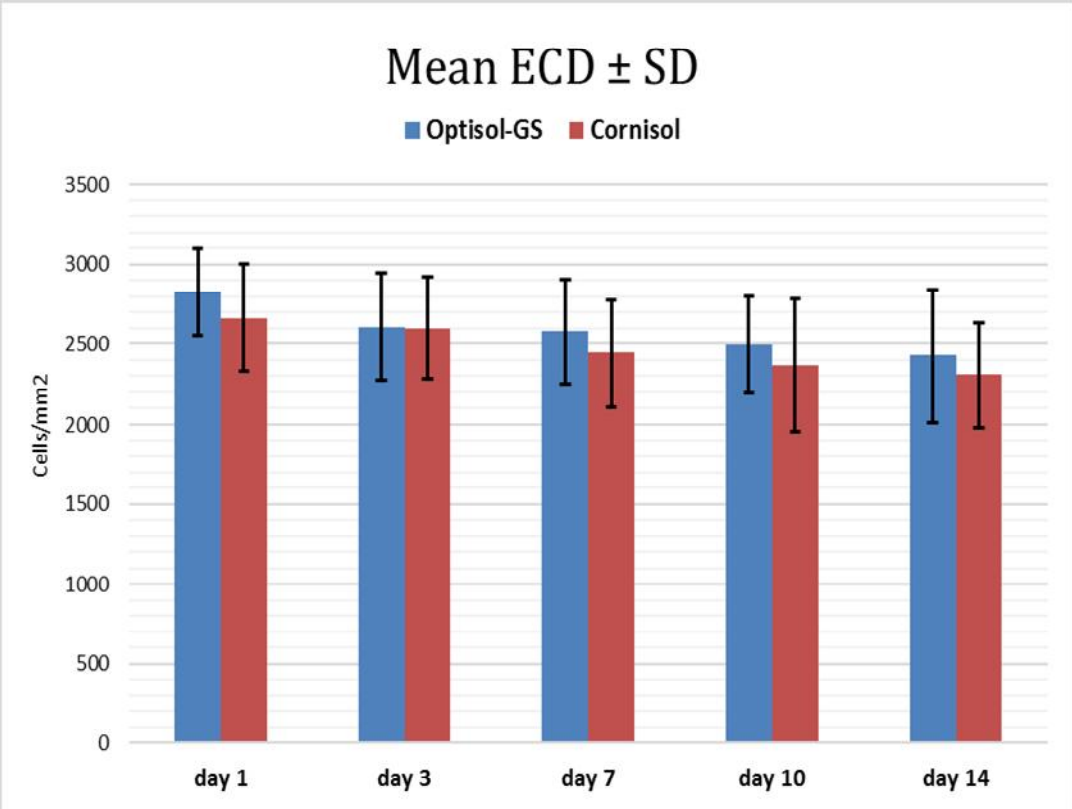


Figure 2.5: Bar graph showing the ECD (±SD) at various days

The endothelial cell loss, represented as a percentage of the baseline count, was 13.46% in the Cornisol group compared to a 14.04% decrease in the Optisol-GS stored tissues (p value = 0.2533).

	Cornisol Mean ECD	ECL (as % of baseline)	Optisol-GS Mean ECD	ECL (as % of baseline)
Baseline (day 1)	2828	-	2667	-
Day 3	2609	2.44	2602	7.74
Day 7	2580	8.36	2444	8.77
Day 10	2499	11.14	2370	11.63
Day 14	2431	13.46	2308	14.04

Table 2.5: Percentage endothelial cell loss over 14 days in the two groups

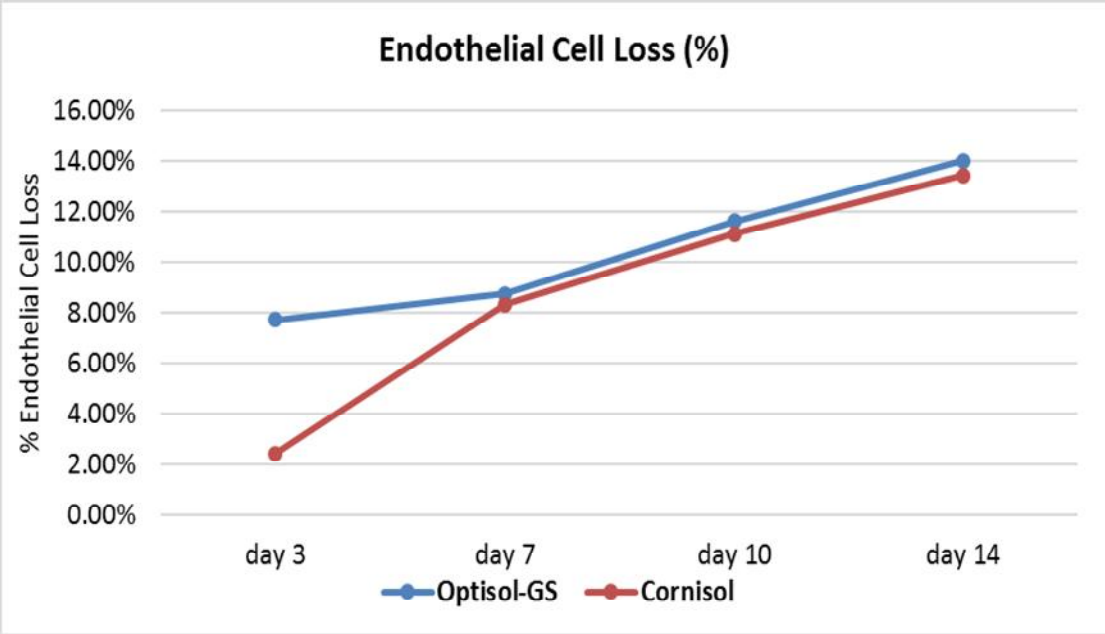


Figure 2.6: Percentage ECL shown as line graph comparing the two media

As denoted by the line graph over time, except the difference on the first day, the endothelial cell loss occurs at similar rates in both the groups.

The coefficient of variance showed an upward trend for till day 7 and then stabilized over the next 7 days in both the groups. In the Cornisol group, the values moved from 41.1 ± 6.5 on day 1 to 44.3 ± 5.9 on day 7 and was 44.6 ± 10.7 on day 14. In Optisol-GS group it increased from 41.3 ± 5.1 to 44.7 ± 5.8 on day 7 and was 43.6 ± 5.5 on day 14. As seen in the following table for each day there was no significant difference between the two groups.

	Cornisol (n)	**Optisol-GS (n)**	**p-value**
Baseline (day 1)	41.1 ± 6.5 (n=25)	41.3 ± 5.1 (n=25)	0.876 (n=25)
Day 3	44.5 ± 8.1 (n=25)	44.4 ± 5.6 (n=25)	0.961 (n=25)
Day 7	44.3 ± 5.9 (n=24)	44.7 ± 5.8 (n=24)	0.934 (n=23)
Day 10	45.7 ± 10.0 (n=21)	44.1 ± 4.9 (n=20)	0.485 (n=20)
Day 14	44.6 ± 10.7 (n=15)	43.6 ± 5.5 (n=24)	0.723 (n=12)

Table 2.6: Mean CV (±SD) over 14 days in the two groups

The hexagonality is inversely related to the CV and therefore, predictably, showed a declining trend in both the groups. In the Cornisol group it started as 51.2 ± 7.5% at baseline and fell to 44.6 ± 8.9%. Compared to that in the Optisol-GS group the baseline was 52.6 ± 6.1% and on day 14 it had decreased to 46.0 ± 4.7%. There was no significant

difference between the two groups on any of the 5 days (see table for p-values).

	Cornisol (n)	Optisol-GS (n)	p-value
Baseline (day 1)	51.2 ± 7.5 (n=25)	52.6 ± 6.1 (n=25)	0.301 (n=25)
Day 3	47.1 ± 1.1 (n=25)	48.4 ± 4.4 (n=25)	0.415 (n=25)
Day 7	47.2 ± 5.2 (n=24)	46.8 ± 4.6 (n=24)	0.627 (n=23)
Day 10	45.6 ± 6.4 (n=21)	45.4 ± 7.6 (n=20)	0.885 (n=20)
Day 14	44.6 ± 8.9 (n=15)	46.0 ± 4.7 (n=24)	0.626 (n=12)

Table 2.7: Mean (± SD) Percentage Hexagonality in the two groups

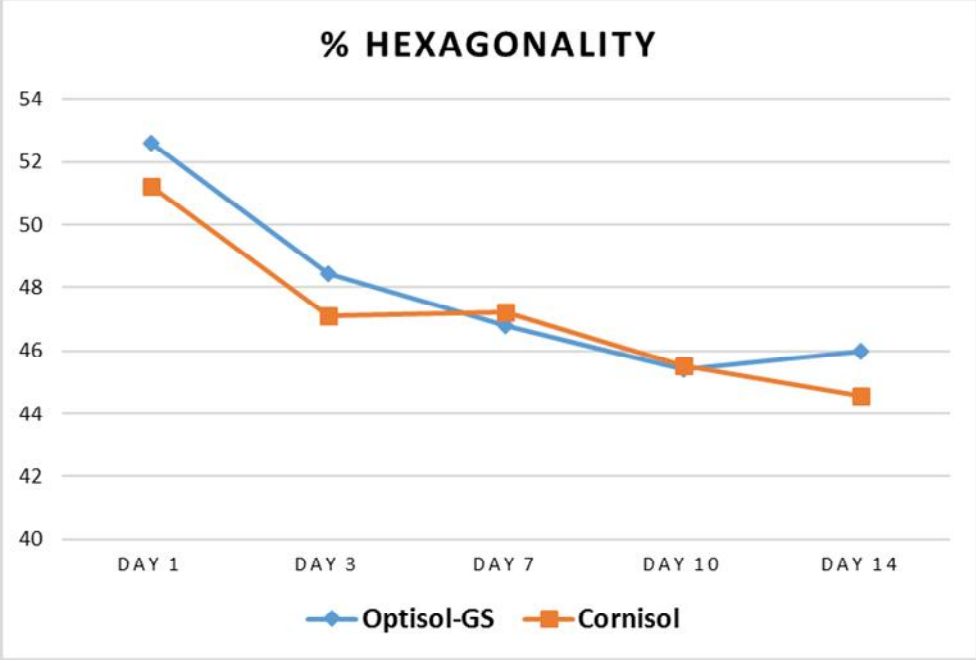


Figure 2.7: Changes in percentage hexagonality shown as a line graph

2.3.4: Histology Findings:

Histology showed adequately stained endothelium in 24 (96%) of tissues in Cornisol group and 23 (92%) of the Optisol-GS tissues. Of the three tissues which did not take up stain properly, two had specular images on the last day and one did not. And two of these tissues were from pseudophakic donors.

Out of the 47 histology slides which were analysed, 42 (89.4%) had an intact endothelial layer. In 5 tissues (3 Cornisol group, 2 Optisol-GS group) there were lots of Descemet's folds seen as linear areas of Alizarin staining suggesting loss of endothelial cells. In most slides few areas isolated drop outs were seen usually located peripherally. Most slides also showed areas of junctional separations. These appear as small alizarin stained areas in the intercellular area where Descemet's membrane is exposed due to separation of the adjacent endothelial cells. Probably this is due to cell shrinkage or pleomorphism (39).

Abnormally stained isolated cells were also seen in most slides, but in 5 tissues (2 Cornisol stored, 3 Optisol-GS stored) there were large confluent areas of abnormal cells. This might suggest some focal endothelial stress. Pleomorphism and polymegathism was not quantified, but as a general observation, it was more predominant in pseudophakic tissues and in old donors.

In the Cornisol group 23 tissues were analysed. An average of 145 cells were counted (± 1.3; range 142 to 147) in each slide. The average area occupied by the cells was found to be 61757 ± 8360 sq. micron. Mean number of abnormally stained cells seen was 0.8 (range 0 to 3) and number of drop out areas was 1.0 (range 0 to 7). The mean endothelial cell density was found to be 2380 ± 287 cells/mm^2 (range 1729 to 2776).

In the Optisol-GS group 24 tissues were analysed. An average of 145 cells were counted (± 1.5; range 142 to 147). The average area of the counted cells was found to be 61260 ± 15815 sq. micron. The average number of abnormally stained cells was 1.0 (range 0 to 4) and the number of drop out areas was 1.8 (range 0 to 8). The mean endothelial cell density was found to be 2330 ± 309 cells/mm^2 (range 1800 to 2781).

In one of the slides in this group, it was not possible to count all the cells as a single contiguous areas due to poor focus and some abnormal cells. In that particular slide, two areas of contiguous cells were counted get the complete number. This is as per the protocol described in the SMAS study(43).

Two-tailed paired t-test was calculated between the groups for 22 paired values of endothelial cell density and p value was found to be 0.28. Additionally intra-group Pearson co-efficient was calculated between the

ECD at baseline and the value obtained by histology analysis. It was found to be 0.49 in the Cornisol group (n=23) and 0.34 in the Optisol-GS group (n=24). Both are positive values and signifies a positive correlation between the SPM count and the histological count.

	Cornisol (n=23)	**Optisol-GS (n=24)**
Cells counted	145 ± 1.3	145 ± 1.5
Area of cells (sq. micron)	61757 ± 8360	61260 ± 15815
Abnormal staining	0.8	1.0
Drop out areas	1.0	1.8
ECD (cells/sq.mm)	2380 ± 287	2330 ± 309
Pearson coefficient	0.49	0.34

Table 2.8: Comparison of the histological analysis with ImageJ

2.4: DISCUSSSION

Cornisol is an indigenous intermediate term hypothermic corneal storage medium which is approved for storing tissues for up to 14 days at 2-8^{o}C(47). It is a chondroitin sulphate containing media which combines the constituents of Optisol-GS and Life4^{o}C. So far there are no published studies comparing Cornisol with other intermediate medium. The data available on the product page(47) is the result of a multi-center trial done before the launch of the product.

Component		**Optisol-GS#**	**Life4^{o}C#**	**Cornisol***
Base Medium	MEM	Yes	Yes	Yes
Glycosaminoglycans	CDS	Yes	Yes	Yes
Buffers	HEPES, Sodium bicarbonate	Yes	Yes	Yes
Deturgescent agent	Dextran-40	Yes	Yes	Yes
Antibiotics	Gentamicin, Streptomycin	Yes	Yes	Yes
Human recombinant insulin		No	Yes	Yes
Glutathione		No	Yes	No
Amino acids		Yes	Yes	Yes
ATP precursors		Yes	Yes	Yes
L-glutamine		No	Yes	Yes
Vitamins, co-enzymes		No	Yes	Yes
Trace elements		No	Yes	Yes
Additional supplements	(anti-oxidants, membrane stabilizers etc.)	No	Yes	No

Table 2.9: Comparison of the components of Optisol-GS, Life4^{o}C and Cornisol. [#Optisol-GS and Life4^{o}C data from Pham et al (25) *Cornisol data from Aurolab website (47)]

This study was designed as a paired comparative design and not as an equivalence or superiority trial. Though the results would be more relevant, we would need many more samples to carry out such a trial. It is probably for the same reason that all the comparative studies done none of the investigators have used a superiority or equivalence design.

Our sample size in each arm was similar to the study done by Price et al(19) (34 vs. 32). The difference can be attributed to the changes in certain assumptions that we made. We were not able to include the target number of samples (25 vs. 34 planned). But in spite of that 25 paired sample is comparable to the previously done in vitro studies. The initial in vitro studies comparing Optisol and DexSol Kaufman et al used 15 pairs(14), Lindstrom used 26 pairs(13), Nelson et al used pairs to compare Chen media vs. Optisol-GS(17), Pham et al (25) studied Life4^{o}C and Optisol-GS using 25 pairs.

Since we used paired samples, the donor profile, the death to preservation time, and the time spent in storage was equal in each group. Which means that for each pair, the Optisol-GS stored tissue was an ideally matched standard control. This eliminated any selection bias.

The slit-lamp findings in both the groups were similar in both the groups. Except one donor, aphakia and pseudophakia was present bilaterally.

So differences in cornea due to surgical intervention was also equally distributed in 24 (96%) of the sample pairs.

The specular parameters showed no significant difference between the Cornisol stored and Optisol-GS stored tissues. This finding was true at baseline and at the four subsequent days. This data comparable to the results obtained by Pham et al in vitro(25) and Price et al in vivo(19) studies to compare Life4^{o}C and Optisol-GS.

The observed endothelial cell loss percentage was 13.46% in Cornisol and 14.04% in the Optisol-GS group. This is little more than the 5±5% reported by Nelson et al for Optisol-GS. Our findings were consistent with Parekh et al(20) where they reported a 8.03±6.6% (vs. 8.77% in our study) at 1 week and further drop of 8.01±6.5% (vs. 5.27% in our study) at end of second week The findings in the Cornisol group was 8.36% and another 5.10% at the end of first and second week respectively. Though not statistically significant, the endothelial cell loss was found to be less in the Cornisol group (especially on day 3 – 2.24% vs. 7.74%). This could be attributed to the addition of components like recombinant human insulin which enhances nutrient uptake by the cells, presence of L-glutamine, vitamins and co-enzymes which help in enhancing the cell metabolism and has some anti-oxidant property.

The changes noted in CV and 6A in both the groups were comparable to results noted by Kanavi et al in comparing Optisol-GS and Eusol-C (CV 39 ± 9 vs. 44 ± 6 that we noted at 1 week and 6A of 50 ± 12 vs. 47 ± 5 in this study). Our findings were also consistent with the study between Chen medium and Optisol-GS by Nelson et al(17) where they noted decreasing hexagonality and increasing co-efficient of variance.

We also noticed that it was not possible to take clear specular microscopy pictures from day 7 onwards and we could get good SPM images for 21 and 15 tissues in Optisol-GS group and for 20 and 14 tissues in the Cornisol group on day 10 and 14 respectively. This could probably be due to some tissue oedema which caused light scattering and loss of transparency. Lindstrom et al noted decreasing number of cells per field of specular image in their extensive study(13). Parekh et al, in their study comparing Optisol-GS with a new storage developmental medium called Cornea Cold(20), noticed similar loss of transparency in tissues stored in either medium (5.6% vs. 11.73% loss of transparency at first and second week for Optisol-GS stored tissues). They also noted a downward trend in the overall morphology of the endothelial cells. This however should not be a cause for concern because most tissues are expected to be utilized before 7 days.

The histological examination revealed a completely intact endothelium in 89.4% of the tissues. This is comparable to the histological studies done by Parekh et al(20) and the electron microscopy studies done by Kaufman et al(14) and Nelson et al(17). Increased polymorphism and pleomorphism were noted but not quantified.

Quantification of endothelial cell density using ImageJ has not been done before for such comparison studies. We used the methodology followed in the Specular Microscopy Ancillary Study subgroup of the Corneal Donor Study(43). ImageJ is an image analysis software developed by the National Institute of Health, USA for biomedical purposes. It has been validated for many studies. There are two published studies where this program was used to analyse the endothelium. Bernard et al used it to quantify area of viable endothelium (48) and Jardine et al used the software to calculate the endothelial cell density and compared it with using Adobe Photoshop(49). We found no significant difference in the ECD in the tissues stored in either group. Also there was a positive correlation between the baseline count and the histological count in each group (Pearson coefficient of 0.49 and 0.34 in Cornisol and Optisol-GS respectively).

In some of the slides, we noticed small round, transparent, refractile bodies, sometimes singly and sometimes in clumps. These were present on the endothelial surface. Slit lamp examination of the media vials did not

reveal any particulate matter. They bear resemblance with starch powder seen under microscope(50) which is a possible contaminant from the powder gloves(51). We are not sure about the clinical significance of glove powder on the endothelium but it can cause TASS in some patients.

The strength of the study lies in its paired design which ensures that each pair goes through the same methodology. This eliminates selection bias. The randomization and masked nature helps to reduce observation bias. Usable tissues were wasted in this study. The tissues were not suitable for surgery because of medical reasons but otherwise they were of optical grade with good endothelial counts at baseline. Therefore we got a practical picture because usually the intermediate media are used to store and transport optical grade tissues in our country.

As with most studies there are certain drawbacks in this one. This was an entirely in vitro study. So we can only predict that the results will hold in a clinical setting. We did not measure the thickness of the corneal tissue. When Optisol was introduced in early 90s, one of the main advantage over DexSol (the most popular intermediate storage then) was that tissues stored were significantly thinner both during storage and in-operative period. We can expect that because both Optisol-GS and Cornisol have the same 2.5%2 Chondroitin sulphate and 1% dextran as the main constituents, the thickness of the stored tissues will be similar. But it would be better to measure it

objectively and compare statistically. Going by the experience of the corneal surgeons using this media, the consensus is that both Cornisol and Optisol-GS stored tissues are much thinner than tissues stored in MK Medium.

We also did not evaluate if the addition of components like human recombinant insulin, L-glutamine and vitamins actually has any bearing on the storage quality.

2.5 CONCLUSION

For all practical purposes, Cornisol seems to be an equivalent and cost-effective substitute for intermediate term storage in Indian and developing country perspective.

Histological analysis and correlation is a very powerful way to support the specular findings. With dual staining it is possible to identify cells with abnormal cell membrane which might appear normal on SPM image. Whereas previous studies only commented on the staining pattern, we quantified the cell count with the ImageJ software and correlated it with the initial findings. This has not been done before in the storage media comparison studies.

As the next step to evaluate Cornisol we suggest that a clinical trial could be undertaken. The parameters to look for would be the intra-storage and intra-operative thickness of tissue, the endothelial cell loss in the transplanted graft over 1 year follow-up and the best corrected visual acuity. Something similar to studies by Lindstrom et al(13) and Price et al(19).

Plate 1: Short and Intermediate-Term Storage Media

Moist Chamber

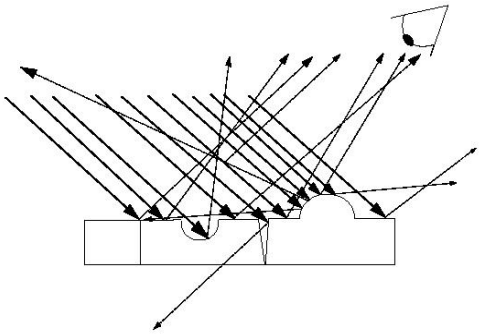
MK Medium

Optisol-GS

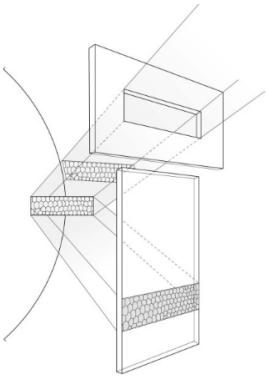
Life4°C

Eusol-C

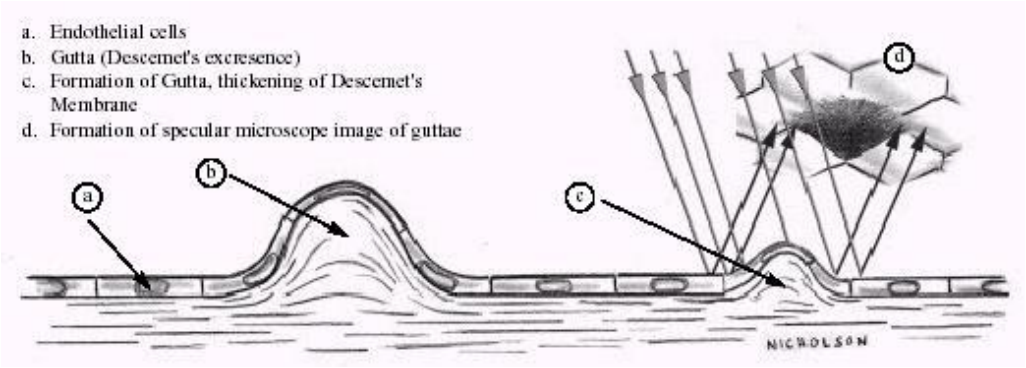
Plate 2: Principles of specular microscopy



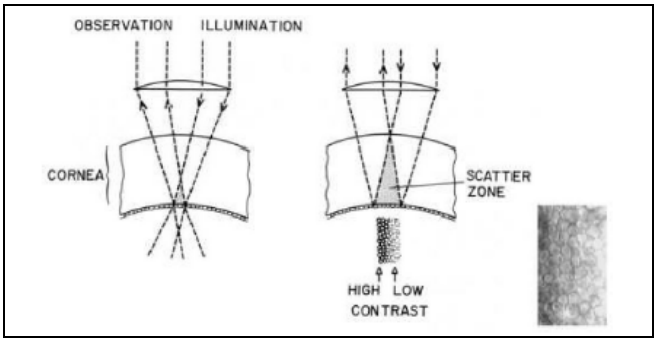
Specular reflection



Specularly reflected rays from the epithelium and endothelium are captured by the device.



How guttae reflect light and appear on specular



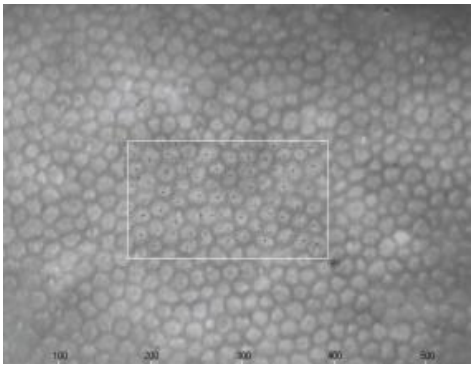
Specular picture depends on the thickness of cornea and width of the light beam

Plate 3: Types of Specular Microscopes

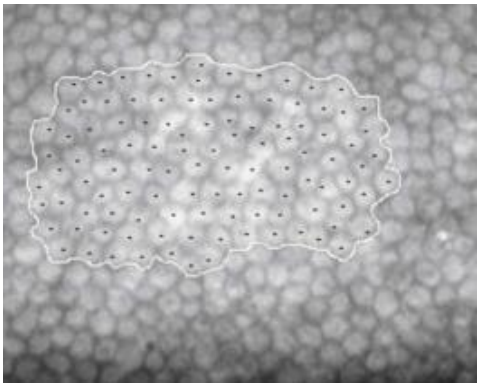
Eye bank specular microscopes have a holder for the vial, x-y movable stage. Pachymeter and media temperature monitors are seen in high-end models.

Clinical specular microscopes are designed to image human subjects

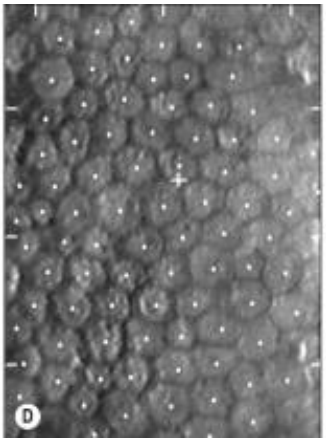
Plate 4: Methods of Specular Morphometric Analysis

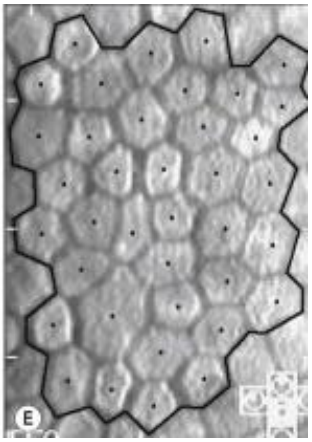


a. Fixed Frame Method

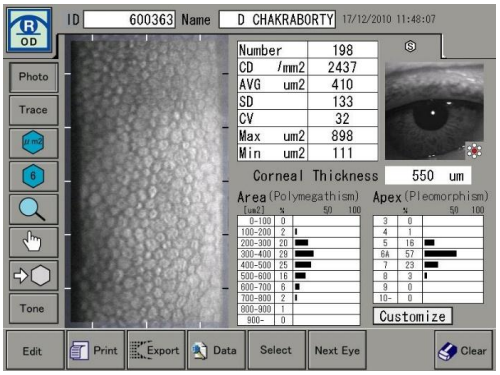


b. Variable Frame Method

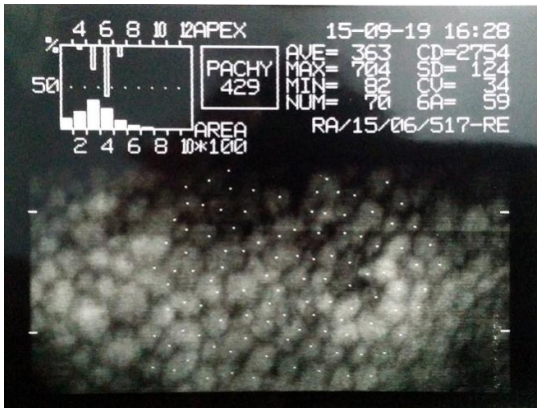




c. Corner Method **d. Center Method** **e. Center-Flex Method**

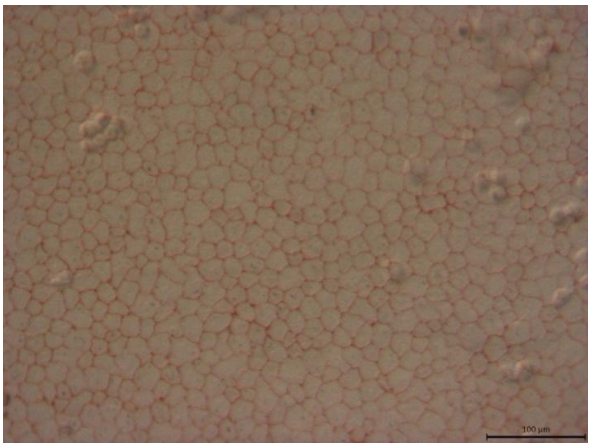


Clinical Specular output



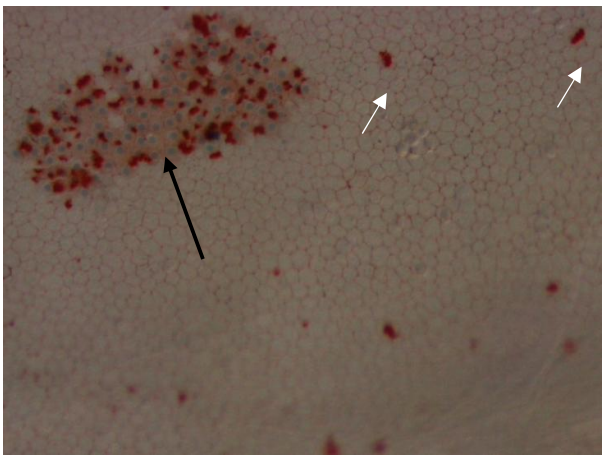
Eye bank Specular Output

Plate 5: Histopathology

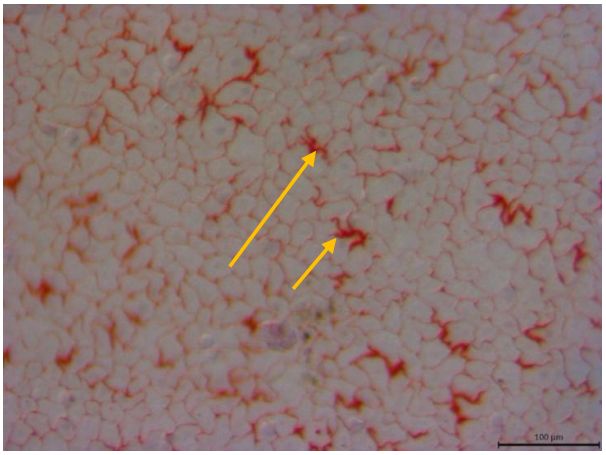


a. Intact epithelial sheet. Alizarin stains the intercellular membranes and trypan blue normally does not stain anything.

Note the transparent refractile bodies.



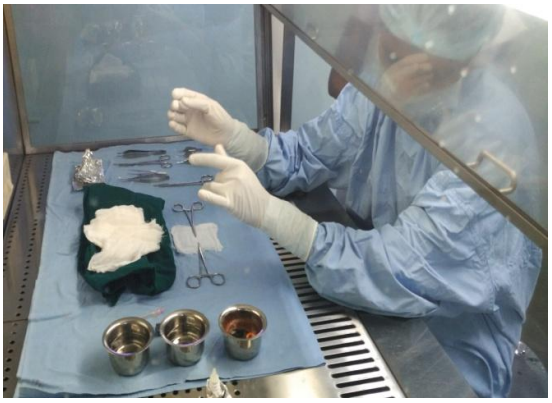
b. Abnormally stained cells (black arrow) and cell drop outs (white arrows). Abnormal cell membranes allow the dyes to center and stains the nuclei and organelles blue and red. Drop out areas are seen as diffusely stained bare Descemet's membrane.



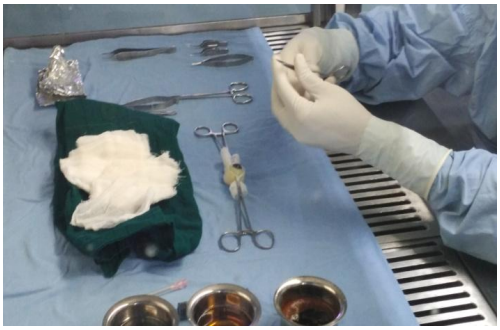
c. Pseudophakic tissues show more pleomorphism and polymorphism. Junctional areas are bare DM where the cells have separated (yellow arrow)

Plate 6: Methodology: Corneo-scleral button excision

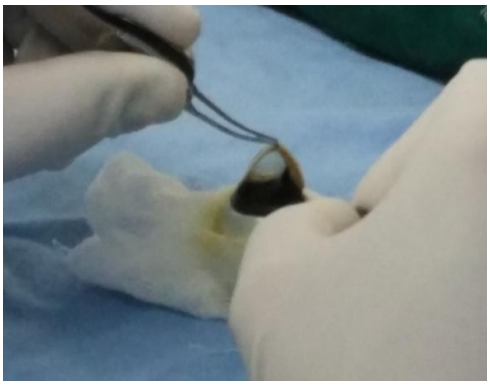
Procedure done in laminar flow



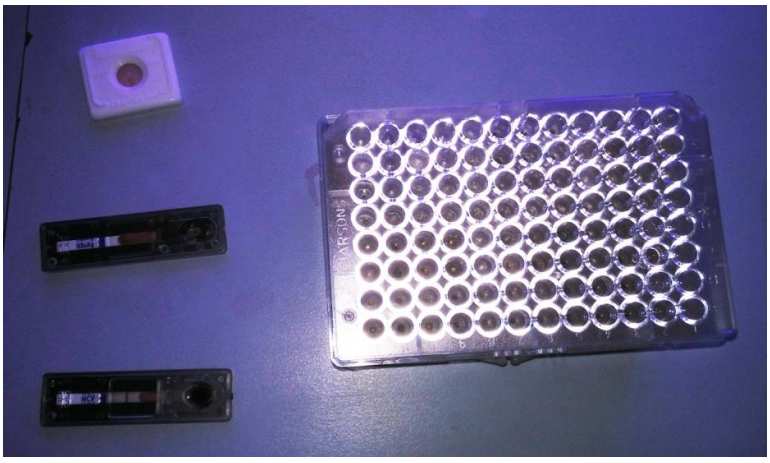
Preparing the instruments



Globe is decontaminated in iodine and antibiotic



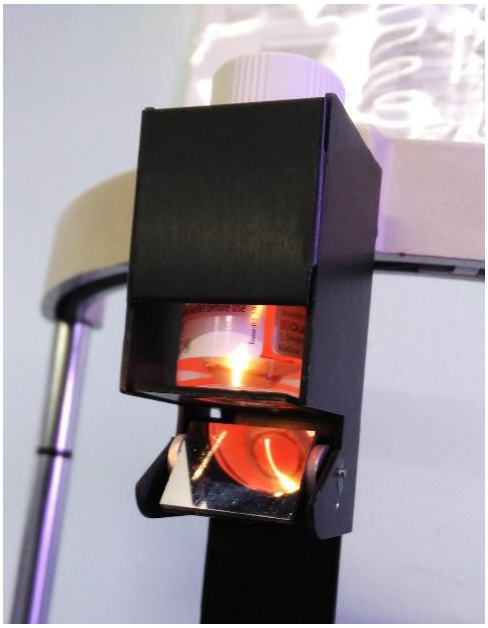
CS button being excised



Serology kits (HIV Tridot, HBV and HCV immunocards and TPHA)

Plate 7: Slit lamp evaluation

Slit lamp attached with holder for media vials

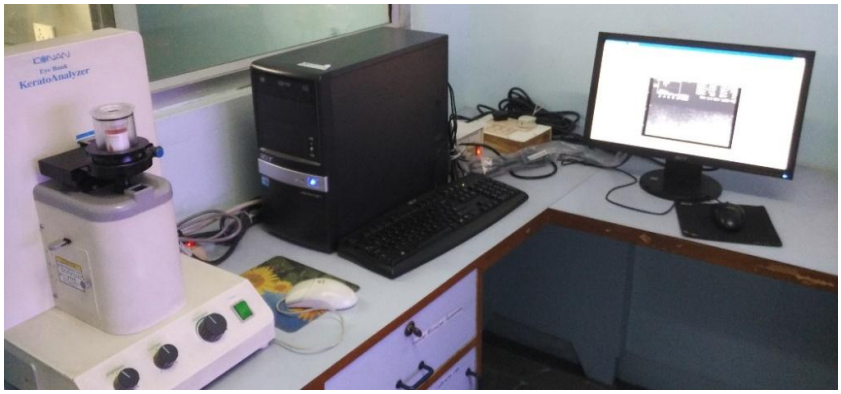


The mirror arrangement allows tissue visualization through the bottom of the vial

Trained eye bank technicians evaluate and grade the tissues

Plate 8: Specular Microscopy

Eye bank specular with holder for media vial

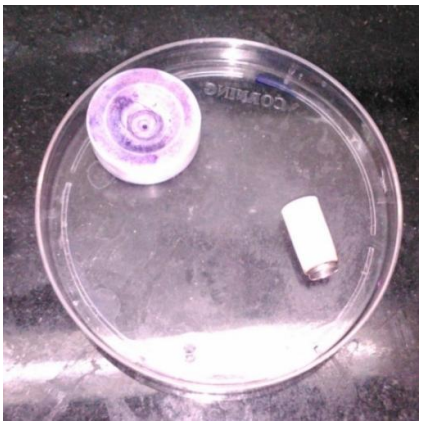


Computer connection allows storage and retrieval of images

In-built software for morphometric analysis

Plate 9: Staining method of tissues

0.2% Alizarin Red **0.25% Trypan Blue**



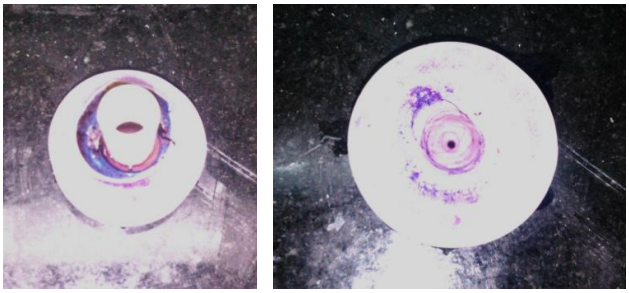
Tissue at 14 days **Teflon block and 7.5mm Trephine**

Staining was done under a laminar flow hood

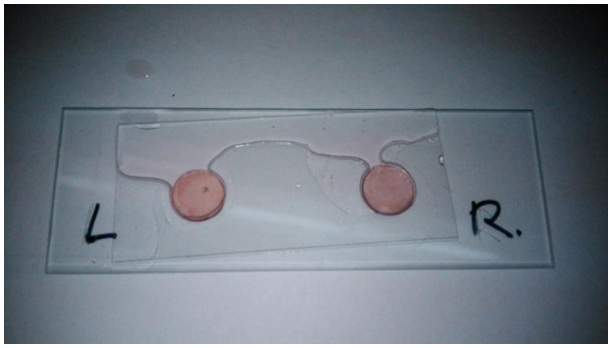
Plate 10: Staining of Tissues contd.

Trypan blue is added drop-wise and allowed to stain for 1:30 minutes and then rinsed with saline

Alizarin Red-S is added next in drops and allowed to stain for 1:30 minutes. Tissue is then rinsed in saline.

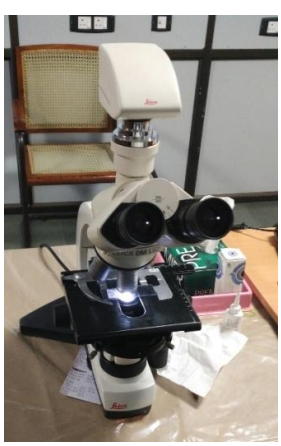


A 7.5 mm button is punched with trepine

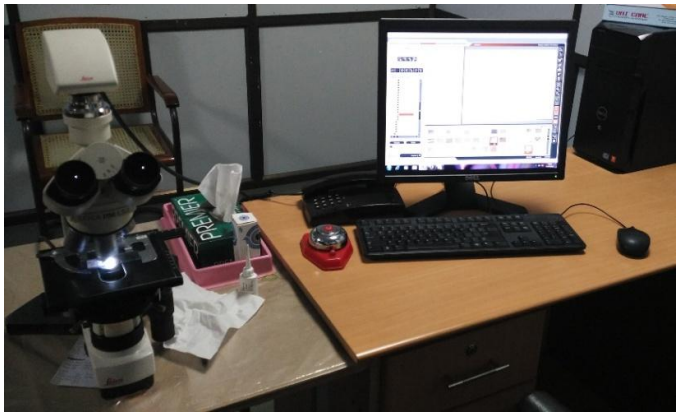


Tissue is mounted in saline and placed under cover slip

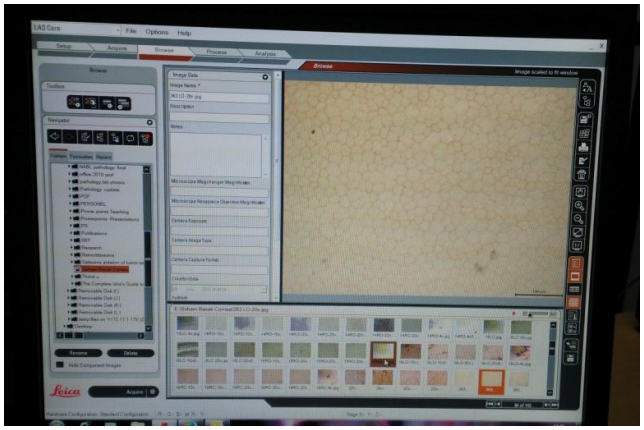
Plate 11: Microscopy



Tissues are examined in Leica microscope with attached camera

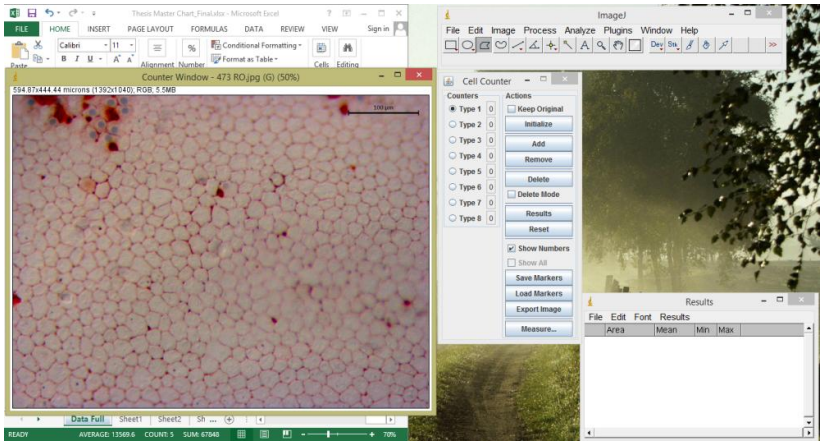


Computer is used to capture the camera images

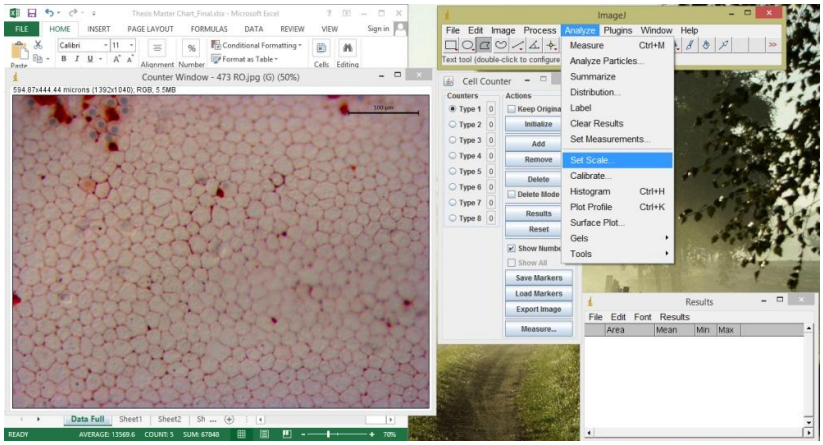


Leica Application Suite Software

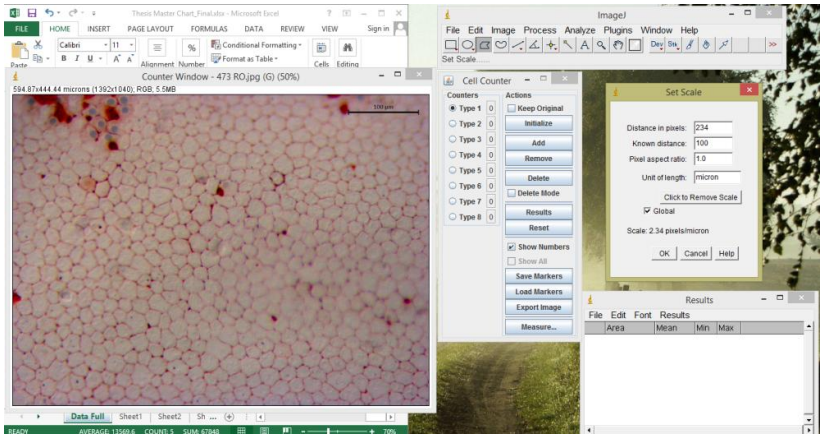
Plate 12: Using ImageJ to analyse the histopathology photos



a. Image is loaded

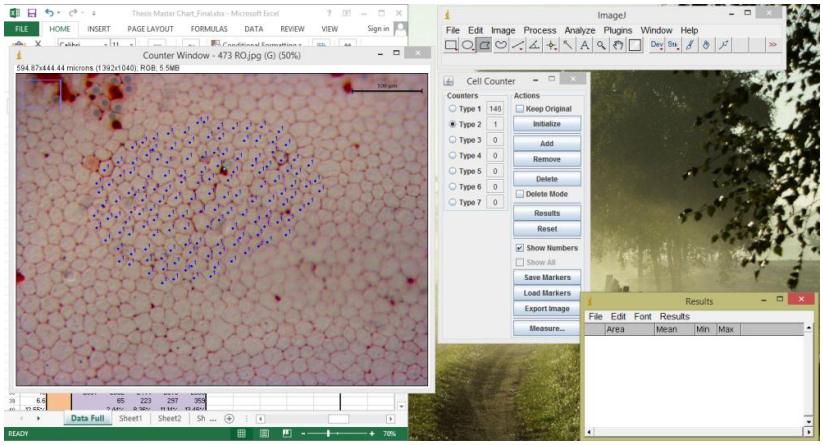


b. Scale adjusted according to magnification

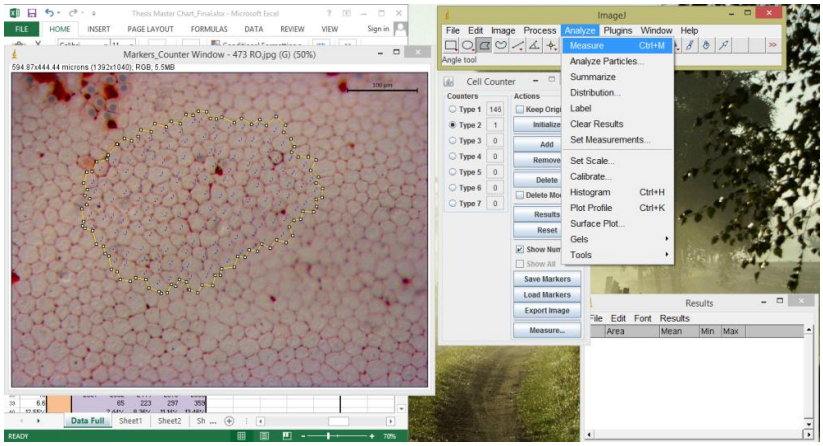


c. For a 200x photo, 100 micron = 234 pixels (2.34pixel/µm)

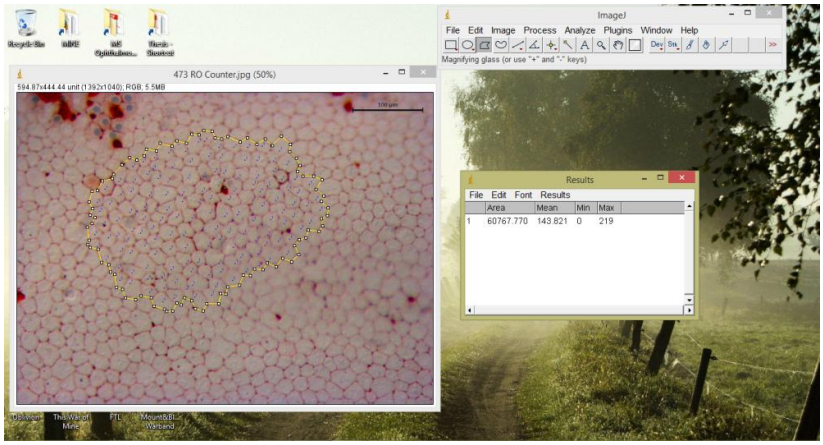
Plate 13: Histopathology analysis contd.



d. 140-150 contiguous cells are counted

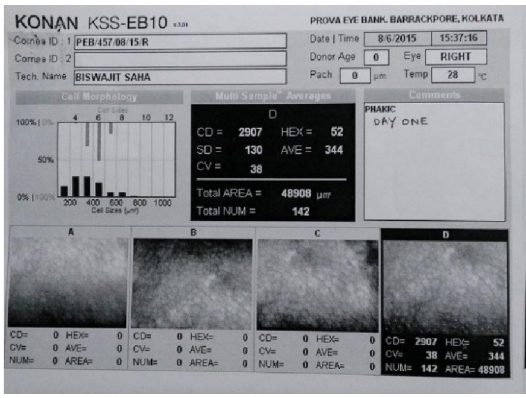


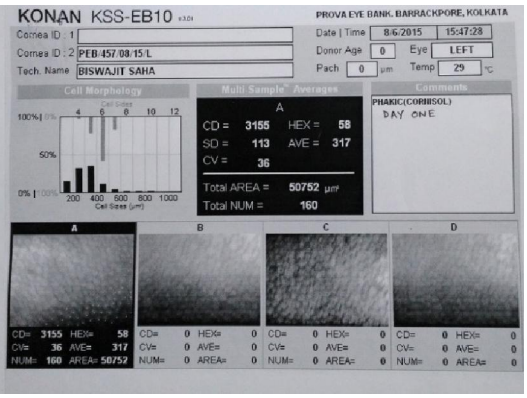
e. Variable frame marked by outlining the marked cells



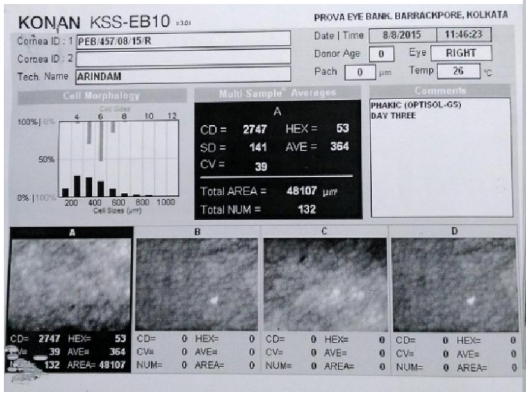
f. Enclosed area is calculated

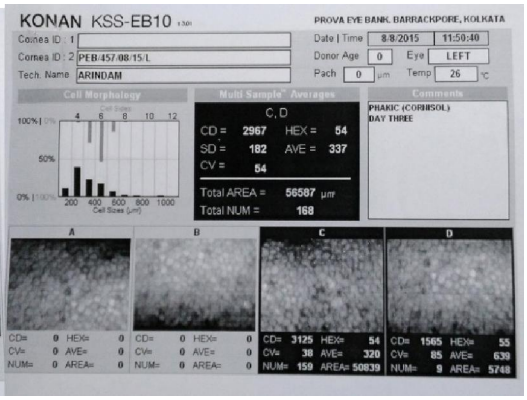
Plate 14: Serial Specular Images of a Sample over 14 days (5 readings)



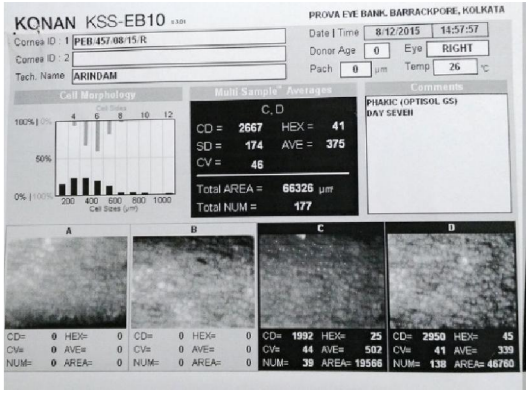


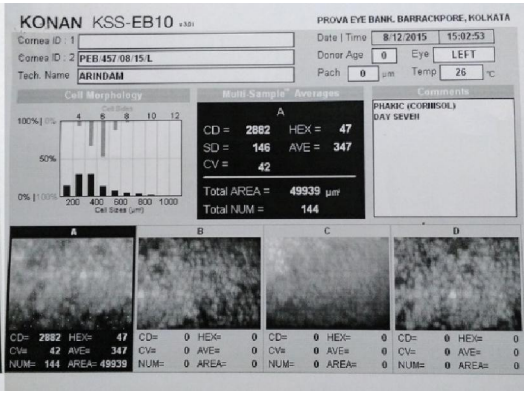
Day 1 Optisol-GS **Day 1 Cornisol**





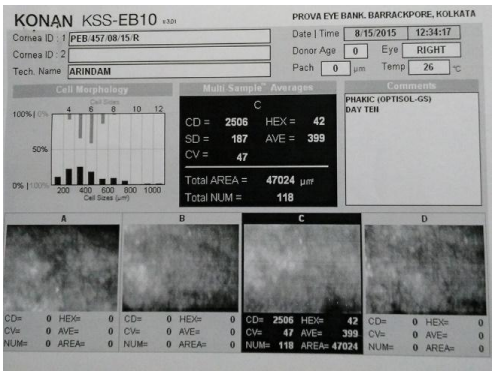
Day 3 Optisol-GS **Day 3 Cornisol**



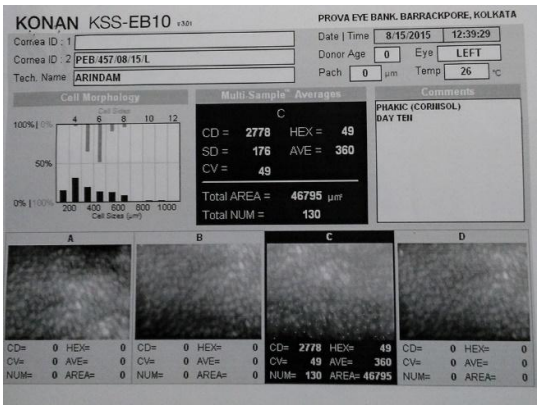


Day 7 Optisol-GS **Day 7 Cornisol**

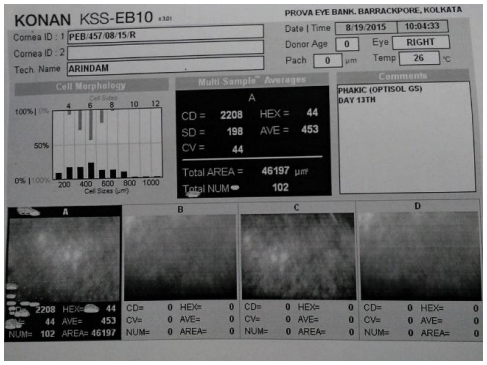
Plate 15: Serial Specular Images of a Sample contd.



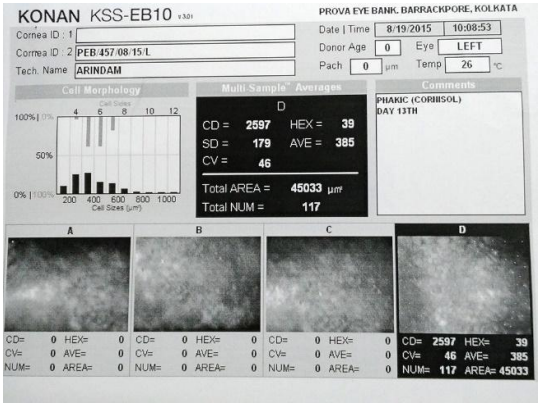
Day 10 Optisol-GS



Day 10 Cornisol



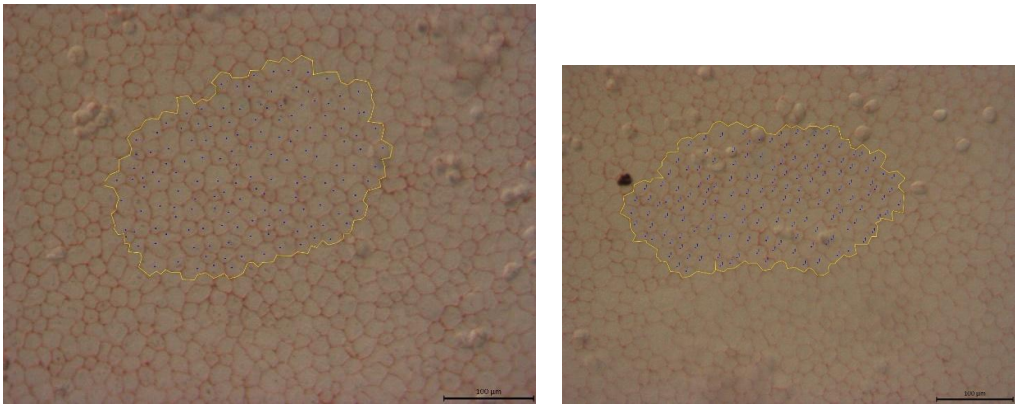
Day 13 Optisol-GS



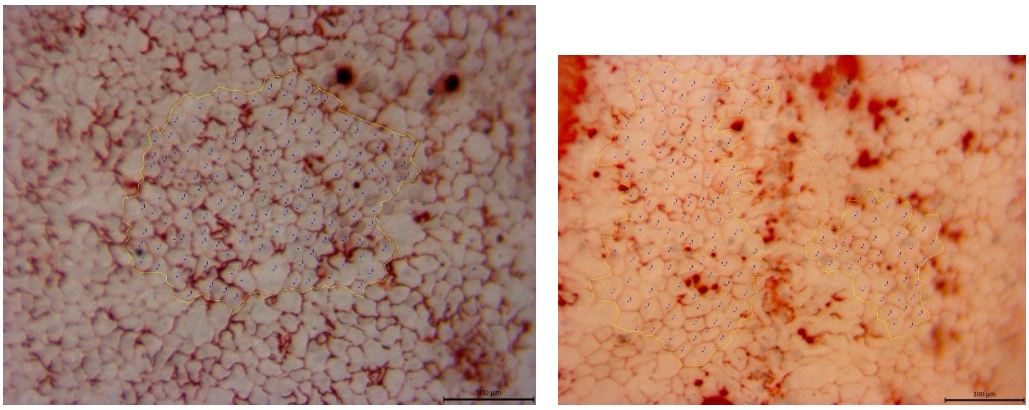
Day 13 Cornisol

Here we notice that in both the tissues stored in Optisol-GS and Cornisol, there is a steady decline in the endothelial count. Also the Co-efficient of variance increases over time associated with an inverse decrease in the percentage hexagonality.

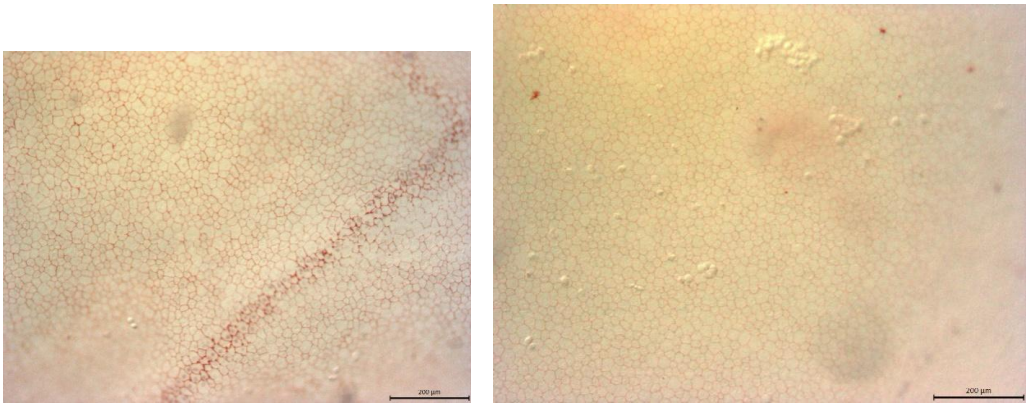
Plate 16: Histology Results



1: Intact endothelial layer in both tissues. Left was Cornisol stored, right was Optisol-GS stored. Yellow border shows counted area.



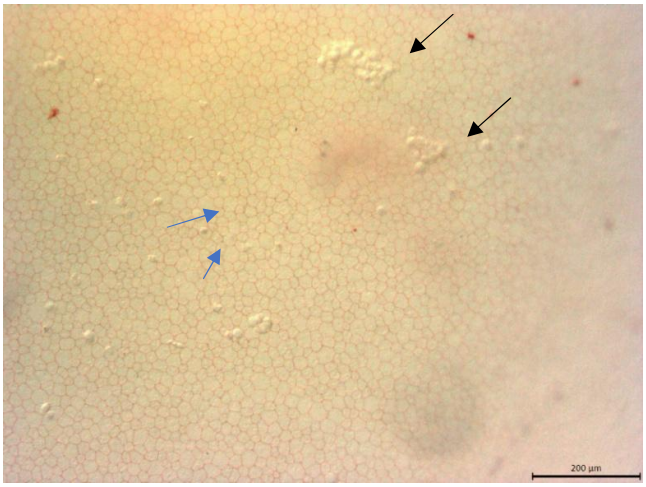
2: Pair of sample from bilateral pseudophakic donor. Note extensive polymorphism and polymegathism. The right Optisol-stored tissue was counted as two separate groups of cells.



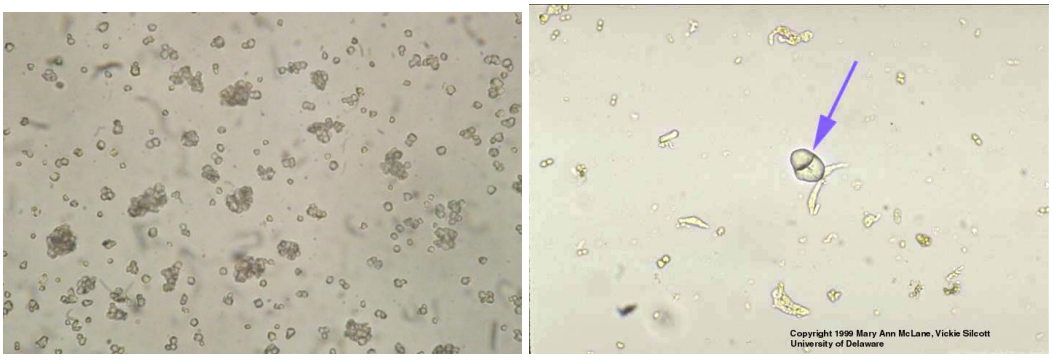
3: The Optisol-GS stored right eye shows staining along a Descemet's fold. Otherwise both tissues show an intact mosaic.

Plate 17: Some peculiar results

Discarded sample: note the clouding and change in colour of the medium. Compare with normal vial on the right.



Refractile bodies (singly and in groups) noted in the slides



Microscopic appearance of starch and glove-powder artefact for comparison

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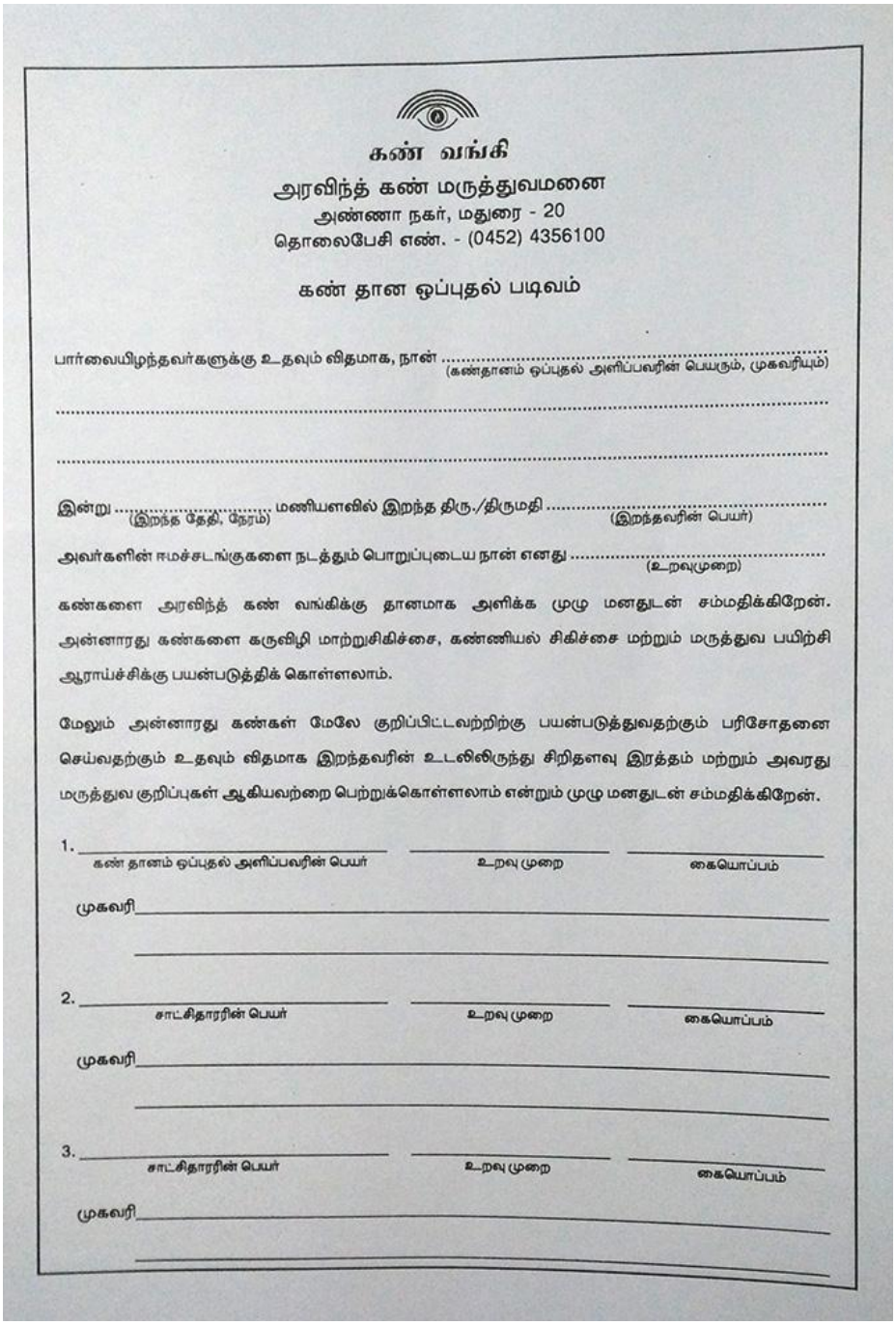
ANNEXURE 1: EYE BANK FORMS



கண் வங்கி

அரவிந்த் கண் மருத்துவமனை
அண்ணா நகர், மதுரை - 20
தொலைபேசி எண். - (0452) 4356100

கண் தான ஒப்புதல் படிவம்

பார்வையிழந்தவர்களுக்கு உதவும் விதமாக, நான் .....
(கண்தானம் ஒப்புதல் அளிப்பவரின் பெயரும், முகவரியும்)

.....

.....

இன்று ..... மணியளவில் இறந்த திரு./திருமதி .....
(இறந்த தேதி, நேரம்) (இறந்தவரின் பெயர்)

அவர்களின் ஈமச்சடங்குகளை நடத்தும் பொறுப்புடைய நான் எனது .....
(உறவுமுறை)

கண்களை அரவிந்த் கண் வங்கிக்கு தானமாக அளிக்க முழு மனதுடன் சம்மதிக்கிறேன். அன்னாரது கண்களை கருவிழி மாற்றுசிகிச்சை, கண்ணியல் சிகிச்சை மற்றும் மருத்துவ பயிற்சி ஆராய்ச்சிக்கு பயன்படுத்திக் கொள்ளலாம்.

மேலும் அன்னாரது கண்கள் மேலே குறிப்பிட்டவற்றிற்கு பயன்படுத்துவதற்கும் பரிசோதனை செய்வதற்கும் உதவும் விதமாக இறந்தவரின் உடலிலிருந்து சிறிதளவு இரத்தம் மற்றும் அவரது மருத்துவ குறிப்புகள் ஆகியவற்றை பெற்றுக்கொள்ளலாம் என்றும் முழு மனதுடன் சம்மதிக்கிறேன்.

1. _____ _____ _____
கண் தானம் ஒப்புதல் அளிப்பவரின் பெயர் உறவு முறை கையொப்பம்

முகவரி _____

_____

2. _____ _____ _____
சாட்சிதாரரின் பெயர் உறவு முறை கையொப்பம்

முகவரி _____

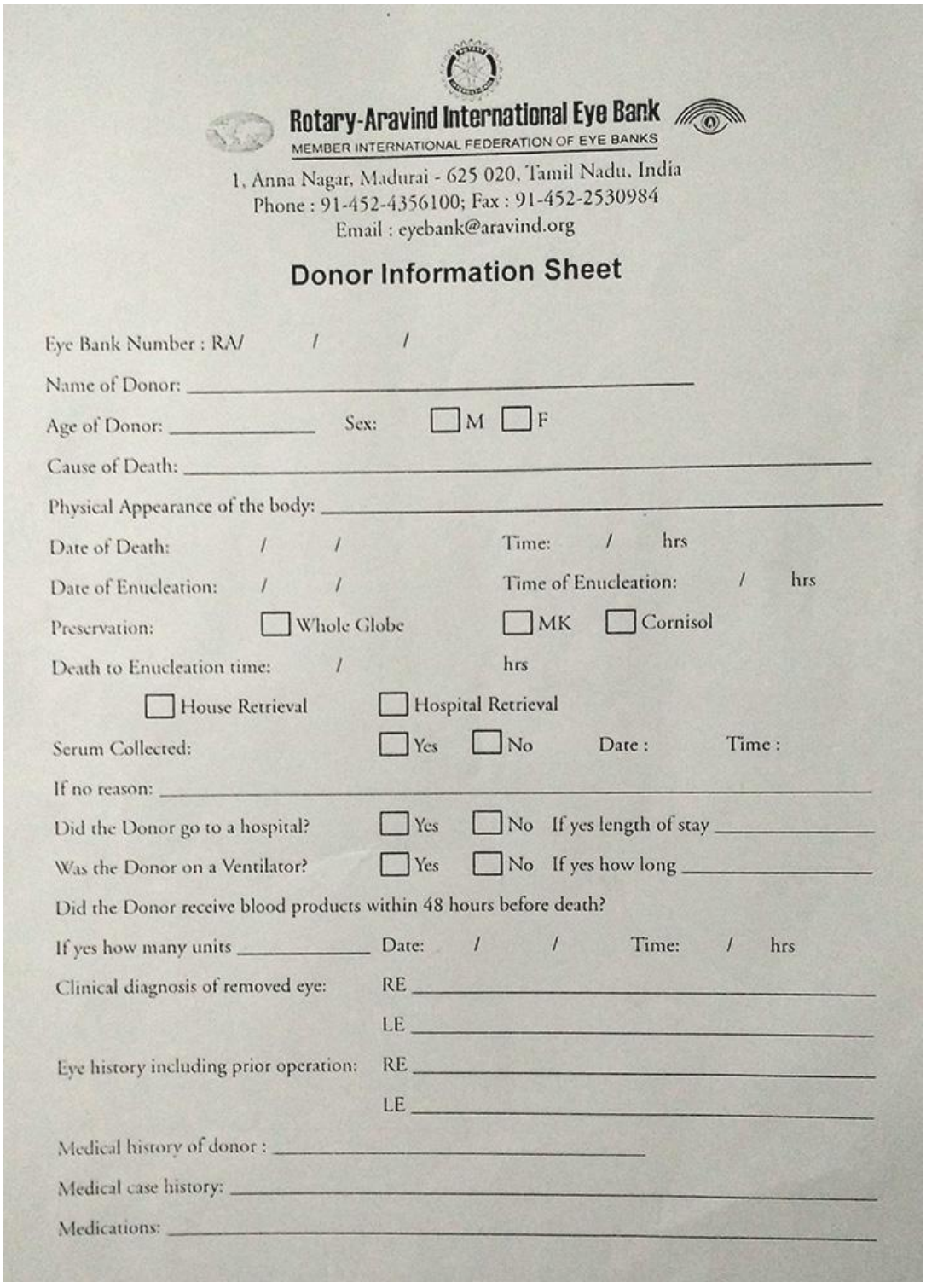
_____

3. _____ _____ _____
சாட்சிதாரரின் பெயர் உறவு முறை கையொப்பம்

முகவரி _____

_____

1: Consent form



Rotary-Aravind International Eye Bank
MEMBER INTERNATIONAL FEDERATION OF EYE BANKS

1, Anna Nagar, Madurai - 625 020, Tamil Nadu, India
Phone : 91-452-4356100; Fax : 91-452-2530984
Email : eyebank@aravind.org

Donor Information Sheet

Eye Bank Number : RA/ / /

Name of Donor: _____

Age of Donor: _____ Sex: ☐ M ☐ F

Cause of Death: _____

Physical Appearance of the body: _____

Date of Death: / / Time: / hrs

Date of Enucleation: / / Time of Enucleation: / hrs

Preservation: ☐ Whole Globe ☐ MK ☐ Cornisol

Death to Enucleation time: / hrs

☐ House Retrieval ☐ Hospital Retrieval

Serum Collected: ☐ Yes ☐ No Date : Time :

If no reason: _____

Did the Donor go to a hospital? ☐ Yes ☐ No If yes length of stay _____

Was the Donor on a Ventilator? ☐ Yes ☐ No If yes how long _____

Did the Donor receive blood products within 48 hours before death?

If yes how many units _____ Date: / / Time: / hrs

Clinical diagnosis of removed eye: RE _____

LE _____

Eye history including prior operation: RE _____

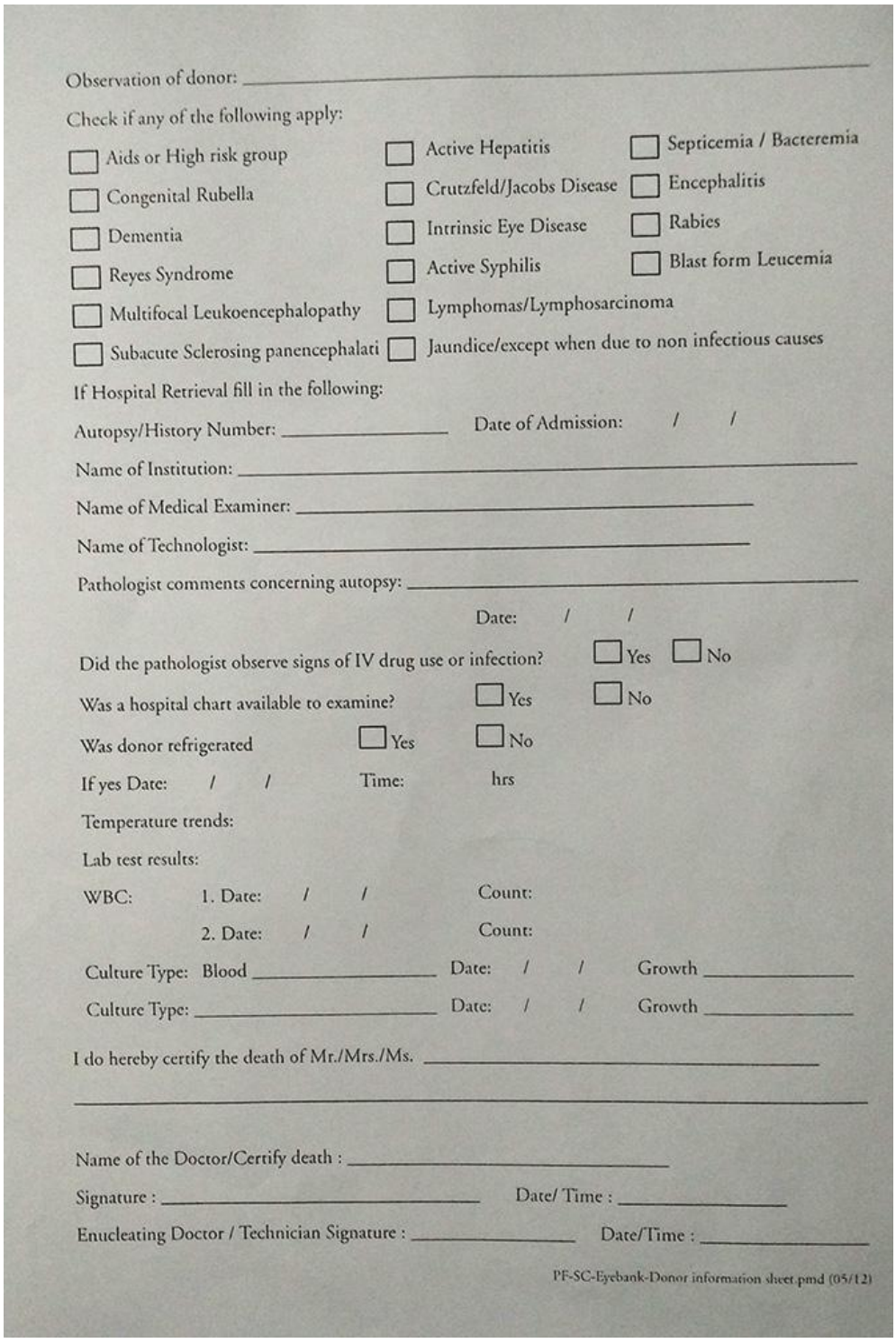
LE _____

Medical history of donor : _____

Medical case history: _____

Medications: _____

2: Donor information



Observation of donor: _____

Check if any of the following apply:

- ☐ Aids or High risk group
- ☐ Congenital Rubella
- ☐ Dementia
- ☐ Reyes Syndrome
- ☐ Multifocal Leukoencephalopathy
- ☐ Subacute Sclerosing panencephalati
- ☐ Active Hepatitis
- ☐ Crutzfeld/Jacobs Disease
- ☐ Intrinsic Eye Disease
- ☐ Active Syphilis
- ☐ Lymphomas/Lymphosarcinoma
- ☐ Jaundice/except when due to non infectious causes
- ☐ Septicemia / Bacteremia
- ☐ Encephalitis
- ☐ Rabies
- ☐ Blast form Leucemia

If Hospital Retrieval fill in the following:

Autopsy/History Number: _____ Date of Admission: / /

Name of Institution: _____

Name of Medical Examiner: _____

Name of Technologist: _____

Pathologist comments concerning autopsy: _____

Date: / /

Did the pathologist observe signs of IV drug use or infection? ☐ Yes ☐ No

Was a hospital chart available to examine? ☐ Yes ☐ No

Was donor refrigerated ☐ Yes ☐ No

If yes Date: / / Time: hrs

Temperature trends:

Lab test results:

WBC: 1. Date: / / Count:

2. Date: / / Count:

Culture Type: Blood _____ Date: / / Growth _____

Culture Type: _____ Date: / / Growth _____

I do hereby certify the death of Mr./Mrs./Ms. _____

_____

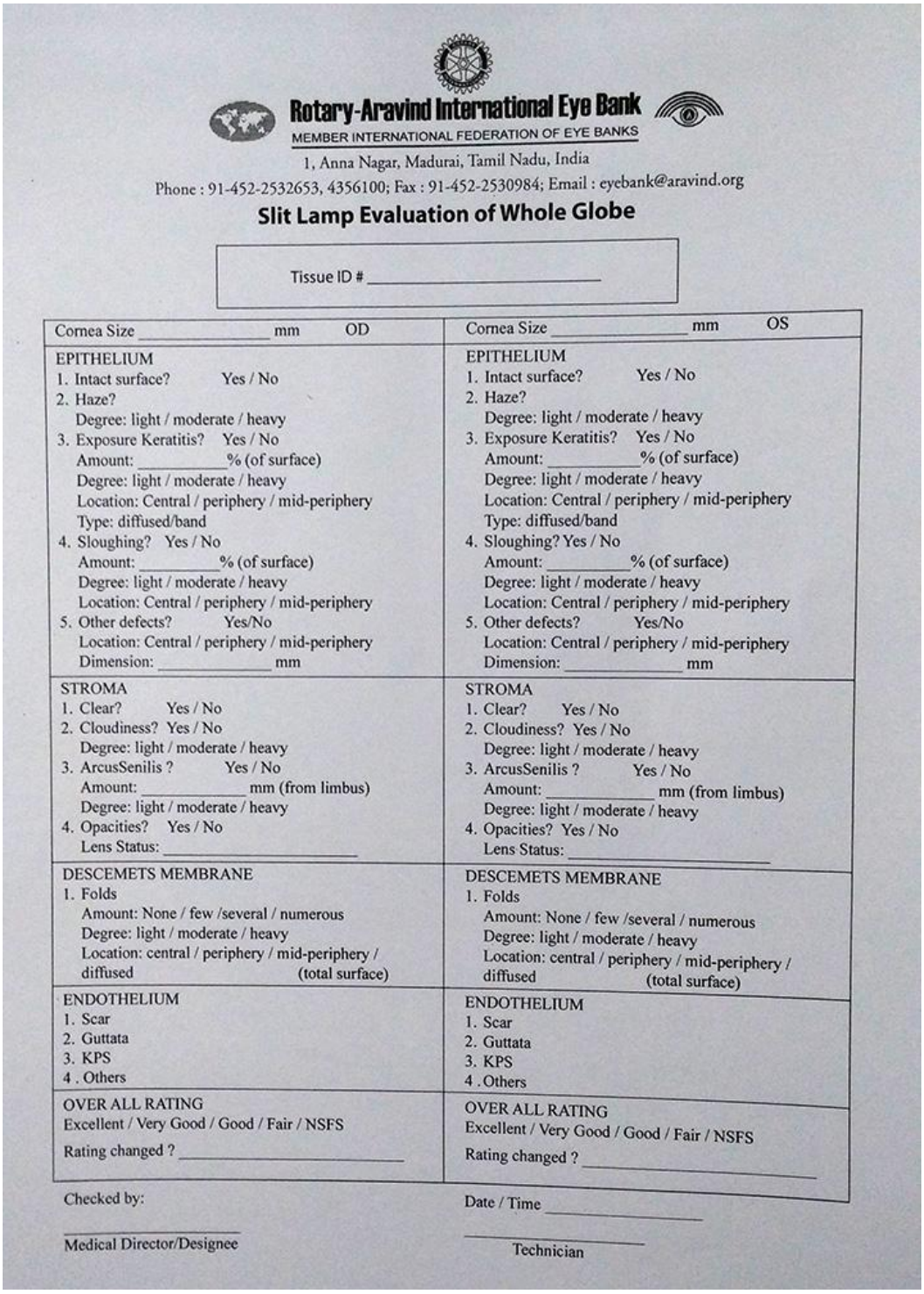
Name of the Doctor/Certify death : _____

Signature : _____ Date/ Time : _____

Enucleating Doctor / Technician Signature : _____ Date/Time : _____

PF-SC-Eyebank-Donor information sheet.pmd (05/12)

3: Donor information (reverse side)



Rotary-Aravind International Eye Bank

MEMBER INTERNATIONAL FEDERATION OF EYE BANKS

1, Anna Nagar, Madurai, Tamil Nadu, India

Phone : 91-452-2532653, 4356100; Fax : 91-452-2530984; Email : eyebank@aravind.org

Slit Lamp Evaluation of Whole Globe

Tissue ID # _____

Cornea Size _____ mm OD	Cornea Size _____ mm OS
EPITHELIUM 1. Intact surface? Yes / No 2. Haze? Degree: light / moderate / heavy 3. Exposure Keratitis? Yes / No Amount: _____% (of surface) Degree: light / moderate / heavy Location: Central / periphery / mid-periphery Type: diffused/band 4. Sloughing? Yes / No Amount: _____% (of surface) Degree: light / moderate / heavy Location: Central / periphery / mid-periphery 5. Other defects? Yes/No Location: Central / periphery / mid-periphery Dimension: _____ mm	EPITHELIUM 1. Intact surface? Yes / No 2. Haze? Degree: light / moderate / heavy 3. Exposure Keratitis? Yes / No Amount: _____% (of surface) Degree: light / moderate / heavy Location: Central / periphery / mid-periphery Type: diffused/band 4. Sloughing? Yes / No Amount: _____% (of surface) Degree: light / moderate / heavy Location: Central / periphery / mid-periphery 5. Other defects? Yes/No Location: Central / periphery / mid-periphery Dimension: _____ mm
STROMA 1. Clear? Yes / No 2. Cloudiness? Yes / No Degree: light / moderate / heavy 3. ArcusSenilis ? Yes / No Amount: _____ mm (from limbus) Degree: light / moderate / heavy 4. Opacities? Yes / No Lens Status: _____	STROMA 1. Clear? Yes / No 2. Cloudiness? Yes / No Degree: light / moderate / heavy 3. ArcusSenilis ? Yes / No Amount: _____ mm (from limbus) Degree: light / moderate / heavy 4. Opacities? Yes / No Lens Status: _____
DESCEMETS MEMBRANE 1. Folds Amount: None / few /several / numerous Degree: light / moderate / heavy Location: central / periphery / mid-periphery / diffused (total surface)	DESCEMETS MEMBRANE 1. Folds Amount: None / few /several / numerous Degree: light / moderate / heavy Location: central / periphery / mid-periphery / diffused (total surface)
ENDOTHELIUM 1. Scar 2. Guttata 3. KPS 4 . Others	ENDOTHELIUM 1. Scar 2. Guttata 3. KPS 4 . Others
OVER ALL RATING Excellent / Very Good / Good / Fair / NSFS Rating changed ? _____	OVER ALL RATING Excellent / Very Good / Good / Fair / NSFS Rating changed ? _____

Checked by:

_____

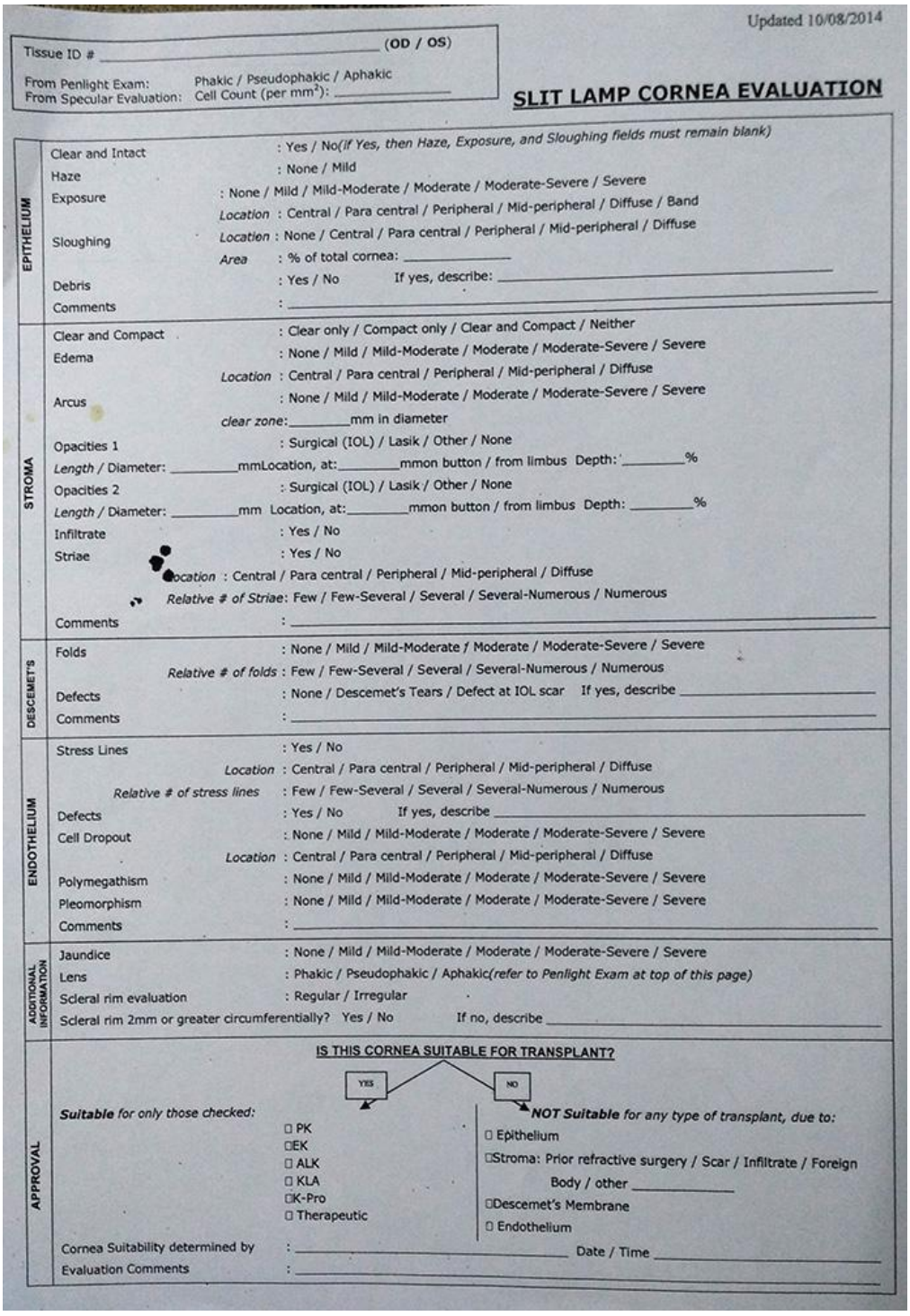
Medical Director/Designee

Date / Time _____

_____

Technician

4: Whole globe evaluation form



Updated 10/08/2014

Tissue ID # _____ (OD / OS)

From Penlight Exam: Phakic / Pseudophakic / Aphakic
From Specular Evaluation: Cell Count (per mm²): _____

SLIT LAMP CORNEA EVALUATION

EPITHELIUM

Clear and Intact : Yes / No *(if Yes, then Haze, Exposure, and Sloughing fields must remain blank)*
Haze : None / Mild
Exposure : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Location : Central / Para central / Peripheral / Mid-peripheral / Diffuse / Band
Sloughing *Location* : None / Central / Para central / Peripheral / Mid-peripheral / Diffuse
Area : % of total cornea: _____
Debris : Yes / No If yes, describe: _____
Comments : _____

STROMA

Clear and Compact : Clear only / Compact only / Clear and Compact / Neither
Edema : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Location : Central / Para central / Peripheral / Mid-peripheral / Diffuse
Arcus : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
clear zone: _____mm in diameter
Opacities 1 : Surgical (IOL) / Lasik / Other / None
Length / Diameter: _____mm Location, at: _____mm on button / from limbus Depth: _____%
Opacities 2 : Surgical (IOL) / Lasik / Other / None
Length / Diameter: _____mm Location, at: _____mm on button / from limbus Depth: _____%
Infiltrate : Yes / No
Striae : Yes / No
Location : Central / Para central / Peripheral / Mid-peripheral / Diffuse
Relative # of Striae: Few / Few-Several / Several / Several-Numerous / Numerous
Comments : _____

DESCEMET'S

Folds : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Relative # of folds : Few / Few-Several / Several / Several-Numerous / Numerous
Defects : None / Descemet's Tears / Defect at IOL scar If yes, describe _____
Comments : _____

ENDOTHELIUM

Stress Lines : Yes / No
Location : Central / Para central / Peripheral / Mid-peripheral / Diffuse
Relative # of stress lines : Few / Few-Several / Several / Several-Numerous / Numerous
Defects : Yes / No If yes, describe _____
Cell Dropout : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Location : Central / Para central / Peripheral / Mid-peripheral / Diffuse
Polymegathism : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Pleomorphism : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Comments : _____

ADDITIONAL INFORMATION

Jaundice : None / Mild / Mild-Moderate / Moderate / Moderate-Severe / Severe
Lens : Phakic / Pseudophakic / Aphakic *(refer to Penlight Exam at top of this page)*
Scleral rim evaluation : Regular / Irregular
Scleral rim 2mm or greater circumferentially? Yes / No If no, describe _____

APPROVAL

IS THIS CORNEA SUITABLE FOR TRANSPLANT?

YES / NO

Suitable *for only those checked:*

- ☐ PK
- ☐ EK
- ☐ ALK
- ☐ KLA
- ☐ K-Pro
- ☐ Therapeutic

NOT Suitable *for any type of transplant, due to:*

- ☐ Epithelium
- ☐ Stroma: Prior refractive surgery / Scar / Infiltrate / Foreign Body / other _____
- ☐ Descemet's Membrane
- ☐ Endothelium

Cornea Suitability determined by : _____ Date / Time _____
Evaluation Comments : _____

5: Slit lamp evaluation of the cornea

ANNEXURE 2: PROFORMA

A prospective, in vitro, randomized study to compare an indigenous intermediate term corneal storage medium with Optisol-GS

Sample Number:

Age: Gender:

Death to Enucleation Time (min):

Moist Chamber to Media Time (min):

Cause of Death:

Reason discarded:

Slit Lamp findings:

	RE	LE
Epithelium		
Stroma		
Descemet		
Endothelium		
Lens status		

RE stored in: Optisol-GS/ Cornisol

LE stored in: Optisol-GS/ Cornisol

Specular Findings:

RE

Day (date)	1	3	7	10	14
CD					
CV					
6A					

LE

Day (date)	1	3	7	10	14
CD					
CV					
6A					

Histology Findings:

	RE	**LE**
Cells counted		
Area in μm^2		
ECD = count/area		
Abnormal staining		
Dropout areas		

Institutional Ethics Committee

(Registration No.ECR/182/Inst/TN/2013 dated 20.04.2013)

12th February 2015

To

Dr. Soham Basak
MS Resident
Aravind Eye Hospital
Madurai

Dear Dr.Soham Basak,

Thesis Title: A Prospective, In Vitro, randomized Study to Compare an Indigenous Intermediate Term Corneal Storage Medium with Optisol-GS

IRB Code: IRB201400108

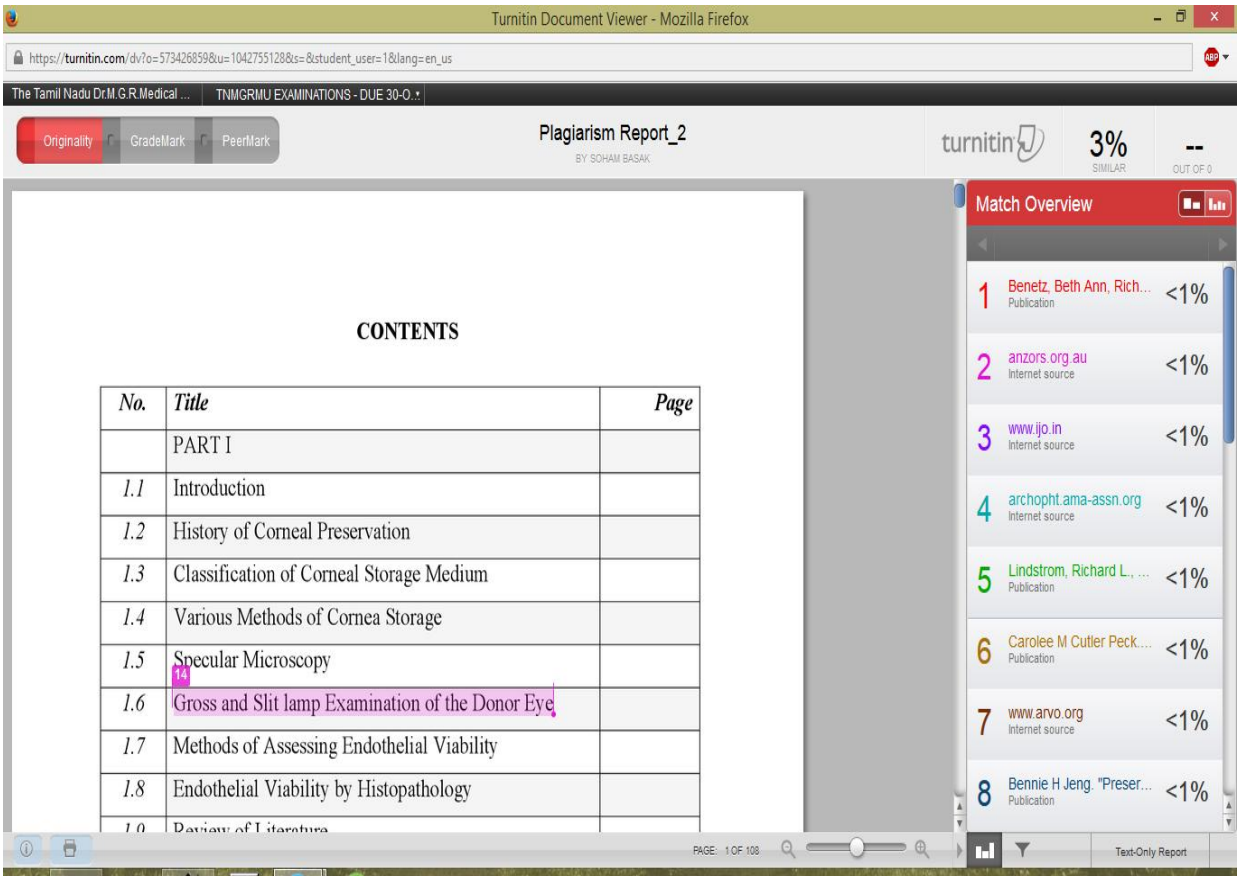
Thank you for submitting your thesis and seeking the approval from the ethics committee. The documents provided by you for consideration which include the thesis protocol and informed consent forms were reviewed for the research methodology and scientific content. The Ethical committee did not find any correction and has recommended the thesis to go ahead in the present form.

Thanking you

Yours Sincerely,

Dr.Lalitha Prajna
Member Secretary
Institutional Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
ARAVIND MEDICAL RESEARCH FOUNDATION
No.1, Anna Nagar, Madurai-625 020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PART I
1.1 Introduction
1.2 History of Corneal Preservation
1.3 Classification of Corneal Storage Medium
1.4 Various Methods of Cornea Storage
1.5 Specular Microscopy
1.6 Gross and Slit lamp Examination of the Donor Eye
1.7 Methods of Assessing Endothelial Viability
1.8 Endothelial Viability by Histopathology
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CONTENTS

No.	Title	Page
	PART I	
1.1	Introduction	
1.2	History of Corneal Preservation	
1.3	Classification of Corneal Storage Medium	
1.4	Various Methods of Corneal Storage	
1.5	Specular Microscopy	
1.6	Slow and Mid Ramp Examination of the Donor Eye	
1.7	Methods of Assessing Endothelial Viability	
1.8	Endothelial Toxicity by Histopathology	
1.9	Review of Literature	
	PART II	
2.1	Aims and Objectives	
2.2	Materials and Methods	
2.3	Results	
2.4	Discussion	
2.5	Conclusion	
2.6	Bibliography	
	ANNEXURE	
	Key bank forms	
	Proforma	
	Institutional Research Board – Approval	
	Plagiarism Report	
	MASTER CHART	

Serial	Tissue ID	Age	Gender	Cause of Death	DtE	EtM	DtP	Reason for Unsuitability
					Time in minutes			
1	**MDU/337**	45	M	Bone Cancer	75	120	195	Cancer with secondaries
2	**MDU/419**	95	F	Natural	150	720	870	Old donor
3	**MDU/446**	78	M	Renal failure	210	180	390	NSFS - Sepsis
4	**CBE/310**	90	M	Natural	90	360	450	Old donor
5	**MDU/447**	87	M	Natural	240	480	720	Old donor
6	**PEB/304**	85	M	CVA	275		275	NSFS - Sepsis
7	**CBE/329**	70	F	Natural	180	480	660	Old donor
8	**MDU/456**	90	M	Cardiorespiratory arrest	210	120	330	Old donor
9	**PEB/309**	65	M	Hepatic encephalopathy	225		225	NSFS - Sepsis
10	**PEB/310**	27	F	Sepsis + Electrolyte Imbalance	320		320	NSFS - Sepsis
11	**MDU/465**	70	M	Bone cancer	330	480	810	Cancer
12	**PEB/324**	36	M	Hepatic encephalopathy	245		245	NSFS - Sepsis
13	**PEB/335**	30	M	Sepsis + Multiorgan failure	205		205	NSFS - Sepsis
14	**MDU/496**	68	F	Natural	130	720	850	Cancer
15	**PEB/349**	87	M	Sepsis + Hepatic Encephalopathy	260		260	NSFS - Sepsis
16	**PEB/358**	62	M	Pneumonia	245		245	NSFS - Sepsis
17	**MDU/517**	78	M	CVA	165	480	645	Dense arcus
18	**MDU/518**	57	F	Lung Cancer	240	600	840	NSFS - Cancer
19	**PEB/363**	35	F	Sepsis + Bleeding diathesis	160		160	NSFS - Sepsis
20	**PEB/370**	72	F	Sepsis + Multiorgan failure	115		115	NSFS - Sepsis
21	**MDU/537**	70	F	Natural	180	150	330	Seropositive HBV
22	**PEB/403**	67	M	Carcinoma base of tongue	175		175	NSFS - Cancer
23	**MDU/583**	67	M	Cardiorespiratory arrest	160	150	310	NSFS - Sepsis
24	**PEB/430**	55	M	CVA	250		250	NSFS - Seropositive HBV
25	**PEB/457**	52	F	Sepsis + Electrolyte Imbalance	265		265	NSFS - Sepsis
26	**PEB/473**	73	M	Cardiorespiratory arrest + Hepatocellular carc	145		145	NSFS - Cancer

Abbreviations used

M = Male; F = Female
CVA = Cerebrovascular accident
NSFS = Not suitable for surgery
RE = Right Eye; LE = Left Eye

DtE = Death to enucleation time
EtM = Enucleation to media time
DtP = Death to preservation time
ECD = Endothlial cell denstiy
CV = Coefficient of variation
HEX = Percentage hexagonality

C&I = Clear and intact
ED = Epithelial Defect
C&C = Clear and compact
ECD in cells/sq.mm
Area in sq.micron

SLE = Slit lamp examintion
SPM = Specular Microscopy
HPE = Histopathology Examination
DM = Descemet's membrane
OS = Optisol; CS = Cornisol

Note: Sample 4 excluded from study

SLE	Optisol-GS Eye					Cornisol Eye				
	Epithelium	**Stroma**	**DM**	**Lens status**	**Endothelium**	**Epithelium**	**Stroma**	**DM**	**Lens status**	**Endothelium**
	Mod ED	Mild edema	Few	Phakic	Mild dropout	Mild ED	Mild edema	Mod folds	Phakic	Mild dropout
	Mild ED	C&C	Mod folds	Pseudophakic	Mild dropout	Mild ED	C&C	Few	Pseudophakic	Mild dropout
	Mild ED	Mod edema	Few	Pseudophakic	Mild dropout	Mild ED	Mod edema	Mod folds	Pseudophakic	Mild dropout
	Mild ED	Mod edema	Mod folds	Phakic	Mod defects	Mild ED	Mod edema	Mod folds	Pseudophakic	Mod defects
	Mild ED	Mild edema	Few	Aphakic	Mild dropout	Mild ED	Mild edema	Few	Aphakic	Mild dropout
	Mod ED	C&C	No fold	Phakic	Mild dropout	Mod ED	C&C	No folds	Phakic	Mild dropout
	Mod ED	Mild edema	Few	Pseudophakic	Clear	Mod ED	Mild edema	Few	Pseudophakic	Clear
	Mild ED	C&C	Few	Phakic	Clear	Mild ED	C&C	Mod folds	Phakic	Clear
	Mild ED	C&C	Few	Phakic	Mild dropout	Mild ED	C&C	Few	Phakic	Mild dropout
	C&I	C&C	No fold	Phakic	Mild dropout	C&I	C&C	No folds	Phakic	Mild dropout
	Mild ED	Mild edema	Few	Pseudophakic	Clear	Mild ED	Mild edema	Few	Pseudophakic	Clear
	Mod ED	C&C	No fold	Phakic	Mild dropout	Mod ED	C&C	No fold	Phakic	Mild dropout
	Mild ED	C&C	Few	Phakic	Clear	Mild ED	C&C	Few	Phakic	Clear
	C&I	C&C	Few	Pseudophakic	Mild dropout	C&I	C&C	No fold	Pseudophakic	Mild dropout
	C&I	C&C	Few	Phakic	Mild dropout	C&I	C&C	Few	Pseudophakic	Mild dropout
	C&I	C&C	Few	Phakic	Mild dropout	C&I	C&C	No folds	Phakic	Mild dropout
	C&I	C&C	Few	Pseudophakic	Mild dropout	C&I	C&C	Few	Pseudophakic	Mild dropout
	C&I	C&C	Few	Phakic	Mild dropout	C&I	C&C	Few	Phakic	Mild dropout
	Mild ED	C&C	No fold	Phakic	Mild dropout	Mild ED	C&C	No fold	Phakic	Mild dropout
	Mild ED	C&C	No fold	Phakic	Clear	C&I	C&C	No fold	Phakic	Clear
	Mild ED	C&C	No fold	Phakic	Mild dropout	Mild ED	C&C	No fold	Phakic	Mild dropout
	Mild ED	C&C	No fold	Phakic	Clear	C&I	C&C	No fold	Phakic	Clear
	Mild ED	C&C	Few	Phakic	Clear	Mild ED	C&C	Few	Phakic	Clear
	Mod ED	C&C	Few	Phakic	Mild dropout	Mod ED	C&C	Few	Phakic	Mild dropout
	Mild ED	C&C	Few	Phakic	Mild dropout	Mild ED	C&C	Few	Phakic	Mild dropout
	C&I	C&C	Few	Pseudophakic	Clear	C&I	C&C	Few	Pseudophakic	Mild dropout
	C&I	C&C	No fold	Phakic	Mild dropout	C&I	C&C	No fold	Phakic	Mild dropout
	Mild ED	Mild edema	Few	Pseudophakic	Mild dropout	Mild ED	Mild edema	Few	Pseudophakic	Mild dropout

SPM	*OS Eye*	ECD					CV					HEX				
		Day 1	*Day 3*	*Day 7*	*Day 10*	*Day 14*	*Day 1*	*Day 3*	*Day 7*	*Day 10*	*Day 14*	*Day 1*	*Day 3*	*Day 7*	*Day 10*	*Day 14*
	LE	2574	2113	2001			47	53	55			56	54	54		
	RE	2347	2018	1988			53	58	58			47	48	45		
	RE	2689	2584	2499	2269	2116	43	46	46	47	49	58	53	53	50	48
	RE	2209					41					50				
	RE	2617	2202	2525	2472		42	37	46	38		46	50	45	50	
	LE	2309	2237	2092		1744	41	42	39		38	49	55	50		44
	LE	3472	3022	2789			47	48	52			44	43	48		
	RE	3215	2415	2604	2173		47	42	49	43		47	52	52	50	
	LE	2564	2475	2331	2597	2631	45	49	45	47	52	53	50	43	51	36
	LE	3115	3049	2801	2832		37	34	38	35		66	57	54	52	
	RE	2949	2118		2369		34	46		50		59	49		35	
	RE	2941	2770	2618	2717	2242	41	45	40	49	46	51	41	50	37	50
	LE	2933	2740	2632	2596	2702	36	51	49	53	37	61	42	42	56	45
	RE	2932	2785	2669	2375	2849	42	47	45	45	46	49	48	44	45	43
	RE	2770	2558	2469	2353	2424	40	42	43	44	32	44	41	46	32	50
	LE	2817	2618	2558	2457		43	49	44	47		51	44	44	37	
	RE	2754	2331	3067	2217	2127	34	44	37	35	44	59	46	45	45	48
	RE	2941	3154	3289	2840	2695	49	47	35	38	43	54	52	51	48	54
	LE	2481	2364	2232	2101	2762	44	45	42	41	45	59	48	47	48	51
	LE	2967	2801	2421	1786	1558	40	46	47	42	38	51	49	47	43	49
	RE	3058	3105	2976	3012	2680	32	34	52	50	52	47	50	49	41	44
	RE	2681	2513	2387	2617	2881	42	43	45	47	43	54	43	38	44	45
	RE	2659	3154	3115	2731		42	39	38	41		45	49	51	39	
	LE	3115	2653	2564	2469		39	44	41	45		51	49	38	44	
	RE	2907	2747	2667	2506	2208	38	39	46	47	44	52	53	41	42	44
	RE	2882	2710	2625	2994	2840	35	40	41	43	45	62	45	46	65	39

CS Eye	ECD					CV					HEX				
	Day 1	Day 3	Day 7	Day 10	Day 14	Day 1	Day 3	Day 7	Day 10	Day 14	Day 1	Day 3	Day 7	Day 10	Day 14
RE	3311	2873	3460			39	47	48			55	50	46		
LE	3003	2745	2326			53	54	57			43	43	40		
LE	2459	2434				60	69				43	37			
LE	2500					37					14				
LE	2016	1996	2336	2174		37	34	47	47		36	43	44	43	
RE	2519	2433	2203		1824	49	39	47		40	51	49	51		32
RE	2304	2747	2457			40	52	54			36	48	40		
LE	2403	2352	2386	2176	1829	44	42	56	52	53	46	39	42	45	43
RE	2545	2336	2232	2201		49	39	48	58		54	40	45	31	
RE	2882	2660	2519	2487		35	37	42	49		57	57	49	51	
LE	2475	2439	2413	2663	2458	29	39	39	42	44	65	50	49	44	42
LE	2849	2639	2532	2538	2677	39	46	43	58	74	50	46	46	46	37
RE	2740	2558	2433	2487	2349	39	43	38	39	40	56	55	45	61	42
LE	2409	2724	2501	2263	2048	49	44	47	47	50	48	42	44	46	43
LE	2532	2381	2252	2020	2311	39	40	46	43	35	44	53	40	45	41
RE	3125	2915	2833	2695		40	35	45	40		57	57	45	49	
LE	2202	2247	1891	1697	2267	41	37	36	34	46	52	40	50	41	40
LE	3144	2808	2921	2389	2496	39	50	36	36	33	52	47	64	47	63
RE	2404	2375	2247	2088	2085	38	40	40	51	48	50	53	53	41	44
RE	2915	2762	2268	1637		38	46	41	42		56	51	50	41	
LE	2949	3355	2352	2608	2412	43	39	43	46	47	47	54	47	47	42
LE	2558	2381	2053	2205	1994	41	44	43	36	38	47	33	45	44	55
LE	2512	3134	2525	3278		39	40	37	30		54	35	54	58	
RE	2415	2070	1972	1942		35	58	49	74		62	55	47	43	
LE	3155	2967	2882	2778	2597	36	54	42	49	46	58	54	47	49	39
LE	2841	2740	2667	3076	2967	37	44	40	40	31	62	46	50	39	61

HPE	OS Eye						CS Eye					
	Eye	Good	Partial	Dropout	Area	ECD	Eye	Good	Partial	Dropout	Area	ECD
	LE	146	3	1	68288	2138	**RE**	144	4	1	60075	2397
	RE	143	2	7	75323	1898	**LE**	144	1	4	61422	2344
	RE	145	0	2	61038	2376	**LE**	147	2	1	79774	1843
	RE	144	2	0	55408	2599	**LE**	144	0	0	68540	2101
	LE	145	0	0	78767	1841	**RE**	144	0	0	72389	1989
	LE	NA					**RE**	145	2	8	72825	1991
	RE	144	2	0	68118	2114	**LE**	146	2	4	81095	1800
	LE	143	1	3	59726	2394	**RE**	146	1	3	54696	2669
	LE	146	0	0	58173	2510	**RE**	145	1	0	54949	2639
	RE	147	0	1	52956	2776	**LE**	142	1	0	55739	2548
	RE	146	0	0	56469	2585	**LE**	145	0	0	54649	2653
	LE	144	0	0	52619	2661	**RE**	143	2	4	54875	2606
	RE	146	1	2	64105	2277	**LE**	147	2	8	66216	2220
	RE	147	1	0	61009	2409	**LE**	145	1	1	71107	2039
	LE	145	1	1	54889	2642	**RE**	145	1	0	56470	2568
	RE	142	0	0	65786	2159	**LE**	NA				
	RE	145	0	0	58819	2465	**LE**	144	0	0	56855	2533
	LE	144	0	0	62341	2310	**RE**	145	0	0	62312	2327
	LE	145	0	2	83887	1729	**RE**	148	1	3	81308	1820
	RE	NA					**LE**	145	0	0	23	2781
	RE	144	2	0	57636	2498	**LE**	147	0	0	57659	2549
	RE	147	2	2	54122	2716	**LE**	146	0	0	58440	2498
	LE	144	0	0	55760	2582	**RE**	143	0	2	73228	1952
	RE	145	2	2	54412	2664	**LE**	145	3	1	56824	2552
	RE	146	0	1	60768	2403	**LE**	147	1	3	58780	2501