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# Tiprotec: A new storage solution for the hypothermic preservation of corneal grafts

## *an experimental study in porcine corneas*

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## Table of contents

## 1. Research hypothesis and aim of the study

Corneal blindness is responsible for approximately 10% of the cases of blindness worldwide (Lamm V et al. 2014). More importantly, in most developing countries corneal diseases represent the second leading cause of blindness (Oliva MS et al. 2012). Corneal transplantation (keratoplasty) is the only effective treatment for most of the disorders leading to irreversible corneal damage and eventually to corneal blindness. A major limitation of this exciting therapeutic modality is, however, the shortage of human donor corneas. For this reason, the appropriate storage and the preservation of the quality of donor corneal grafts are of paramount importance.

For the storage of donor corneoscleral disks three methods are today available: hypothermia (cold storage), organ culture and cryopreservation. Since cryopreservation (preservation in extremely low temperatures ranging from $-80^0$C to $-196^0$C) is only rarely used (Brunette I et al. 2001), hypothermic storage at $2\text{-}6^0$C and organ culture preservation at $37^0$C are the main methods used for eye banking worldwide. Hypothermic (cold) storage is rather simple and inexpensive but the preservation period is limited to 7-10 days (Powers RM et al. 2016). On the other hand, organ culture is technically more demanding and more expensive but it extends the storage period up to 4-6 weeks (Pels E and Schuchard Y 1983).

Since both corneal storage and preservation methods have specific advantages and disadvantages – none of them is superior to the other – a method which unifies the advantages and avoids the disadvantages of both cold storage and organ culture is highly desirable. Tiprotec (Dr F Köhler Chemie, Bensheim, Germany) is a potassium-enriched, *N*-acetylhistidine buffered and amino acid-fortified preservation solution augmented with the iron chelators deferoxamine and 3,4- dimethoxy-N-methyl-benzohydroxamic acid. It has been exclusively used until now as a medium for the storage of vascular grafts. In experimental settings it has shown excellent performance in the preservation of pig and rat aorta, as well as rat mesenteric artery. Endothelial integrity in particular, as assessed by the degree of endothelial-dependent and endothelial-independent relaxation, seems to be maintained in cold storage from 4 to 14 days (Wille T et al. 2008, Zatschler B et al. 2009, Veres G et al. 2016). A hypothermic storage solution based on Tiprotec has shown a protective effect to other type of cells (hepatocytes) (Pless-Petig G et al. 2012). These findings have been further confirmed in human tissue. Garbe et al. showed that Tiprotec was superior to the traditionally used media histidine-tryptophan-ketoglutarate, 0.9%

NaCl and phosphate-buffered saline solution for the hypothermic storage of isolated rings of human internal mammary artery for up to 2 weeks (Garbe S et al. 2011). In accordance to the above findings, Wilbring et al reported that after 96 hours of cold storage endothelial dependent relaxation of intraoperative isolated saphenous vein segments was largely preserved if they were stored in a Tiprotec solution (Wilbring M et al. 2013). More recently, Tiprotec demonstrated protection of endothelial integrity even under deep freezing (up to $-80^0$C) conditions (Von Bomhard A et al. 2016). A favorable effect in the preservation of human hepatocytes was also recently confirmed by Pless-Petig et al who reported a significant improvement in the rate of cell attachment and in the degree of metabolic activity after hypothermic storage with the use of a modified Tiprotec solution. (Pless-Petig et al. 2016).

In this experimental study, we used for the first time Tiprotec as a solution for corneal preservation and hypothermic storage of porcine corneal grafts. We compared the resultant endothelial cell morphology and viability with this obtained after preservation of the ex-vivo corneas with both standard techniques: conventional cold storage (using Eusol-C) and organ culture (using culture medium II [K II]).

# 2. Introduction

## 2.1. A brief history of corneal transplantation

The initial concept of corneal transplantation or keratoplasty should be attributed to the Greek physician Galen (130-200 AD) who first proposed *abrasion corneae,* a form of superficial keratotomy, as a method to restore transparency in ulcerated and scarred corneas (Moffat SL et al. 2005).

Several centuries later, after the first microscopic observation of the cornea from the Dutch microbiologist Antonie van Leeuwenhoek became available (Moffat SL et al. 2005), the idea of replacing the opaque cornea with a transparent material became popular. The French surgeon Guillaume Pellier der Quengsy (1750-1835) was the first to describe the method of keratoprosthesis in 1789 during the French Revolution (Chirila TV et al. 1999), while in 1796 Erasmus Darwin, the grandfather of Charles, suggested the trephination as a method to remove the opaque cornea with the expectation that it will heal later with a transparent scar ((Darwin E 1796).

Experimentation with corneal transplantation started however much later, in the beginning of the 19th century. The German physician Karl Himley (1772-1837) from Göttingen was

the first to suggest in 1813 that opaque animal corneas could be replaced from corneas of other animals (Crawford AZ et al. 2013), while his student Franz Reisinger (1768-1855) performed hundreds of unsuccessful experimental corneal transplantations in rabbits and chickens (Reisinger F 1814). Despite repeated failures, the hope that the procedure is feasible was revived in 1837 by the Irish surgeon Samuel Bigger who, during his captivity by Bedouins in Egypt, performed the first reported successful transplantation of an allogeneic cornea into the blind eye of a pet gazelle (Bigger SLL 1837). This achievement inspired the American ophthalmologist Richard Kissam who performed the first recorded therapeutic corneal xenograft on a human in 1838. The recipient was a young Irishman and the donor was a 6-month-old pig. The graft remained transparent for a couple of weeks (Kissam R 1844).

The first successful human allograft was finally reported in 1905. It was performed by the private practice ophthalmologist Eduard Zirm (1887-1948) in Olmutz near Prague (Zirm EK 1906). The recipient was a 45-year-old farm laborer who had sustained severe bilateral alkali burns and the living donor was an 11-year-old boy whose eye was enucleated due to penetrating injury to the sclera. Although the graft on the right eye failed, the graft on the left eye survived and lead to a significant improvement in the patient's visual acuity. This was a significant milestone, since the cornea became the first solid tissue ever to be transplanted successfully. It was also the first case of full-thickness corneal grafting known today as ``penetrating keratoplasty`` (PKP). Based on Zirm´s technique several ophthalmologists performed full-thickness corneal grafting with the use of corneas from enucleated eyes of living donors over the subsequent 30 years. Three of them deserve special mention. The Russian ophthalmologist Vladimir Filatov (1875-1956) who first reported that cadaver corneas stored in a moist chamber at 4 C° could successfully be used as donor material (Filatov VP 1937), the American ophthalmologist Richard Paton (1902-1984) who, based on Filatov's idea, established the first eye bank in 1944 (Payne JW 1980) and the Spanish Ramon Castroviejo (1904-1989) who innovated several surgical instruments and improved this way the graft technique (DeVoe AG 1987). Additionally, achievements in other fields especially in microbiology, in immunology and in surgical techniques offered further support for the development and the final establishment of this exciting treatment modality (Medawar PB 1948, Billingham RE et al. 1956).

### 2.2. The indications of corneal transplantation

The indications of keratoplasty have substantially changed since the era of the first corneal transplantation in 1905. The initial indication was the treatment of irreversible corneal scaring and haziness as a consequence of various infectious insults: bacterial, fungal, trachomal and viral (herpes simplex). In many countries of the developing world these infectious diseases remain indeed one of the most important reasons for corneal blindness and subsequent keratoplasty (Whitcher HP et al. 2001). In the western world, however, these initial indications have substantially subsided thanks to the development of preventive hygiene measures, and due to the new antibiotic therapies with drops and ointments (Thomas PA and Geraldine P 2007). They have been largely replaced by the bullous keratopathy and the graft failure after previous transplantation attempts (Arentsen JJ et al 1976).

New indications, like keratoconus and Fuchs' endothelial dystrophy have also emerged in the more recent years, especially after 2000. These diseases are now the main indications for corneal transplantation, in Germany, as well as in other developed countries. Finally, other corneal diseases such as xerophthalmia, the iridocorneal endothelial syndrome, ocular trauma, corneal ulcer and other corneal dystrophies are indeed rarer but important indications for a corneal transplantation (Damji KF 1990, Kang PC 2005, Pahor D et al. 2007, Wang J et al. 2013, Le R 2016).

### 2.3. The preservation of human conreas

#### 2.3.1. The importance of corneal endothelium in corneal transplantation

The human cornea is a transparent avascular structure covering the front part of the eye globe. Its main function is to transmit and focus the light to the retina to generate vision. Its transparency needs to be maintained for optimal vision. The cornea is structured into well-organized layers, and each layer has its own importance in maintaining the viability and transparency of the tissue. From the anterior to the posterior cornea, the human corneal tissue consists of a stratified epithelium, Bowman's layer, the stroma, Descemet's membrane, and a mosaic-like patterned monolayer of hexagonal endothelial cells (Nishida T 2005) **(Figure 1)**. The epithelium is a self-renewing layer and harbors a resident stem cell population at its periphery. However, the stroma and endothelium are usually quiescent and so far have not been considered to regenerate (Polisetti N et al. 2013). The corneal endothelium that lines the posterior corneal surface is derived from the neural crest during embryologic development (Bahn CF et al. 1984). Human endothelial cell

density is approximately 6000 cells/mm² during the first month of life (Bahn CF et al. 1986) but decreases to about 3500 cells / mm² by the age of five years (Nucci P et al. 1990). A further decrease at a slower pace continues throughout life so that at the age of 85 the mean cell density is 2300 cells/mm² (Yee RW et al. 1985). There is evidence that the endothelial cells possess proliferative capacity, since in tissue culture they can be induced to divide. In vivo, however, they are arrested in the G1 phase of the cell cycle constructing this way a non-replicable monolayer (Joyce NC 2003).

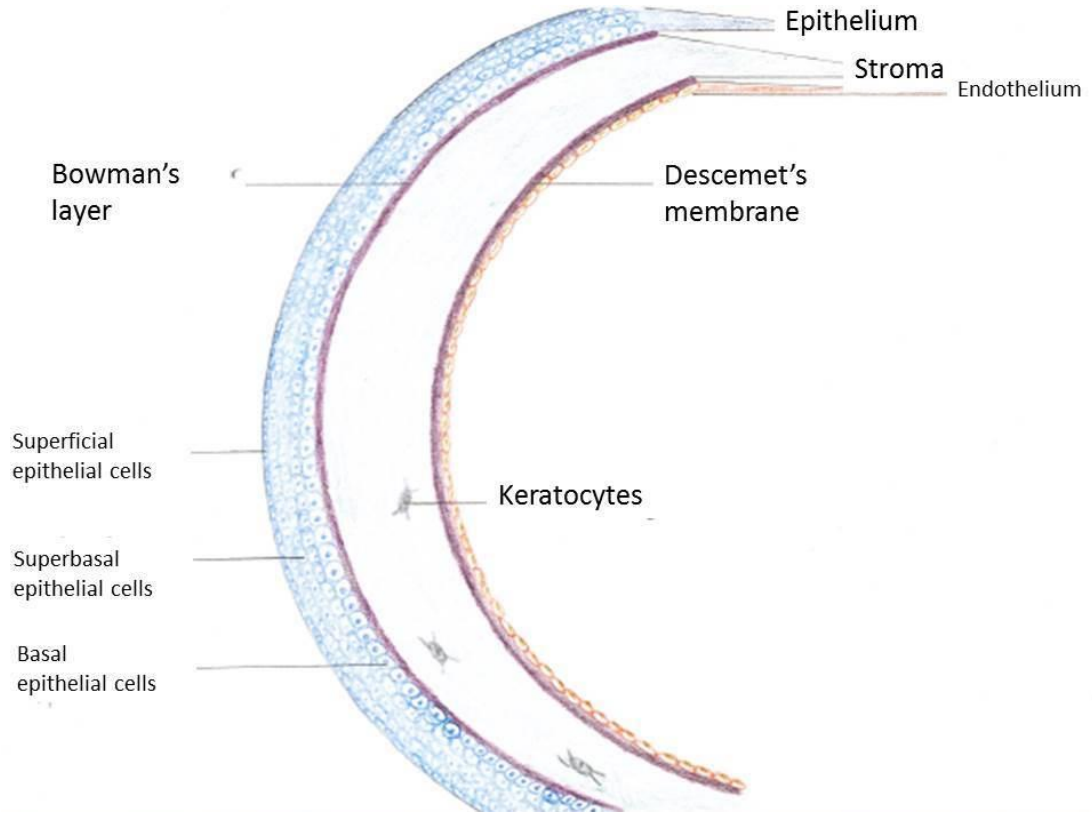



**Figure 1. The human cornea**

The Descemet membrane is formed from collagen secreted form the endothelial cells. At the time of birth, the Descemet membrane is approximately 3 μ thick and consists of collagen in a banded pattern. Throughout life, endothelial cells continue to secrete collagen added to the Descemet membrane but in a non-banded pattern. Nevertheless, if the endothelial cells are stressed or damaged, they may secrete collagen added to the posterior part of the Descemet membrane in a banded fashion (Johnson DH et al. 1982). The transparency of the cornea is mainly the result of the ultrathin structure, the crystalline organization and the restriction in the range of distances between adjacent stromal collagen fibrils. These particular properties of the stromal collagen in combination

with the extreme thickness of the other corneal layers prevents almost completely light scattering. However, if the cornea swells, disruptions of the spacing among the collagen fibrils ensues with a consequent significant scattering of the light and lose of corneal transparency (Meek K and Knupp C 2015). The corneal endothelium plays a critical role in the regulation of stromal hydration by behaving as a semi-permeable barrier to the movement of fluids and nutrients. This property is due to the presence of intercellular gap junctions and tight junctions at the apical membrane of endothelial cells. Therefore, fluids and nutrients can leak in the para-cellular space and enter the stroma. On the other hand, endothelial cells prevent excessive fluid entrance in the stroma and subsequent corneal swelling by actively pumping ions and drawing this way osmotically water from the stroma to the aqueous humor. Endothelial cell loss or endothelial dysfunction has as a consequence the inability to efficiently pump fluid out of the stroma, resulting in stromal and epithelial edema and eventually in loss of corneal clarity and visual acuity (Bourne WM 2003, Mergler S et al. 2007).

Since endothelial cells cannot actively divide in vivo, the only way to preserve the proper corneal function, despite the normal age-related or the accelerated disease- or trauma-related endothelial loss, is by expansion of the existing cells. In the case of age associated decline, the remaining endothelial cells are usually enough to maintain a sufficient barrier and pump function. However, if the density falls below a critical value of 500 to 1000 cells/mm², then the function of corneal endothelium becomes compromised and stromal edema ensues (Peh GS et al. 2011).

Given the unique role of endothelium in the maintenance of corneal function, it is not surprising that the main goal of corneal graft preservation is to minimize corneal endothelial loss, which independent of the method of preservation reaches 10-30%. This renders up to 40% of preserved donor corneas eventually unsuitable for transplantation, aggravating further the problem of graft shortage (Mergler S and Pleyer U 2007). This decline in endothelial cell density of the donor corneas is directly related to the length of storage time (Pels E and Schuchard Y 1983, Bourne WM et al. 2001), and has been mainly attributed to apoptosis (Albon J et al. 2001). After transplantation, endothelial loss of corneal grafts continues at a pace faster than that of normal corneas to the point that by 3 years 53% of the preoperative endothelial cell density is lost (Bourne WM et al. 1994). This process can end up to a condition called late endothelial failure, characterized by graft swelling and haziness unresponsive to the treatment with corticosteroids. Late endothelial failure accounts for the majority of late graft failures (Ing JJ et al. 1998). It has

been shown, that the initial postoperative endothelial cell density is inversely related to late endothelial failure (Nishimura JK et al. 1999), suggesting that preservation methods, by minimizing initial endothelial loss, may have a very significant contribution to a favorable long term outcome of keratoplasty.

### 2.3.2. Methods of corneal preservation

Since cornea is an avascular structure, selection criteria for corneal donors are less restrictive than for vascularized tissues. Nevertheless, several infectious diseases have been transmitted through corneal grafts (Eastlund T 1995, Armitage WJ et al. 2009), underlying thus the importance of careful testing the donors according to the medical standards guidelines issued from the European Eye Bank Association (EEBA 2016) and the Eye Bank Association of America (EBAA 2015). The same guidelines define also the methods the eye banks should use to assess the quality of donor corneas. The most important component of this quality control is the assessment of endothelial cell density and viability. This is performed with the use of specular or confocal light microscopy (Price M and Price F 2012). Regarding the age of the donor, although most eye banks in America prefer donors 65 years old or younger, this is recently changing given the results of the Cornea Donor Study which has shown that the five years success rate of corneal transplantation was not lower if the donors were 65-75 years old (Mannis MJ et al. 2013). On the other hand, European eye banks tend to accept grafts form donors even older than 75 years. The minimum acceptable endothelial cell density also varies among eye banks. According to the 2010 European Eye Bank Association Report, 70% of the eye banks accept as minimum density 2000 cells / mm² while the others have a minimum ranging from 2100 to 2500 cells / mm² (Claerhout I et al. 2010). In general, a cornea with an endothelial density of 2.200 cells / mm² should include a sufficient number of viable cells in order to retain its transparency for the next 25 years (Armitage WJ et al. 2003). Nevertheless, the long term surviving of the graft is also dependent form several other factors like the allograft rejection and the recipient diagnosis (Patel SV 2011).

The initial method of corneal preservation was the storage of the whole globe in the “moist chamber”, a moistened pot at 2-6 C° as prescribed by Filatov in 1935 (Filatov VP 1937). This type of storage was however limited by the availability of metabolic substrates and buildup of metabolic waste products in the aqueous humor. It was, for this reason, replaced by the storage of corneas excised from the eye along with a rim of sclera (a corneoscleral disk or button). The storage of grafts in serum was introduced in the

1960s and the serum was soon replaced by a synthetic solution whose composition mimicked that of aqueous humor. A revolutionary turn followed in early 1970s when McCarey and Kaufman reported the development of a modified tissue medium (McCarey-Kaufman [M-K] medium) in which human corneas with viable endothelium could be preserved at 4°C for at least 4 days (McCarey BE and Kaufman HE 1974). M-K medium contained TC199, Earle's salts, HEPES buffer and gentamicin, and remained the standard corneal preservation medium for some 15 years. The application of this method gave the opportunity to keratoplasty to become a scheduled surgery. As underlined by Wilson and Bourne in their 1989 major review on corneal preservation, "this allowed the patient to better plan for the transplant and for the surgery to be performed when a well-trained regular team of operating personnel was available to assist a well-trained surgeon." (Wilson SE and Bourne WM 1989). Corneas stored at 4°C in M-K medium remained thin and clear and could be transported in polystyrene containers with ice. This solution was subsequently superseded by other commercial preparations, such as KSol and Optisol, containing osmotic agents to limit corneal tissue swelling, and offering extended preservation times of a week or ten days. Nevertheless, MK-medium is still in use in the developing countries due to its low cost and simplicity of production (Armitage WJ 2008).

For the storage and preservation of corneoscleral disks three methods are today available: hypothermia (cold storage), organ culture and cryopreservation. Of these, only cryopreservation permits the storage of ocular tissue indefinitely. Indeed, cryopreserved corneas have been successfully transplanted in several occasions in the past (McCarey BE and Kaufman HE 1974, Ehlers N 1982, Kaufmann HE et al. 1996, Brunette I et al. 2001). More recently, they have been used in Deep Lamellar Endothelial Keratoplasty (Chen W et al. 2010) and as a tectonic graft for perforated corneas (Jang JH and Chang SD 2011). Cryopreservation, however, has been associated with variable and unpredictable rates of endothelial loss (Halberstadt M et al. 2003). As a result, cryopreserved corneas are been currently used only occasionally in emergency situations, when the aim is to save the eye (Brunette I et al. 2001).

#### 2.3.2.1. The hypothermic (cold) storage

Hypothermic (cold) storage is the most common method of corneal preservation in the USA and in most Asian countries (Powers RM et al. 2016). It is based on the principle that cold reduces metabolic cellular demand. On the other hand, cooling has also

deleterious effects on cells, such as the suppression of active transport of ions across cell membranes leading to water influx and cellular edema, and the disruption of calcium homeostasis and proton exchange leading to acidosis (Armitage WJ 2011). As a consequence, the storage time is limited to a maximum of 10-14 days. This time span has been claimed by the manufacturers of the newer storage solutions which have largely replaced the first hypothermic solution, the M-K medium which permitted a maximum storage of only 4 days. These are: the modified M-K medium, the K-Sol, the Dexol, the Liquorol, the Optisol (GS and plus), the Chen medium, the Eusol-C (Al.Chi.mia, Padova, Italy), the Cornisol (Aurolab, Madurai, India) and the newest Life $4^0$C (Numedis Inc. Minnesota, USA). It should be noted that, despite the extended time limits offered by the manufacturers, most eye banks prefer to keep corneas no more than 7-10 days in hypothermic conditions, due also to the fact that corneal epithelium is less well than endothelium preserved, and the extend of epithelial defects after transplantation increases with storage time (Armitage WJ 2008).

The technique of hypothermic storage is rather simple and inexpensive. The corneas are stored in vials and refrigerated at 2-$6^0$ C. The vials may allow inspection of corneal endothelium with specular microscopy. During the storage period the corneas remain thin and they are readily available for surgical use. All the hypothermic storage media consist of a tissue culture medium supplemented with deturgescent agents like dextran and chondroitin sulfate to prevent corneal swelling. Chondroitin sulfate is considered the most crucial ingredient, since it presumably plays an important role in the intracellular redox system as an antioxidant and as a membrane stabilizer. It was the addition of chondroitin sulfate to tissue culture media which lead from the M-K medium to the development of the newer solutions. Other additives include antibiotics, energy sources, antioxidants, membrane stabilizers and growth factors. All storage solutions are commercially available and ready for use (Pels E et al. 2008).

From all the above storage media, Optisol GS (from Chiron Ophthalmics Irvin, CA, until 1997 and after that form Bausch & Lomb Inc., Rochester, NY, USA) remains the most popular. It was introduced in 1991 as a hybrid of K-Sol, Dexol and CSM (culture storage medium). It contains: 2.5% chondroitin sulfate, 1% dextran, Fe, 14 vitamins, amino acids, cell metabolites, antioxidants and precursors of adenosine triphosphate (Szafilk J et al. 2000). Studies have repeatedly shown a high percentage of clear grafts and a low percentage of endothelial loss after corneal transplantation (Lindstrom RL et al. 1992, Wagoner MD et al. 2005). Moreover, other commercially available hypothermic storage

media (Cornisol, Life $4^0$C and Eusol-C) have shown no superiority against Optisol-GS in comparative studies (Soni NG et al. 2015, Kanavi MR et al. 2015, Basak S et al. 2016).

Since the donor's eye is usually contaminated, decontamination of the corneal graft is an essential part of the storage process. Antibiotics (mainly gentamycin) are for this reason added in all storage solutions. However, antibiotics are more effective when the bacteria are more metabolically active, which certainly does not happen under hypothermic conditions. Nevertheless, preoperative warming of the donor corneas enhances the decontaminating effect of the antibiotics which have been stored in the tissue during the hypothermic storage period (Pels E et al. 2008). On the other hand, hypothermic storage solutions do not routinely contain antifungal agents even though most postkeratoplasty endophthalmitis and keratitis cases are of fungal origin. Recent studies suggest that the addition of the antifungal agent amphotericin B in Optisol GS significantly improves the activity against the contamination with Candida species albeit with the expense of increased toxicity against the corneal endothelial cells (Layer N et al. 2014).

The main advantages of hypothermic storage are the simplicity of the technique and the low cost due to minimal equipment requirements and minimal handling. The storage solutions are readily available and easy to transport to procurement sites making possible the recovery of corneas from donors even in remote areas. As a consequence, the availability of corneas is dramatically increased, while the eye bank processing can be performed without sophisticated infrastructure and with minimal training of the personnel.

#### 2.3.2.2. The storage in organ culture medium

Summerlin and colleagues were the first to report the preservation of corneas in organ culture medium at $37^0$ C for 4 weeks (Summerlin WT et al. 1973). This was shortly followed by the successful transplantation of organ cultured corneas by Doughman and colleagues (Doughman DJ et al. 1976). This preservation method, although pioneered by American ophthalmologists, predominates now days in Europe and in Australia. Of the 62 eye banks included in the 2010 European Eye Bank Association Directory, 47 used organ culture, 9 used hypothermic storage and 6 used both methods (Mannis MJ et al. 2013). Organ culture allows a significantly longer period of preservation which is typically 4 weeks (Pels E and Schuchard Y 1983), although successful transplantations with the use of corneas preserved even for 7 weeks have also been reported (Ehlers H et al. 1999). The extended storage period comes, however, at a cost of a more complicated technique in comparison to hypothermia. The corneas are stored in an incubator at 30-$37^0$C. The

storage solution consists of tissue culture medium (most commonly Eagle's essential medium), supplemented with 2-10% fetal or newborn calf serum, antibiotics (mostly penicillin and streptomycin) and antifungal agents (amphotericin B) (Armitage WJ 2008). The presence of calf serum has raised concerns about possible transmission of Creutzfeldt-Jacobs prion protein during the periods of disease outbreak, and alternative animal product-free solutions have been successfully tested (Bednarz J et al. 2001, Smith VA and Johnson T 2010, Hempel B et al. 2011). Nevertheless, the common practice of including calf serum in the organ culture medium has not been substantially changed. The majority of the eye banks change the culture medium every 1-2 weeks while the rest keep the same solution for the whole storage period. Since dehydration macromolecules are ingested from corneal cells at these storage temperatures, they are not added in the solution. Therefore, the cornea swells to about twice its normal thickness. The corneal swelling has to be reversed before its use for transplantation. The corneal grafts are placed for this reason in a solution containing dextran. The same solution is used for the transport of the cornea. The extend of deswelling depends on the dextran concentration which varies between 4-8% in the different banks and the time varies also from less to one up to seven days. All the above minor differences in handling techniques are nevertheless associated with similar results in terms of graft outcomes (Armitage WJ 2008). The possible toxic effect of dextran due to unexpected penetration into the graft tissue along with the difficulty to prepare the solution has triggered recently the search for an alternative macromolecule (Smith V and Johnson T 2012). Despite the initial encouraging results, the use of dextran solution remains the standard method for the reversal of corneal swelling prior to transplantation. The inspection of corneal endothelium under specular microscopy is not feasible with organ culture. Therefore phase contrast or bright field light microscopy is necessary. To visualize the endothelial cells, swelling of the intercellular space with a hypotonic solution is required and it should be performed under aseptic conditions (Sperling S 1986). The swelling is very transient, lasting only a few minutes and is dependent on the storage time and on the particular composition of the storage medium (Thuret G et al. 2004). The whole process is technically demanding and requires experienced observers for the interpretation of the images. Regarding microbiological safety, organ culture appears superior to hypothermic storage since the antibiotics and antifungal agents included in the storage solution are far more effective at 30-37$^0$C. In addition, contaminated tissue will be easily recognized since the micro-organisms will grow rapidly in this temperature. Screening through the

examination of a sample of the medium normally takes place after 7 days in culture and just before the transplantation. So the risk of transplanting contaminated tissue is minimized since up to 3.5% of the corneas in organic culture have been reported to be discarded due to contamination (Pels L 1997). These corneas would probably have been distributed for transplantation if they had been stored under hypothermic conditions. The incidence of endophthalmits has been reported as low as 0.1% after storage in organ culture medium (Armitage WJ 2008). The main advantage of organ culture is the length of storage period. This 4 week period permits the implementation of microbiological testing for safety, as well as, a more thorough evaluation of the corneal endothelium also after vital staining such as with tryphan blue for the more accurate recognition of necrotic cells. Given the great variability regarding endothelial loss among stored corneas, organ culture is considered a ``stress test`` for the recognition of those graft with irreversibly affected vitality (Böhnke M 1991). This way the most suitable corneas for each procedure can be selected. Moreover, the capacity of corneal endothelium for self-repair is maintained only with organ culture (Nejepinska J et al. 2010). On the other hand, maintenance in organ culture medium it is definitely a more complicated preservation method with a higher cost compared to the hypothermic storage **(Table 1).** Nevertheless, despite their differences, the few studies which have compared the two methods in terms of clinical outcome and of post-operative decline in endothelial cell density have demonstrated similar results showing this way no definite superiority of one method against the other (Rijneveld WJ et al. 1992, Rijneveld WJ et al.2008).

**Table 1. Hypothermic storage versus Storage in Organ Culture Medium**

| **Hypothermic Storage (2-6$^0$C)** | **Storage in Organ Culture Medium (30-37$^0$C)** |
|---|---|
| Most common storage method in USA and in Asia | Most common storage method in Europe and in Australia |
| Storage period: 7-10 days | Storage period: 4-6 weeks |
| Offers thin grafts ready to use | Pre-operative deswelling of the graft is necessary |
| Minimal bacterial growth in low temperatures. | At 30-37$^0$C the graft contamination becomes |

| | |
|---|---|
| Antibiotics added provide coverage after intra-operative warming.<br><br>Antifungal agents are not added in the solution | more obvious and the antibiotics and the anti-mycotics are more effective.<br><br>The risk of transplanting contaminated tissue is reduced |
| The more easily transported hypothermic medium to procurement sites facilitates getting more donor corneas from remote areas. | More thorough evaluation of the stored corneas permits the selection of the most suitable cornea for each procedure |
| Simplicity in equipment, no need for sophisticated staff training, lower cost | Technical complexity, need for qualified staff, higher cost |

# 3. Material and methods

## 3.1. Experimental design

This prospective, in vitro, 3-armed parallel study was performed with the use of 90 porcine corneas (examined for their endothelial quality and transparency) randomly selected for preservation with the 3 storage methods (30 corneas pro method): organ culture (in K II-medium), cold storage (in Eusol-C) and cold storage (in Tiprotec; all solutions supplemented with dextran). After 10 and 30 days of cultivation/storage, the corneas were examined for their sterility, endothelial quality and transparency and then returned to their respective cultivation/storage medium. After 30 days, the experiment ended for 20 randomly selected corneas per group (of these, 3 corneas from each group were submitted to histological and imuunohistochemical analyses). The remaining corneas (10 from the Tiprotec and 10 from the Eusol-C group) were submitted to organ culture in K II for 10 more days to test the corneas for their regenerative capacity at favorable conditions (aiming to "simulate" their transplantation). The remaining 10 corneas from the organ culture group served as controls and were also further cultured in K II for 10 days. After that, a final examination of sterility, endothelial quality and transparency was performed. Moreover, one randomly selected cornea pre group was submitted to histological and immunohistochemical analysis). The experimental design is depicted in Figure 2; all methods mentioned above are described below in detail.

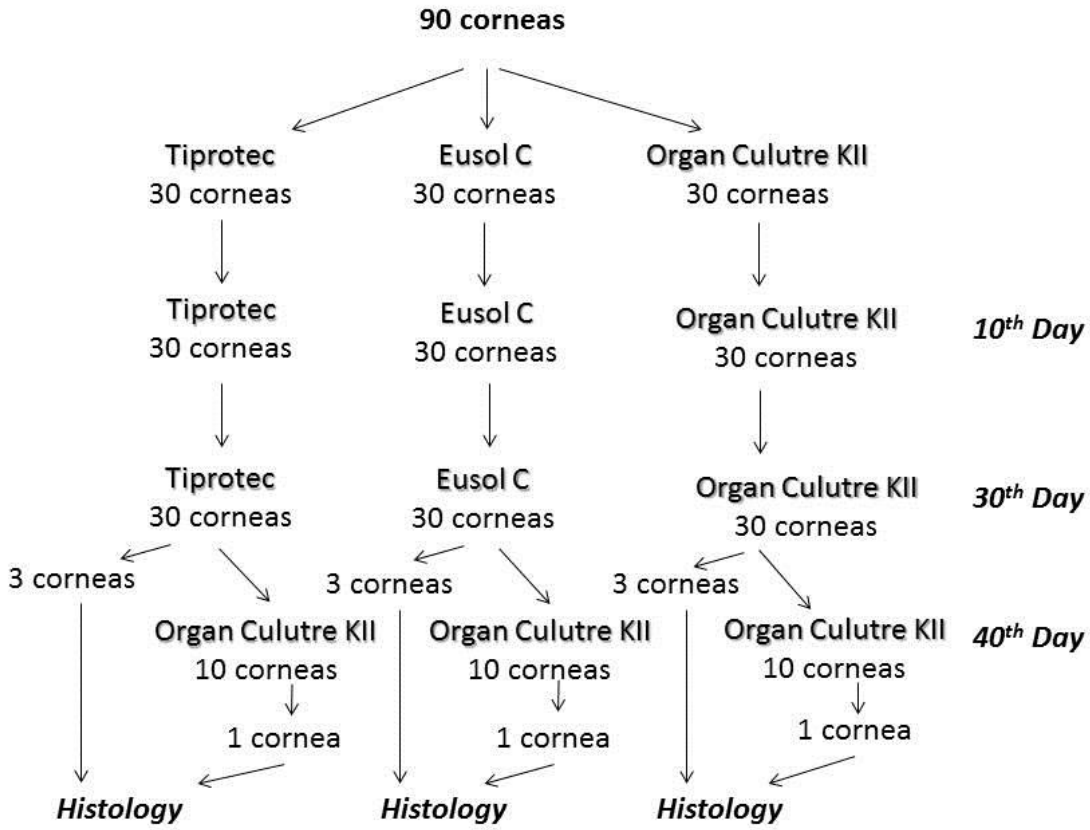



**Figure 2: The experimental design**

### 3.2. Storage solutions.

Three storage solutions were tested in this study: Culture medium K II, Eusol-C and Tiprotec. Tiprotec (Dr F Köhler Chemie, Bensheim, Germany) is a potassium-enriched, N-acetylhistidine buffered solution, containing amino acids (L-asparagine acid, L-tryptophane, glycine, alanine and a-ketoglutaric acid) and iron-chelators (deferoxamine mesilate and N-hydroxy-3,4-dimethoxy-N-methyl-benzohydroxamic acid). The three main components of Tiprotec are: the basic solution (containing all the above mentioned ingredients), the glucose solution and the lyophilisate. Prior to the distribution to the corneal chambers, the glucose solution was mixed with the lyophylisate and the mixture was subsequently injected into the basic solution. The final storage medium was prepared by mixing 3g of dextran 500 powder with 50ml of the Tiprotec solution **(Table 2)**.

**Table 2. Composition of Tiprotec**

| **Ingredient** | **Concentration (mg/l)** |
|---|---|
| NaCl | 850 |
| KCl | 5640 |
| $MgCl_2.6H_2O$ | 1690 |
| $Na_2HPO_4$ | 150 |
| $CaCl_2.2\ H_2O$ | 10 |
| N-acetylhistidine. $H_2O$ | 6710 |
| Tryptophane | 420 |
| a-ketoglutaric acid | 300 |
| L-asparaginic acid | 690 |
| Glycine | 780 |
| Alanine | 460 |
| Deforxamine mesilate | 53 |
| 3,4 Dimethoxy-N-methylbenzhydroxic acid | 3,5 |
| Glucose.$H_2O$ | 1980 |

Eusol-C, a solution developed from the Company ALCHIMIA (Ponte S. Nicolo, Italy) for the hypothermic storage of corneas, contains dextran, sodium pyruvate, glucose, amino acids, mineral salts, vitamins, gentamicin, HEPES buffer solution, bicarbonate and phenol red. Phenol red acts as a pH-Indicator **(Table 3)**.

**Table 3. Composition of Eusol-C**

| **Ingredient** | **Concentration (mg/l)** |
|---|---|
| NaCl | 6800 |
| KCl | 400 |
| $MgCl_2.7H_2O$ | 200 |
| $Na_2HPO_4.H_2O$ | 140 |
| $CaCl_2$ | 200 |
| $NaHCO_3$ | 2200 |
| D-glucose | 1000 |
| Phenol red | 10 |
| L-histidine. $HCl.H_2O$ | 42 |
| L-tryptophane | 10 |
| L-arginine.HCl | 126 |
| L-cystine | 24 |
| L-glutamine | 292 |
| L-isoleucine | 52 |
| L-leucine | 52 |
| L-lysine.HCl | 73 |
| L-methionine | 15 |
| L-phenylalanine | 32 |
| L-threonine | 48 |
| L-tryptophane | 10 |
| L-tyrosine | 36 |
| L-valine | 46 |
| Folic acid | 1 |

| | |
|---|---|
| Cholin.Cl | 1 |
| Nicotinamide | 1 |
| D-Ca-pantothenate | 1 |
| Pyridoxal.HCl | 1 |
| Thiamine.HCl | 1 |
| Riboflabin | 0,1 |
| Myo-inositol | 2 |

Organic culture medium K II (Biochrom GmbH, Berlin, Germany), the storage solution currently used in most corneal banks in Germany, is a base solution containing Minimal Essential Medium (which contains more than 20 amino acids) and Earle's Salts **(Table 4)**. 50 ml of Culture Medium K II had to be mixed with 1,6 ml filtered fetal calf serum before been distributed into the corneal chambers.

**Table 4. Composition of Culture Medium K II**

| **Ingredient** | **Concentration (mg/l)** |
|---|---|
| NaCl | 6800 |
| KCl | 400 |
| $MgCl_2.7H_2O$ | 200 |
| $Na_2HPO_4.H_2O$ | 140 |
| $CaCl_2$ | 200 |
| $NaHCO_3$ | 2200 |
| D-glucose | 1000 |
| Phenol red | 10 |
| L-histidine. $HCl.H_2O$ | 42 |
| L-tryptophane | 10 |

| | |
|---|---|
| L-arginine.HCl | 126 |
| L-cystine | 24 |
| L-glutamine | 292 |
| L-isoleucine | 52 |
| L-leucine | 52 |
| L-lysine.HCl | 73 |
| L-methionine | 15 |
| L-phenylalanine | 32 |
| L-threonine | 48 |
| L-tryptophane | 10 |
| L-tyrosine | 36 |
| L-valine | 46 |
| Folic acid | 1 |
| Cholin.Cl | 1 |
| Nicotinamide | 1 |
| D-Ca-pantothenate | 1 |
| Pyridoxal.HCl | 1 |
| Thiamine.HCl | 1 |
| Riboflabin | 0,1 |
| Myo-inositol | 2 |

### 3.3. Procedure of obtaining and preserving the corneal grafts.

The first step was the excision of the corneoscleral buttons from enucleated porcine eyes in vitro **(Figure 3)**. This was done manually under sterile conditions after the preparation of the bulbous for excision with Betaisodona solution (100ml of Betaisodona solution contain 10g of Povidon-Iodine while other ingredients include: glycerole, nonoxinole 9, dinatriumhydrogenphosphate, citric acid, sodium hydroxide and potassium iodine) and washing afterwards with NaCl 0,09% solution. The excision of the button was then

performed with a 17mm-trephine. After the final preparation of the storage solutions, every three randomly selected corneoscleral buttons were placed in the special containers filled with K II (for organ culture), in the containers with Eusol-C (for conventional cold-storage) and in the containers filled with Tiprotec (for Tiprotec cold-storage). The containers with K II were placed in an incubator at 36 °C whereas the containers with Tiprotec and Eusol-C were placed in the refrigerator at 4°C. This procedure was repeated 10 times, leading to a final sample of 90 corneoscleral discs stored up to a maximum of 30 days while a subset of corneas of the three storage groups was submitted to organ-culture (in KII-media) for further 10 days (as described in experimental design).

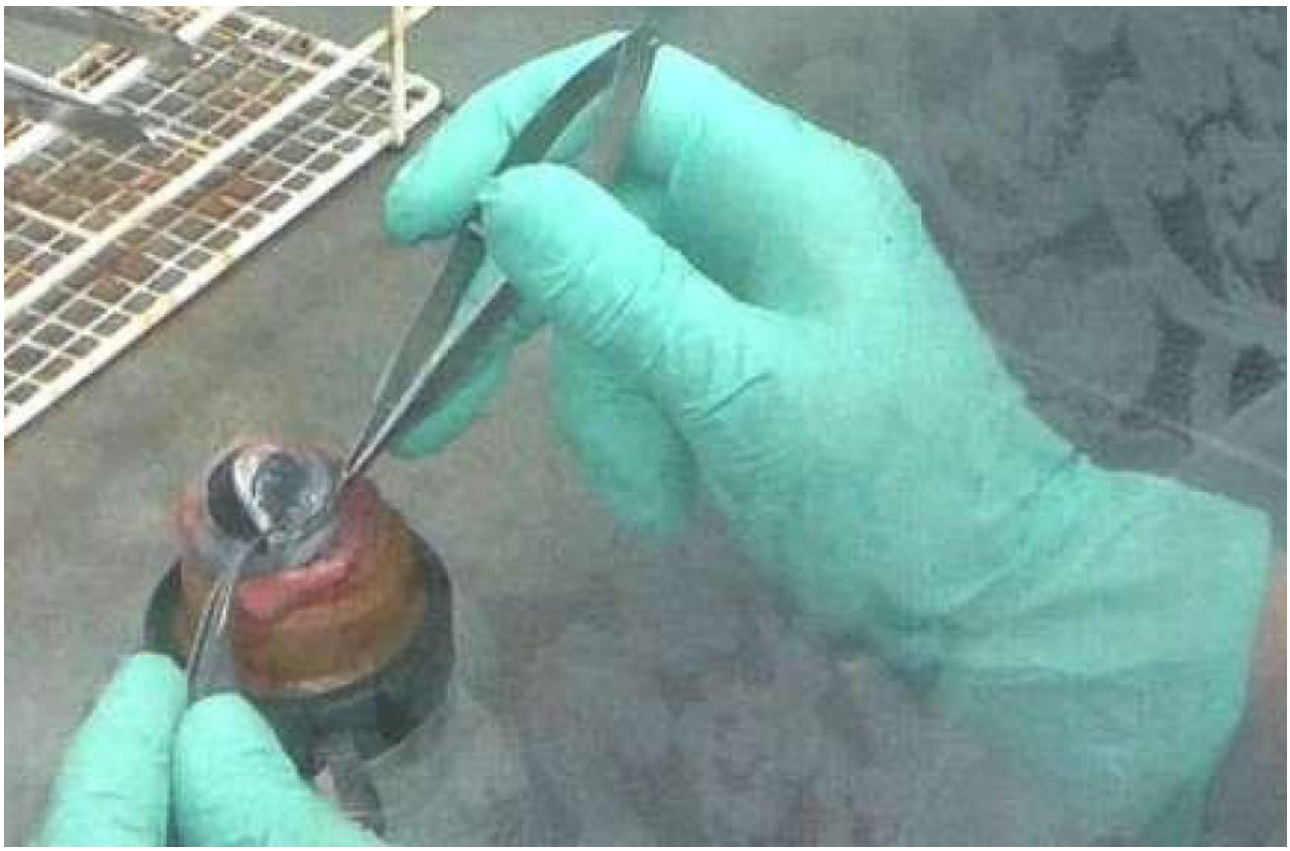


**Figure 3: The excision of the corneoscleral button from the enucleated porcine eye**

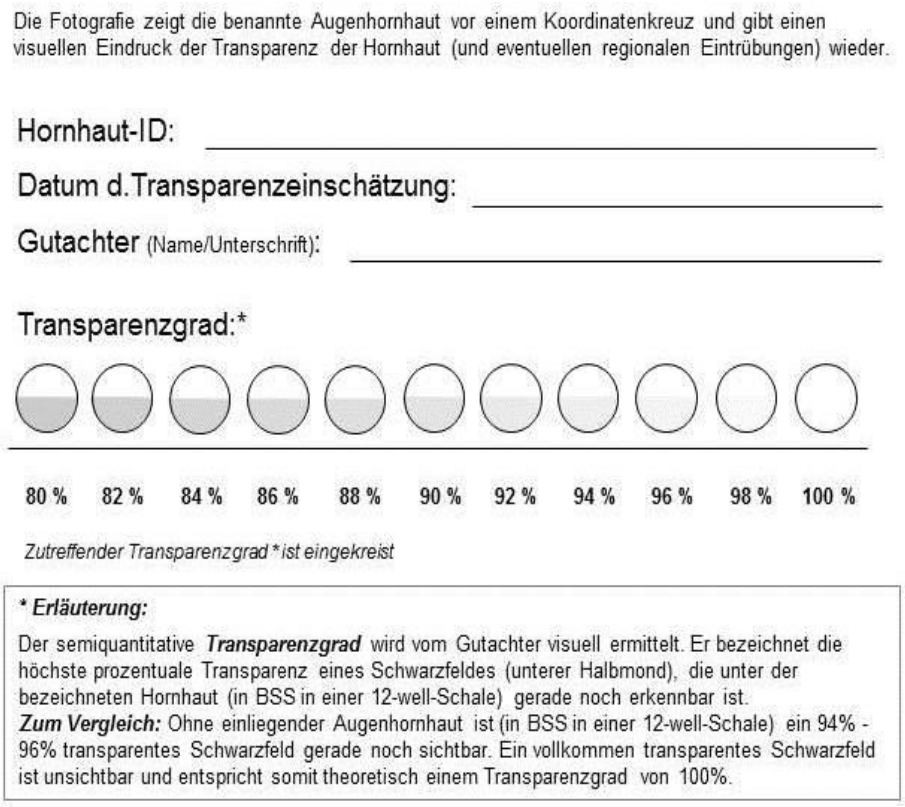
### 3.4. Optical macro- and microscopic evaluation of corneal tissue quality

The following parameters of corneal tissue quality were assessed by one dedicated examiner:

1. Corneal transparency

2. Corneal endothelial density.
3. Additional endothelial morphometric parameters (i.e. the number of hexagonal or pentagonal cells and the presence of intracellular vacuoles)

Prior to storage each corneal button was examined as follows: The endothelium was made visible after osmotic preparation with balanced salt solution (BSS) and then inspected. The transparency of each cornea was assessed by laying the tissue on top of each consecutive circle of a specific self-developed and previously validated visual scale **(Figure 4).** The transparency of the particular corneal graft was graded (and expressed as percentage) according to the visibility of lower gray semicircles of the scale.


Die Fotografie zeigt die benannte Augenhornhaut vor einem Koordinatenkreuz und gibt einen visuellen Eindruck der Transparenz der Hornhaut (und eventuellen regionalen Eintrübungen) wieder.

Hornhaut-ID: _____

Datum d.Transparenzeinschätzung: _____

Gutachter (Name/Unterschrift): _____

Transparenzgrad:*

80 % 82 % 84 % 86 % 88 % 90 % 92 % 94 % 96 % 98 % 100 %

*Zutreffender Transparenzgrad * ist eingekreist*

***** Erläuterung:***

Der semiquantitative ***Transparenzgrad*** wird vom Gutachter visuell ermittelt. Er bezeichnet die höchste prozentuale Transparenz eines Schwarzfeldes (unterer Halbmond), die unter der bezeichneten Hornhaut (in BSS in einer 12-well-Schale) gerade noch erkennbar ist.
***Zum Vergleich:*** Ohne einliegender Augenhornhaut ist (in BSS in einer 12-well-Schale) ein 94% - 96% transparentes Schwarzfeld gerade noch sichtbar. Ein vollkommen transparentes Schwarzfeld ist unsichtbar und entspricht somit theoretisch einem Transparenzgrad von 100%.

**Figure 4: The scale used for the assessment of corneal transparency**

This initial assessment was followed by a detailed examination of the corneas under a binocular inverted phase-contrast microscope (Leitz Fluvert FU - Phasenkontrast-Mikroskop, Leica Microsystems, Wetzlar, Germany), at a magnification of x 200. To determine the endothelial cell density, cell counting was performed in the middle of the

graft according to the following method: The endothelium was photographed with the use of a digital camera (3CCD, Color Vision Camera, Donpisha/Sony, Berlin, Germany) and of a printer (P91 Thermodrucker, Mitsubishi Electric Europe B.V., Barcelona, Spain) attached to the microscope. Endothelial cells were then counted in a 0,01 $m^2$ area (Schroeter J and Rieck P 2009) and extrapolated to the dimensional unit „cells per $mm^2$". This procedure was repeated three times, and the mean of the three measurements was used as a measure of the endothelial cellular density of the particular cornea. The additional qualitative assessment of corneal endothelium was based on cell morphology and the presence of vacuoles. A randomly selected 9 $mm^2$ area was defined in each picture. Twenty endothelial cells out of this particular area were assessed regarding their shape (pentagonal and hexagonal cells). The percentage of cells containing vacuoles was also defined. Finally, microbiological testing and discard of the contaminated cornea was performed after their extraction from the bulbi.

Ten days later the corneoscleral buttons where taken out of their containers under sterile conditions, were placed in BSS and all the above described thorough evaluation of corneal endothelial integrity was repeated. Microbiological screening was carried out once again. After this evaluation the corneoscleral buttons from the K II group were stored in new containers with freshly prepared K II, since this is the procedure regularly followed in most of the corneal banks when using organic culture as their preferred storage method. The corneoscleral discs from the other two groups were placed in their old containers. This procedure was repeated once again on the $30^{th}$ day of storage. After the last microbiological screening, 10 corneoscleral discs from each group were randomly selected and submitted to organ-culture (in K II-media) for further 10 days (as described in *experimental design*) and submitted subsequently to a last examination as described above. After their final examination, all corneas were placed in flasks filled with paraformaldehyde.

### 3.5. Histological analysis of corneal tissue

Histological studies were performed in 12 randomly selected corneas. Three corneas of each group from the pool of grafts preserved for 30 days and one cornea of each group from the pool of grafts preserved for 40 days were examined. Three freshly excised corneal grafts were used as controls. The analysis was performed by an independent experienced observer blinded to the mode of preservation of each cornea.

### 3.5.1. Tissue preparation, staining and light microscopy

Each corneal button submitted to histological analysis was fixed in 4% formaldehyde and embedded in paraffin. The paraffin blocks were frozen at -11$^0$C for at least 10 min and subsequently were cut in consecutive 3μm sections with the use of a microtome. These tissue sections were subsequently placed on glass slides which were dried in an incubator at 60$^0$C.

Haematoxylin and eosin staining of the slides was performed according to the following procedure:

1. Deparaffinization and rehydration with graded alcohol series: The slide was immersed in xylol solution (3 times for 5 minutes each time). After that it was transferred for 2 minutes to ethanol 100%, then through 96% and 80% ethanol respectively for 2 minutes each.
2. Haematoxylin-eosin staining. After the initial preparation the slide was in haematoxylin immersed and left for 10 minutes. Subsequently, it was washed under tap water for 10 minutes and then was incubated for 10 more minutes with eosin. Finally, it was again washed and dehydrated with graded alcohol series: Immersion twice in 80% ethanol for 30 seconds, then twice in ethanol 96% for 30 seconds and finally three times in ethanol 100% for 30 seconds. At the end, the specimen was left three times in xulol solution for one minute.

The evaluation of the stained tissue slides was performed microscopically applying histopathologic standard procedures.

The following parameters of corneal quality were examined:

1. The degree of epithelial dissociation
2. The degree of epithelial thickness
3. The degree of basal cell contact
4. The overall endothelial quality
5. The overall corneal quality

The assessment of the above parameters was done semi-quantitatively with the use of arbitrary defined ordinal scales (0-3 or 1-3 grades) used routinely in our laboratory.

### 3.5.2. Immunohistochemistry

Four corneas of each study group were subjected to immunohistochemical staining aiming to the detection of epithelial cells expressing Ki-67 antigen, a nuclear protein indicating cell proliferation, according to the following procedure:

1. *Deparaffinization and rehydration with graded alcohol series:* The slide was immersed in xylol solution (3 times for 5 minutes each time). After that it was immersed consecutively for 2 minutes in ethanol 100%, then in ethanol 96% and finally in ethanol 80%
2. *Antigen retrieval to unmask the antigenic epitope:* The slide was incubated with Tris/EDTA/Citrate-Basis buffer at three different PH values: 6 / 7.8 / 9 at 1210C for 5 minutes. After that it was rinsed twice with distilled water and finally with TBST-buffer solution (a mixture of tris-buffered saline and polysorbate 20) for 5 minutes
3. *Block of endogenous peroxidase activity which can lead to high background staining:* The slide was incubated in the humidified chamber with "ready to use" peroxidase-blocking solution for 10 minutes. After that it was rinsed with TBST-buffer solution two times for 5 minutes each.
4. *Staining:* To demonstrate Ki-67 antigen, a nuclear protein preferentially expressed during the active phases of the cell cycle, the slide was incubated with the Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1 antibody (Dako Denmark AS, Glostrup, Denmark) at a dilution 1:100 at $37^0$C for 1 hour. It was rinsed, subsequently, with TBST-buffer solution two times for 5 minutes each. This initial step was followed by incubation with the Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) reagent at $37^0$C for 30 minutes. This reagent is a peroxidase-conjugated polymer, which also carries antibodies to rabbit and mouse immunoglobulins. This step was followed again by rinsing with TBST-buffer solution two times for 5 minutes each. The reaction was finally made visible by loading the slide onto a Dako instrument and staining with the use of the dedicated template/protocol for Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse , code K5007. After monitoring for 10 minutes, a time span usually enough to obtain an acceptable staining intensity, the slide was immersed once in distilled water. Counterstaining of the nucleus was at the end accomplished after immersion in haemalum (a mixture of haematoxylin –Dako S2020 1:5 with distilled water) for 3 minutes and rinsing for 5 minutes afterwards with tap water. This final procedure stains the cell nuclei blue, which provides a contrast to the brown color of the DAB chromogen for better visualization of tissue morphology.
5. *Dehydration and mounting:* The slide was placed twice for 30 seconds in ethanol 80%, then twice for 30 seconds in ethanol 96%, after that three times for 30

seconds in ethanol 100% and afterwards three times for 1 minute in xylol solution. Finally the slide was mounted with coverslip und viewed on microscope. The extend of Ki67 staining was recorded using a 3-grade system, based on the percentage of positively stained nuclei.

## 4. Statistical analysis

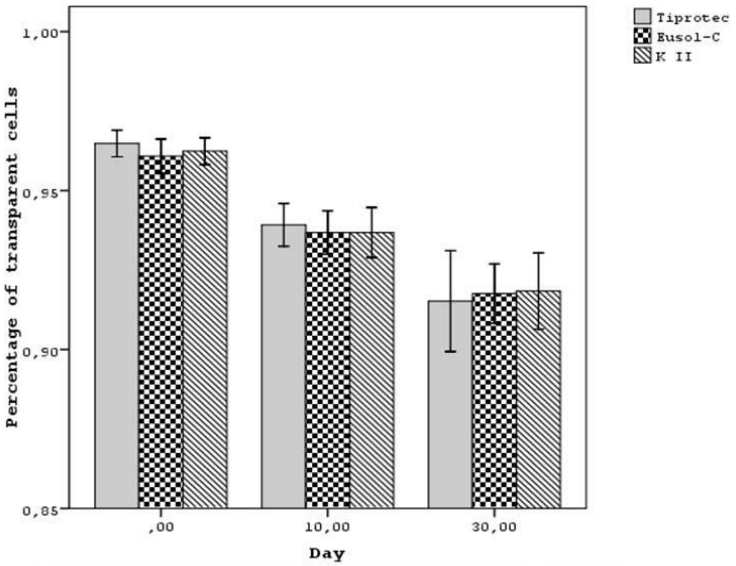
The analysis was performed separately in the data for the samples preserved for 30 days as well as in those from the samples whose storage was extended up to 40 days. The later represent a separate analysis for simulated – transplanted Tiprotec and Eusol-C preserved corneas (compared to the organ-cultured corneas). The data from histological evaluation were also separately analyzed. Numeric data are presented as mean values ± their standard errors (SEM). Categorical variables are presented as percentages (%). All numeric variables were tested for normality with the Shapiro-Wilk test. Given the fact that some variables did not demonstrate normal distribution, further analysis was performed with the use of non-parametric tests. To compare percentages we had to transform them according to the arcsine transformation which is commonly used for proportions. This consists of taking the arcsine of the square root of each proportion. After this arcsine transformation further analysis was as above performed with the use of non-parametric tests. Differences within the same group at subsequent time points were tested with the Friedman's test while differences between the three different preservation media groups were tested with the Kruskal-Wallis H test. Additional comparisons were performed, when necessary, between two variables from different media groups with the use of Mann-Whitney test and between paired variables (belonging to the same media group) with the use of Wilcoxon test. A p-value $< 0.05$ was considered statistically significant. Data analysis was performed with the use of the statistical software SPSS-version 19.0 (SPSS Inc, Chicago, Ill).

# 5. Results

Five samples (16,6%) from the group of Tiprotec had to be discarded due to contamination detected at the first evaluation (i.e after 10 days of storage). No further contamination was noted in the rest of the corneal buttons during the whole storage period.

## 5.1. Quality of corneas after 30 days of storage.

The degree of endothelial transparency was significantly reduced over time with all preservation media, without any significant difference among the three media groups at any point of time. More specifically, a mean transparency loss of 2.65% for the corneas preserved in Tiprotec was observed as compared to 2.36% for those in Eusol-C und 2.73% in K II after the first 10 days of storage. These percentages were increased to 5.14% for Tiprotec, 4.31% for Eusol-C und 4.71% for K II after 30 days **(Figure 5)**.



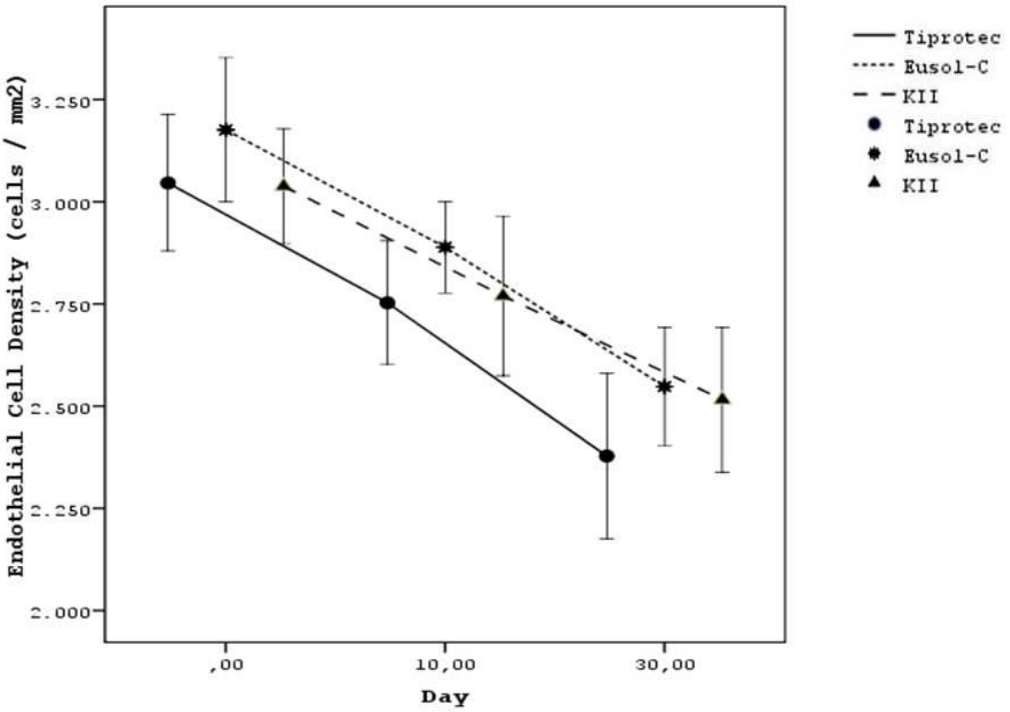

| | Day 1 | Day 10 | Day 30 | p* |
|---|---|---|---|---|
| Tiprotec | 96,48±0,2 % | 93,92±0,3 % | 91,52±0,7 % | **0.000** |
| Eusol-C | 95,73±0,2 % | 93,47±0,3 % | 91,60±0,4 % | **0.000** |
| KII | 96,00±0,2 % | 93,27±0,3 % | 91,47±0,6 % | **0.000** |
| p** | 0,126 | 0,423 | 0,704 | |

p*: statistical significance calculated with Friedman test after arcsin transformation of the percentages

p**: statistical significance calculated with Kruskal-Wallis H test after arcsin transformation of the percentages

**Figure 5: The degree of corneal transparency (%) after 30 days of storage**

A significant reduction in endothelial cell density was also observed with all three preservation media after 30 days of storage. Nevertheless, the differences in the number of viable cells among the three preservation media groups were not found significant on the 10th or on the 30th day of preservation **(Figure 6).**

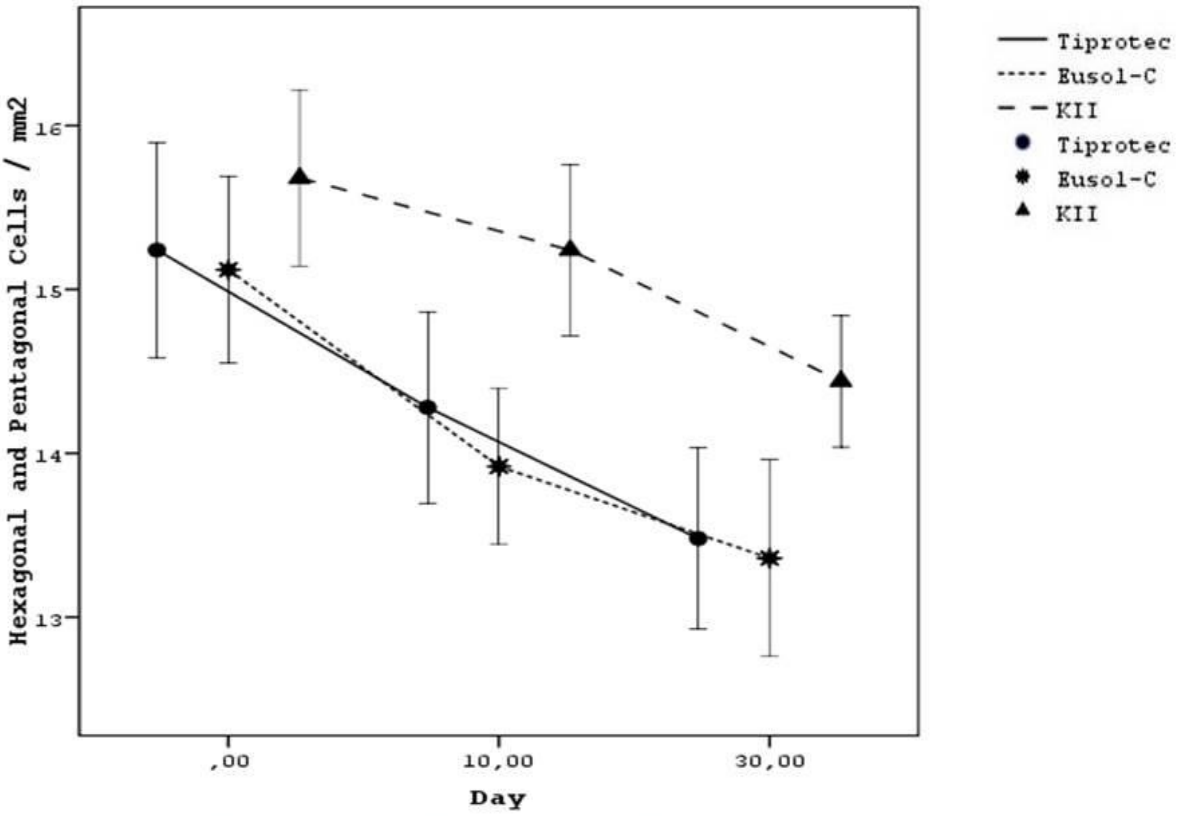



| | Day 1 | Day 10 | Day 30 | p* |
|---|---|---|---|---|
| Tiprotec | 3046±83,4 | 2752,9±75,8 | 2378±100,8 | **0.000** |
| Eusol-C | 3153,3±78,4 | 2855,6±55,9 | 2565±63,2 | **0.000** |
| KII | 3025±64,8 | 2756,6±83,2 | 2550±81,7 | **0.000** |
| p** | 0,658 | 0,514 | 0,516 | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 6. Endothelial cell density (cells/mm$^2$) after 30 days of storage**

The number of hexagonal and pentagonal cells was also significantly reduced overtime in all media groups. Of note, the number of hexagonal and pentagonal cells on the 10th and at the 30th day was found significantly higher in the K II group in comparison to both the Tiprotec (p:0.027 and p:0.007 respectively) and the Eusol-C groups (p:0.000 and p:0.000 respectively) **(Figure 7).**

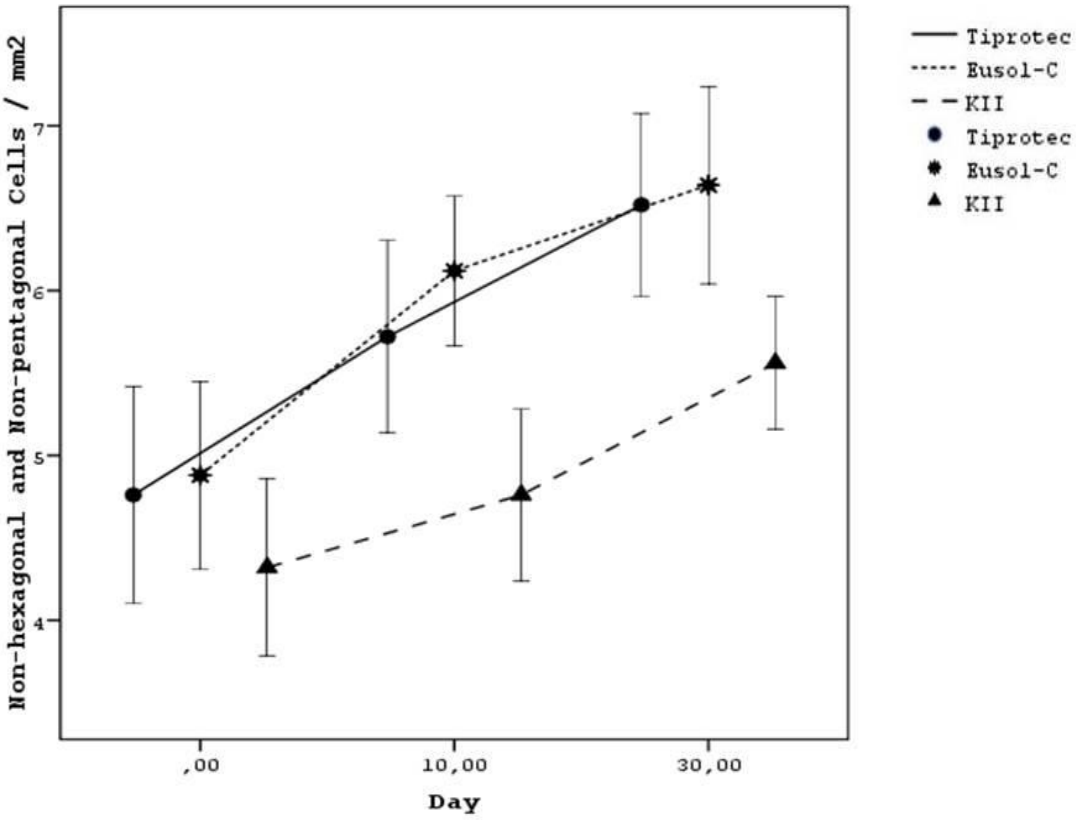



| | Day 1 | Day 10 | Day 30 | p* |
|---|---|---|---|---|
| Tiprotec | 15,2±0,32 | 14,2±0,29 | 13,4±0,27 | **0.000** |
| Eusol-C | 15 ±0,27 | 13,8±0,22 | 13 ±0,28 | **0.000** |
| KII | 15,8 ±0,23 | 15,1±0,22 | 14,4 ±0,17 | **0.000** |
| p** | 0,159 | 0,001 | 0,001 | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 7. Number of hexagonal and pentagonal cells / mm$^2$ after 30 days of storage**

As expected, a significant increase in the number of cells of other types was noted in all three media groups overtime. In accordance to the previous findings about the hexagonal and the pentagonal cells, the number of cells without hexagonal or pentagonal morphology was significantly lower in the K II group both at the 10$^{th}$ and at the 30$^{th}$ day of preservation in comparison to the Tiprotec (p:0.027 and p:0.007 respectively) and the Eusol-C groups (p:0.000 and p:0.000 respectively) **(Figure 8).**



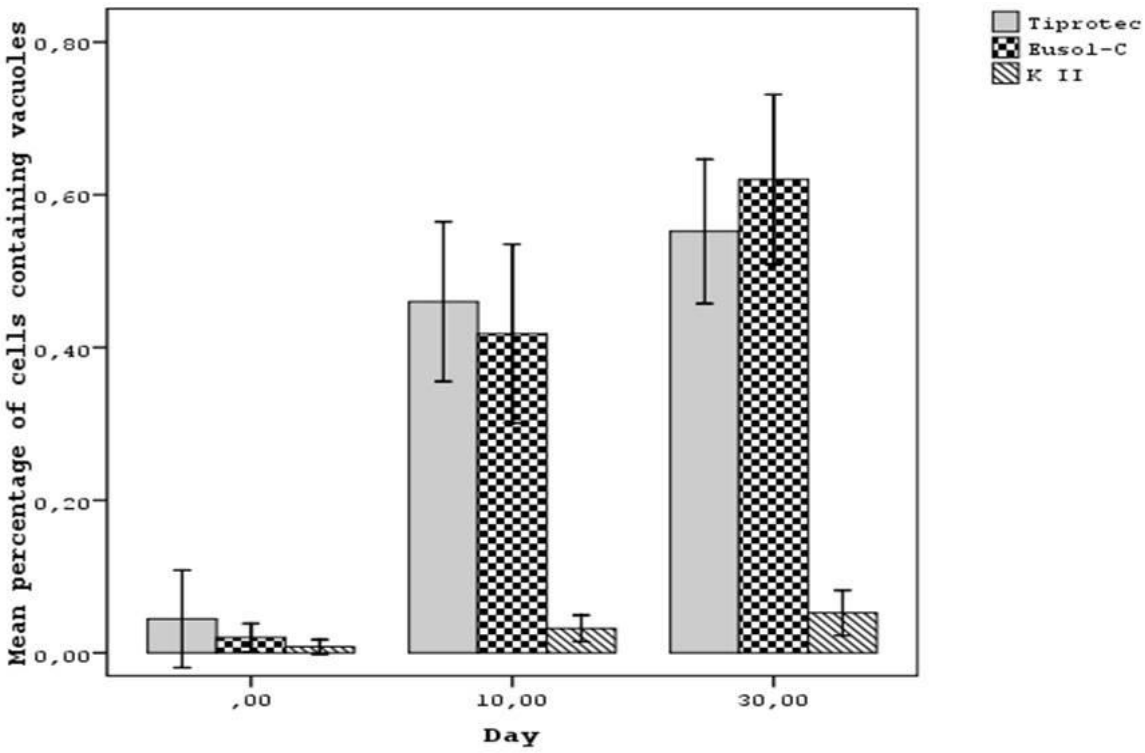

| | Day 1 | Day 10 | Day 30 | p* |
|---|---|---|---|---|
| Tiprotec | 4,7±0,32 | 5,7±0,29 | 6,5±0,27 | **0.000** |
| Eusol-C | 4,9 ±0,27 | 6,1±0,22 | 6,9 ±0,28 | **0.000** |
| KII | 4,2 ±0,23 | 4,8±0,22 | 5,5 ±0,17 | **0.000** |
| p** | 0,159 | 0,001 | 0,001 | |

p*: statistical significance calculated with Shapiro-Wilk test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 8. Number of non-hexagonal / pentagonal cells / mm$^2$ after 30 days of storage**

Finally, the percentage of vacuoles containing cells was significantly increased overtime in all media groups, although in the K II group this trend was significantly lower both at the 10$^{th}$ and at the 30$^{th}$ day of preservation in comparison to the Tiprotec (p:0.000 and p:0.000 respectively) and the Eusol-C groups (p:0.000 and p:0.000 respectively) **(Figure 9).**

Of note no difference was found between the Tiprotec and the Eusol-C groups both at the 10$^{th}$ (p:0.343) and at the 30$^{th}$ (p:0.213) preservation day.




| | Day 1 | Day 10 | Day 30 | p* |
|---|---|---|---|---|
| Tiprotec | 4,4±3,1 % | 46±5,2 % | 55,2±4,7 % | **0.000** |
| Eusol-C | 2,1±0,8 % | 39,5±5,1 % | 62,3±4,7 % | **0.000** |
| KII | 0,6±2,1 % | 3,1±0,7 % | 5±1,2 % | **0.000** |
| p** | 0,184 | 0,000 | 0,000 | |

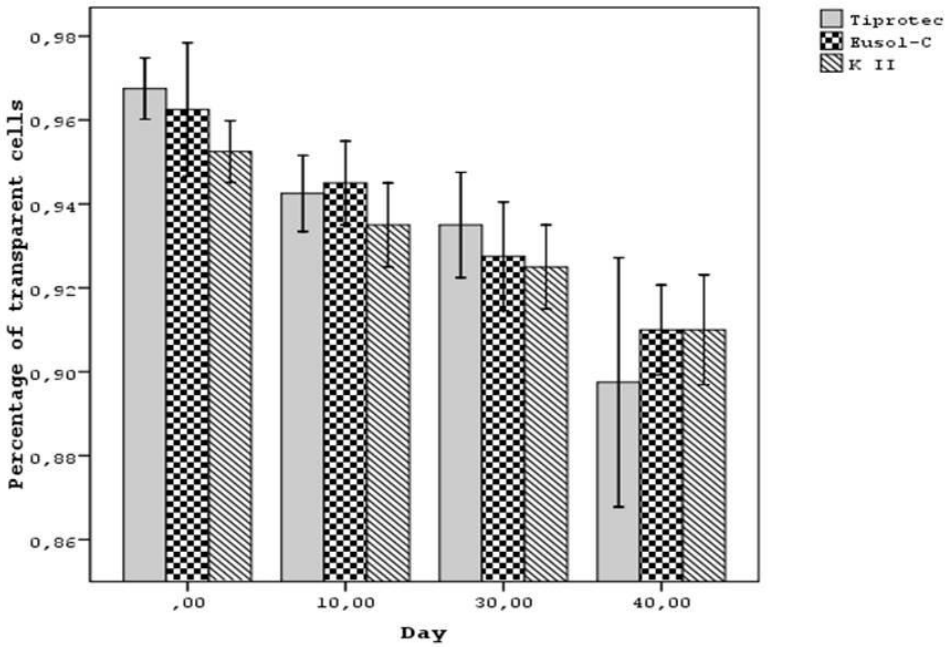
p*: statistical significance calculated with Friedman test after arcsin transformation of the percentages
p**: statistical significance calculated with Kruskal-Wallis H test after arcsin transformation of the percentages

**Figure 9. The percentage of endothelial cells containing vacuoles after 30 days of storage**

### 5.2. Quality of corneas of regenerative capacity testing subset (after 10 further days in organ culture)

After the submission of 10 corneas from the Tiprotec and the Eusol groups respectively to organ culture in K II (transit to conditions more favorable to the cellular biology ``simulating`` corneal transplantation; see also figure 1), the degree of transparency of these corneas showed the same significant decline over time. More specifically, a transparency loss of 2.58% for the corneas preserved in Tiprotec was observed as compared to 2.01% for those in Eusol-C und 2.5% in K II after the first 10 days of storage. These percentages were increased to 3.35% for Tiprotec, 3.61% for Eusol-C and 3.4% for K II after 30 days.

Finally after 40 days of storage loss of transparency reached 7.23% in the Tiptrotec group, 5.2% in the Eusol-C group and 5.85% in the K II group **(Figure 10).**

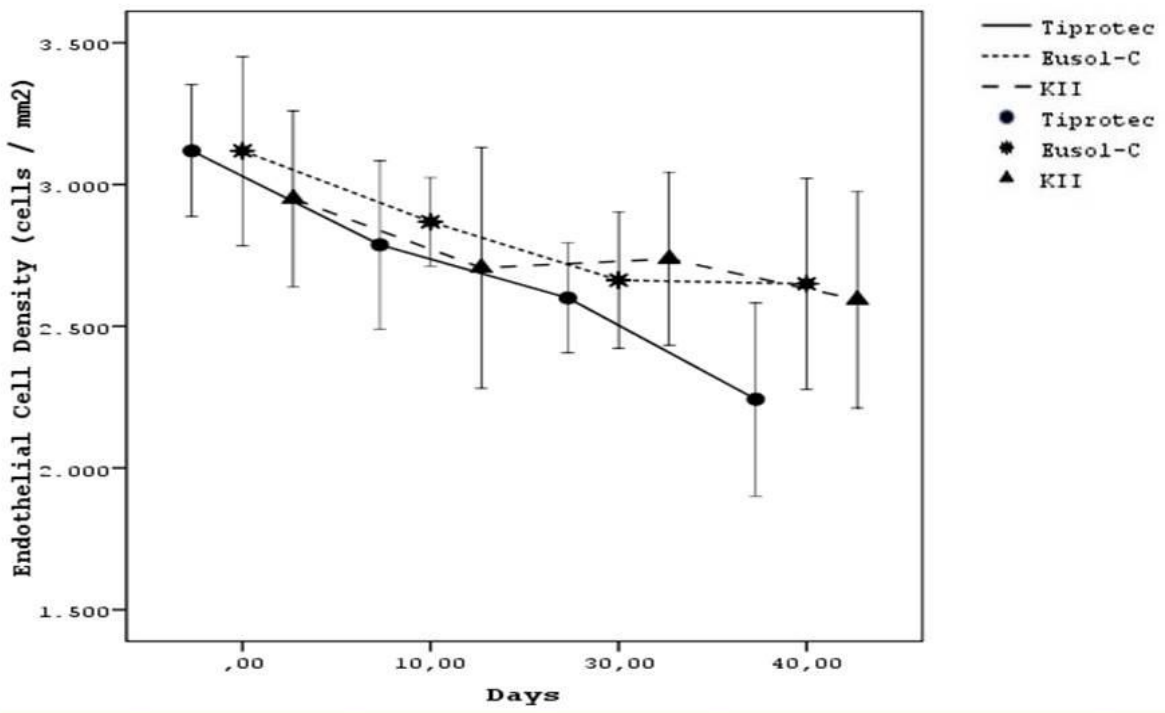



| | Day 1 | Day 10 | Day 30 | Regeneration phase in organ culture (simulation of transplantation) | p* |
|---|---|---|---|---|---|
| Tiprotec | 96,75±1 % | 94,25±1,2 % | 93,5±1,7 % | 89,75±4,2 % | **0.000** |
| Eusol-C | 96±2,1 % | 94,2±1,4 % | 92,6±1,6 % | 91,2±1,3% | **0.000** |
| KII | 95,6±1,2 % | 93,±1,7 % | 92,2±1,4 % | 90±3,8 % | **0.000** |
| p** | 0,260 | 0,165 | 0,319 | 0,715 | |

p*: statistical significance calculated with Friedman test after arcsin transformation of the percentages
p**: statistical significance calculated with Kruskal-Wallis H test after arcsin transformation of the percentages

**Figure 10. The degree of corneal transparency (%) after 40 days of storage**

Regarding endothelial density, a significant overtime reduction was observed only in the groups of Tiprotec and Eusol-C. Nevertheless, the differences in the number of endothelial cells among the three storage media groups were in accordance to the previous findings not significant after 10 , 30 or 40 days of preservation **(Figure 11).**

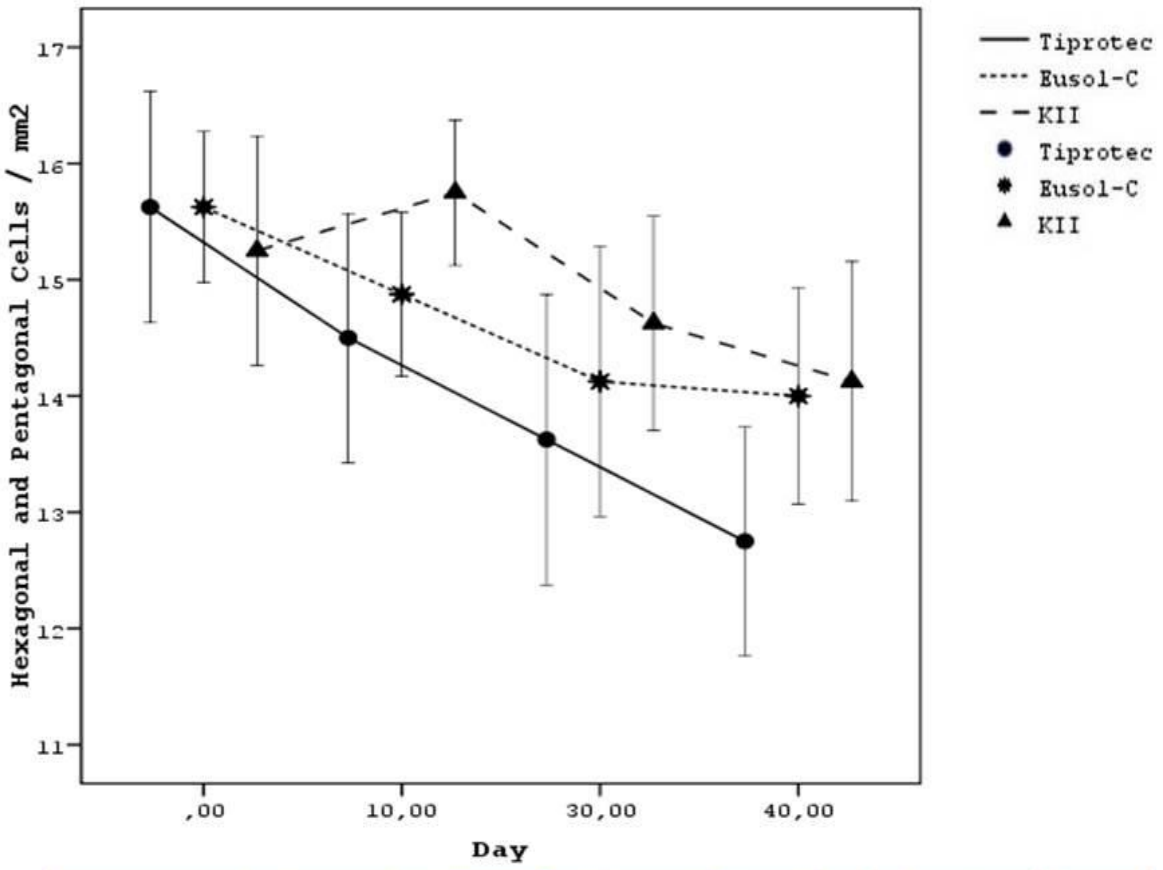



| | Day 1 | Day 10 | Day 30 | Regeneration phase in organ culture (simulation of transplantation) | p* |
|---|---|---|---|---|---|
| Tiprotec | 3118,75±328,34 | 2787,5±420,67 | 2600±273,86 | 2242,5±481,38 | **0.000** |
| Eusol-C | 3190±442,09 | 2886±271,54 | 2580±348,16 | 2615±474,37 | **0.025** |
| KII | 3030±424,39 | 2750,6±542,11 | 2750±407,56 | 2655±592,75 | 0.315 |
| p** | 0,879 | 0,559 | 0,638 | 0,165 | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 11. Endothelial cell density (cells / mm$^2$) after 40 days of storage**

A significant decline in the number of hexagonal and pentagonal cells was again observed in all media groups. Contrary to the previous results the differences among the three media groups did not reach statistically significance at the 10$^{th}$, 30$^{th}$ or 40$^{th}$ day of storage. **(Figure 12).**

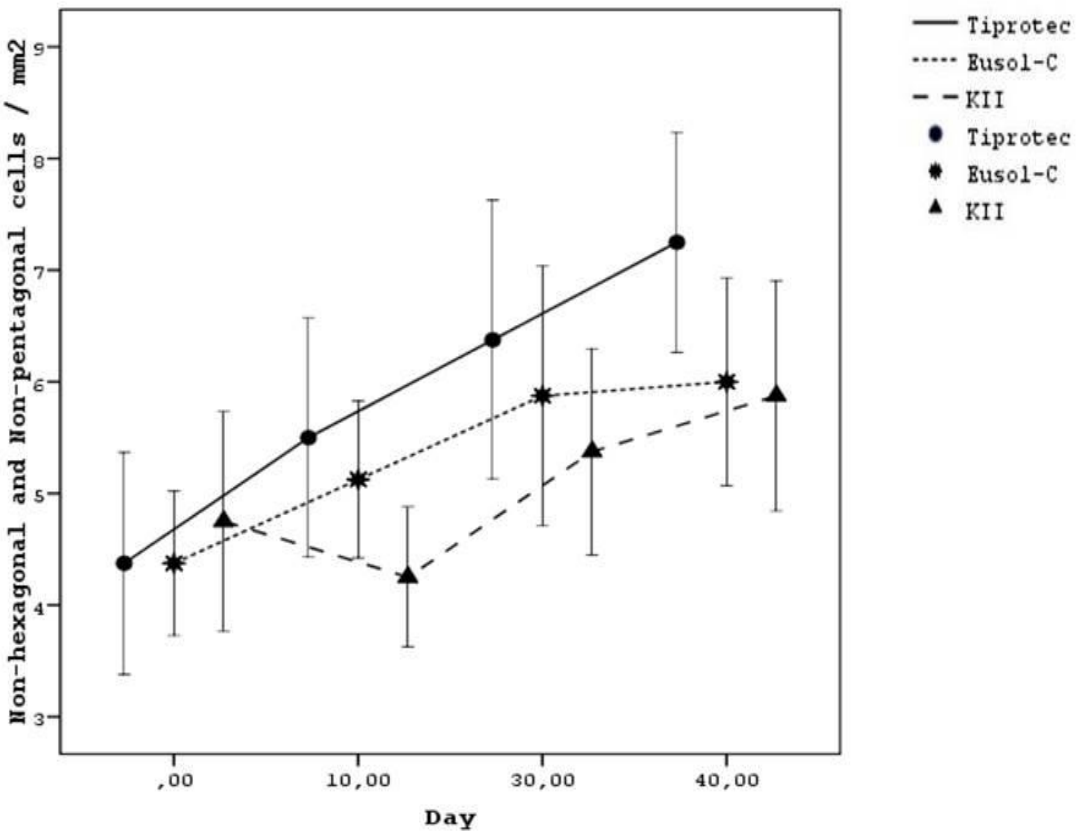



| | Day 1 | Day 10 | Day 30 | Regeneration phase in organ culture (simulation of transplantation) | p* |
|---|---|---|---|---|---|
| Tiprotec | 15,62±1,4 | 14,5±1,51 | 13,62±1,76 | 12,75±1,38 | **0.000** |
| Eusol-C | 15,2 ±1,22 | 14,5±1,17 | 13,8 ±1,61 | 13,7 ±1,33 | **0.004** |
| KII | 15,4 ±0,34 | 15,6±0,96 | 14,6 ±1,17 | 14,1 ±1,37 | **0.001** |
| p** | 0,682 | 0,092 | 0,364 | 0,133 | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 12. Number of hexagonal and pentagonal cells / mm$^2$ after 40 days of storage**

A significant increase in the number of cells with non-hexagonal or non-pentagonal shape was also noted in all three groups overtime. The differences among the groups were not significant at any point of time **(Figure 13).**



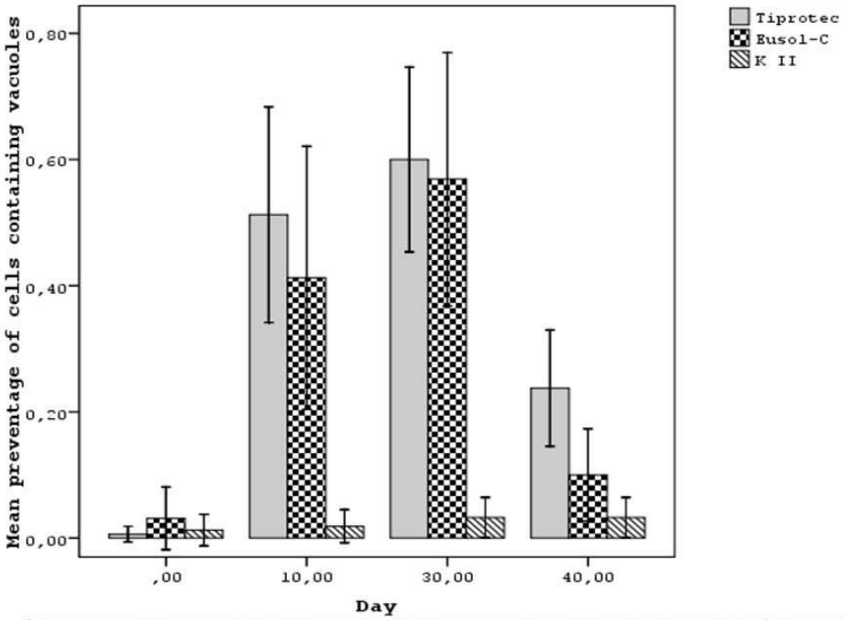

| | Day 1 | Day 10 | Day 30 | Regeneration phase in organ culture (simulation of transplantation) | p* |
|---|---|---|---|---|---|
| Tiprotec | 4,37±1,40 | 5,50±1,51 | 6,37±1,76 | 7,25±1,38 | **0.000** |
| Eusol-C | 4,8±1,22 | 5,4±1,17 | 6,2±1,61 | 6,3±1,33 | **0.004** |
| KII | 4,6±1,34 | 4,4±0,96 | 5,4±1,17 | 5,9±1,37 | **0.001** |
| p** | 0,682 | 0,092 | 0,364 | 0,133 | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 13. Number of non-hexagonal / pentagonal cells / mm$^2$ after 40 days of storage**

Finally, the percentage of vacuoles containing cells was significantly increased overtime in all media groups (p:0.000 for Tiprotec, p:0.000 for Eusol-C and p:0.0016 for K II respectively). In the K II group this trend was again significantly lower at the 10$^{th}$, at the 30$^{th}$ and at the 40$^{th}$ day of preservation in comparison to the Tiprotec and the Eusol-C groups (p:0.000, p:0.000 and p:0.0012 respectively) **(Figure 14).** No differences were found again between the Tiprotec and the Eusol-C groups both at the 10$^{th}$ (p:0.408) ,30$^{th}$ (p:0.879) or 40$^{th}$ (p:0.237) preservation day. Interestingly, the comparison between the

30th and 40th day of presentation revealed a significant reduction in the percentage of vacuoles both for Tiprotec (p: 0.012) and Eusol-C (p:0.005) groups.




| | Day 1 | Day 10 | Day 30 | Regeneration phase in organ culture (simulation of transplantation) | p* |
|---|---|---|---|---|---|
| Tiprotec | 0,6±1,7 % | 51,2±24,1 % | 60±20,7 % | 23,7±13% | **0.000** |
| Eusol-C | 4±6,5 % | 42±26,9 % | 61,5±26,8 % | 15±14,1 % | **0.000** |
| KII | 1,5±3,3 % | 3±4,2 % | 4,1±4,5 % | 4,1±4,5 % | **0.016** |
| p** | 0,333 | **0,000** | **0,000** | **0,012** | |

p*: statistical significance calculated with Friedman test
p**: statistical significance calculated with Kruskal-Wallis H test

**Figure 14. The mean percentage of endothelial cells containing vacuoles after 40 days of storage**

### 5.3. Results of histological analysis

In comparison to the control corneas, the preserved ones showed a significantly higher degree of epithelial dissociation (p: 0.041) and a significantly lower degree of epithelial thickness (p: 0.049). On the other hand, the parameters of endothelial quality were not found significantly different among the control and the preserved corneas **(Table 5, Figures 15-16).** Of note, none of the above parameters were found significantly different when the comparison was restricted only among the three media groups whether including **(Table 6)** or not **(Table 7)** the regenerative capacity subset of samples (i.e. the samples preserved for 40 days)**.**

**Table 5. Comparison of data from histological analysis including the control corneas**

| | **Epithelial Dissociation** *(Scale:0-3)* | **Basal Cell Contact** *(Scale:0-3)* | **Epithelial Thickness** *(Scale:0-3)* | **Enothelial Quality** *(Scale:0-3)* | **Ki 67 Rate** *(Scale:0-3)* | **Cornea Quality** *(Scale:1-3)* |
|---|---|---|---|---|---|---|
| ***Control*** | ***0,0±0,0*** | ***3,0±0,0*** | ***3,0±0,0*** | ***3,0±0,0*** | ***3,0±0,0*** | ***1,0±0,0*** |
| **Tiprotec** | 2,25±0,95 | 1,5±1,29 | 1,5±1 | 2,0±1,41 | 1,5±1,73 | 2,25±0,5 |
| **Eusol-C** | 1,75±1,25 | 2,25±0,95 | 2,0±0,81 | 2,0±0,0 | 2,25±1,5 | 2,0±0,81 |
| **KII** | 1,0±0,0 | 3,0±0,0 | 1,25±0,5 | 2,0±0,0 | 0,87±0,48 | 2,25±0,5 |
| p* | **0,041** | 0,084 | **0,049** | 0,067 | 0,227 | 0,065 |

p*: statistical significance calculated with Kruskal-Wallis H test

**Table 6. Comparison of data from histological analysis among the corneas stored in the 3 different media (control corneas not included).**

| | **Epithelial Dissociation** | **Basal Cell Contact** | **Epithelial Thickness** | **Enothelial Quality** | **Ki 67 Rate** | **Cornea Quality** |
|---|---|---|---|---|---|---|
| **Tiprotec** | 2,25±0,95 | 1,5±1,29 | 1,5±1 | 2,0±1,41 | 1,5±1,73 | 2,25±0,5 |
| **Eusol-C** | 1,75±1,25 | 2,25±0,95 | 2,0±0,81 | 2,0±0,0 | 2,25±1,5 | 2,0±0,81 |
| **KII** | 1,0±0,0 | 3,0±0,0 | 1,25±0,5 | 2,0±0,0 | 0,87±0,48 | 2,25±0,5 |
| p* | 0,167 | 0,111 | 0,352 | 0,670 | 0,554 | 0,829 |

p*: statistical significance calculated with Kruskal-Wallis H test

**Table 7. Comparison of data from histological analysis among the corneas stored in the 3 different media up to 30 days (control corneas not included).**

| | **Epithelial Dissociation** | **Basal Cell Contact** | **Epithelial Thickness** | **Enothelial Quality** | **Ki 67 Rate** | **Cornea Quality** |
|---|---|---|---|---|---|---|
| **Tiprotec** | 2,00±1,00 | 2,00±1,00 | 2,00±0,00 | 2,66±0,57 | 2,00±1,73 | 2,00±0,00 |
| **Eusol-C** | 2,33±0,57 | 2,00±1,00 | 2,33±0,57 | 2,00±0,00 | 3,00±0,00 | 2,00±1,00 |
| **KII** | 1,00±0,00 | 3,00±0,00 | 1,33±0,57 | 2,00±0,00 | 0,66±0,28 | 2,33±0,57 |
| p* | 0,100 | 0,230 | 0,110 | 0,102 | 0,144 | 0,740 |

p*: statistical significance calculated with Kruskal-Wallis H test

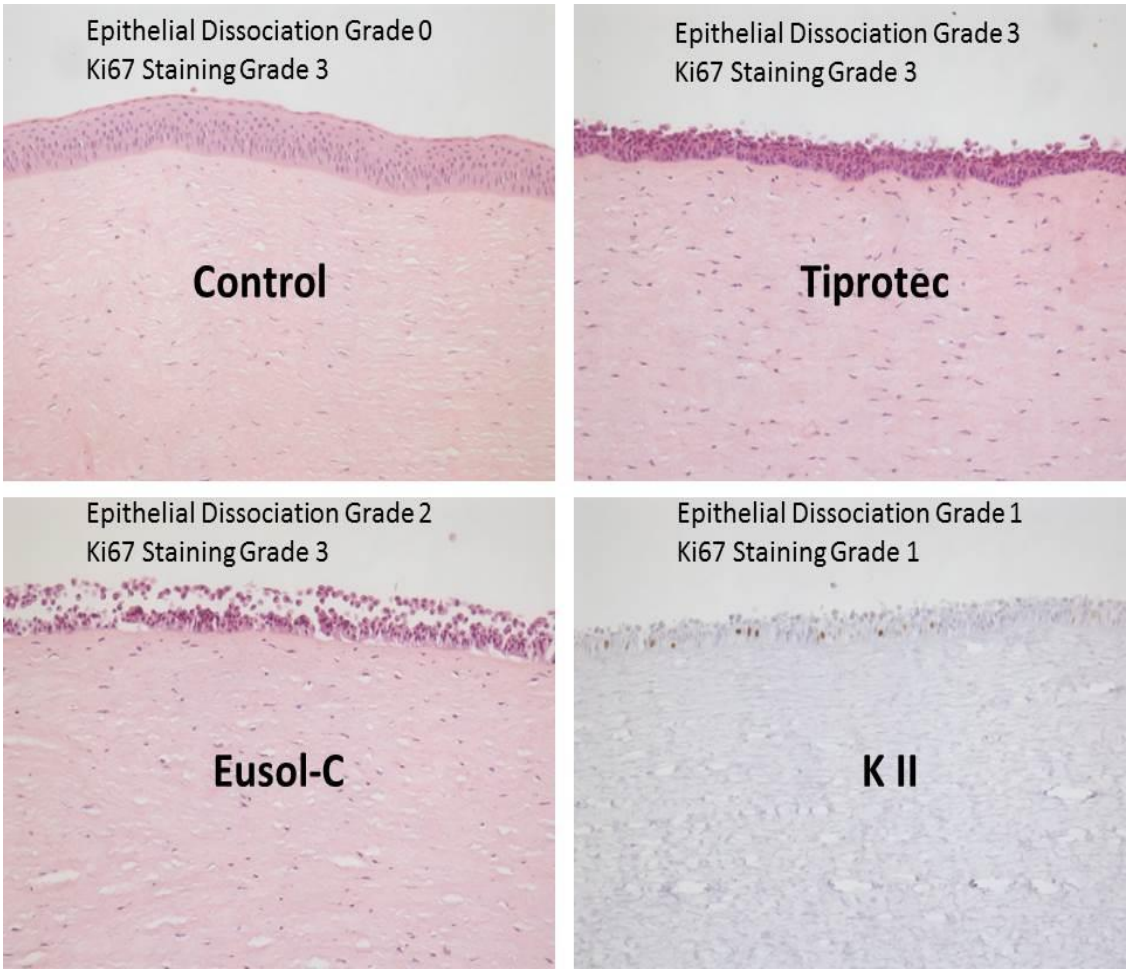



**Figure 15. Various degrees of epithelial dissociation (scale: 0-3) and of Ki67 staining of epithelial cells (scale: 0-3) in samples preserved for 30 days in the three storage media**

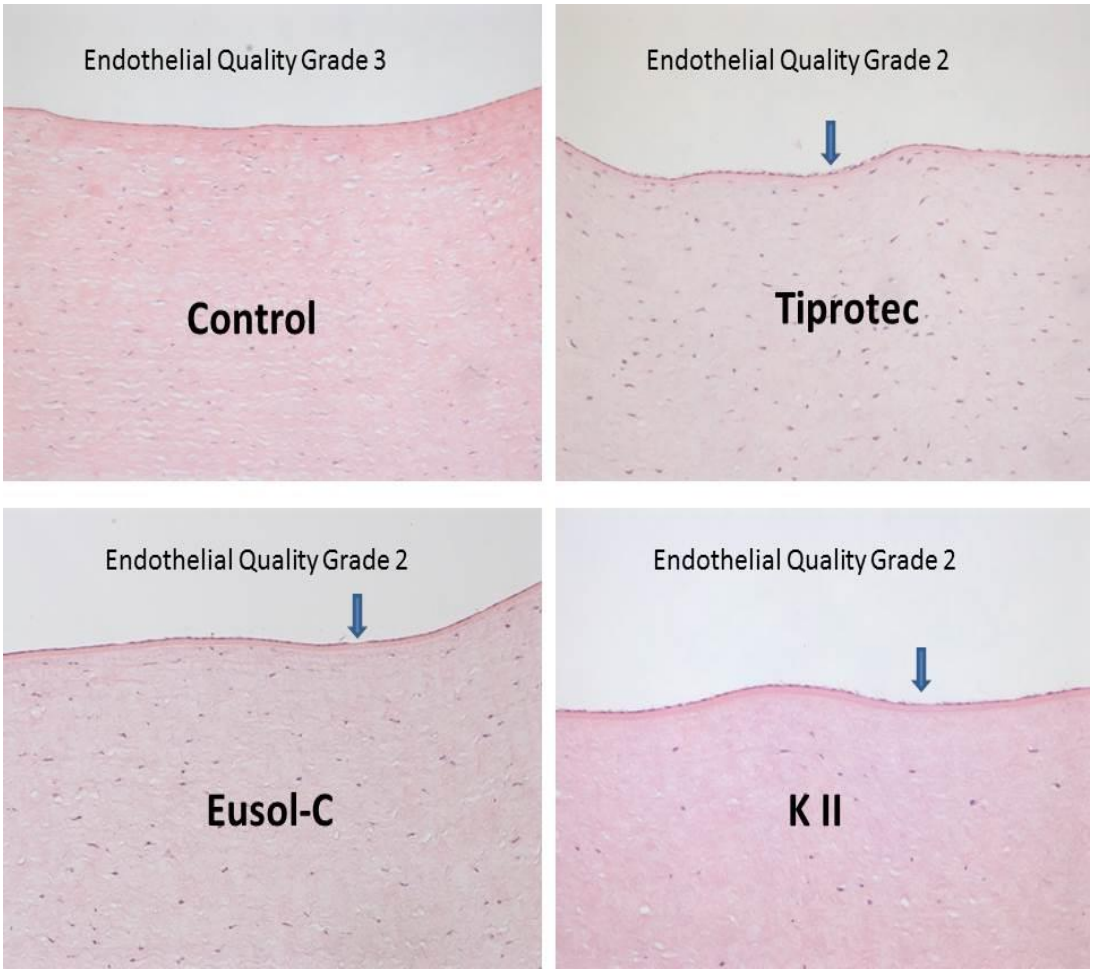



**Figure 16. Slightly reduced quality of corneal endothelium as shown by the intermittent loss of endothelial continuity (arrows) in samples preserved for 30 days in the three storage media (scale: 0-3).**

## 6. Discussion

In the present experimental study, the hypothermic storage with both solutions demonstrated a mild inferiority regarding the preservation of endothelial integrity in comparison to the storage in organ culture medium K II at $36^0$C. Although some corneas stored in Tiprotec (a storage solution we used extrapolating its known beneficial effect in the preservation of vascular endothelium) tended to display slightly poorer endothelial quality parameters compared to Eusol-C stored corneas (especially at extended storage for 30 days), these differences were not significant. Hence, Tirpotec appears to be similarly effective as the commercially available and widely used solution Eusol-C for the hypothermic storage of porcine corneal grafts at $4^0$C, perhaps even for an extended

storage period. Nevertheless, a progressive decline in both endothelial viability and quality was observed with all preservation media as expected (see below).

To our knowledge this was the first study to assess the effectiveness of Tiprotec as a storage solution for the hypothermic preservation of corneal tissue. Tiprotec has been exclusively used until now as a medium for the storage of vascular grafts. Several components of the Tiprotec solution have been associated with its cytoprotective effect. The potassium it contains terminates the resting potential of the cell membranes leading to decreased $O_2$ consumption and accordingly to decreased metabolic demands of the cell (Preusse CJ et al, 1981). In addition, the high extracellular potassium levels it creates prevent the potassium outflow from the cells and the resultant apoptosis (D'Alessandro AM et al. 1994, Lang F et al. 2005).

Moreover, to prevent the iron-dependent formation of reactive oxygen species, which have been implicated in cold induced injury, the iron-chelators deferoxamine and LK-614 have been also included in the Tiprotec solution (Wu S et al. 2009). Glucose is also part of the solution and it acts as a substrate for the glycolytically highly active endothelial cells (Mertens S et al. 1990). Glycine, alanine, aspartate and a-ketoglutarate have been associated with the prevention of pore formation in the cellular membrane induced by hypoxia and resulting in sodium influx and cellular edema (Wu S et al. 2009). Finally its mildly acidotic pH has also demonstrated protective properties against cellular injury (Nishimura Y et al. 1998).

We observed a constant over time decline in endothelial cell density: after 30 days, 15.7% of the endothelial cells were lost at organ culture, while cold-storage corneas displayed moderately higher endothelial losses (21.9% for Tiprotec and 18.6% for Eusol-C). These observations are congruent with findings reported by other investigators. In an older study, Nelson et al. compared Optisol-GS with Chen medium for the hypothermic preservation of 3 pairs of donor corneas. A median endothelial loss of 11% after 14 days of storage in Optisol-GS was reported (Nelson LR et al. 2000). In a more recent study, Basak et al who compared the storage media Optisol-GS with Cornisol in the hypothermic preservation of human corneas, reported a 13.5% vs 14.5 % decline in corneal endothelial cell density after 10 days of storage (Basak S and Prajna NV 2016). Consistent are also the findings of Parekh et al who found an endothelial loss at 7 days 9.4% vs. 8.0% and at 14 days 6.45% vs. 8% when they compared Optisol-GS with Cornea Cold as hypothermic preservation media of donor corneas (Parekh M et al. 2014). On the other hand, Kanavi et al, demonstrated a significantly lower percentage of endothelial loss after 7 days of

hypothermic storage (3.04% with Optisol-GS und 2.82% with Eusol-C) (Kanavi MR et al. 2015) while Sibayan et al reported an extraordinary high percentage of endothelial loss (25.84%) after 10 days of hypothermic storage in Optisol-GS albeit in a small sample of 21 human corneas (Sibayan ASB et al. 2015). Similar results have been reported not only for the hypothermic storage but also for the organ culture. After 21 days of storage, human endothelial cells isolated from one organ cultured cornea demonstrated a 26.7% decline in cell density as shown by Bednarz et al (Bednarz J et al. 2001). A decrease of endothelial density of about 20% within the first 2 weeks of storage in organ culture medium was also reported by Hempel et al (Hempel B et al. 2001). A high rate of decline in endothelial density after 5 weeks of storage of donor corneas in organ culture was also shown by Smith et al (Smith VA and Johnson T, 2010). These corneas however, had been already stored for a mean time of 10 days before their culture and this could have affected their initial density and the subsequent viability of their endothelium. More recently, Parekh et al showed also a higher degree of endothelial loss (35%) after 30 days of storage with the organ culture method (Parekh M et al. 2015). Nevertheless, their study had two unique features: the use of cadaveric and not suitable for transplantation corneas together with the combination of endothelial cell density and cell mortality in one variable. On the other hand, a similar to our findings 10% decay of endothelial density after a mean storage period of 9 days was reported by Acquart et al in a large series of human corneal grafts organ cultured in $31^0$C (Acquart S et al. 2010).

Beyond the general agreement about the progressive endothelial decay with either storage strategy, the variation in the degree of endothelial loss reported can be explained not only from differences in the technical details of storage (Thuret G et al. 2004) and in the materials selected for preservation, but also form diversities in counting methods. The later appears to be a real problem in the everyday practice of eye banking since its can affect the reproducibility and the accuracy of endothelial cell density determination (Thuret G et al. 2003, Lass JH et al. 2005). Finally, additional sources of variation could have been the intra- and inter-observer variability which, according to Benetz et al can also be attributed to factors unique to the donor corneas such as the cause of death, death to preservation time, state of the epithelium, degree of striae in the Descemet membrane and tissue temperature at the time of imaging (Benetz BA et al. 2006). Our controlled experimental setting have compensated for most but not all of the above sources of error. In any case, our counting method has been repeatedly validated and is currently the standard practice in our research laboratory.

In addition to endothelial cell density, several morphometric characteristics of endothelial cells have been associated with the quality of corneal grafts. In our study, two of these parameters were examined: the degree of pleomorphism (i.e. deviation of hexagonal or pentagonal shape) together with the percentage of vacuolization. We found a small but significant increase in pleomorphism over time in all cornea groups. This degree of deviation from normal shape was lower for the organ cultured group at least for the corneas preserved up to 30 days.

Similar morphometric changes have been reported by several other investigators. A significant decrease in the percentage of hexagonal cells of organic cultured corneas after a mean storage period of 9 days was found in the study of Acquart et al (Aquart S et al. 2015). On the other hand, Kanavi et al reported a non-significant decrease in hexagonal cells after 7 days of hypothermic storage (Kavani MR et al. 2015). A similar decrease in hexagonality after 14 days of hypothermic storage was demonstrated in the study of Basak et al (Basak S and Prajna NV 2016). Others have used these particular morphological characteristics as components of more complex morphometric variables and arbitrary constructed quality scales. In the previously mentioned study by Parekh et al, for example, a progressive deterioration in endothelial morphology after a 4 week hypothermic storage was found. For the assessment of morphology the authors used a scoring system including several variables such as changes or disappearance of intercellular margins, changes in cellular shape and size and dystrophic cellular changes (Parekh et al. 2014).

The smaller sample size of the corneas of the regeneration capacity subgroup (corneas put in organ culture for additional 10 days after 30 days in cold storage and controls let in parallel prolonged organ culture for 40 days respectively) might have not allowed the morphometric changes to reach statistically significant levels in this group.

While vacuolization of endothelial cells was clearly lower in organ cultured corneas both after 30 and 40 days of storage, the reduction in the number of cells with vacuoles of the Tiprotec and the Eusol-C groups after their organ-cultivation could obviously be attributed to their recovery after the submission to the more favorable environment. It is well known that endothelial recovery is possible only in organ cultured grafts (Nejepinska J et al. 2010). Our finding may indicate that this can also happen even if the organic cultured corneas have been previously preserved under hypothermic conditions. Similar findings were reported several years ago by Camposampiero et al who showed a significant improvement in several parameters of endothelial integrity in human corneas

cultured for a mean of 19 days after a 7-10 days hypothermic storage (Camposampiero D et al. 2003). Nevertheless, more evidence about the effect of such a dual storage procedure upon endothelial integrity is still lacking, while reports about the self-repairing potential of transplanted endothelium are increasingly appearing in the literature (De Bogert DV et al. 2018).

Regarding vacuoles, a general agreement exists that their presence is associated with poorer endothelial quality **(Figure 17)**. The pathogenic mechanism underlying their formation is unclear. It has been suggested that they are associated with a phagocytic remodeling intracellular response to cells stress (Pels E and Schuchard Y 1984), while more recently they have been characterized as indicators of apoptotic cascades leading to cellular death (Armitage WJ 2011). An approximately doubling of significant vacuolization after 7 days of hypothermic storage was also reported by Kanavi et al (Kavani MR et al. 2011). In another more recent study, Hermel et al demonstrated a significant positive correlation between vacuolization and endothelial loss in organ cultured human donor corneas (Hermel M et al. 2017). Interestingly, despite the obvious importance of vacuoles in every day practice of eye banking, vacuolization has not been systematically included in the studies about corneal graft preservation, may be due to uncertainties about their pathogenic role and the lack of a typical reference image to facilitate their recognition.

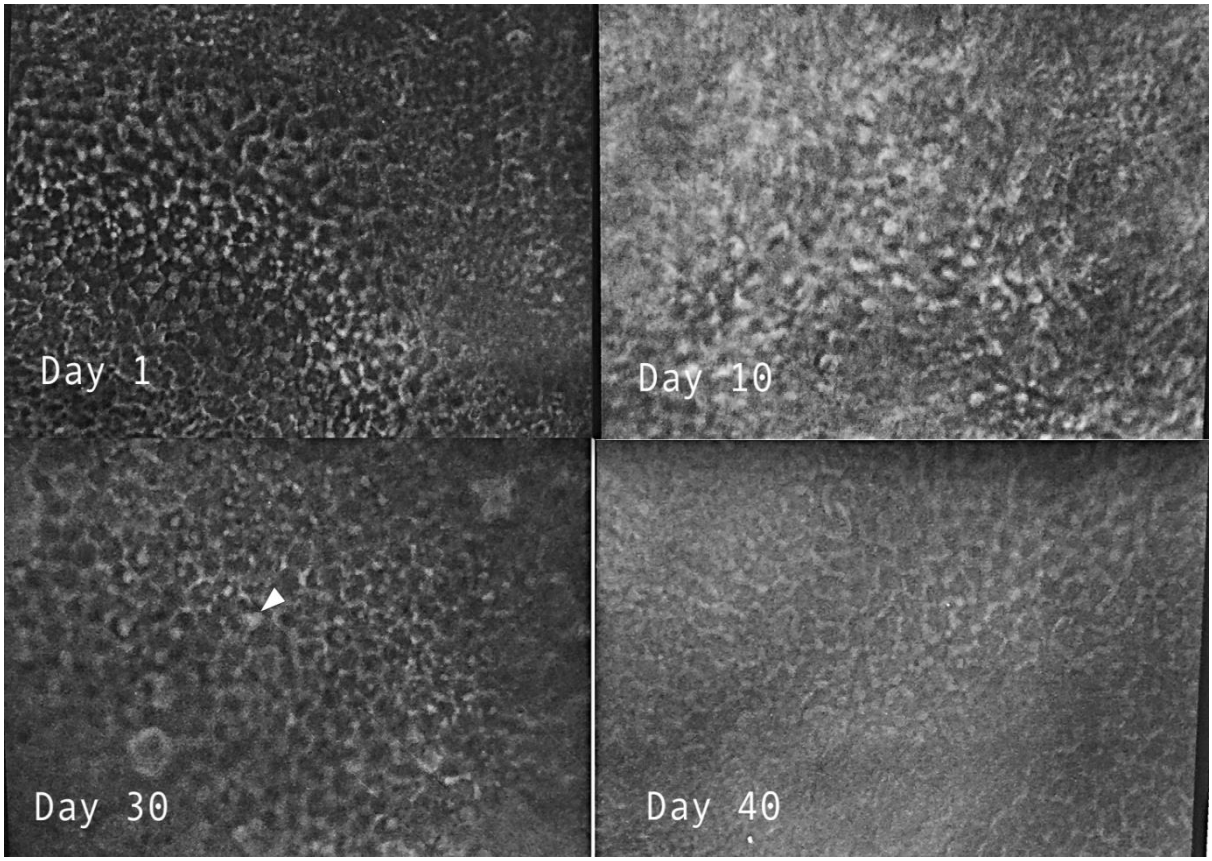



**Figure 17. Corneal endothelial cells from a specimen preserved in Eusol-C. The arrowhead indicates the formation of a vacuole after 30 days of storage.**

The degree of corneal transparency is another, more global index of corneal quality. High degree of transparency is essential to maintain clear vision. The corneal stroma with its particular structural organization of collagen fibers plays a pivotal role in the maintenance of corneal transparency. In addition, the intact endothelium preserves corneal stromal dehydration through a sodium-potassium pump ($Na^+$-$K^+$-ATPase), preventing this way corneal swelling and maintaining corneal transparency (Bonanno JA 2012). A minimal loss of transparency, independent from the storage medium used, was observed in our study even in the regenerative capacity subgroup after 40 days of storage. Parekh et al, however, reported a more significant transparency loss, up to approximately 20%, in their donor corneas preserved in the hypothermic storage solutions Optisol-GS and Corld-Cornea for up to 30 days (Parekh M et al. 2014). This significant decline in transparency happened despite the presence of dextran which as a major dehydrating agent preserves endothelial transparency in both solutions. Dextran was also included in our storage solutions, so that the discrepancy with our findings can be explained only from

differences in the material (human corneas unsuitable for transplantation with an initial transparency of approximately 70% vs our porcine corneas) and in the method of measurement they used. A higher degree of transparency loss (6-10%) compared to our findings was reported by the same authors after 30 days of corneal storage in organ culture media (Parekh M et al. 2015). Due to differences in methodology their data are not quantitatively comparable to ours. It is important at this point to underline the complexity in the molecular structure and the physical properties of human cornea associated with its high degree of transparency and the consequent gross diversity in the methods used to assess it both in vivo and ex vivo (Meek KM and Knupp C 2015). The later poses difficulties in the eye banks worldwide, so that the development of a simple standardized method for transparency measurements remains highly demanded (Parekh M et al. 2014).
The only moderate (and mostly insignificant) difference among the three storage media was further confirmed by the results of the histological analysis. Beyond the general impression of endothelial or overall corneal quality, the percentage of epithelial cell nuclei positively stained with the proliferation marker Ki67 was not found different among the three media. Corneal epithelium plays a very important role in graft survival after transplantation (Jeng BH 2006). Unfortunately, the effect of storage upon its viability has attracted significantly less attention compared to endothelium. Although the primary aim of this study was the evaluation of corneal endothelium under three different storage conditions, histological analysis offered some additional hints about the effect of storage upon epithelial and basal cells. A significant increase in the dissociation of epithelial cells and a significant decrease in the thickness of corneal epithelium in comparison to control corneas were found in all media groups. These findings are in agreement with those of previous reports. In a study conducted by Soni et al a significant increase in epithelial defects of corneal grafts preserved for 17 days in hypothermic storage was demonstrated (Soni NG et al. 2015),. A very meticulous assessment of changes in corneal epithelium of donor corneas after initial hypothermic storage and subsequent transfer to organ culture was performed by Haug et al (Haug K et al. 2013). They described a loss of superficial layers of the epithelium along with detachment of basal cells. Of note, a tendency for epithelial regeneration was observed in the corneas transferred form the hypothermic storage to organic culture. Former studies had shown a significantly higher degree of epithelial loss. Greenbaum et al reported a near complete loss of all epithelial layers of human corneas after 4 days of storage in Optisol GS or in Dexsol (Greenbaum 2004), while Spelsberg et al found a 57% epithelial loss after 4 days of storage in organ culture

medium (Spelsberg H et al. 2002). Their findings however, could not be confirmed by our data and by the above mentioned more recent studies.

The major limitation of our study is the unblinded macro- and microscopic assessment of the corneas from a single examiner. The inherent inter-observer variability in morphometric studies has led to the development of specific semiquantitative methods or computer algorithms (Saad HA et al. 2008) for the counting and qualitative assessment of corneal endothelial cells. The use of one of these methods from two blinded observers could potentially have increased the validity of our results. It should be noted however, that even the automatic software programs remain problematic and their superiority against manual counting has not been proved (Hirneiss C et al. 2007, Huang J et al 2018). On the other hand, our histological analysis was performed in a totally blinded manner and although only a subgroup of corneas was examined, the findings supported those of the macro- und microscopic assessment of the whole sample.

The staining with tryphan blue is a common practice in many eye banks using organ culture as the preferred storage method. Dying endothelial cells with permeable membranes are strongly stained by tryphan blue. We used instead the presence of vacuoles, an acceptable but more subjectively and less commonly assessed index of endothelial cell viability. Our method could have, this way, led to an underestimation of endothelial loss due to inclusion of several necrotic cells in the calculation or endothelial density. However, overestimation of endothelial cell density occurs even after the staining with tryphan blue an as shown by Pipparelli et al (Pipparelli et al. 2011) who proposed a combination of cell labeling and image analysis to determine the pool of viable endothelial cells. Nevertheless, an uneven distribution of this potential measurement bias among our three study groups is highly unlikely, so that it has most probably not affected the comparisons among them.

Finally, any extrapolation of our experimental findings to humans should not be done unless they are confirmed by further studies in human corneas.

The reason for the high rate of contamination detected in the early phase of storage only in the Tirpotec group should be attributed to the lack of antibiotics in this storage solution in contrast to the other media. Since the rich composition of storage fluids in nutrients favors their contamination by bacteria and fungi, the addition of antibiotics is of paramount importance and has shown to effectively prevent the development of bacterial endophthalmitis in graft recipients (Hassan SS et al. 2005). The use of Tiprotec as a new

corneal graft storage medium should be considered only after the addition of antibiotics offering a broad-spectrum antimicrobial coverage.

In conclusion, we showed in this experimental study that the cryopreservation medium Tiprotec, used until now for the preservation of vascular grafts, was only mildly inferior to the medium Eusol-C for the hypothermic storage of corneal tissue for an extended period of time up to 30 days. In comparison to organ culture with culture medium K II, both Tiprotec and Eusol-C were found less effective in preserving both endothelial cell quality, as assessed by the morphometric analysis, and endothelial viability, as assessed by the degree of vacuolization at least up to the $30^{th}$ day of storage. However, both Tiprotec- and Eusol-C-preserved corneas demonstrated a certain capacity to recover after their submission in organ culture. In addition, Tiprotec was not inferior in preserving corneal epithelium in comparison to both Eusol-C and organ culture medium K II. Since this is the first report about the potential use of Tiprotec as a medium for the hypothermic storage of porcine corneas, further studies in human corneas will be needed to confirm our findings and to support its use as an alternative solution in the everyday practice of eye banking.

## 7. Abstract

**Introduction:** Corneal transplantation (keratoplasty) is the only effective treatment for most of the disorders leading to irreversible corneal damage and eventually to corneal blindness. For the storage and preservation of donor corneoscleral disks three methods are today available: hypothermia (cold storage), organ culture and cryopreservation. Since cryopreservation is only rarely used, hypothermic storage at $4^0$C and organ culture with preservation at $37^0$C are the main methods used for eye banking worldwide.

**Aim of the study:** Both corneal storage and preservation methods have specific advantages and disadvantages – none of them is superior to the other. A method which could demonstrate all the advantages and avoid the disadvantages of both cold storage and organ culture would be thus highly desirable. We performed an experimental study in porcine corneas to assess the use of Tiprotec, a new storage solution for corneal preservation and cold storage. We compared the resultant endothelial cell morphology and viability with this obtained after preservation of the ex-vivo corneas with both standard

techniques: conventional cold storage (using Eusol-C) and organ culture (using culture medium II [K II]).

**Methods:** Ninety porcine corneas were randomly selected for preservation with the 3 storage methods (30 corneas pro method): organ culture (in K II-medium), cold storage (in Eusol-C) and cold storage (in Tiprotec; all solutions supplemented with dextran). Endothelial quality and transparency were examined microscopically on the 1$^{st}$, 10$^{th}$ and 30$^{th}$ day of storage. A subset of 30 corneas (10 corneas from each group) was further submitted to organ culture for 10 more days to test the corneas for their regenerative capacity at favorable conditions (aiming to "simulate" their transplantation). At the end of the experiment 12 randomly selected corneas were submitted to histological and immunohistochemical analysis.

**Results:** After 30 days of storage both endothelial transparency and endothelial cell density were significantly reduced with all preservation media without any significant differences among them. Similarly, a significant decrease was observed in the number of hexagonal and pentagonal cells along with a significant increase in the number of other types of endothelial cells. Of note, these changes were of lower magnitude in the organ cultured corneas. Finally, the percentage of vacuoles containing cells was significantly increased overtime in all media groups, although in the organ culture group this trend was significantly lower both at the 10$^{th}$ and at the 30$^{th}$ day of preservation in comparison to the Tiprotec (p:0.000 and p:0.000 respectively) and the Eusol-C group (p:0.000 and p:0.000 respectively). Similar were the findings in the subset of corneas submitted in organ culture for 10 more days. However, the endothelial cell density remained relatively stable in the organ culture group and the corneas stored in Eusol-C seemed to be stabilized after their transition into organ culture. Interestingly, the comparison of cold storage corneas before and after their subjection to organ culture revealed a significant reduction in the percentage of vacuoles both for Tiprotec (p: 0.012) and Eusol-C (p:0.005) groups. Finally, in the small subset of corneas submitted to histological analysis no significant differences were found among the three groups regarding both their endothelial and epithelial quality.

**Conclusions:** The cryopreservation medium Tiprotec, used until now for the preservation of vascular grafts, was found only mildly inferior to the medium Eusol-C for the hypothermic storage of corneal tissue for an extended period of time up to 30 days. In comparison to organic culture with culture medium KII, both Tiprotec and Eusol-C were

found less effective in preserving endothelial cell quality, as assessed by the morphometric analysis, and endothelial cell viability, as assessed by the degree of vacuolization at least up to the 30$^{th}$ day of storage. However, both Tiprotec- and Eusol-C-preserved corneas demonstrated a certain capacity to recover after their submission in organ culture. In addition, Tiprotec was not inferior in preserving corneal epithelium in comparison to both Eusol-C and organic culture medium K II. Since this is the first report about the potential use of Tiprotec as a hypothermic storage medium, further studies in human corneas will be needed to confirm our findings and to support its use as an alternative solution in the everyday praxis of eye banking.

## 8. Zusammenfassung

**Einleitung:** Hornhauttransplantation, auch als Keratoplastik benannt, ist die einzige wirksame Behandlung für die meisten Krankheiten, die irreversiblen Hornhautschäden verursachen und schließlich zu Hornhautblindheit führen. Zur Lagerung und Konservierung von Spenderhornhautscheiben stehen heute drei Methoden zur Verfügung: Hypothermie (Kühllagerung), Organkultur und Kryokonservierung. Da die Kryokonservierung nur selten angewendet wird, sind die Kühllagerung bei $4^0$ C sowie die Organkultur bei $37^0$ C die wichtigsten Methoden, die weltweit für den Augenbanking eingesetzt werden.

**Ziel der Studie:** Beide Methoden der Hornhautkonservierung haben konkrete Vor- sowie Nachteile – keine ist der anderen überlegen. Eine Methode, die alle die Vorteile hätte und die Nachteile sowohl der Kühllagerung als auch der Organkultur vermeiden könnte, wäre daher erwünschst.

Wir führten eine experimentelle Studie an Schweine-Hornhäuten durch, um die Verwendung von Tiprotec, eines neuen Kryopräservationsmedium zu untersuchen.

Zu diesem Zweck wurden die Morphologie sowie die Lebensfähigkeit der Endothelzellen der in Tiprotec gelagerten Hornhäute mit der von Hornhäuten die mit zwei Standardtechniken d.h. konventionelle Kühllagerung in Eusol-C sowie Organkultur in Kulturmedium K II erhalten wurden verglichen.

**Methoden:** Neunzig Schweinehornhäuten wurden nach dem Zufallsprinzip zur Konservierung mit den drei Speichern Methoden ausgewählt (30 Hornhäute pro Methode): Organkultur (in Kulturmedium K II), Kühllagerung (in Eusol-C) und

Kühllagerung (in Tiprotec; Dextran wurde in alle Lösungen hinzugefügt). Die endotheliale Qualität und Transparenz wurden am 1., 10., und 30. Lagerungstag mikroskopisch untersucht. Eine Untergruppe von 30 Hornhäuten (10 Hornhäute pro Gruppe) wurde für 10 weitere Tage der Organkultur unterzogen, um die Hornhäute auf ihre Regenerationsfähigkeit unter günstigen Bedingungen (mit dem Ziel, ihre Transplantation zu simulieren) zu testen. Am Ende des Experiments wurden 12 zufällig ausgewählte Hornhäute einer histologischen und immunohistochemischen Analyse unterzogen.

**Ergebnisse:** Nach 30 Tagen von Lagerung waren sowohl die Endotheltransparenz als auch die Endothelzellendichte in allen Konservierungsgruppen signifikant reduziert, ohne dass es signifikante Unterschiede zwischen ihnen gab. In ähnlicher Weise wurde eine signifikante Abnahme der Anzahl von hexagonalen und pentagonalen Zellen zusammen mit einer signifikanten Zunahme der Anzahl von Endothelzellen anderer Morphologie beobachtet. Bemerkungswerteweise waren diese Veränderungen für die organkulturierte Hornhäute signifikant niedriger. Schließlich waren die vakuolenhaltige Zellen in allen Mediengruppen über die Zeit signifikant vermehrt, obwohl dieser Trend in der Organkulturgruppe sowohl am 10. als auch am 30. Tag der Konservierung im Vergleich zur Tiprotec (p:0.000 und p:0.000 beziehungsweise) und Eusol-C (p:0.000 und p:0.000 beziehungweise) deutlich geringer ausfiel. Ähnlich waren die Befunde in der Hornhaut-Untergruppe, die in der Organkultur für 10 weitere Tage eingereicht wurde. Die Endothelzellendichte blieb jedoch in der Organkulturgroup relativ stabil und die in Eusol-C gespeicherten Hornhäute schienen nach ihrem Übergang in die Organkultur stabilisiert zu sein. Interessanteweise zeigte der Vergleich von Kühllagerungshornhäuten vor und nach der Organkultivierung eine signifikante Reduktion des Vakuolenanteils sowohl bei Tiptotec (p: 0.012) als auch bei Eusol-C (p:0.005) Gruppen. In den Hornhäuten, die einer histologischen Analyse unterzogen wurden, wurde schließlich keine signifikante Unterschied zwischen den drei Gruppen hinsichtlich ihrer endothelialen sowie epithelialen Qualität gefunden.

**Zusammenfassung:** Das bisher zur Konservierung von Gefäßgewebe verwendete Kühllagerungsmedium Tiprotec wurde für die hypothermische Lagerung von Hornahautgewebe über einen längeren Zeitraum bis zu 30 Tagen nur geringfügig weniger effektiv als Esuol-C gefunden. Im Vergleich zur Organklultur mit dem Kulturmedium K II erwiesen sich sowohl Tiprotec als auch Eusol-C als weniger wirksam bei der Erhaltung der endothelialen Zellqualität, wie durch die morphometirsche Analyse beurteilt wurde,

sowie bei der Endothelzellvitalität als Grad der Vakuolisierung mindestens bis zu der 30. Tag der Lagerung gemessen wurde. Jedoch zeigten sowohl Tiprotec- als auch Eusol-C-konservierte Hornhäute eine bestimmte Fähigkeit, sich nach ihrer Verabreichung in der Organkultur zu erholen. Darüber hinaus war Tirpotec sowohl dem Eusol-C als auch dem organischem Kulutrmedium K II beim Erhalt des Hornhautepithels nicht unterlegen. Da dies der erste Bericht über die mögliche Verwendung von Tiprotec als hypothermisches Speichermedium ist, werden weitere Studien erforderlich sein, um unsere Ergebnisse zu bestätigen und die Verwendung von Tiprotec als eine alternative Lösung in der täglichen Praxis des Augenbankwesens zu unterstützen.

## 9. Abbreviations

**PKP**: Penetrating Keratoplasty

**M-K medium** : McCarey-Kaufman medium

**K II**: Culture Medium II

**BSS**: Balanced Salt Solution

## 10. Tables



## 11. Figures

## 12. References


Acquart S, Gain P, Zhao M, Gavet Y, Defreyn A, Piselli S, Garraud O, Thuert G**. Endothelial morphometry by image analysis of corneas organ cultured at 31$^0$C. Invest Ophthalmol.** Vis Sci 2010;51 (3):1356-1364

Albon J, Tullo AB, Aktar S, Boulton ME.
**Apoptosis in the endothelium of human corneas for transplantation.**
Invest Opthalmol Vis Sci 2000 ;41 (10):2887-2893

Arentsen JJ, Morgan B, Green WR.
**Changing indications for keratoplasty.** Am J Ophthalmol 1976;81 (3):313-318

Armitage WJ.
**Developments in corneal preservation.** In Krieglstein GK, Weinreb RN (editors) Corneal and external eye disease. Springer-Verlag Berlin Heidelberg 2008:101-109

Armitage WJ.
**Assessment of corneal quality by eye banks.** J Ophthalmic Vis Res 2011;6 (1):3-4

Armitage WJ.
**Preservation of human cornea.** Transfus Med Hemother 2011;38 (2):143-147

Armitage WJ, Dick AD, Bourne WM.
**Predicting endothelial cells loss and long-term corneal graft survival.** Invest Ophthalmol Vis Sci 2003;44 (8):3326-3331

Armitage WJ, Tullo AB, Ironside JW.
**Risk of Creutzfeldt-Jakob disease transmission by ocular surgery and tissue transplantation.** Eye (Lond) 2009;23 (10):1926-1930

Bahn CF, Falls HF, Varley GA, Meyer RF, Edelhauser HF, Bourne WM. **Classification of corneal endothelial disorders based on neural crest origin.** Ophtalmology 1984;91 (6):558-563

Bahn CF, Glassman RM, MacCallum DK, Lillie JH, Meyer RF, Robinson BJ, Rich NMl.
**Postnatal development of corneal endothelium.** Invest Ophthalmol Vis Sci 1986;27 (1):44-51

Basak S, Prajna NV.

**A prospective, in vitro, randomized study to compare two media for donor corneal storage**. Cornea 2016,35 (9):1151-1155

Bednarz J, Doubilei P, Wollnik P, Engelmann K.

**Effect of three different media on serum free culture of donor corneas and isolated human corneal endothelial cells.** Br J Ophthalmol 2001;85 (12):1416-1420

Benetz BA, Gal RL, Ruedy KJ, Rice C, Beck RW, Kalaijan AD, Lass GH.
**Specular microscopy ancillary study methods for donor endothelial cell density determination of donor cornea study images.** Curr Eye Res 2006;31 (4):319-327

Bigger SLL.
**An inquiry into the possibility of transplanting the cornea, with the view of relieving blindness caused by several diseases of that structure**. J Med Soc Dublin 1837;11: 408-417

Billingham RE, Brent L, Medawar PB.
**The antigenic stimulus in transplantation immunity.** Nature. 1956 (4532);178:514-9

Böhnke M.

**Donor tissue for keratoplasty. Report of experiences by the Hamburg cornea bank.** Klin Monbl Augenheilkd 1991;198 (6):562-571

Bourne WM.

**Biology of the corneal endothelium in health and disease.** Eye 2003;17 (8):912-918

Bourne WM, Hodge DO, Nelson LR.

**Corneal endothelium five years after transplantation.** Am J Ophthalmol 1994;118 (2):185-196

Bourne WM, Nelson LR, Maguire LJ, Baratz KH, Hodge DO.

**Comparison of Chen medium and Optisol-GS for human corneal preservation at 4°C. Results of transplantation.** Cornea 2001;20 (7):683-686

Bonanno JA.

**Molecular mechanisms underlying the corneal endothelial pump.** Exp Eye Res 2012;95 (1):2-7

Brunette I, Le François M, Tremblay MC, Guertin MC.

**Corneal transplant tolerance of cryopreservation.** Cornea 2001;20 (6):590-596

Camposampiero D, Tiso R, Zanneti E, Ruzza A, Bruni A, Ponzin D.

**Improvement of human corneal endothelium in culture after prolonged hypothermic storage.** Eur J Ophthalmol 2003;13 (9-10):745-751

Chen W, Lin Y, Zhang X, Wang L, Liu M, Liu J, Ye Y, Sun L, Ma H, Qu J.

**Comparison of fresh corneal tissue versus glycerin-cryopreserved corneal tissue in deep anterior lamellar keratoplasty.** Invest Ophthalmol Vis Sci 2010;51 (2):775-781

Chirila TV, Hicks CR.

**The origins of the artificial cornea: Pellier de Quengsy and his contribution to the modern concept of keratoprosthesis.** Gesnerus 1999;56 (1-2):96-106

Claerhout I, Maas H, Pels E.

**European Eye Bank Association Directory Report, 18th ed.2010.** www.europeraneyebanks.org

Crawford AZ, Patel DV, McGhee CNJ.

**A brief history of corneal transplantation: From ancient to modern.** Oman J Ophthalmol 2013;6 (Suppl 1):S12-S17

D'Alessandro AM, Southard JH, Love RB, Belzer FO.

**Organ preservation.** Surg Clin North Am 1994;74 (5):1083-1095

Damji KF, Rootman J, White VA, Dubord PJ, Richards JS.

**Changing indications for penetrating keratoplasty in Vancouver, 1978–87.** Can J Ophthalmol 1990;25 (5): 243-248

Darwin E.

**Zoonomia, or, the laws of organic life.** Dublin: Printed for P. Byrne, 1796-1800

De Bogert DV, Ni Dhubhghaill S, Koppen C, Tassignon MJ, Zakaria N.

**A review of the evidence for in vivo corneal endothelial regeneration.** Surv Ophthalmol 2018;63 (2):149-165

DeVoe AG.

**Ramon Castroviejo.** Trans Am Ophthalmol Soc 1987; 85:6-8

Doughman DJ, Harris JE, Schmitt MK.

**Penetrating keratoplasty using $37^0$C organ cultured cornea.** Trans Sect

Ophthalmol Am Acad Ophthalmol Otolaryngol 1976;81 (5):778-793

Eastlund T.
**Infectious disease transmission through tissue transplantation: Reducing the risk through donor selection.** Cell Tranplant 1995;4 (5):455-477

EBAA. **Medical Standards.** Published by: EBAA 1015 18th Street, NW, Suite 1010, Washington, DC 20036, USA October 2011. http://restoresight.org/wp-content/uploads/2011/11/Medical-Standards-October-2011.pdf

EEBA. **Minimum Medical Standards.** Revision 2. Operative from 1.1.2016. http://eeba.eu/downloads/EEBA%20Minimum%20Medical%20StandardsRev02-2016.pdf

Ehlers H, Ehlers N, Hjortdal JO.
**Corneal transplantation with donor tissue kept in organ culture for 7 weeks.** Acta Ophthalmol Scand 1999;77 (3):277-278

Ehlers N, Sperling S, Olsen T.
**Post-operative thickness and endothelial cell density in cultivated, cryopreserved human corneal grafts.** Acta Ophthalmol (Copenh) 1982;60 (6):935-944

Filatov VP.
**Transplantation of the cornea from preserved cadavers' eyes.** Lancet 1937;229:1395-1397

Garbe S, Zatschler B, Müller B, Dieterich P, Ebner A, Rauen U, Matschke K, Deussen A.
**Preservation of human artery function following prolonged cold storage with a new solution.** J Vasc Surg 2011;53 (4):1063-70

Greenbaum A, Hasanay SM, Rootman D.

**Optisol vs Dexsol as storage media for preservation of human corneal epithelium.** Eye 2004;18 (5):519-524

Halberstadt M, Böhnke M, Athmann S, Hagenah M.

**Cryopreservation of human donor corneas with dextran.** Invest Opthalmol Vis Sci 2003;44 (12):5110-5115

Hassan SS, Wilhelmus KR.

**Eye-banking risk factors for fungal endophthalmitis compared with bacterial endophthalmitis after corneal transplantation.** Am J Ophthalmol 2005;139 (4):685-690

Haug K, Azqueta A, Johnsen-Soriano S, Shahdadfar A, Drolsum LK, Moe MC, Roger MT, Romero FJ, Collins AR, Nicolaissen B.

**Donor cornea transfer from Optisol GS to organ culture storage: a two-step procedure or increase donor tissue lifespan.** Acta Ophthalmol 2013;91 (3):219-225

Hempel B, Bednarz J, Engelmann K.

**Use of a serum-free medium for long-term storage of human corneas. Influence on endothelial cell density and corneal metabolism.** Graefes Arch Clin Exp Ophtalmol 2001;239 (10):801-805

Hermel M, Salla S, Fuest M, Walter P.

**The role of corneal endothelial morphology in graft assessment and prediction of endothelial loss during organ culture of human donor corneas.** Acta Ophthalmol 2017;95 (2):205-210

Hirneiss C, Schumann RG, Grüterlich M, Welge-Luessen UK, Kampik A, Neubauer AS.

**Endothelial cell density in donor corneas: a comparison of automatic software programs with manual counting.** Cornea 2007;26 (1):205-210

Ing JJ, Ing HH, Nelson LR, Hodge DO, Bourne WM.

**Ten-year postoperative results of penetrating keratoplasty.** Ophthalmology 1998;105 (10):80-83

Jang JH, Chang SD.

**Tectonic deep anterior lamellar keratoplasty in impending corneal perforation using cryopreserved cornea.** Korean J Ophthalmol 2011;25 (2):132-135

Jeng BH.

**Preserving the cornea: corneal storage media.** Curr Opin Ophthalmol 2006;17 (4):332-337

Johnson DH, Bourne WM, Campbell RJ.

**The ultrastructure of Descemet's membrane. II. Aphakic bullous keratopathy.** Arch Ophthalmol 1982; 100 (12): 1948-1951.

Joyce NC.

**Proliferative capacity of the corneal endothelium.** Pro Retin Eye Res 2003;22 (3):359-389

Kanavi MR, Javadi MA, Chamani T.

**Specular microscopic features of corneal endothelial vacuolation.** J Opthalmic Vis Res 2011;6 (1):5-7

Kanavi MR, Ali Javadi MA, Chamani T, Fahim P, Javadi F.

**Comparing quantitative and qualitative indices of the donated corneas maintained in Optisol-GS with those kept in Eusol-C.** Cell Tissue Bank 2015;16 (2):243-247

Kang PC, Klintworth GK, Kim T, Carlson AN, Adelman R, Stinnett S, Afshari NA .
**Trends in the indications for penetrating keratoplasty, 1980–2001.** Cornea 2005;24 (7):801-803

Kaufmann HE, Escapini H, Capella JA, Robbins JE, Kaplan M.

**Living preserved corneal tissue for penetrating keratoplasty.** Arch Ophthalmol 1966;76(4):471-476

Kissam R.
**Ceratoplastice in man.** N Y J Med 1844; 2: 281-282

Lamm V, Hara H, Mammen A, Dhaliwal D, Cooper DK.
**Corneal blindness and xenotransplantation.** Xenotransplantation 2014;21 (2):99-114

Lang F, Foller M, Lang KS, Lang PA, Ritter M, Gulbins E, Verenivov A, Huber SM.
**Ion channels in cell proliferation and apoptotic cell death.** J Membr Biol 2005; 205(3):147-157

Lass JH, Ruedy KJ, Benetz BA, Beck RW, Baratz KH, Holland EJ, Kalajian A, Kollman C, Manning FJ, Mannis MJ, McCoy K, Montoya M, Stulting D, Xing D; Cornea Donor Study Group.
**An evaluation of image quality and accuracy of eye bank measurement of donor cornea endothelial cell density in the Specular Microscopy Ancillary Study.**
Ophtalmology 2005;112 (3):431-440

Layer N, Cevallos V, Maxwell AJ, Hoover C, Keenan JD, Jeng BH.

**Efficacy and safety of antifungal additives in Optisol-GS corneal storage medium.** JAMA Ophthalmol 2014;132 (7):832-837

Le R, Yucel N, Khattak S, Yucel YH, Prud'homme GJ, Gupta N.

**Current indications and surgical approaches to corneal transplants at the University of Toronto: A clinical-pathological study.** Can J Ophthalmol 2016;52 (1):74-79

Lindstrom RL, Kaufman HE, Skelnik DL, Laing RA, Lass JH, Musch DC, Trousdale MD, Reinhart WJ, Burris TE, Sugar A, Davis RM, Hirokawa K, Smith T, Gordon JF .

**Optisol corneal storage medium.** Am J Opthalmol 1992;114 (3):345-356

McCarey BE, Kaufman HE.

**Improved corneal storage.** Invest Ophtalmol Vis Sci 1974;13 (3):165-173

Medawar PB.

**Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye.** Br J Exp Pathol. 1948;29 (1):58-69

Meek K, Knupp C.

**Corneal structure and transparency.** Prog Retin Eye Res 2015;49:1-16

Mergler S, Pleyer U.

**The human corneal endothelium: New insights into electrophysiology and ion channels.** Prog Retin Eye Res 2007;26 (4):359-378

Mertens S, Noll T, Spahr R, Krutzfeldt A, Piper HM.
**Energetic response of coronary endothelial cells to hypoxia.** Am J Physiol 1990;258 (3):H689-694

Moffat SL, Cartwright VA, Stumpf TH.
**Centennial review of corneal tranplantation.** Clin Experiment Ophthalmol 2005;33 (6):642-657

Nejepinska J, Juklova K, Jirsova K.
**Organ culture, but not hypothermic storage, facilitates the repair of the corneal endothelium following mechanical damage.** Acta Ophthalmol 2010:88 (4):413-419

Nelson LR, Hodge DO, Bourne WM.
**In vitro comparison of Chen medium and Optisol-GS medium for human corneal storage.** Cornea 2000;19 (6):782-787

Nishida T. Cornea. In: Krachmer J, Mannis M, Holland E, editors.
**Cornea: Fundamentals, Diagnosis and Management.** Vol 1. Philadelphia, PA: Elsevier-Mosby 2005: 3-26

Nishimura Y, Romer LH, Lemasters JJ.
**Mitochondrial dysfunction and cytoskeletal disruption during chemical hypoxia to cultured rat hepatic sinusoidal endothelial cells: the PH paradox and cryoprotection by glucose, acidotic PH and glycine.** Hepatology 1998;27 (4):1039-1049

Nishimura JK, Hodge DO, Bourne WM.
**Initial endothelial cell density and chronic endothelial loss rate in corneal transplants with late endothelial failure.** Ophthalmology 1999;106 (10):1962-1965

Nucci P, Brancato R, Mets MB, Shevell SK.
**Normal endothelial cell density range in childhood.** Arch Ophtalmol 1990;108 (2):247-248

Oliva MS, Schottman T, Gulati M.
**Turning the tide of corneal blindness.** Indian J Ophthalmol 2012;60 (5):423-427

Parekh M, Salvalaio G, Ferrari S, Amoureux MC, Albrecht C, Fortier D, Ponzin D.
**A quantitative method to evaluate the donor corneal tissue quality used in a comparative study between two preservation media.** Cell Tissue Bank. 2014;15 (4):543-554

Parekh M, Ferrari S, Ruzza A, Pugliese M, Ponzin D, Salvalaio G.
**A portable device for measuring donor corneal transparency in eye banks.** Cell Tissue Bank 2014;15 (1):7-13

Parekh M, Ferrari S, Salvalaio G, Ponzin D.
**Synthetic versus serum-based medium for corneal preservation in organ culture: a comparative study between 2 different media.** Eur J Ophthalmol 2015;25 (2):96-100

Pahor D, Gracner B, Falez M, Gracner T.
**Changing indications for penetrating keratoplasty over a 20-years period, 1985-2004.** Klin Monbl Augenheilkd 2007;224 (2):110-114

Patel SV.

**Graft survival after penetrating keratoplasty.** Am J Ophthalmol. 2011;151 (3): 397-398

Payne JW.
**New directions in eye banking.** Trans Am Ophthalmol Soc 1980;78:983-1026

Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS.
**Human corneal endothelial cell expansion for corneal endothelium transplantation: An overview.** Transplantation 2011;91 (8):811-819

Pels L.
**Organ culture: the method of choice for preservation media.** Br J Ophthalmol. 1997;81 (7):523-525

Pels E, Schuchard Y.
**Organ-culture preservation of human corneas.** Doc Ophtalmol 1983;56 (1-2):147-153

Pels E, Schuchard Y.
**The effects of high molecular weight dextran on the preservation of human corneas.** Cornea 1984-1985;3 (3):219-227

Pels E, Beele E, Claerhout I.
**Eye bank issues: II. Preservation techniques: warm versus cold storage.** Int Ophthalmol 2008;28 (3):155-163

Pipparelli A, Thuret G, Toubeau D, He Z, Piselli A, Lefevre S, Gain P, Muraine M.
**Pan-corneal endothelial viability assessment: application to endothelial grafts predissected by eye banks.** Cornea 2011;52 (8):6018-6025

Pless-Petig G, Singer B, Rauen U.
**Cold storage of rat hepatocyte suspensions for one week in a customized cold storage solution-Preservation of cell attachment and metabolism.** PLoS One 2012;7 (7):e 40444

Pless-Petig G, Broering R, Canbay A, Sauer IM, Paul A, Rauen U.

**Kalterung und Kryokonservierung primarer humaner Hepatozyten in modifizierter TiProtec-Loesung.** Z. Gastroenterol 2016;54 (12): 1343-1404

Polisetti N, Joyce NC.

**The culture of limbal stromal cells and corneal endothelial cells.** Methods Moll Biol 2013;1014: 131-139

Powers RM, Linden JV.

**Tissue Banking**. In: Rossi's Principles of Transfusion Medicine. Simon TL, McCullough J, Snyder EL, Solheim BG, Strauss RG (Editors). Wiley Blackwell 2016:474

Preusse CJ, Gebhard MM, Bretschneider HJ.

**Myocardial "equilibration processes" and myocardial energy turnover during initiation of artificial cardiac arrest with cardioplegic solution- reasons for a sufficiently long cardioplegic perfusion**. Throrac Cardiovasc Surg 1981;29 (2):71-76

Price M, Price F.

**Monitoring and maintaining endothelial cell health. Expert advice on diagnostic tests and their clinical relevance.** Ophthalmology Management 2012; 16: 40-44

Reisinger F.

**Die Keratoplastik, ein Versuch zur Erweiterung der Augenheilkunde.** Bayerische Annalen 1824;1:207

Rijneveld WJ, Beekhuis WH, Van Rij G, Rinkel-van Driel B, Pels E.

**Clinical comparison of grafts stored in Mc Carey-Kaufman medium at $4^0$C and in corneal organ culture at $31^0$C.** Arch Ophthalmol 1992;110 (2):203-205

Rijneveld WJ, Remeijer L, van Rij G, Beekhuis H, Pels E.

**Prospective clinical evaluation of McCarey-Kaufman and organ culture cornea preservation media: 14-year follow-up.** Cornea 2008:27 (9):996-1000

Saad HA, Terry MA, Shamie N, Chen ES, Friend DF, Holiman JD, Stoeger C.

**An easy and inexpensive method for quantitative analysis of endothelial damage by using vital dye staining and Adobe Photoshop software.** Cornea 2008;27 (7):818-824

Schroeter J, Rieck P.

**Endothelial evaluation in the cornea bank.** Dev Opthalmol 2009;43:47-62

Shi W, Liu M, Gao H, Li S, Wang T, Xie L.

**Penetrating keratoplasty with small-diameter and glycerin-cryopreserved grafts for eccentric corneal perforations.** Cornea 2009;28 (6):631-637

Sibayan ASB, Garcia-Arenal CP, Corpus KD, Manlongat JRG, Ibanez MBB, Padilla DB, Dy Liacco JU.

**Serial endothelial cell count of donor corneal buttons in Optisol-GS.** Procedia Chemistry 2015;14:394-397

Smith VA, Johnson T.

**Evaluation of Megacell II MEM as a storage medium for corneas destined for transplantation.** Ophthalmic Res 2010;43 (1):18-25

Smith VA, Johnson T.

**Evaluation of an animal product-free variant of MegaCell$^{TM}$MEM as a storage medium for corneas destined for transplantation.** Ophtalmic Res 2010;43 (1):33-42

Smith V, Johnson T.

**Identification and evaluation of a thinning agent compatible with MegaCell DCS, an animal product-free corneal storage medium.** Graefes Arch Clin Exp Ophthalmol 2012;250 (12):1777-1786

Soni NG, Hoover CK, Da Silva H, Jeng BH.

**Preservation of the corneal epithelium in different corneal storage media.** Cornea 2015;34 (11):1400-1403

Spelsberg H, Reinhard T, Sundmacher R.

**Hornhautepithelschädigung bei langer Transplantatverwildauer in dextranhaltigem Organkulturmedium eine prospektive Studie.** Klin Monbl Augenheilkd 2002;219 (6):417-421

Sperling S.

**Evaluation of the endothelium of human donor corneas by induced dilatation of intercellular spaces.** Graefes Arch Clin Exp Ophthalmol 1986;224 (5):428-434

Summerlin WT, Miller GE, Harris JE, Good RA.

**The organ-cultured cornea: an in vitro study.** Invest Ophthalmol Vis Sci 1973;12 (3):176-180

Szafilk J, Liberek I, Brix M.

**Corneal storage methods.** Transplantation Proceedings 2000;32 (6):1424-1425

Thomas PA, Geraldine P.

**Infectious keratitis.** Curr Opin Infect Dis 2007;20 (2):129-141

Thuret G, Manissolle C, Acquart S, Le Petit JC, Campos-Guyotat L, Doughty MJ, Gain P.

**Is manual counting of the corneal endothelial cell density in eye banks still acceptable? The French experience.** Br J Ophthalmol 2003;87 (12):1481-1486

Thuret G, Manissolle C, Herrag S, Deb N, Campos-Guyotat L, Gain P, Acquart S.

**Controlled study of the influence of storage medium type on endothelial assessment during corneal organ culture.** Br J Ophthalmol 2004;88 (4):579-581

Veres G, Hegedűs P, Barnucz E, Schmidt H, Radovits T, Zöller R, Karck M, Szabó G.

**TiProtec preserves endothelial function in a rat model.** J Surg Res 2016;200 (1):346-355

Von Bomhard A, Elsässer A, Ritschl M, Schwarz S, Rotter N.

**Cryopreservation of endothelial cells in various cryoprotective agents and media-vitrification versus slow freezing methods.** PLoS One 2016;11 (2):1-15

Wagoner MD, Gonnah el-S.

**Corneal graft survival after prolonged storage in Optisol – GS.** Cornea 2005;24 (8):976-979

Wang J, Hasenfus A, Schirra F, Bohle RM, Seitz B, Szentmary N.

**Changing indications for penetrating keratoplasty in Homburg / Saar form 2001 to 2010-histopathology of 1,200 corneal buttons.** Graefes Arch Clin Exp Ophthalmol 2013;251 (3):797-802

Whitcher HP, Srinivasan M, Upadhyay MP.

**Corneal blindness: a global perspective.** Bull World Health Organ 2001;79 (3):214-221

Wilbring M, Tuqtekin SM, Zatschler B, Ebner A, Reichenspurner H, Kappet U, Matschke K, Deussen A.

**Preservation of endothelial vascular function of saphenous vein grafts after long-time storage with a recently developed potassium-chloride and N-acetylhistidine enriched storage solution.** Throrac Cardiovasc Surg 2013;61 (8):656-62

Wille T, de Groot H, Rauen U.

**Improvement of the cold storage of blood vessels with a vascular preservation solution. Study in porcine aortic segments.** J Vasc Surg 2008;47 (2):422-31

Wilson SE, Bourne WM.

**Corneal preservation.** Surv Ophthalmol 1989:33 (4);237-259

Writing Committee for the Cornea Donor Study Research Group, Mannis MJ, Holland EJ, Gal RL, Dontchev M, Kollman C, Raghinaru D, Dunn SP, Schultze RL, Verdier DD, Lass JH, Raber IM, Sugar J, Goronov MS, Sugar A, Stulting RD, Montoya MM, Penta JG, Benetz BA, Beck RW.

**The effect of donor age on penetrating keratoplasty for endothelial disease: graft survival after 10 years in the Cornea Donor Study.** Ophthalmology 2013; 120 (12):2419-2427

Wu S, Wohlschlaeger J, de Groot H, Rauen U.

**Evaluation of a modified HTK solution containing the new iron chelator LK 614 in an isolated rat liver perfusion model.** J Invest Surg 2009;22 (5):340-347

Yee RW, Matsuda M, Schultz RO, Edelhauser HF.

**Changes in the normal corneal endothelial cellular pattern as a function of age.** Curr Eye Res 1985;4 (6):671-678

Zatschler B, Dieterich P, Muller B, Kasper M, Rauen U, Deussen A.

**Improved vessel preservation after 4 days of cold storage: experimental study in rat arteries.** J Vasc Surg 2009;50 (2):397-406

Zirm EK.

**Eine erfolgreiche totale keratopastik.** J Refract Corneal Surg 1906; 5: 258-261

## 13. Danksagung

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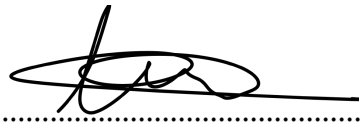
## 14. Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

## 15. Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift:  .....