Charles University in Prague
Faculty of Science
Department of Animal Physiology and Developmental Biology

THE INFLUENCE OF THE STORAGE CONDITIONS ON THE QUALITY OF HUMAN CORNEAS PREPARED FOR GRAFTING

Jana Nejepínská

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THESIS LEADER:

Mgr. Kateřina Jirsová, PhD

Laboratory of the Biology and Pathology of the Eye

1st Faculty of Medicine, Charles University in Prague, Czech Republic

Ocular Tissue Bank, General Teaching Hospital, Prague, Czech Republic

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ABSTRACT

Corneal quality and its preparation for keratoplasty can determine significantly its functionality and survival of the graft. Corneas are generally stored in media at reduced temperature (hypothermic storage) or at 31-37 °C (tissue culture storage). Among the important factors for corneal graft survival is the presence of Fas ligand in the corneal endothelial cells. The quality of the storage medium could be significantly influenced by metabolites of the stored tissue. Nitric oxide produced by corneal cells can have cytoprotective or cytotoxic effects depending upon its concentration.

The aims of this study were to examine the behaviour of injured corneas (by the induction of a lesion in the corneal endothelium) under tissue culture or hypothermic conditions, and to characterize the repair process and its rate using histological, microscopic and morphometric approaches. Real-time RT-PCR and immunohistochemistry were used to detect the changes in the expression and localization of Fas ligand depending on time of storage and storage conditions. Nitric oxide production by corneas stored up to 3 weeks in tissue culture or hypothermic media was followed using the Griess reaction.

Our results show that the repair process occurs only during tissue culture storage. A lesion area of 1 mm^2 was fully repaired within 5 days; however, the endothelial cell density in the wounded area continues to increase up to 3 weeks post-injury. These results indicate that corneas with presence of dead cells or with endothelial defects should be stored under tissue culture conditions. The repair kinetics enable us to estimate the storage times the storage times that are sufficient for repairing larger defects of the corneal endothelium.

We discovered that Fas ligand is maintained on the plasma membrane for at least 3 weeks of tissue culture storage. Fas ligand is expressed in the endothelium of corneas stored under hypothermic conditions, as well, but partial inhibition of protein transport to the plasma membrane occurs.

We determined the level of nitric oxide in corneas stored under tissue culture or hypothermic conditions and found that these concentrations are lower that those that could cause cytotoxic effects, especially in the endothelial cells.

The results of this work have practical consequences that can be useful for further improving of tissue quality for keratoplasty and increasing our understanding of the behaviour of corneas stored under different conditions and for different lengths of time.

ABSTRAKT

Kvalita rohovkového štěpu a způsob jeho přípravy pro transplantaci může výrazně ovlivnit funkčnost a přežívání transplantátu. Rohovky se téměř výhradně uchovávají v médiích při snížené teplotě (hypotermické skladování), nebo při 31–37 °C (tkáňové kultury). Jedním z důležitých faktorů, podílejících se na přežívání štěpu, je přítomnost Fas ligandu v endotelových buňkách rohovky. Kvalita skladovacího média může být výrazně ovlivněna i metabolity uchované tkáně. Cytoprotektivně či naopak cytotoxicky může v závislosti na koncentraci působit oxid dusnatý produkovaný buňkami rohovky.

Cílem této práce bylo na modelové situaci (indukce léze v rohovkovém endotelu), zjistit, jakým způsobem se chovají poškozené rohovky uchované metodou tkáňových kultur či v hypotermických podmínkách a charakterizovat proces obnovy rohovkového endotelu, včetně rychlosti její reparace. Reparace byla charakterizována pomocí histologických, mikroskopických a morfometrických přístupů. Pomocí real-time RT-PCR a imunohistochemické metody byly sledovány změny v expresi a lokalizaci Fas ligandu v závislosti na typu skladovacího média a délce uchování rohovky. Koncentrace oxidu dusnatého produkovaného rohovkou skladovanou až po dobu tří týdnů metodou tkáňových kultur i v hypotermických podmínkách byla určena pomocí Griessovy reakce.

Zjistili jme, že k reparaci endotelu dochází pouze v rohovkách uchovávaných metodou tkáňových kultur. Léze o ploše 1 mm^2 je zcela reparována během 5 dní, ale ke zvyšování density endotelových buněk v postižené oblasti dochází v době do 3 týdnů po poškození. Tyto výsledky ukazují, že rohovky s přítomností mrtvých buněk, či s chybějícími oblastmi endotelových buněk je třeba uchovávat metodou tkáňových kultur. Kinetika reparace umožňuje odhad délky uchování, dostatečně dlouhé pro reparaci rozsáhlejších defektů rohovkového endotelu.

Zjistili jsme, že exprese i přítomnost Fas ligandu v plasmatické membráně zůstává zachována po dobu 3 týdnů uchování v tkáňových kulturách. Fas ligand je exprimován i v rohovkách skladovaných v hypotermických podmínkách, dochází však k částečné inhibici transportu proteinu na plasmatickou membránu.

Stanovili jsme hladinu oxidu dusnatého produkovaného buňkami rohovek uchovávaných v tkáňových kulturách i hypotermických podmínkách a zjistili jsme, že tyto koncentrace jsou nižší než koncentrace, které by mohly vyvolat poškození rohovky, zejména endotelu.

Výsledky této práce jsou přínosné z praktického hlediska, jejich uplatnění může vést k dalšímu zvýšení kvality tkáně připravované pro transplantaci rohovky. Získané informace, rozšiřují i znalosti struktury a metabolismu rohovky v závislosti na typu použitého skladování.

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1. ABBREVIATION LIST

6A	hexagonality
ACAID	anterior chamber-associated immune deviation
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pairs
cAMP	cyclic adenosine triphosphate
CCTS	Collaborative corneal transplantation studies
CD	cluster of differentiation
cGMP	cyclic guanosine triphosphate
CV	coefficient of variability
DAF	decay accelerating factor
dNTP	deoxyribonucleoside triphosphate
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
ECD	endothelial cell density
ECDA	endothelial cell density of alive cells
ECDD	endothelial cell density of dead cells
EEBA	European eye bank association
E-MEM	Eagle's minimal essential medium
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FADD	Fas-associated protein with death domain
FasL	Fas ligand
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gp	glycoprotein
GSH	glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
IFN-γ	interferon gamma
IL	interleukin

iNOS	inducible nitric oxide synthase
MCP	membrane cofactor protein
MEM	minimal essential medium
MHC	major histocompatibility complex
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NF-κB	nuclear factor - κB
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
OTB	ocular tissue bank
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
TGF-β	tumor growth factor beta
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
UPL	Universal ProbeLibrary
UV	ultra violet

2. INTRODUCTION

Tissue selection and its proper storage are among the key factors for corneal graft survival. The crucial parameter of corneal quality is the density of endothelial cells. In view of the lack of regenerative capacity of the corneal endothelium, it is necessary to guarantee the storage conditions that can at least maintain, or better improve corneal quality.

Although corneal transplantation is the most successful form of solid tissue transplantation, with the success rate exceeding 90 % in low risk cases, in high risk patients the success rate decreases to less than 30 %. For this reason, it is essential to characterize all factors that could negatively influence the graft quality or its survival.

The purpose of this study was to characterize different factors (capacity of the corneal repair, Fas ligand presence in the endothelium, and the level of nitric oxide in the storage medium) that could influence the state of human corneas stored under tissue culture or hypothermic conditions and to find the optimal media for tissue preservation for corneal grafting.

This aims of this study were:

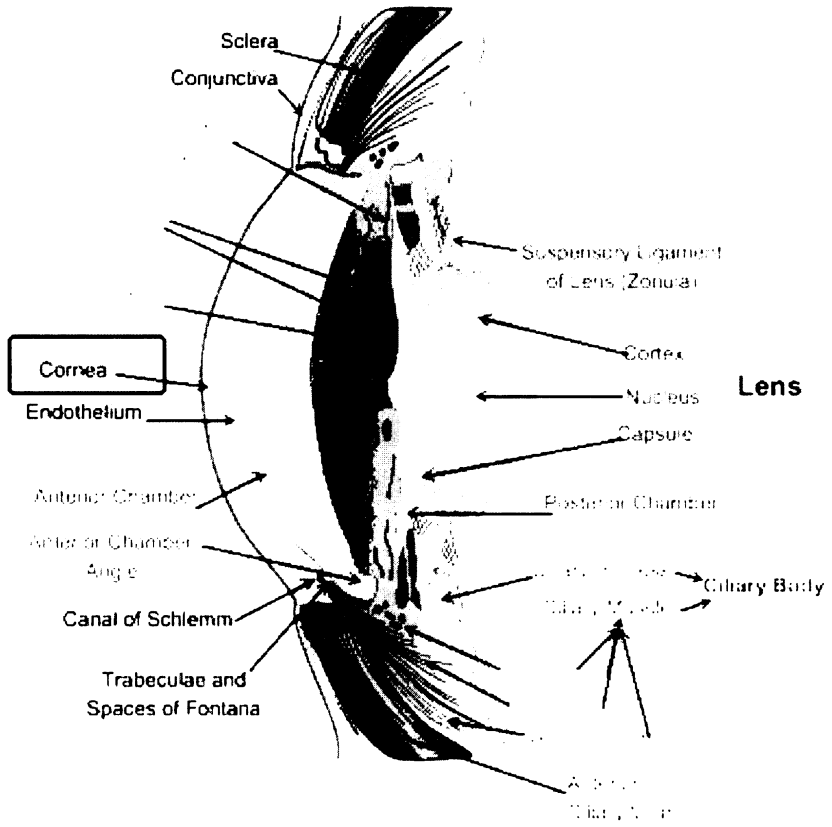
1) To evaluate the reparative capacity of corneas with mechanically induced endothelial damage stored under tissue culture or hypothermic conditions.
2) To determine the expression and localization of the Fas ligand molecule in fresh corneas and in those stored under tissue culture or hypothermic conditions for different periods of time.
3) To assess the level of nitric oxide in the storage medium produced by corneas stored under tissue culture or hypothermic conditions for different periods of time.

3. LITERATURE REVIEW

3.1. CORNEA

The cornea is a transparent avascular tissue exposed to the external environment (Fig. 1). The anterior surface is covered by a tear film and the posterior surface communicates with the aqueous humour. The transparent cornea is continuous with the opaque sclera and the semitransparent conjunctiva. The transition zone between the cornea and the sclera constitutes the limbus, which is highly vascularised and contains a reservoir of stem cells. The cornea is one of the most heavily innervated and most sensitive tissues in the body, with a density of nerve endings about 300 to 400 times greater than in the skin (ROZSA and BEUERMAN 1982).

Figure 1: The anterior segment of the eye; the cornea and surrounding tissue (www.visionsource-brighteyes.com).



The adult human cornea measures 11–12 mm horizontally and 9–11 mm vertically. It is approximately 0.5 mm thick in the centre, and its thickness increases toward the periphery up to about 0.7 mm (MISHIMA 1968). The cornea has the greatest curvature at the centre, and its refractive power is 40-44 dioptres, constituting about

two-thirds of the total refractive power of the eye. The optical properties of the cornea are determined by its transparency, surface smoothness, contour, and refractive index (NISHIDA 2005).

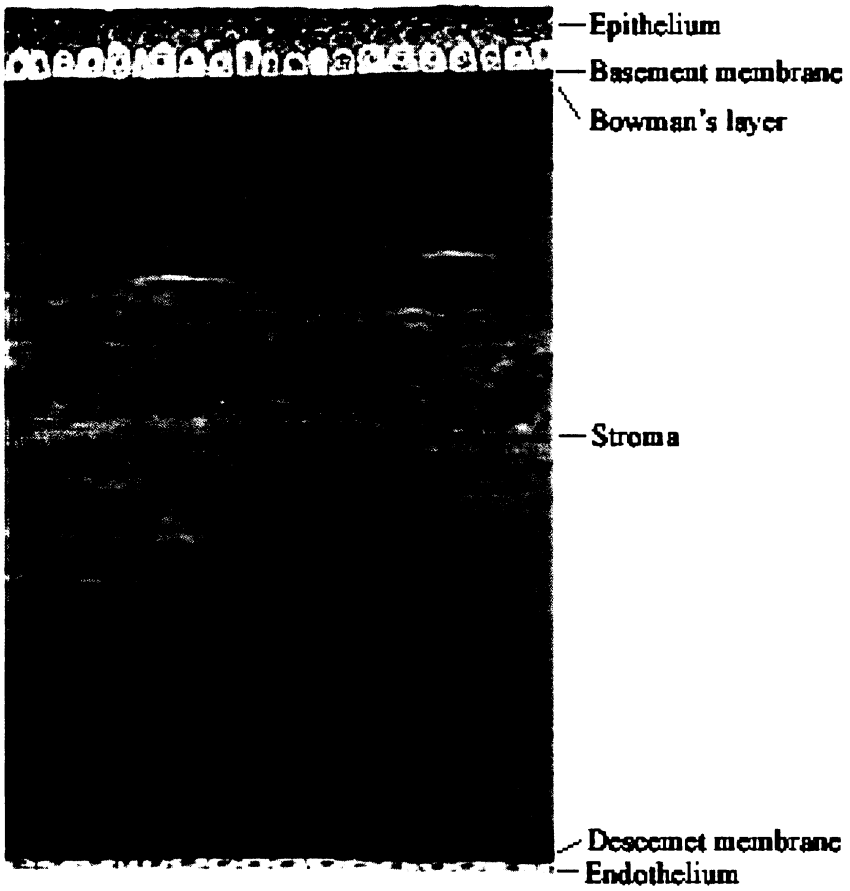
The main functions of the cornea are to enable the entry of light to the inner parts of the eye and to protect the anterior chamber of the eye against mechanical damage and the entry of infectious agent (NISHIDA 2005).

Despite the fact that the cornea is one of the least vasculated tissues in the body, the corneal epithelial and endothelial cells are metabolically active and thus need to be supplied by nutritients. Glucose is supplied by diffusion from the aqueous humour to the whole cornea, even to the epithelial cells. In contrast, oxygen is harvested by the tear fluid, which absorbs it from the air, while a small proportion of oxygen is obtained by diffusion from the aqueous humour and limbal circulation (RILEY 1969, WEISSMAN *et al.* 1981).

3.1.1. Corneal histology

The cornea consists of three different cellular layers (the epithelium, the stroma, including an acellular Bowman's layer, and the endothelium) and two basement membranes (the basement membrane of the epithelium and Descemet's membrane that is the basement membrane of the endothelium) (Fig. 2). Epithelial cells are derived from the epidermal ectoderm, whereas keratocytes and endothelial cells are of neuroectodermal origin. All components of the cornea interact with each other to maintain the integrity and function of the tissue (NISHIDA 2005).

Figure 2: The corneal layers (OTB archive).



Epithelium

The corneal epithelium consists of five or six cellular layers with a thickness of about 50 µm, which represents about 10 % of the total corneal thickness. The outer part is composed of 1-2 layers of superficial cells, followed by 2-3 layers of wing cells and a monolayer of columnar basal cells that adhere to the basement membrane.

The function of the epithelium is to protect the cornea from microbial attack and, with the aid of the tear film, to contribute to the maintenance of the optically smooth corneal surface. Cellular components of the epithelium also play an important role in corneal immunology. Dendritic Langerhans cells are abundant at the periphery but are sparsely present in the central region of the normal cornea (GILLETTE *et al.* 1982). These antigen-presenting cells express HLA class II molecules, and their number can be increased in ocular inflammation or reduced by treatment with corticosteroids. Langerhans cells mediate the immunological rejection of a corneal graft (WHITSETT and STULTING 1984).

Basement membrane of the epithelium

The basal cells of the epithelium adjoin to the 40-60 nm-thick basement membrane and secrete the components necessary for its formation, the most important ones being collagens IV and VII, laminin, fibronectin, heparan sulphate proteoglycans and fibrin (BERMAN *et al.* 1983). The main function of the basement membrane is to fix the polarity of the epithelial cells.

Bowman's layer

Between the epithelial basement membrane and the stroma of human cornea, there is a 12-μm thick acellular layer called Bowman's layer. It is a random arrangement of collagen fibres and is considered to be the anterior portion of the corneal stroma. It is not regenerated after injury and many mammals do not have it at all, so the physiological role of Bowman's layer has not been fully elucidated (NISHIDA 2005).

Stroma

The largest portion of the cornea, more than 90 %, constitutes the stroma. The majority of stroma consists of extracellular matrix (collagens, proteoglycans), while only 2-3 % of total stromal volume is occupied by the keratocytes (OTORI 1967). Many characteristics of the cornea, such as its physical strength, stability and shape, are largely attributable to the anatomical and biochemical properties of the stroma. As an example, the regular arrangement of collagen fibres into parallel lamellae in the stroma provides the transparency of the cornea (MAURICE 1984).

Descemet's membrane

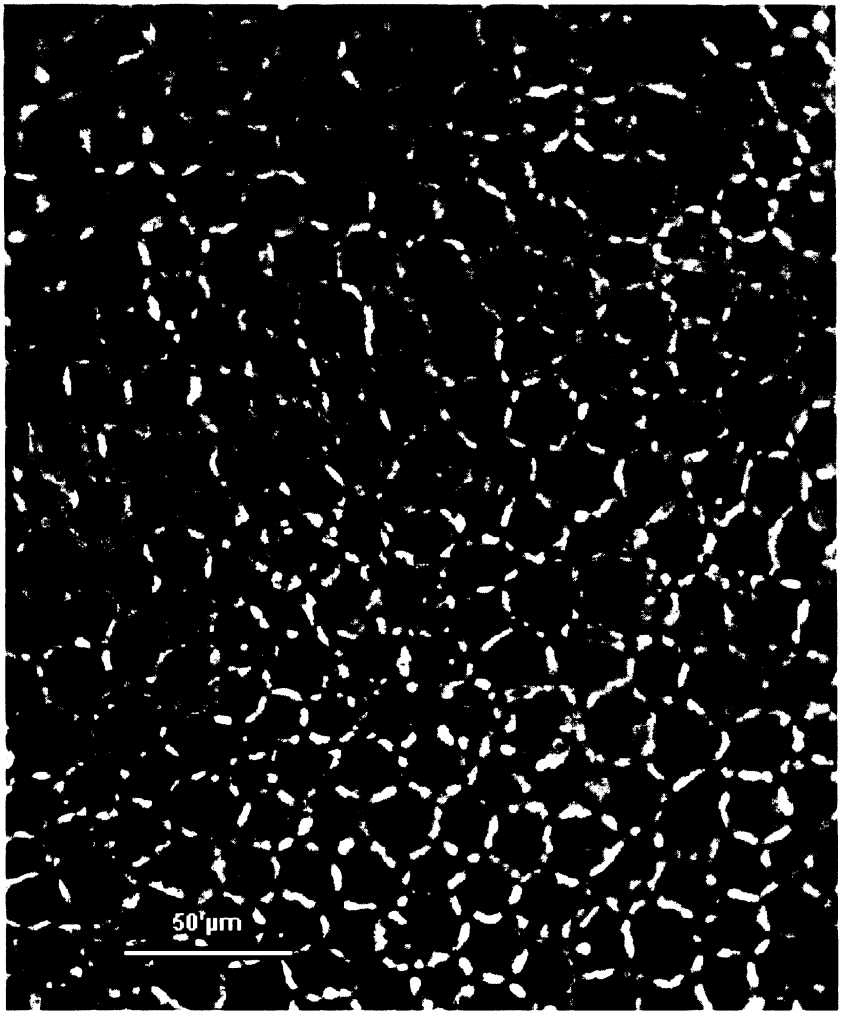
Descemet's membrane is the basement membrane of the corneal endothelium. It gradually increases in thickness from birth (3 μm) to adulthood (8-10 μm), is stratified into thin layers, and does not regenerate. It prevents the influx of the aqueous humour to the stroma and consequent corneal edema (NISHIDA 2005).

Endothelium

The corneal endothelium is a monolayer of cells that covers the posterior surface of Descemet's membrane. These cells are usually hexagonal (Fig. 3), about 20 μm in diameter and 5 μm thick. Endothelial cells contain a large nucleus and abundant cytoplasmic organelles, including mitochondria, endoplasmic reticulum, free ribosomes, and Golgi apparatus, indicating that cells are metabolically active and secretory. The

anterior surface of cells is flat and adjoins to Descemet's membrane, whereas the posterior surface forms microvilli that bulge into the anterior chamber to maximize the area exposed to aqueous humour (NISHIDA 2005).

Figure 3. The hexagonal cells in healthy corneal endothelium (a phase contrast micrograph) (OTB archive).



Corneal endothelial cells stop proliferating in humans (as well as in monkeys and cats and in contrast to rabbits) shortly after birth (NISHIDA 2005). The human newborn has about 6,000 endothelial cells per mm^2 and this number decreases to approximately 4,000 cells per mm^2 at the age of 3-4 years. After this, the number of endothelial cells decreases by about 0.6 % per year (LAULE 1978). Various pathological states, such as inflammatory reactions, corneal dystrophies or intraocular surgery, also lead to a decrease in the endothelial cell count (BOURNE 2001).

The loss of endothelial cells for any reason results in the enlargement of the remaining neighbouring cells and their spreading to cover the defective area, without cell division. This enlargement of some cells causes a variation in cell size, called **polymegatism**. Another parameter that characterizes the condition of the endothelium is termed **pleomorphism** and refers to the different shape of cells. In the normal healthy cornea, more than 50 % of cells are hexagonal, but after endothelial injury a decrease in hexagonality occurs. Both of these characteristics are important qualitative markers of the condition of the corneal endothelium. The coefficient of variation is the most sensitive index of corneal endothelial dysfunction and an increasing hexagonality index is a feature of endothelial wound healing (NISHIDA 2005).

The most important physiological function of the endothelium is regulating the water content of the corneal stroma by Na^+/K^+-dependent ATPase and Na^+/H^+ exchanger, both expressed in the basolateral membrane. The osmotic gradient between the stroma and aqueous humour prevents the influx of water into the stroma, which would lead to edema formtion and impaired vision (NISHIDA 2005).

3.2. KERATOPLASTY

Keratoplasty or corneal transplantation is the operative replacement of the recipient's corneal tissue by donor tissue. It is the oldest, the most common, and the most successful form of solid tissue transplantation in the world. In the United States more than 46,000 keratoplasties are performed per year (WILHELMUS *et al.* 1995), in the Czech Republic more than 500 keratoplasties every year (FILIPEC 2000).

The major goals of keratoplasty are to improve vision, to maintain the integrity of the globe and in some cases to decrease pain. The most frequent indications for keratoplasty are: keratoconus, pseudophagic bullous keratopathy, corneal dystrophies and degenerations, and scarring caused by infection or trauma (FORRESTER and KUFFOVA 2004).

In contrast to other tissue transplantation, no systemic immunosupression must be used; only topical corticosteroids are given up to 3 years post-transplantation to prevent graft failure. Another difference compared to most organ transplantations is that AB0 compatibility is not performed routinely. HLA-DR-matching is performed in about 10 % of grafted corneas (EEBA Directory 2007).

3.2.1. History of corneal grafting

The first attempts to replace pathological or damaged corneas are known from the 18th and 19th centuries. Many types of procedures were used, and the first successful allograft keratoplasty was performed in 1835 on a pet gazelle (BIGGER 1837). A lot of experiments with xenografts and artificial grafts was attempted, as well, but the results were largely unsuccessful (MOFFATT *et al.* 2005).

The first successful human keratoplasty was performed more than one hundred years ago on 7th December 1905 in Olomouc by Dr. Eduard Konrad Zirm. He performed two penetrating corneal transplants on a man who had sustained severe bilateral alkali burns more than 1 year earlier by working with lime. As the donor tissue, an eye globe was retrieved from 11 years old boy, who suffered from sympathetic ophthalmia after an intraocular metallic foreign body injury (ZIRM 1906). Although the graft on the right eye (from the peripheral part of the donor's cornea) failed and had to be removed, the left graft (from the central cornea) remained clear. The success using this technique was not repeated for about two decades by surgeons around the world, or

even by Zirm himself. The lamellar graft was more likely to use in subsequent years if any graft was performed (LAIBSON 1996).

The modern era of corneal transplantation started in the 1950s. The main progress stemmed from improvements in surgical technique, the development of very fine sutures and instruments, a better understanding of the immunology and pathophysiology of corneal grafts, and improved eye-banking methods. The use of antibiotics helps to prevent corneal graft infection, while anti-inflammatory drugs enable rejection to be controlled. Even today, immunological rejection is the greatest limiting factor to successful corneal grafting (MOFFATT *et al.* 2005).

3.2.2. Corneal grafting

There are 2 types of corneal transplantation, penetrating and lamellar keratoplasty. Both methods are performed today, according to corneal pathology of the recipient.

Penetrating keratoplasty

In penetrating keratoplasty, the full thickness of the cornea is grafted to the recipient (Fig. 4A). This method was used in the first successful human transplantation and this model is, with some modifications, still used today in more than 90 % of patients. The disadvantage of full thickness grafting is the need of suturing to prevent shifting of the graft and to make a watertight connection. There is also higher risk of endoophthalmitis from contaminated donor tissue or from possible contamination during surgery. (FORRESTER and KUFFOVA 2004).

Lamellar keratoplasty

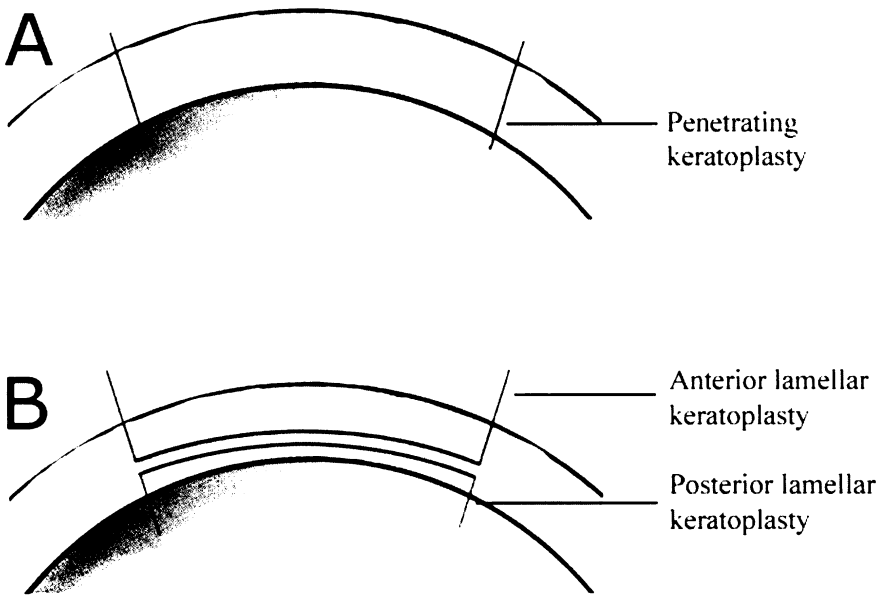
In contrast, in lamellar keratoplasty, only a partial thickness of the corneal graft is used in the surgery. Anterior or posterior lamellar keratoplasty (Fig. 4B) is performed, depending on which corneal structure needs to be replaced. The patient's part of the cornea is removed and replaced by donor tissue. By performing **anterior lamellar keratoplasty**, it is possible to remove just the epithelium (onlay lamellar keratoplasty) or the epithelium with the anterior part (2/3) of the stroma (inlay lamellar keratoplasty) (FORRESTER and KUFFOVA 2004).

On the other hand, **posterior lamellar keratoplasty** can be performed when the patient's endothelium is insufficient. It enables posterior corneal tissue grafting, while leaving the recipient's anterior cornea intact (MELLES *et al.* 1998). Deep lamellar

endothelial keratoplasty (DLEK) is a procedure in which a posterior lamella of the cornea is implanted into a round pocket in the recipient cornea (MELLES 2000, 2002, TERRY 2001, 2004). This technique was subsequently refined, finally by stripping off Descemet's membrane and the endothelium and placing the donor lamellar graft on the bare recipient stroma. This procedure has been named Descemet stripping endothelial keratoplasty (DSEK). Initially, the donor lamellar disc was created with a manual dissection, now the process is facilitated by the use of a blade microtome (Descemet stripping automated endothelial keratoplasty - DSAEK) (PRICE and PRICE 2005).

The number of lamellar keratoplasties increases sharply in recent years. The advantage of this approach is that it is less risky as no or a few sutures are necessary, because the graft is embedded tightly in the host cornea (CULBERTSON 2006).

Figure 4: Diagram of types of corneal grafting. Penetrating keratoplasty (A), anterior and posterior lamellar keratoplasty (B).



3.3. PREPARATION OF CORNEA FOR GRAFTING

Although the first transplantation in 1905 was performed using live donor's fresh cornea, in the early 1930s, the need for corneas led in the use of cadaver corneas, first reported by FILATOV (1935). The first eye bank was established in 1959 in New York by Dr. Townley Patton (FORRESTER and KUFFOVA 2004), which, together with the development of corneal storage media and tissue evaluation led to conditions under which corneal grafting become a more common and standard procedure.

3.3.1. Tissue preparation

After death, it is necessary to process the cornea as soon as possible to maintain its characteristics and viability. This process starts either with an eye globe removal from the body (enucleation) or corneal retrieval (corneal rim excision).

In the latter case the tissue is placed in storage medium and in this "close system" the tissue is kept until the time for grafting. Whole eyes are decontaminated (distilled water, 0.5 % povidon-iodine, or solution of antibiotics) and examined with a slit lamp to detect any corneal abnormalities, such as the presence of corneal scars or neovascularization. At the same time the donor's blood sample is serologically tested to avoid the use of corneas infected with hepatitis B or C, HIV or syphilis.

In the case of whole globes removal, the corneoscleral rims are trephined under sterile conditions in a biohazard hood. The examination of the cornea is normally performed using light microscopy after endothelial treatment by trypan blue dye and sucrose. The trypan blue solution (usually 0.1-0.5 %) enables the observer to distinguish between the viable and nonviable cells (blue stained nuclei) and to count the percentage of dead cells. The sucrose solution (usually 0.9 %) causes the swelling of the endothelial cell borders (PELS and VAN DER GAAG 1984-1985) and facilitates distinguishing individual cells in order to calculate the endothelial cell density and to detect abnormalities in the shape and size of the endothelial cells as well as other endothelial abnormalities (binucleated cells). Besides these main qualitative and quantitative parameters of the corneal endothelium, the condition of the epithelium is reviewed as well.

Subsequently, the cornea is placed into sterile preservative medium and stored in a medium-dependent manner until the surgery. In the tissue culture method, the cornea

is reassessed before transplantation and microbiology tests are performed (PELS and SCHUCHARD 1993).

3.3.2. Corneal preservation

History of corneal preservation

In 1935, V. P. Filatov reported the successful storage of whole globes in tightly stoppered glass jars in an ice chest at 4 °C for 20-56 hours, which was basically the moist chamber technique (FILATOV, 1935). This method of corneal storage quickly became very popular, remained in common usage until three decades ago for corneal storage up to 48 hours and is occasionally used until nowadays.

Many other approaches have been attempted since that time, but considered as unsatisfactory until the introduction of M-K medium in 1974 (MCCAREY and KAUFMAN 1974). This medium allowed the storage of donor tissue up to 3-4 days at 4 °C and enabled keratoplasty to be a scheduled, rather than an emergency, procedure. Many storage media built upon the successes of M-K were established since that time, such as K-Sol, CSM, and Dexsol. All these liquids were supplemented with chondroitin sulphate to prevent corneal swelling and allowed for storage of donor corneas for 7-10 days (WILSON and BOURNE 1989).

At the present time, the most widely used hypothermic storage medium, especially in the United States, is Optisol-GS (LINDSTROM et al. 1992). In Europe the tissue culture method is preferred type of storage with particular media used, in some banks the both types of storage are performed.

Methods of corneal storage

The sudden arrest of aqueous humour formation after death and the depletion of nutrients and oxygen supply to the eye for variable length of time, especially at room temperature, results in initial damage to the corneal cells by autolysis (BASU and HASANY 1974). Corneal preservation medium can maintain the health of living corneal cells and can prolong their survival for a significant period. The time from death to enucleation and the placement of the cornea into storage medium should therefore be as short as possible.

The major goals of corneal preservation are (1) to maintain the highest possible numbers of viable endothelial cells, keratocytes and epithelial cells using the simplest

preservation medium and (2) to detect and prevent microbial contamination of the donor tissue. (FORRESTER and KUFFOVA 2004). Two main methods for corneal preservation, hypothermic and tissue culture, are currently in use, each providing specific benefits and disadvantages.

Hypothermic storage

The storage under hypothermic conditions is used in the United States and 43 % of European banks associated with the European eye bank association (EEBA) (EEBA Directory 2007). In some eye banks, both storage methods are used. The excised corneoscleral button is placed in biologically defined low-temperature medium (4-8°C) and stored under these conditions until transplantation. In this closed system, all corneal examinations, such as biomicroscopy (slit lamp examination) or specular microscopy, are performed. The aim of this storage is to minimize or even to inhibit cellular processes that take place in the cornea under physiological conditions. This allows conserving the state of the cornea as long as possible. The main corneal characteristic that should be preserved is high number of viable endothelial cells.

The first successful method for storing corneaoscleral button in a chemically defined medium at 4 °C was M-K medium (MCCAREY and KAUFMAN 1974), which extended storage time to approximately 4 days. Several solutions are used for hypothermic storage today; widely known are Optisol-GS (Bauch&Lomb, USA), Eusol-C (Alchimia, Italy), and Licorol (Opsia Pharma, France). All these commercially available media have a similar composition: nutrients (proteins, amino acids), energy-rich molecules (ATP), antibiotics and antimycotics (streptomycin, gentamycin, neomycin, amphotericin) and osmotically active substances (chondroitin sulphate and/or dextran). The latter molecules act to maintain clarity and physiological thickness of the cornea, which permits better evaluation and easier manipulation of the donor tissue at the time of the surgical procedure and also assists earlier visual rehabilitation (KOMURO *et al.* 1999).

Optisol-GS is the most widely used medium in the United States today and allows for corneal storage up to 14 days at 4°C, although the average storage time is 4-5 days (WILHELMUS *et al.* 1995). This corneal storage medium is based on tissue culture medium 199, Eagle's balanced salt solution, and minimal essential medium (MEM). It contains 2.5 % chondroitin sulphate, dextran, HEPES buffer, various ATP precursors,

and an enhanced antimicrobial system containing gentamycin (100 µg/ml) and streptomycin sulphate (200 µg/ml) (KAUFMAN *et al.* 1991).

Tissue culture storage

Tissue culture is the preferred storage method; 83 % of European eye banks associated with the EEBA use this type of corneal storage (EEBA Directory 2007). The primary aim of tissue culture has been to keep the tissue alive, but the potential of the tissue culture technique lies in the possibility to improve the quality of the donor tissue in specific ways (EHLERS *et al.* 1994).

Corneal storage under tissue culture condition is more technically difficult and time-consuming compared to hypothermic storage. After the assessment of the cornea, the corneoscleral button is suspended in a sterile culture medium, which is usually changed every week. The storage temperature is close to physiological conditions, ranging between 31 °C and 37 °C, depending on the particular type of medium. The maximum storage time also differs in particular media, ranging from 28 up to 48 days (FRUEH and BÖHNKE 1995). However, most European banks do not store corneas for more than 35 days (EEBA Directory 2007). This relatively long period of storage enables the creation of better transplantation schedules and better matching between donor cornea and recipient, if necessary.

During storage the cornea loses its clarity and the corneal stroma swells. To restore transparency and physiological thickness, the cornea is transferred a few days before grafting to a medium that consists of mostly the same components as the storage medium with the addition of 5 % dextran. Dextran is osmotically active and enables the corneal endothelium to return to its original thickness (PELS and SCHUCHARD 1993).

There are many types of tissue culture media for corneal storage, usually prepared directly in the eye banks. They are all based on E-MEM (Eagle's minimal essential medium) and contain many nutrients, necessary for corneal metabolism, antibiotics, fungistatics and 2-10 % foetal calf serum, which improves the survival of endothelial cells (PELS and SCHUCHARD 1993). Tissue culture media are also commercially available, e.g. CorneaMax (Eurobio, France), Inosol (Chauvin Bausch&Lomb, France).

The main advantage of long-term preservation is the extension of the donor pool due to the longer storage time possible. Such long-term preservation also offers the possibility of microbiological testing during the storage, which minimizes the risk of a contaminated cornea grafting. There are standard tests for bacteria and fungi; bacteria

can be also detected by turbidity and changes in medium colour due to pH changes of phenol red indicator. The microbiologic cultivation of culture media is performed using agar blood, trypsin-soya broth, and thioglycolate broth to detect any media contamination. Another advantage is the chance to reassess the state of the cornea before transplantation. This is important mainly when the cornea is difficult or impossible to evaluate during its initial assessment due to edema or exposition keratopathy (PELS 1997)

Another advantage of tissue culture lies in reducing the number of antigen-presenting cells, mostly HLA-DR-positive Langerhans cells in the corneal epithelium and dendritic cells in the stroma (PELS and VAN DER GAAG 1984-1985).

On the other hand, a trained personnel is needed and there is the risk of possible alterations in the components of foetal calf serum. In addition, bovine spongiform encephalitis is a concern when using foetal calf serum (FRUEH and BOHNKE 2000). Due to this risk, new media with no presence of serum have been developed. Animal compound-free medium with synthetic deswelling agent poloxamer 188 (Lutrol F68, Germany) is the new approach to eliminate the use of foetal calf serum (THURET *et al.* 2005).

Moist-chamber storage

The moist-chamber technique was the first method introduced in this field and it remains the simplest and least expensive of all storage techniques. This method enables the storage of the whole eye globe or the excised cornea in a sterile jar filled with a saturated moist atmosphere at 4 °C. If the time from the death of the donor to the enucleation of the donor's eye is short (from 4 to 6 hours), the donor eyes can be stored up to 48 hours (CHU 2000).

Moist-chamber storage is advantageous in many situations, particularly in developing countries, where the need for donor corneas is greatest and the benefits of tissue culture laboratories and bacteriologically controlled areas are not generally available to the eye banks. The aim is to collect as many donor eyes as possible within a short time after the donor's death for emergency rather than elective surgery. This method also enables modifying of the excision technique of the graft to obtain tissue (the cornea as well as the conjunctiva) for special types of grafting (BASU 1995).

The most important criticism of the moist-chamber approach is that the endothelium is exposed to post-mortem changes in the aqueous humour, so this method

has been widely replaced by the preservation of the isolated cornea in various solutions kept under organ culture conditions at 31-37 °C or cold storage at 4 °C (EHLERS *et al.* 1994).

3.3.3. Criteria of donor tissue suitability for grafting

To maximalize the chance of a successful keratoplasty, it is necessary to use a donor cornea with appropriate characteristics. For this reason, criteria have been developed and improved every year, although they differ between individual eye banks.

Donor age and donor tissue quality

The minimal endothelial cell density suitable for transplantation is considered to be 2,000-2,300 cells per mm^2. Corneas with less than 1,000 cells per mm^2 lose their capacity to pump water from the stroma, swell and lose their clarity. Because there is a gradual decrease in endothelial cell density with age (0.6 % per year), corneas from donors older than 85 years are generally not used for grafting, although there are is no age limit in some eye banks (EEBA Directory 2007).

On the other hand, infant corneas are not usually used either because of their different refractive properties (the average curvature is about 50 dioptres) (FORRESTER and KUFFOVA 2004), as well as the stronger immunological response that occurs after grafting of this immunologically immature tissue.

Corneas can also be excluded because of an opacity or scar in the optic centre, synechiae, signs of senile changes in the endothelium, marks of refractive surgery *etc.* (BRIGHTBILL 1995).

Time from death to enucleation and media preservation

After death, uveal and other intraocular tissues decompose into the aqueous humour and generate a highly toxic environment for corneal endothelial cells (EHLERS *et al.* 1994). For this reason, the time until the eye globe removal and processing should be as short as possible. As a maximal time for corneal retrieval, 24 hours *post mortem* is commonly accepted; the temperature at which the body is stored after death has also an influence (PELS AND SCHUCHARD 1993).

Serology

An infection of the donor cornea may not only cause post-operative complications (endoophthalmation) and graft failure, but there is a danger of life-threatening agent transmission. Donation of corneal tissue is thus excluded when the cause of death is unknown or in circumstances that could place the tissue recipient at risk from disease or infection.

Together with careful anamnesia inspection, the donor's blood is tested for the presence hepatitis B (HbsAg), hepatitis C (HCV and HbC antibody), AIDS (HIV 1 and HIV 2 antibodies), and syphilis (ASIMAKIS *et al.* 1996). From January 2007 the Czech Republic is the only country in the world where additional testing for the presence of pathogenic prions has to be performed.

Risk factors

Although keratoplasty is the most successful form of solid tissue transplantation, due to the immune privilege of the anterior chamber of the eye, there are some risk factors influencing its success rate. These factors include the primary patient diagnosis and the state of the patient's cornea (a previous graft failure, vascularisation of the cornea or the corneal graft, the presence of adhesions in the anterior segment, and glaucoma or uveitis in the transplanted eye or corneal graft), the quality of the donor tissue, and the surgical technique used (MAGUIRE *et al.* 1994).

The size of the transplanted corneal button is also important. The lowest risk occurs when using corneal graft approximately 7-7.5 mm in diameter; it has been shown that either a smaller or larger than average graft is more likely to fail (WILLIAMS *et al.* 1992).

The influence of HLA-DR glycoprotein on graft survival has been confirmed by Collaborative corneal transplantation studies (MAGUIRE *et al.* 1994). Because dendritic cells act to stimulate T_H-cells, HLA-matching, especially in HLA-DR (PELS and VAN DER GAAG 1984-1985), may prevent immune rejection in high-risk cases, especially in patients with systemic diseases. On the other hand, it is highly probable that no or little connection is between HLA-A and HLA-B glycoproteins or the AB0 blood group system and the success or failure of a graft (MAGIURE *et al.* 1994).

The age of the recipient has a great influence. Interestingly, the recipients younger than 40 years of age have an elevated risk of graft failure. The risk decreases abruptly between the fourth and fifth decade of life; the main reason is probably the impairment of the immune system with age. On the other hand, very old patients are also likely to suffer a graft failure (MAGUIRE *et al.* 1994).

3.3.4. Corneal failure

Although the success rate of keratoplasty is generally greater than 90 % in the second year after transplantation in low risk cases, in high risk recipients the success rate is much lower, between 10 and 30 % (HILL 1994).

Primary graft failure

Primary graft failure generally happens soon after transplantation because of poor corneal graft quality either due to poor preservation or surgical trauma (WILLIAMS *et al.* 1992). It is a process in which the cloudy corneal graft does not clear and stay edematous within two months following surgery (the postoperative period) (WILLIAMS *et al.* 1992). The factors leading to primary failure have not been precisely determined; however, a common characteristic feature of primary graft failure is extensive destruction of the endothelial cells.

Later graft failure occurs due to immune rejection, inappropriate wound healing of the eye surface, infection or the recurrence of primary corneal diseases.

Immune rejection

Immune rejection remains the main cause of graft failure, responsible for approximately 31 % of all graft failures. The remaining failures are caused by the recurrence of previous pathology, secondary infection, epithelial problems and other factors. Corneal rejection can be caused by the infiltration of host cells into the epithelium, the stroma or the endothelium. It can be reversible (rejection episode), followed by full recovery, or irreversible, usually preceded by one or more rejection episodes and leading to graft failure (FORRESTER and KUFFOVA 2004).

Epithelial rejection is characterized by the development of a rejection line that represents the replacement of the donor epithelium by the host epithelial cells, but is usually reversible. On the other hand, endothelial rejection lines are more serious. They usually develop close to an area of vascularization and are composed of advancing lymphocytes and macrophages, which progress across the cornea and destroy the healthy endothelium. This process illustrates the risk posed by a vascularised cornea and the significance of the immune privilege in the anterior chamber of the eye, which is impaired in this case (PLSKOVA 2002).

Keratolysis

Postoperative keratolysis (corneal melting) is a type of acute corneal graft failure that may occur after almost all intraocular operations. It can be associated with infectious, inflammatory or trophic causes, especially in less developed counties as a result of malnutrition. The process is started by an epithelial defect, when the rapid self-regenerative capacity of the epithelium fails. The exposed stroma is next attacked by immune mediators and matrix metalloproteinases, and melting is accelerated by inflammatory cells (VERMA 2005).

The corneal melting is a rapid process that can occur at any time, and often occurs again and again after re-keratoplasty. It often happens in patients with systemic collagen diseases, recurrent defects of the epithelium, limbal stem cell deficiency, an ocular burn, or after nonsteroidal anti-inflammatory drug treatment. It occurs mostly in patients who suffer from rheumatoid arthritis (VIVINO *et al.* 2001). Some patients may suffer from keratolysis without any obvious reasons.

Late endothelial failure

After penetrating keratoplasty, a chronic loss of endothelial cells is routinely observed. By definition, it is a continuous decrease of endothelial cell density over time in the absence of any clinically evident immune reaction. The annual rate of cell loss is 7.8 % on average from the third to the fifth year after keratoplasty (BOURNE *et al.* 1994). The decay of endothelial cell density is exponential and compatible with the finding that only 35 % of the initial graft endothelial cells are left after 10 years (NISHIMURA *et al.* 1999).

For this reason, corneal failure many years after keratoplasty can occur. The contributing factors are advanced donor age, a long death to excision time interval, and relatively low endothelial cell density (BÖHRINGER *et al.* 2002).

3.4. IMMUNOLOGICALLY PRIVILEGED SITES

It was recognized almost 140 years ago that in some parts of the body the immune system appears not to function and these sites were termed "immunologically privileged". This term was firstly used by Sir Peter Medawar and is now applied to the sites and tissues of the body, in which the full immunodestructive response is modified to protect critical organs, such as the brain, the testis, the uterus, the trophoblast, and the anterior chamber of the eye (MEDAWAR 1945, 1948).

In these sites allogeneic or xenogeneic tissue grafts enjoy prolonged survival in comparison to other areas and create an exception to the conventional laws of transplantation. In addition, certain allogeneic tumours can survive and proliferate in these locations, and the inoculation of an antigen into an immunologically privileged site leads to a deviated immune response (FORRESTER and KUFFOVA 2004).

3.4.1. Immune privilege in the cornea

In the eye even minor episodes of inflammation can result in impaired vision or even blindness, if the inflammation proceeds unchecked. For this reason, infectious organisms and tumour cells are not excluded from tissue, to ensure that protective immunity against potential pathogens would not act against essential eye structures, as a side-effect.

The immune privilege is not simply a passive process involving physical barriers; rather it is an active procedure that uses an important natural mechanism to induce cell death in potentially dangerous infiltrating lymphoid and myeloid cells. There are a number of components contributing to this unique environment, all of which are necessary to maintain the immune privilege and secure corneal allograft survival (STREILEIN 1997).

A list of passive and active mechanisms of immune privilege is shown in Tab. 1.

Table 1: Features of immune-privileged sites (STREILEIN 1997)

Passive
Blood-tissue barriers
Deficient efferent lymphatic drainage
Tissue fluid that drains into blood vasculature
Reduced expression of MHC class I and II on parenchymal cells
Active
Constitutive expression of cell surface molecules that modulate immune effector cells and molecules:
atypical class Ib
Fas ligand
CD59, MCP, DAF - inhibitors of complement
Immunosuppressive microenvironment containing:
transforming growth factor - beta
alpha - melanocyte stimulating hormone
vasoactive intestinal peptide
calcitonin gene-related peptide
free cortisol
IL-1 receptor antagonist

Passive features of the immune privilege

Passive features represent the stable components and aspects of the eye that function by a mechanical sequestration of tissue and reduced antigen presentation.

- The **blood-ocular barrier**, residing at the edge of the cornea (among the iris vessels, the epithelium of the ciliary body, the retinal pigment epithelium, and the retinal vessels) stringently regulates the access of blood-borne cells and molecules into the eye. By this potent barrier, immune effectors within the blood stream, including sensitised T-cells and antibodies, are largely, but not completely, excluded from the eye (STREILEIN 1997).
- In addition, the eye, at least its internal compartments, lacks **lymphatic drainage** pathways. This mechanism disables the entrance of lymphocytes and thus their encounter and sensitising with ocular antigens. Instead of classical blood vessels, intraocular fluids are drained via the trabecular meshwork that

exports ocular antigens into the canals of Schlemm and the venous circulation (STREILEIN 1997).

- Besides these microanatomical features, parenchymal cells of the eye poorly express class I and class II molecules encoded within the **major histocompatibility complex** (MHC) (NIEDERKORN 1999). Since MHC molecules are required for T-lymphocytes to detect antigenic substances, reduced expression of these molecules on ocular cells reduces the ability of T-cells to detect antigens within the ocular compartments. Corneal epithelial and stromal cells express under normal conditions low levels of MHC gp I antigens and do not express MHC gp II antigens (WHITSETT and STULTING 1984). MHC class II antigens are found mainly on Langerhans cells, limbal cells (the limbus is a transition zone between the cornea and the conjunctiva) (FORRESTER and KUFFOVA 2004), and dendritic cells present in the corneal stroma (HAMRAH *et al.* 2003). However, HLA class II antigens are widely distributed on many cell types in pathological processes, especially on dendritic cells (PEPOSE *et al.* 1985).

Active features of the immune privilege

Active features of immune privilege correspond to molecules that are uniquely expressed by ocular cells and molecules that profoundly influence the behaviour of cells of the immune system. Active processes regulate both the induction of immunity to antigens introduced into or arising from the eye and the expression of immunity in the eye.

- **Membrane inhibitors of activated complement components** (decay accelaerating factor, membrane cofactor protein, CD59) interfere with the completion of the complement cascade and therefore prevent immune-mediated cell lysis.
- **Fas ligand** binds to its co-receptor Fas, which is expressed on activated T-cells and polymorphonuclear leucocytes. By this mechanism, Fas ligation induces the apoptosis of antigen-specific T-cells that encounter an antigen within the eye (NIEDERKORN 1999).
- **Immunosuppressive cytokines and neuropeptides** (transforming growth factor-beta 2, alpha-melanocyte stimulating hormone, vasoactive intestinal

peptide, calcitonin gene-regulated peptide, free cortisol and interleukin-1 receptor antagonist), which are present in the soluble intraocular microenvironment, suppress and regulate immune cell function (STREILEIN 1997).

Anterior chamber-associated immune deviation

The anterior chamber-associated immune deviation (ACAID) is a form of tolerance induction in the eye; however, similar altered immune responses have been described in other immunologically privileged organs (FORRESTER and KUFFOVA 2004). ACAID can be defined as a dynamic down-regulation of delayed-type hypersensitivity (DTH) to antigens that enter the anterior chamber of the eye. This term conveys the concept that the ocular immune response is deviated away from a T_H1 pathway toward a T_H2-like pathway (NIEDERKORN 1999). Although it is believed that ACAID is an adaptation for protecting normal ocular tissues from non-specific inflammatory injury produced by DTH responses in the eye, it is essential for corneal allograft survival.

3.5. FAS – FAS LIGAND SYSTEM IN TRANSPLANTATION BIOLOGY

The expression of Fas ligand (FasL) has important implications for the maintenance of immune privilege and for suppressing corneal allograft rejection. The encounter between the cells of the corneal graft expressing FasL and immunocompetent inflammatory cells, such as macrophages, monocytes, neutrophils, or activated T cells, expressing Fas, leads to subsequent apoptosis of the inflammatory cells and corneal graft survival (GRIFFITH *et al.* 1995).

3.5.1. Characterization of Fas and Fas ligand

In 1989, a cell surface receptor that has the property of killing cells was found. Four years later, SUDA *et al.* (1993) identified the ligand that triggers cell death by binding to this surface receptor. These molecules of crutial importance were named FAS and FAS ligand (www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=134638).

Molecular biology of Fas and Fas ligand

Both Fas receptor (also called CD95 or Apo-1) and Fas ligand (also called CD178 or CD95L) were initially characterized as membrane-anchored proteins, suggesting that direct cell-cell (juxtacrine) interaction is necessary for activation of the Fas system (TAKAHASHI *et al.* 1994). FasL is a type II-membrane protein belonging to the tumour necrosis factor family, while Fas receptor is a type I-membrane protein (glycoprotein) and belongs to the tumour necrosis factor/nerve growth factor receptor family (www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=134638). Later it was demonstrated that FasL is expressed as a soluble cytokine by metalloproteinase-mediated release in some cell types (TANAKA *et al.* 1995) and that a soluble variant of Fas receptor also exists. Therefore, the effects of the Fas system may be mediated by paracrine or autocrine mechanisms, as well (MOHAN *et al.* 1997).

Fas and Fas ligand mediated functions

Fas and FasL have been characterized best in the immune system, in which they are involved in the downregulation of immune reactions, as well as in T cell-mediated cytotoxicity, through the induction of apoptosis (NAGATA and GOLSTEIN 1995). The spectrum of Fas ligand-mediated functions is very wide, including both beneficial and

deleterious effects: FasL-interactions play an important role in human carcinogenesis (ZHANG *et al.* 2005), Fas/FasL-mediated apoptosis is important for skin homeostasis (HILL *et al.* 1999), FasL may contribute to the immune privilege of tumours (HAHNE *et al.* 1996). Fas/FasL-mediated apoptosis is necessary for angiogenesis inhibition (VOLPERT *et al.* 2002).

The presence of FasL in the cornea helps to protect the anterior chamber of the eye against immune attack and, as a beneficial side-effect, prevents corneal graft rejection (STUART *et al.* 1997); allogenic FasL-deficient graft is promptly rejected. Both immune privilege and allograft survival are partially provided by the induction of apoptotic cell death of Fas-positive cells infiltrating FasL-positive corneal tissue (either original or grafted) (HORI *et al.* 2000).

Mechanism of Fas-induced cell death

Fas protein binding together with an integral membrane ligand (FasL) induces Fas oligomerization and recruitment of the adaptor molecule, Fas-associated protein with death domain (FADD). FADD sequentially activates pro-caspases 8 and 3 and caspase-3 then acts on several cellular substrates (KRAMMER 2000), ultimately leading to chromatin aggregation, cell fragmentation into apoptotic bodies and cleavage of the other cell components (EARNSHAW 1995).

3.5.2. Fas and Fas ligand expression

Fas is expressed on a variety of cell types throughout the body, such as lymphocytes and neutrophils, and also in the liver, the lungs, the ovary, and the heart, where its function is unclear (www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=134638).

In contrast, Fas ligand is expressed predominantly on activated T-cells, but was found also in nonlymphoid tissues, including the testis, the spleen, and the thymus (GRIFFITH *et al.* 1995).

Fas ligand expression in the eye

There is abundant FasL expression throughout the eye, including the retina, the iris, the ciliary body, and the cornea (GRIFFITH *et al.* 1995). FasL is strategically placed at or near areas that comprise the blood-ocular barrier, as well as in locations where there is an opportunity for potential interaction between ocular tissue and inflammatory cells (STUART *et al.* 1997).

Fas mRNA and proteins are expressed in all three major cell types of the cornea (epithelial, endothelial, and keratocytes) (WILSON *et al.* 1996). Similarly, FasL mRNA was detected in all three major cell types of the cornea. However, FasL protein has not been detected in keratocytes (GRIFFITH *et al.* 1995, WILSON *et al.* 1996). The lack of its detection in fresh-frozen human corneas was explained by posttranscriptional regulation of Fas ligand synthesis in keratocytes (MOHAN *et al.* 1997). FasL expression in the corneal endothelium and epithelium suggests its importance in controlling inflammatory cells that would enter the cornea from the conjunctiva or the anterior chamber (STUART *et al.* 1997).

Mechanisms of avoiding spontaneous activation

The co-expression of Fas and FasL by the same cell raises the question of why these cells do not undergo spontaneous autocrine or paracrine cell death. The systems modulating Fas are complex and there are likely to be many mechanisms regulating Fas activation it many sites including the cornea; the most important ones are mentioned below:

- The soluble forms of Fas are secreted as membrane bound form antagonists.
- A second signal may be required for Fas activation (TAN and HUNZIKER 2003).
- The ligands and receptors are segregated to different cell membranes in polarized cells or to intracellular compartments, e.g. FasL is preserved in secretory lysosomes in cytotoxic T-lymphocytes.
- In addition to ligand and receptor compartmentalization, the cytosolic signalling components themselves are also compartmentalized, contributing to the suppression of spontaneous death signalling.

Mechanisms to avoid the spontaneous activation of Fas/FasL system are essential for cell physiology. In several pathological conditions, including inflammatory responses, graft rejection, renal disorders or physical tissue damage, apoptosis correlates with increased expression levels of Fas or FasL or both. This higher expression of Fas or FasL could lead to spontaneous cell death of healthy cells (TAN and HUNZIKER 2003).

3.6. NITRIC OXIDE

3.6.1. Biosynthesis of nitric oxide

In mammalian cells, nitric oxide (NO) arises from the enzymatic oxidation of terminal nitrogen of the amino acid L-arginine, according to the reaction:

1 L-arginine + 1 O_2 + 1,5 NADPH → 1 NO + 1 citrulline (FEIHL *et al.* 2001).

The responsible enzymes are proteins of a heme family known as NO synthases (NOS). All members of this family share a similar homodimeric structure, where each monomer consists of a reductase domain and an oxygenase domain, separated by a 30-amino acid sequence for the attachment of the Ca^{2+}-binding protein calmodulin. Enzymatic activity requires the presence of additional cofactors, namely flavin mononucleotid, tetrahydrobiopterin, and heme (FEIHL *et al.* 2001).

There are three known isoforms of NOS; each are products of different genes, but are highly conserved (FORSTERMANN *et al.* 1995): neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). The expression of nNOS and eNOS is constitutive, although it can be regulated and they are produced by many tissues. nNOS is expressed typically in the cerebellum, the skeletal muscle and the myenteric plexuses. eNOS is ubiquitous in the vascular endothelium, but may also be found in the placenta, kidney tubular epithelial cells, and neurons (FEIHL *et al.* 2001).

In contrast, iNOS is present in few locations under normal physiological conditions, such as the respiratory epithelium, the gravid uterus or the ileal mucosa. However, it can be induced in countless tissues. iNOS expression has been demonstrated in different cell types, such as macrophages, neutrophils, fibroblasts, vascular endothelial and smooth muscle cells, the endocardium, the myocardium, the renal tubular epithelium, mesangial neurons, and astrocytes (FEIHL *et al.* 2001).

There are many agents capable of inducing iNOS. The most important ones are: UV light, cAMP-elevating agents, ozone, trauma, bacterial products (lipopolysacharide, enterotoxins, lipoteichoic acid), and pro-inflammatory cytokines, including IL-1, IFN-γ, and TNF-α. Bacterial products and pro-inflammatory cytokines act synergistically in promoting expression through signalling pathways that involve (but are not restricted to) activation of NF-κB. In contrast, many agents may oppose cytokine induction of

iNOS. These include anti-inflammatory cytokines such as IL-10 or TGF-β (FEIHL *et al.* 2001).

Calmodulin binding is absolutely necessary for enzymatic activity in all isoforms of NOS. In the case of eNOS and iNOS, relatively high concentrations of calcium ions, in the range of 0.1-1 μM, are needed for binding (FORSTERMAN et al. 1995). This feature of the constitutive isoforms is crucial to the role of nitric oxide in signalling, because it allows the intermittent production of NO by cells in response to Ca^{2+} variations. In contrast, iNOS is able to bind calmodulin at the nanomolar concentrations normally found in the cytosol in the basal state. Thus, after the expression of iNOS, NO can be produced at rates that may be only limited by the availability of substrates and cofactors (DEDKOVA and BLATTER 2002).

3.6.2. Characteristics and effects of nitric oxide

In the physiological state, nitric oxide is a gas with a lipophilic character, which allows it an unhindered passage through cellular membranes and enables its biological role as a second messenger (JENG *et al.* 2002). At the same time, depending on the rate, timing, spatial distribution, and chemical environment, NO as a free radical can transform into various reactive nitrogen species and can act as an indirect toxic effector molecule (WINK and MITCHELL 1998, LIAUDET *et al.* 2000). These various properties of NO can be divided into direct and indirect effects.

Direct effects of nitric oxide

Direct effects serve to promote homeostasis and occur at the low (nanomolar) concentrations that typically result from the regulated activation of constitutive NOS isoforms. In vivo, an essential metabolic fate of NO that limits its local concentration is its diffusion into erytrocytes, where it oxidizes the ferrous iron of oxyhemoglobin to yield the nitrate anion and methemoglobin. In the physiological state, this mechanism is sufficient to keep the concentration of nitric oxide in the nanomolar range, at least in nonhydrophobic compartments, i.e. outside biological membranes, and direct effects of NO will be prevalent under these conditions (BECKMAN and KOPPENOL 1996).

The best characterized effect is the activation of soluble guanylyl cyclase, leading to the formation of cGMP (DENNINGER and MARLETTA 1999). cGMP in turn interacts with several downstream elements, including cGMP-dependent proteinkinases, cGMP-regulated phosphodiesterases, and cGMP-gated ion channels. These pathways

lead to many types of physiological responses, such as vasodilatation (MARIN and RODRIGUEZ-MARTINEZ 1997), the reduced adhesion of platelets and leukocytes (KUBES *et al.* 1991, RADOMSKI *et al.* 1993) or signal transduction in the nervous system (CHRISTOPHERSON and BREDT 1997).

Nitric oxide also downregulates the activity of two key enzymes in oxidative metabolism, cytochrome oxidase (BROWN 1999), which catalyses the terminal step in the mitochondrial electron transport chain, and mitochondrial aconitase (GADNER *et al.* 1997), which plays an essential role in the citric acid cycle.

In contrast with this potentially deleterious action, NO may be highly protective against oxidative stress: it is able to reduce highly toxic hypervalent metal-O_2 complexes produced from the interaction of hydrogen peroxide with heme (JOURD'HEUIL *et al.* 1998), it inhibits the iron-catalyzed generation of hydroxyl radicals (MILES *et al.* 1996), it reacts with lipid hydroperoxyl radicals to terminate chain lipid peroxidation (WINK and MITCHELL 1998), and it is able to scavenge the superoxide radical (RUBBO *et al.* 1994).

Indirect effects of nitric oxide

In contrast to direct effects, indirect effects occur at the high (micromolar) concentrations of nitric oxide that are usually achieved during the continuous NO production by iNOS. Many of these effects can be deleterious to the host and occur after the reaction of NO with oxygen and its derivates (FEIHL *et al.* 2001).

In aqueous solutions, NO decays to nitrite by a reaction with molecular oxygen. The intermediates of this reaction are nitric dioxide (NO_2) and dinitric trioxide (N_2O_3), a potent nitrosating species. The production of N_2O_3 can lead to the formation of S-nitrosothiols and carcinogenic N-nitrosamines. S-nitrosylation of proteins is an ubiquitous regulatory reaction comparable to phosphorylation and S-nitrosothiols appear to play significant roles in a large number of biological processes, such as ion channel activation, ryanodine receptors (STOYANOVSKY *et al.* 1997), cardiatic L-type Ca^{2+} channels (CAMPBELL *et al.* 1996), Ca^{2+}-dependent K+-channels (BOLOTINA *et al.* 1994) and GAPDH inhibition (MOHR *et al.* 1999), which leads to glycolysis suppression and can cause cell death in conditions such as tissue hypoxia.

Furthermore, reduced glutathione (GSH), due to its high affinity for both reactive nitrogen and oxygen species, is a central biomolecule involved in the cellular defence against nitrosative and oxidative stress (WINK and MITCHELL 1998). Depletion of

GSH has been shown to markedly increase NO-dependent cytotoxicity (WALKER *et al.* 1995). S-nitrosothiols may inhibit several enzymatic pathways involved in the maintenance of the GSH pool and it appears that in circumstances associated with high NO production, nitrosative stress enhances the cellular susceptibility to oxidant-mediated damage, providing an important cycle of cytotoxic amplification in inflammatory conditions (FEIHL *et al.* 2001).

Another harmful molecule, peroxynitrite, forms in the reaction of NO and the superoxide radical (O_2^-). This highly reactive oxidative species has a half-life of only about 1 second, but this is sufficient to oxidize biomolecules, namely DNA, thiols, lipids and mitochondrial enzymes, or to inhibit enzymes and other proteins by nitration reactions. It is now known that most of the deleterious effects on cellular energetics previously attributed to nitric oxide are in fact mediated by peroxynitrite (BROWN 1999).

3.6.3. Nitric oxide and apoptosis

The effects caused by NO in programmed cell death can be literally antagonistic. NO has been shown to protect cells against apoptotic death by mechanism including the up-regulation of anti-apoptotic proteins, e.g. Bcl-2 and heme oxygenase-1, as well as the inhibition of pro-apoptotic events, such as mitochondrial release of cytochrome c and caspase-3 activation (SHEN *et al.* 1998, KIM *et al.* 1999, LIAUDET *et al.* 2000).

In sharp contrast, NO can induce apoptosis (SHEN *et al.* 1998, KIM *et al.* 1999, LIAUDET *et al.* 2000) in a variety of cell lines, including macrophages (ALBINA *et al.* 1993), vascular endothelial cells (LOPEZ-COLLAZO *et al.* 1997) and ventricular myocytes (PINSKY *et al.* 1999). Recent data indicate that peroxynitrite rather than nitric oxide itself may be responsible for NO-dependent apoptosis, with possible mechanisms involving DNA injury and mitochondrial damage.

3.6.4. Nitric oxide and inflammation

Nitric oxide may exert either pro- or anti-inflammatory effects, depending on its concentration and certain circumstances. As an example, NO has quite a complex effect on the biosynthesis of prostanoid mediators of inflammation; it can either inhibit or stimulate cyclooxygenase (SALVEMINI 1997, GOODWIN *et al.* 1999) or prostacyclin synthase (WADE and FITZPATRICK 1997).

Nitric oxide has major interactions with the pathways of gene expression controlled by the transcription factor NF-κB. Both activation (UMANSKY *et al.* 1998, KALRA *et al.* 2000) and inhibition (UMANSKY *et al.* 1998, DELATORRE *et al.* 1999) of NF-κB activity have been described. While modest up-regulation of NO production at an early stage of inflammation would stimulate NF-κB activity and thus stimulate the inflammatory response, higher concentrations during subsequent phases would serve as a feedback mechanism and oppose the unlimited expression of genes regulated by NF-κB (FEIHL *et al.* 2001).

To summarize the biological effects of nitric oxide, there is a wide range from physiological regulation and cell protection, to cytotoxicity, depending on the amount of the molecule produced and the specific chemical environment. This ambivalent character occurs both in direct and indirect effects, as well as in its role in apoptosis and inflammation.

3.6.5. Nitric oxide in the cornea

As the cornea is a transparent avascular tissue that allows light to transit to the more posterior structures, it is constantly exposed to a wide spectrum of light, including the UV range. The cornea absorbs most of the UV light entering the eye and is consequently susceptible to damage from reactive oxygen species. There are a number of mechanisms to minimize the impact of this stress factor. From 20 to 40 % of the soluble corneal proteins are isoenzymes of aldehyde dehydrogenase, which directly absorbs UV light and removes cytotoxic aldehydes produced by UV-induced lipid peroxidation (ABEDINIA *et al.* 1990, GONDHOWIARDJO *et al.* 1991). Furthermore, the cornea is rich in antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, all of which help in the removal of free radicals and reactive oxygen species (RAO *et al.* 1987).

In the eye, NO serves as a mediator in diverse processes, but it can also cause cytotoxic effects via peroxynitrite formation. There are 2 types of NOS expressed in the cornea. eNOS can be found both in normal and diseased corneas, in the epithelium and the endothelium, but is not apparent in stromal cells. In contrast, iNOS expression is associated with oxidative damage that occurs in common human corneal disorders. This isoform was found in epithelial, stromal, and endothelial cells of corneas with bullous

keratopathy, Fuchs' dystrophy or keratoconus, but was absent or minimal in normal corneas (BUDDI *et al.* 2002, SAGOO *et al.* 2004).

The corneal stroma has a tendency to absorb water and to swell due to its charge characteristics. The corneal endothelial pump, Na^+/K^+ ATPase, is localized to the lateral endothelial cell membrane (GEROSKI and EDELHAUSER 1984) and helps the outward movement of water to the anterior chamber by the activation of the NO/cGMP pathway (MCKEE *et al.* 1994). This suggests that basal levels of NO are necessary for water transport and essential to prevent corneal swelling (YANAGIYA *et al.* 1997) and that NO products may also decrease intraocular pressure (BEHAR-COHEN *et al.* 1996).

On the other hand, when this beneficial level is exceeded, the deleterious effects of NO and its products occur.

Nitric oxide and corneal storage for grafting

As NO has both beneficial and harmful effects, depending on its concentration, the level of NO in corneal storage medium could be a significant factor influenced the quality of corneas intended for grafting. A little knowledge is about the quantity of NO produced by the cornea under physiological or pathological conditions.

KIM *et al.* (2002) measured the NO concentration in the tear fluid and aqueous humour under normal and various NO-related inflammatory conditions and summarized the critical NO concentration (Tab. 2). The normal concentration was 0.36 ± 0.08 µM in tears and 4.12 ± 0.95 µM in the aqueous humour. However, the harmful concentration of NO for corneal cells, especially the corneal endothelium has not been determined, yet.

Table 2: Possible functional roles according to the critical NO level in tear and aqueous humour (KIM *et al.* 2002).

	Normal	**Normal wound healing (inflammation)**	**Pathological inflammation**
Concentration ratio	1	1.5 – 2.5	3 - 10
Function	Physiologic	Defence mechanism Cell survival signalling Apoptosis	Tissue damage Oxidative stress Nitrosative stress DNA damage, *etc.*

A ratio of 1.5-2.5 of nitric oxide value, assuming the normal nitric oxide value to be 1, may play a defensive role, whereas much higher concentrations of NO (3 to 10 fold of nitric oxide value), may induce tissue damage.

An excessive concentration of NO in corneal storage medium may cause cell damage and a decrease of tissue quality for grafting. However, the levels of NO in the culture medium of corneas stored under tissue culture storage have not been detected yet.

Changes in the concentration of NO in corneas under hypothermic storage were studied by JENG *et al.* (2002). This group found that NO is continuously released by human corneas during storage in Optisol-GS medium before transplantation, until an equilibrium (2.77 μM) is reached. Hovewer, the rates, at which NO was produced, was different for each cornea, ranging between 0.5 μM to 2.5 μM in first 30 days of storage.

The study also indicates that the diffusion of nitrites and nitrates present in corneal tissue before storage has a negligible contribution to the increase of NO levels, suggesting that majority of NO in the corneal storage media is produced by corneal cells during storage.

4. MATERIALS AND METHODS

4.1. MATERIALS

All experiments were performed using human corneas from deceased donors obtained from the Ocular Tissue Bank Prague. The use of human corneas adhered to the tenets of the Declaration of Helsinki. The corneas not suitable for corneal grafting due to unacceptable parameters for keratoplasty, positive serology results, indications of former corneal injury or surgery or low endothelial cell density (the detection of FasL and NO only) were used.

The globes were enucleated by the eye bank staff, decontaminated with 0.5 % povidone-iodine, neutralized with 2 % natrium thiosulphate, and examined biomicroscopically.

The corneoscleral rims were trephined under sterile conditions in a biohazard hood, cornea was assessed and stored under tissue culture or hypothermic conditions. Some corneas were further processed without any storage.

4.2. REPAIR OF THE CORNEAL ENDOTHELIUM UNDER TISSUE CULTURE OR HYPOTHERMIC CONDITIONS

4.2.1. Treatment of corneas (Experimental design)

Twelve corneal pairs unsuitable for transplantation (reasons other than endothelial quantity or quality) were used for the study. The whole bulbi were obtained from donors (mean age 67 ± 12 years, range 47-81 years) less than 24 hours after death. After decontamination, the eye bulbi were examined by slit lamp, and then corneoscleral rings were obtained by trephination under sterile conditions in a biohazard hood. Corneas with a calculated endothelial cell density (ECD) per mm^2, percentage of dead cells (DC), endothelial cell density of live cells (ECDA) per mm^2, coefficient of variation of cell area (CV) and percentage of hexagonal cells (6A), were used.

Only corneas with an ECDA higher than 2000 cells/mm^2 and with similar endothelial parameters between paired corneas were used: ECD differed by no more than 10 %, the percentage of DC did not differ by more than 1.6 %. The number of Descemet's folds did not differ by more than three.

The assessment was performed after the corneal endothelium was treated with 0.12 % trypan blue in PBS and 0.9 % sucrose using light imicroscopy (Olympus CKX-41, Japan) and an camera (Olympus C-3040, Japan). The ECD, CV and 6A were assessed from 1 central (0.3 mm^2) and 4 non-overlapping pericentral phase contrast photographs (magnification of 200x). The ECD of dead cells was assessed from bright field photographs of the same parts of the cornea at a magnification of 100x to include a larger area for assessing dead cells, and the percentage of dead cells was calculated. All photographs were processed by a semi-automated Lucia computer analysis system to obtain average values of the ECD, ECDA percentage of DC, CV and 6A separately in the central and pericentral parts of the cornea.

After the initial assessment, the central part of each cornea was mechanically damaged using a special metal rod (Fig. 5). The flat end of the rod (0.79 mm^2) was gently pressed for 15 seconds onto the central part of the endothelial surface to induce injury. Subsequently, the damaged corneas were treated with trypan blue and sucrose and photographs were taken again. The size of the wounds varied between corneas from 0.95 -1.25 mm^2 (mean 1.14 mm^2).

Subsequently, one cornea of each pair was stored in tissue culture (31°C) and the other was placed in hypothermic storage (4°C) in a random manner.

Figure 5: Metal rod for experimentally made lesion in the corneal endothelium (1.0 mm in diameter, 0.785 mm^2).

4.2.2. *Incubation media*

Optisol-GS medium (Bausch&Lomb Inc., USA) was used for the storage of corneas under hypothermic conditions. It is a sterile, buffered culture media, which is enhanced with polypeptides, an osmotic agent (dextran), chondroitin sulfate, gentamicin sulfate, streptomycin, and phenol red indicator. Twenty ml are used per cornea.

Tissue culture medium (prepared in the ocular tissue bank) was used for the storage of corneas at 31 °C. The medium is based on EMEM (Earle's Minimum Essential Medium) with addition of glycine, HEPES (25 mM) (AppliChem GmbH, Germany), $NaHCO_3$ (2.24 g/l) and foetal calf serum (2 %). The medium also contains an antibiotic-antimycotic solution of penicillin G (100 U/ml), streptomycin sulphate (100 µg/ml), and amfotericin B (Fungizone) (0.25 µg/ml or 0.85 % NaCl). The medium is stored at –20 °C and/or at 4 °C up to 14 days before usage. Fifty ml is used for storage, and the medium is changed every week.

4.2.3. *Assessment of corneas*

Corneas were reassessed on day 7, 14, 21, and 28 (culture medium only) after approximately 12-hour deswelling in a tissue culture medium containing 5 % dextran (room temperature), using an inverted microscope (Olympus CKX-41).

The ECD of the pericentral part was assessed from 4 non-overlapping phase contrast photographs (0.30 mm^2 each) taken from the part of the corneal endothelium adjacent to the damaged area at a magnification of 200x, ECD of central part was evaluated from 1 photograph taken in the centre. The ECDD was assessed from bright field photographs (1.26 mm^2 each) of the same parts of the cornea at a magnification of 100x.

The photographs were taken and processed by a semi-automated Lucia computer analysis system 4.8 to obtain the ECD, ECDD, CV and 6A values for the pericentral and central areas separately.

4.2.4. *Measurement of the lesion repair*

To observe the repair process and its rate, 5 corneas stored in tissue culture medium without dextran were observed every day until the lesion with dead cells completely disappeared. Bright field photograph of the lesion was taken at a magnification of 100x

and the damaged area was assessed using a Lucia computer analysis system. The average repair rate was determined using Lucia computer analysis system.

4.2.5. Staining of the corneal endothelium

Two more corneas were used to confirm the induction of corneal damage: immediately after the injury, the endothelium was stained with alizarin red S to visualize the denuded Descemet´s membrane and any remaining dead cells (TAYLOR and HUNT 1981). The method was combined with trypan blue staining of dead the cell nuclei.

Corneas were rinsed in 0.9 % NaCl solution and the endothelium was incubated with 0.2 % alizarin red S (pH 4.20) for 2 minutes. Corneas were rinsed again in NaCl solution and the endothelium was incubated with 0.15 % trypan blue in PBS for 2 minutes. Corneas were rinsed once more in NaCl solution and observed with an inverted microscope and bright field photographs were taken at a magnification 100x or 200x.

4.3. FAS LIGAND DETECTION

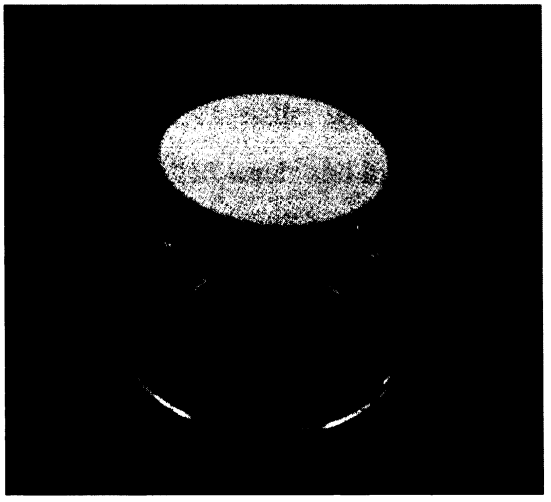
4.3.1. Impression cytology of the corneal endothelium

Corneas excised from the eye globes using trephine (12 mm in diameter) were used fresh or after 7, 14 or 21 days of storage in tissue culture medium or medium for hypothermy as described in section 4.2.

Corneal endothelial cells were obtained from the cornea by their impression onto Millicell membrane (Millicell-CM, 0.4 μm culture plate insert, 12 mm diameter, Millipore Corp., USA) (Fig. 6). The corneal endothelium consists of a cell monolayer and can be easily separated from the neighbouring with Bowman's layer.

First, the three legs at the edge of the membrane plastic holder were cut with forceps. Corneas were washed in phosphate buffered saline (PBS) and 3 imprints of the endothelium were taken from about 1/3 of corneal surface by pushing the sterile membrane onto the endothelial layer. The membranes with imprints were laid back in package and stored at -70°C until use.

Figure 6: The Millicell membrane on a plastic holder.



4.3.2. Quantification of Fas ligand expression in human corneal endothelium using real-time RT-PCR

Twenty-one corneal endothelium imprints were used for the experiment. The samples were divided into 7 groups, according to storage conditions (fresh, 7, 14, 21 days of

tissue culture storage, 7, 14, 21 days of hypothermic storage). Three independent experiments in duplicates were preformed .

RNA isolation using the phenol-chloroform method

RNA isolation from corneal endothelial cells was performed using TRI Reagent (Molecular Research Center, Inc., USA), according to the manufacturer's instruction.

Millicell membranes with imprinted endothelial cells were released mechanically from the plastic holder and immersed in 400 µl of TRI Reagent in a microtube. The tubes were snap frozen at –70 °C and then defrosted in room temperature to enhance homogenisation. Membranes were disrupted by homogenisation sticks.

The homogenate was subsequently incubated for 5 minutes at room temperature to permit the complete dissociation of the nucleoprotein complexes, mixed with 100 µl of chloroform and shaken vigorously. The resulting mixture was stored for 15 minutes at room temperature and then centrifuged at 12,000 g for 15 minutes at 4 °C.

Four µl of glycogen was put into fresh tubes, the upper aqueous phase of sample (with RNA) was added and stirred. 200 µl of isopropanol was added to precipitate RNA and the mixture was shaken vigorously. The tubes were incubated for 10 minutes at room temperature and centrifuged at 12,000 g for 8 minutes at 4 °C.

After centrifugation the supernatant was removed and the RNA pellet was washed with 75 % ethanol (800 ml), followed by 5-minut centrifugation at 7,500 g at 4 °C.

Ethanol was discarded and the RNA pellet was briefly air-dried. The RNA was solubilized in 20 µl of RNase-free distilled water and incubated at 55 °C for 15 minutes.

RNA isolation using a NucleoSpin RNA II isolation kit

For improved RNA purity, RNA isolation from corneal endothelial cells was performed using a NucleoSpin RNA II isolation kit (Bioké, Netherlands), according to the manufacturer's instructions.

A Millicell membrane was mixed with 350 µl of buffer RA1 and disrupted by homogenisation sticks, followed by addition of 3.5 µl of mercaptoethanol and vortexing vigorously.

The lysate was filtered through NucleoSpin Filter units in a collecting tube by centrifuging for 1 minute at 11,000 g.

Three hundred and fifty µl of 70 % ethanol was added to the homogenized lysate and mixed by vortexing. The mixture was placed in a NucleoSpin RNA II column in a centrifuge tube and centrifuged for 30 seconds at 8,000 g.

Three hundred and fifty µl of membrane desalting buffer was added to the sample mixture and centrifuged at 11,000g for 1 minute to dry the membrane.

DNA was digested by 10 µl of DNase I and 90 µl of DNase reaction buffer mixture. The DNase mixture was applied directly onto the centre of the column membrane and incubated at room temperature for 15 minutes followed by washing with 200 µl of buffer RA2 and centrifugation for 30 seconds at 8,000 g.

Six hundred µl of buffer RA3 was added and centrifuged for 30 seconds at 8,000 g. The membrane was dried completely by 250 µl of RA3 and centrifugation for 2 minutes at 11,000 g. The column was placed into a nuclease-free microcentrifuge tube.

RNA was eluted in 20 µl of RNase-free water and centrifuged at 11,000g for 1 minute.

RNA quality evaluation and concentration determination

Gel electrophoresis was performed to evaluate the RNA integrity using 1 % agarose gel (Serva Electrophoresis GmbH, Germany) in TBE buffer (TRIS, boric acid, EDTA solution), with the addition of ethidium bromide.

The RNA sample was mixed with loading dye (0.25 % bromophenol blue, 0.25 % xylencyanol FF) and glycerol. A standard 100 bp ladder (Serva Electrophoresis, Germany) was run with each experiment. The RNA was visualized on a UV-transiluminator (Ultra-Lum, Inc., USA) due to ethidium bromide intercalation.

Spectrophotometry was performed to determine the RNA concentration of the samples. The samples were diluted 1:50 and absorbance was measured at 260 nm, 280 nm, and 320 nm wavelengths to evaluate RNA purity using a Helios Gamma spectrophotometer (Thermo Spectronic, United Kingdom). The RNA concentration was calculated using "Spectrophotometric measurement of nucleic acids' concentration" software (www.molbiol.ru).

Reverse transcription-PCR

RNA was transcribed to cDNA using SuperScript II reverse transcriptase according to the manufacturer's instruction (Invitrogen Corp., USA). To standardize the PCR reaction, the RNA content was adjusted to 1 μg of RNA in each tube.

A mixture of random hexamers, dNTP, RNA, and PCR water was incubated in a thermal cycler (MyCycler, Bio-Rad Laboratories, USA) for 5 minutes at 65 °C. The tubes were chilled to 25 °C, 5X-first strand buffer, DTT and RNaseOUT were added, and the mixture was incubated in the thermal cycler for 5 minutes at 25 °C. Reverse transcriptase was added and the vials were incubated in the thermal cycler for 99 minutes at 42 °C. The reverse transcriptase was inactivated by heating to 70 °C for 15 minutes (Tab. 3).

Table 3: Reaction mixture content and volume used for reverse transcription.

Reagent	Volume [μl]	Manufacturer
Random hexamers (500μg/ml)	1	Promega Corp., USA
dNTP	2	AB Gene, USA
RNA	1 μg	
PCR water	According to RNA volume	
5X first strand buffer	4	Invitrogen Corp., USA
DTT (0,1M)	2	Invitrogen Corp., USA
RNaseOUT	1	Invitrogen Corp., USA
Superscript II reverse transcriptase (200U/μl)	0.5	Invitrogen Corp., USA

Gradient PCR and other optimization procedures

Optimization of the primer annealing temperature was performed using a gradient PCR method. PCR reactions with different annealing temperatures (50-66 °C) were performed using a thermal cycler. Taq purple polymerase (Top Bio, Czech Republic) was used as the enzyme and the reaction was vizualized in 2 % agarose gel.

Optimal primer and magnesium chloride concentrations were chosen using PCR reactions with different concentrations of the reagents by the real-time PCR method and a Rotor Gene 2000 instrument (Corbett Research, Australia). Combi Taq Hot Start polymerase was used as the enzyme and the amplification product was visualized by Sybr green intercalation dye.

Real-time PCR with Sybr green product visualization

The expression of FasL mRNA was assessed by the real-time PCR method using β-actin as an endogenous housekeeping gene (Tab. 4). The experiments were performed on a Rotor Gene 2000 instrument. FasL and β-actin gene expressions were detected by a Sybr green visualization stain that intercalates into the bases of double-strand DNA. One pair of β-actin primers and 3 pairs of FasL primers were designed using Primer3 Output software.

Primer sequences:

Beta actin (sense primer): **ggcatcctcaccctgaagta**

Beta actin (antisense primer): **aaggtctcaaacatgatctgggt**

Fas ligand 1 (sense primer): **tggccttgtgatcaatgaaa**

Fas ligand 1 (antisense primer): **tcatcttcccctccatcatc**

Fas ligand 2 (sense primer): **tcttccacctacagaaggag**

Fas ligand 2 (antisense primer): **ttaaatgggccactttcctc**

Fas ligand 3 (sense primer): **gcagcccttcaattacccat**

Fas ligand 3 (antisense primer): **cagaggttggacagggaagaa**

Table 4: Reaction mixture contents and volumes used for real-time PCR for β-actin or FasL detection.

Reagent	Volume [µl]	Manufacturer
dNTP	0.2	AB Gene, United Kingdom
Buffer-complete	2	Top Bio, Czech Republic
Taq polymerase (Hot Start) (1 U/µl)	1	Top Bio, Czech Republic
Sense primer (β-actin or FasL) (8 µM)	1	Generi Biotech, Czech Republic
Antisense primer (β-actin or FasL) (8 µM)	1	Generi Biotech, Czech Republic
Sybr green	0.2	Top Bio, Czech Republic
$MgCl_2$ (25mM)	2	Top Bio, Czech Republic
PCR water	10.6	Top Bio, Czech Republic
cDNA	2	

Real-time PCR with UPL probe product visualization

The expression of FasL mRNA was assessed by the real-time PCR method using β-actin as an endogenous housekeeping gene (Tab. 5 and 6). The experiments were performed on a Rotor Gene 2000 instrument (Corbett Research, Australia). FasL and β-actin gene

expressions were detected by UPL Probes. Intron-spanning primers and probes were designed using Roche-applied-science software (www.roche-applied-science.com).

Primer sequences:

Beta actin (sense primer): **ccaaccgcgagaagatga**

Beta actin (antisense primer): **ccagaggcgtacagggatag**

Fas ligand (sense primer): **ccaaccgcgagaagatga**

Fas ligand (antisense primer): **ccagaggcgtacagggatag**

Hot Start Taq polymerase was activated for 2 minutes at 95 °C. Fifty cycles were performed with denaturation for 20 seconds at 95 °C, and primer hybridisation, together with annealing and fluorescence acquiring at 60 °C for 60 seconds. The amplification was terminated at 4 °C.

Table 5: Reaction mixture contents and volumes for β-actin real-time PCR amplification.

Reagent	Volume [µl]	Manufacturer
dNTP	0.2	AB Gene, United Kingdom
Buffer-complete	2	Top Bio, Czech Republic
Taq polymerase (Hot Start) (1 U/µl)	1	Top Bio, Czech Republic
Sense primer (β-actin) (8 µM)	1	Generi Biotech, Czech Republic
Antisense primer (β-actin) (8 µM)	1	Generi Biotech, Czech Republic
UPL probe #64	0,2	Roche Diagnostics GmbH., Germany
$MgCl_2$ (25mM)	2	Top Bio, Czech Republic
PCR water	10.6	Top Bio, Czech Republic
cDNA	2	

Table 6: Reaction mixture content and volume for Fas ligand real-time PCR amplification.

Reagent	Volume [µl]	Manufacturer
dNTP	0,2	AB Gene, United Kingdom
Buffer-complete	2	Top Bio, Czech Republic
Taq polymerase (Hot Start) (1U/µl)	1	Top Bio, Czech Republic
Sense primer (FasL) (8 µM)	1	Generi Biotech, Czech Republic
Antisense primer (FasL) (8 µM)	1	Generi Biotech, Czech Republic
UPL probe #20	0,2	Roche Diagnostics GmbH., Germany
$MgCl_2$ (25mM)	3	Top Bio, Czech Republic
PCR water	9,6	Top Bio, Czech Republic
cDNA	2	

Each individual experiment was performed in duplicate and the reaction efficiency for each gene was estimated by comparative analysis provided by Rotor Gene software version 6. To calculate the ratio of target gene to housekeeping gene expression, the relative quantification model with efficiency correction (PFAFFL 2001) was employed (Fig. 7).

Figure 7: Mathematical model of the relative expression ratio in real-time PCR (PFAFFL 2001).

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(control - sample)}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(control - sample)}}$$

4.3.3. Immunocytochemical detection of Fas ligand in human corneal endothelial cells

Endothelial cells from forty-one corneas, either freshly harvested or stored for 7, 14, or 21 days in tissue culture or hypothermic medium, were used for the experiment, approximately 6 corneas for each time period and medium type.

Indirect fluorescent immunocytochemistry

FasL localization in the corneal endothelium was detected using a "dropping method".

Membranes with impressed endothelial cells were fixed in acetone for 1 minute. The membranes with cells were released from the plastic holder (due to incubation with acetone), placed cell side up on round 12-mm coverslip and rinsed five times in PBS for 3 minutes. Permeabilization in 0.02 % Triton X 100 (Sigma-Aldrich, Inc., USA) in PBS for 7 minutes was performed and the membranes were rinsed five times in PBS for 3 minutes.

The specimens were incubated with primary antibody (Rabbit anti human FasL, Q-20, 1:50, Santa Cruz Biotechnology, Inc., USA) diluted with 1 % bovine serum albumin (BSA) and incubated for 1 hour at room temperature. The membranes were then rinsed five times in PBS for 3 minutes.

The secondary antibody (FITC conjugated donkey anti rabbit – IgG, 1:200, Jackson Immuno Research Laboratories, Inc., USA) diluted with 1 % BSA was applied to the membranes. The membranes were incubated in the dark for one hour at room temperature and rinsed five times in PBS in dark, too.

The membranes were transferred to an underlying slide and overlain with a cover slip, using Vectashield-propidium iodide mounting medium (Vector laboratories, Inc., USA) to visualize the cell nuclei.

Negative control specimens (primary antibody omitted) were run with each experiment.

Specimen examination

The slides were examined by fluorescent microscopy (Olympus, BX-51, Japan) and the photographs at 200x or 400x magnification were taken using a Vosskühler VDS CCD-1300 camera (VDS Vosskühler GmbH, Germany). The micrograph were analysed using the Lucia 4.8 programme (Laboratory Imaging, Czech Republic). The localization of the signal was determined in a minimum of 500 cells per specimen.

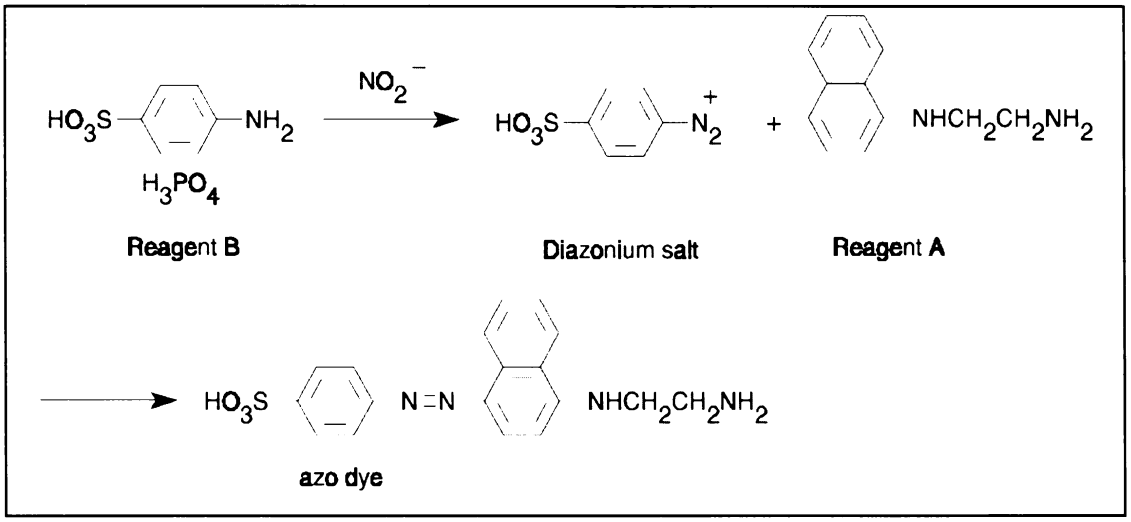
4.4. QUANTIFICATION OF NITRIC OXIDE RELEASE BY CORNEAS IN STORAGE MEDIUM

4.4.1. Griess reaction

As NO nitrite and nitrate are stable breakdown products of NO, the nitrite/nitrate concentration in the storage media is directly correlated with NO production. Nitrite concentration can be assessed using a spectrophotometric assay based on the Griess reaction (Griess reagent kit for nitrite determination, product information, Sigma-Aldrich, Inc., USA) (Fig. 8). The concentration of nitrates can be measured by their conversion to nitrites by (NAD[P]H) nitrate reductase, according to the reaction:

$$NO_3^- + FAD + NADPH \rightarrow NO_2^- + NADP^+$$

Fig. 8: Principle of nitrite quantitation using the Griess reaction. Formation of the azo dye is detected via its absorbance at 548 nm.



Experimental design

Fourteen corneas were used for the experiment. Seven were placed in tissue culture medium (50 ml) and stored at 31 °C, and 7 corneas were placed in medium for hypothermic storage (20 ml) at 4 °C. The composition of the storage media is described in section 4.1. Two hundred and fifty µl of medium was removed under sterile conditions in a biohazard hood after a defined storage time in tissue culture medium or Optisol-GS storage medium. The samples were stored at -20 °C until nitric oxide detection was performed.

The media of corneas with the mechanically induced lesion were used in the experiment, to evaluate the influence of cell reparation to nitric oxide production. Four corneas stored in tissue culture medium and four corneas stored in medium for hypothermy were used for this experiment.

To ensure that the phenol red pH indicator did not interfere with the colorimetric detection, tissue culture medium without a cornea was incubated under the same conditions, and processed in the same manner.

Quantitative assay for nitrite and nitrate levels

Fifty µl of sample, blank, or nitrite standard solution were placed in a 96-well plate. Fresh storage medium was used as a blank, and sodium nitrite solution diluted in storage medium in decreasing concentrations of sodium nitrite (100 µM –0.78 µM) was used as a standard.

The samples were incubated with 40 µM NADPH, 14 mU nitrate reductase (Sigma-Aldrich, Inc., USA), and 20 mM TRIS (pH 7.6) (Serva Electrophoresis GmbH, Germany). The reaction was terminated after 5 minutes by dilution with 48 µl of distilled water (MISKO *et al.* 1993).

The Griess reagent A (N-(1-Naphthyl)ethylenediamine dihydrochloride) and Griess reagent B (Sulphanilic acid in phosphoric acid solution) were mixed in equimolar quantities and 100 µl of Griess reagent A and B (Sigma-Aldrich, Inc., USA) mixture was added to each sample. The colour change was measured using an ELISA reader (Sunrise Remote, Tecan, Austria) with a 540 nm absorbance filter and a 620 nm reference absorbance filter. Data were processed using Kim 32 microplate software (Daniel Kittrich-Software Production, Czech Republic).

5. RESULTS

5.1. REPAIR OF THE CORNEAL ENDOTHELIUM UNDER TISSUE CULTURE OR HYPOTHERMIC CONDITIONS

The mean ECD of corneas subsequently stored under tissue culture conditions was 2765 ± 349 and 2834 ± 381/mm^{2}in the central and pericentral areas, respectively. The mean ECD in the central area before damage was 2677 ± 404 and 2760 ± 389/mm^2 in the pericentral area of corneas later stored under hypothermic conditions. The mean percentage of dead cells of all examined corneas was 0.45 ± 0.52 % (ranged from 0 to 1.84 %).

Immediately after inducing an injury in the central cornea, a bluish denuded Descemet's membrane with remnant dead endothelial cells was observed, with little differences between corneas.

As was confirmed on two corneal pairs, the extent of damage completely covered the surface that was touched by the flat end of the metal rod. No live endothelial cells remained at the site of injury (Fig. 9).

In the region adjacent to the wound (pericentral area), the mean ECD did not change significantly. In this area, the percentage of dead cells increased to 2.58 % or 0.73 % in corneas destined for storage under tissue culture or hypothermic conditions, respectively. In some of the corneas, bluish areas (spots) of different shapes, containing no dead cells, were observed in the areas adjacent to the wound.

The mean values of ECD (± SD), the percentage of dead cells, CV, and 6A in the central and pericentral endothelium of fresh corneas and those stored under tissue culture or hypothermic conditions for different time periods are shown in Tab. 7 and Tab. 8.

The presence of cell debris indicated the location of lesions in the central endothelium, observed using bright field microscopy after 7 days storage in tissue culture. No or few dead cells were present in the damaged areas or in adjacent areas. The original damaged area was fully repaired by enlarged cells of irregular shape; ECD in this central part was significantly decreased (1762 ± 267/mm^2) as was 6A (45.4 ± 7.6), while CV was increased (19.6 ± 3.6) (Fig. 10A). A number of elongated cells were observed shifting centripetally to the site of the former lesion (Fig. 10B). The endothelial morphology of fresh and stored corneas is shown in Fig. 11 and 12.

Seven days of storage does not lead to repair in corneas stored under hypothermic conditions. The bluish-colored wounds of the corneal endothelium, indicating the presence of dead cells, were clearly visible, with no changes in their extent compared to their state immediately after the injury. The area adjacent to the wound was cloudy, but the endothelial cell mosaic was relatively unchanged compared to that before and just after damage. One hundred per cent and 0.7 % of dead cells were present centrally and pericentrally, respectively. ECD in the pericentral area was 2635 ± 388/mm^2.

After 14 days, no signs (presence of dead cells or cell debris) of the injury was observed in tissue culture stored corneas. The locations of the original wounds could be identified only by the presence of larger and irregularly shaped cells. ECD was 1933 ± 354 in the central and 2478 ± 425/mm^2 in the pericentral area. The percentage of dead cells did not exceed 0.1 %.

Twenty-one days of tissue culture storage led to complete repair of the endothelium. The mean ECD was slightly increased in the central endothelium (2162 ± 417/mm^2) and decreased pericentrally (2389 ± 424/mm^2). A similar condition of the corneas could be observed 28 days after wounding.

The corneas stored under hypothermic conditions became cloudy; the pericentral ECD could be assessed only in 10 corneas on day 14 (2523 ± 363/mm^2) and 5 corneas on day 21 (2505 ± 465/mm^2). The extent of the central injury was unchanged compared to the previous assessments, with blue spots indicating the endothelial lesion. In addition to this damage, 1.5 and 8.4 % of dead cells were detected pericentrally on days 14 and 21.

The mean surface of damaged area was 1.14 ± 0.1 mm^2 (range 1.03 – 1.25 mm^2) immediately after the injury. The extent of the injury was significantly decreased up to 84, 43, and 11 % of original values after 24, 48 and 72 hours, respectively (Table 9). The spot with dead cells was observed in one cornea (0.04 mm^2, i.e. 3.2 % of the original injury) after 4 days in tissue culture. No dead cells were present at the place of former wound indicating by rest of cell debris at 6 other corneas. The centres were fully repaired by enlarged cells of irregular morphology.

Figure 9: Bright field micrograph of the corneal endothelium immediately after the induction of the lesion. The extent of the damage was complete over all of the injured surface. Alizarin red S staining. Scale bar represents 50 μm.

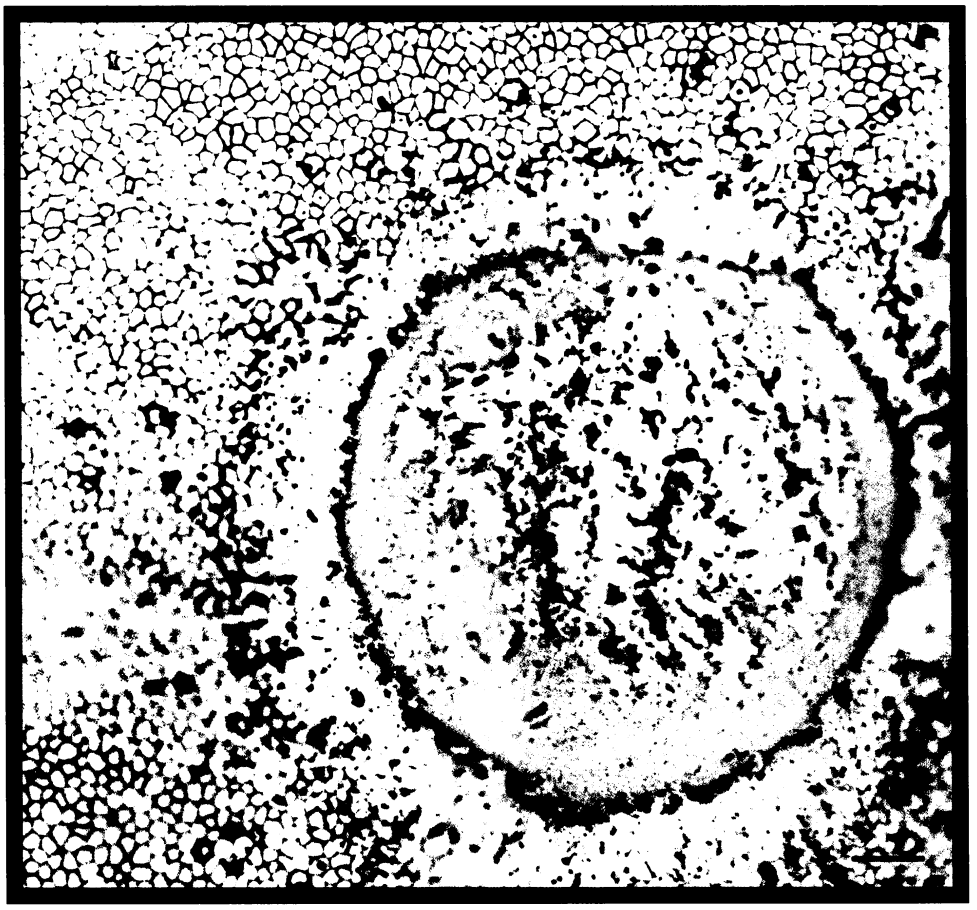


Table 7: The quantitative and qualitative endothelial parameters of the central (injured) endothelium of fresh corneas and corneas stored under tissue culture or hypothermic conditions. No... number of cornea, ± SD...standard deviation.

Central endothelium	Tissue culture storage						Hypothermic storage					
	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A
before lesion	400/P	2266	2266	0,00	22,02	51,85	400/L	2232	2200	1,45	19,22	53,19
	401/L	2558	2558	0,00	15,36	55,10	401/P	2517	2517	0,00	16,40	46,84
	402/L	2149	2149	0,00	20,32	58,06	402/P	2011	2011	0,00	25,04	50,00
	697/L	2448	2448	0,00	14,21	67,35	697/P	2281	2281	0,00	8,80	20,00
	700/P	2983	2983	0,00	13,33	65,96	700/L	2974	2974	0,00	21,06	60,00
	727/L	3221	3209	0,40	16,15	63,43	727/P	3481	3472	0,25	16,26	60,32
	728/P	2716	2688	1,03	13,51	44,12	728/L	2519	2446	2,92	20,23	48,28
	768/L	3249	3249	0,00	18,45	60,71	768/P	3072	3072	0,00	20,81	46,81
	769/L	2898	2884	0,47	18,60	50,00	769/P	2809	2799	0,34	18,35	58,49
	771/L	2930	2893	1,28	23,84	55,88	771/P	2665	2632	1,26	22,92	45,79
	773/L	2921	2921	0,00	17,02	58,33	773/P	2863	2863	0,00	15,37	56,00
	800/P	2839	2826	0,43	12,85	61,70	800/L	2699	2672	1,01	14,00	63,46
average		**2765**	**2756**	**0,30**	**17,14**	**57,71**		**2677**	**2662**	**0,60**	**18,21**	**50,77**
±SD		349	376	0,45	3,59	6,79		404	395	0,91	4,37	11,44
7 days after lesion	400/P	1668	1657	0,68	20,12	44,30						
	401/L	1391	1391	0,00	16,33	33,33						
	402/L	1703	1697	0,38	23,83	37,10						
	697/L	1552	1552	0,00	15,08	41,67						
	700/P	1904	1902	0,13	26,98	45,95						
	727/L	2095	2072	1,11	21,85	50,00						
	728/P	1500	1488	0,80	16,56	56,00						
	768/L	1853	1826	1,47	15,12	55,81						
	769/L	1541	1541	0,00	19,49	35,42						
	771/L	2316	2316	0,00	21,42	51,16						
	773/L	1912	1912	0,00	19,27	50,00						
	800/P	1708	1706	0,14	18,56	44,29						
average		**1762**	**1755**	**0,39**	**19,55**	**45,42**						
±SD		267	366	0,51	3,60	7,55						

Central endothelium		Tissue culture storage					Hypothermic storage					
14 days after lesion	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A
	402/L	1729	1729	0,00	23,47	65,00						
	697/L	2091	2091	0,00	18,37	46,03						
	700/P	2004	2002	0,10	22,95	34,62						
	727/L	2174	2167	0,33	23,63	38,89						
	728/P	1639	1635	0,24	17,01	44,83						
	768/L	2492	2474	0,74	13,62	36,47						
	769/L	1721	1717	0,23	18,75	46,97						
	771/L	2491	2477	0,55	20,48	61,90						
	800/P	1912	1911	0,04	20,31	41,10						
average		**1933**	**1928**	**0,21**	**20,07**	**48,04**						
±SD		354	349	0,24	3,02	10,61						
21 days after lesion	400/P	1666	1651	0,86	24,01	37,50						
	401/L	1405	1405	0,00	22,01	42,86						
	402/L	1766	1766	0,00	16,77	52,38						
	697/L	2403	2403	0,00	18,99	38,33						
	700/P	2087	2087	0,00	19,72	45,71						
	727/L	2543	2542	0,06	18,17	45,00						
	728/P	2302	2300	0,07	14,87	36,17						
	768/L	2875	2868	0,22	20,01	50,00						
	769/L	2005	2005	0,00	18,32	52,75						
	771/L	2548	2542	0,22	21,28	42,42						
	773/L	2287	2286	0,03	18,81	55,70						
	800/P	2056	2056	0,00	27,72	45,95						
average		**2162**	**2388**	**0,12**	**20,06**	**45,40**						
±SD		417	424	0,25	3,39	6,35						
28 days after lesion	768/L	1843	1842	0,02	19,24	50,82						
	769/L	1297	1289	0,62	23,52	57,14						
	771/L	2320	2318	0,07	17,29	50,00						
	773/L	2230	2189	1,78	20,08	54,62						
	800/P	1756	1756	0,00	20,30	36,46						
average		**1889**	**2327**	**0,50**	**20,09**	**49,81**						
±SD		595	635	0,53	5,94	14,31						

Table 8 (next page): The quantitative and qualitative endothelial parameters of the pericentral endothelium of fresh corneas and corneas stored under tissue culture or hypothermic conditions. No...number of cornea, ± SD...standard deviation

Pericentral endothelium		Tissue culture storage						Hypothermic storage				
	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A
before lesion	***400/P***	2528	2528	0,00	21,04	44,07	***400/L***	2365	2327	1,58	19,94	36,45
	401/L	2575	2575	0,00	11,73	57,78	***401/P***	2524	2524	0,00	14,88	46,67
	402/L	2049	2039	0,48	15,33	56,46	***402/P***	2174	2163	0,49	17,44	56,40
	697/L	2547	2531	0,63	14,15	62,50	***697/P***	2455	2447	0,33	14,67	61,59
	700/P	2904	2904	0,00	16,35	52,40	***700/L***	2932	2932	0,03	16,01	60,91
	727/L	3339	3338	0,04	19,64	52,82	***727/P***	3625	3624	0,03	16,77	59,86
	728/P	2864	2812	1,84	18,63	46,21	***728/L***	2635	2625	0,38	19,25	41,33
	768/L	3428	3397	0,91	17,22	54,52	***768/P***	3113	3113	0,00	16,22	54,59
	769/L	2979	2946	1,10	15,63	53,26	***769/P***	2855	2853	0,07	16,85	46,43
	771/L	3137	3109	0,89	19,28	51,82	***771/P***	2862	2850	0,44	18,82	56,74
	773/L	2908	2898	0,34	13,48	58,82	***773/P***	2986	2986	0,00	15,13	55,96
	800/P	2749	2740	0,32	18,92	54,29	***800/L***	2601	2580	0,80	20,15	55,97
average		**2834**	**2818**	**0,55**	**16,78**	**53,75**		**2760**	**2752**	**0,35**	**17,18**	**52,74**
±SD		381	376	0,56	2,82	5,08		389	395	0,47	1,95	8,10
immediately after lesion	***400/P***	2444	2413	1,30	22,28	60,80	***400/L***	2346	2334	0,48	19,83	38,89
	401/L	2450	2382	2,77	11,55	59,46	***401/P***	2492	2490	0,10	17,92	63,73
	402/L	1998	1980	0,89	18,90	49,00	***402/P***	2046	1985	2,98	16,76	64,96
	697/L	2483	1986	20,01	17,18	58,14	***697/P***	2576	2520	2,16	17,57	57,14
	700/P	2995	2994	0,01	17,60	59,41	***700/L***	2919	2916	0,12	13,60	59,38
	727/L	3318	3307	0,31	17,14	51,28	***727/P***	3575	3575	0,00	17,47	52,74
	728/P	2845	2814	1,08	18,61	55,15	***728/L***	2845	2814	1,08	18,61	55,15
	768/L	3342	3341	0,05	19,07	40,35	***768/P***	3279	3279	0,00	17,53	42,72
	769/L	2811	2777	1,19	14,44	61,21	***769/P***	2858	2856	0,08	20,28	56,82
	771/L	3195	3161	1,08	18,11	39,47	***771/P***	2913	2902	0,36	17,84	53,70
	773/L	3048	3031	0,54	18,73	56,39	***773/P***	3105	3090	0,48	14,61	52,30
	800/P	2712	2665	1,70	17,06	51,69	***800/L***	2589	2565	0,92	18,18	56,87
average		**2803**	**2738**	**2,58**	**17,56**	**53,53**		**2795**	**2777**	**0,73**	**17,52**	**54,53**
±SD		407	467	5,54	2,62	7,47		418	431	0,94	1,89	7,55
7 days after lesion	***400/P***	2231	2228	0,13	18,23	36,36	***400/L***	2249	2240	0,40	19,04	53,66
	401/L	2343	2343	0,00	18,14	44,12	***401/P***	2348	2344	0,16	17,12	46,51
	402/L	1913	1912	0,03	18,30	43,43	***402/P***	2159	2136	1,07	15,07	41,38
	697/L	2218	2217	0,03	18,90	50,47	***697/P***	2409	2407	0,55	21,89	21,59
	700/P	2551	2550	0,03	22,24	41,67	***700/L***	2995	2995	0,00	17,19	46,00
	727/L	3124	3124	0,01	17,24	53,64	***727/P***	3347	3324	0,67	16,07	45,75
	728/P	2729	2728	0,03	19,85	39,08	***728/L***	2242	2196	2,02	18,44	48,15
	768/L	3088	3083	0,17	20,42	47,79	***768/P***	2873	2861	0,41	19,17	45,34
	769/L	2525	2525	0,01	20,02	45,24	***769/P***	2630	2624	0,25	21,83	46,11
	771/L	2847	2842	0,15	17,87	39,13	***771/P***	2994	2958	1,20	17,84	53,70
	773/L	2309	2309	0,03	18,02	44,21	***773/P***	2977	2953	0,82	15,75	58,71
	800/P	2386	2385	0,04	21,26	54,85	***800/L***	2398	2374	1,03	16,37	51,98
average		**2522**	**2520**	**0,06**	**19,21**	**45,00**		**2635**	**2618**	**0,72**	**17,98**	**46,57**
±SD		366	366	0,06	1,54	5,79		388	388	0,56	2,21	9,21

Pericentral endothelium		Tissue culture storage						Hypothermic storage				
	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A	No.	ECD [mm2]	ECDA [mm2]	DC [%]	CV	6A
14 days after lesion	***400/P***	1931	1931	0,02	23,42	44,74	***400/L***	2209	2041	7,63	12,33	66,67
	401/L	2337	2335	0,05	18,30	63,55	***401/P***	2175	2148	1,21	18,21	47,62
	402/L	1894	1890	0,23	23,69	49,69	***402/P***	2102	2101	0,06	15,77	45,83
	697/L	2177	2177	0,01	17,37	51,91	***697/P***	2238	2229	0,42	16,93	41,46
	700/P	2752	2752	0,00	18,99	46,15	***700/L***	3017	2952	2,15	18,40	37,50
	727/L	2799	2791	0,29	18,65	46,37						
	728/P	2320	2320	0,02	23,60	52,05						
	768/L	3403	3400	0,09	15,53	49,69	***768/P***	2864	2864	0,00	20,28	44,70
	769/L	2702	2701	0,05	20,28	51,91	***769/P***	2496	2496	0,02	18,73	44,35
	771/L	2762	2758	0,16	17,71	35,71	***771/P***	2924	2919	0,16	16,41	63,64
	773/L	2367	2365	0,12	16,73	49,06	***773/P***	2899	2899	0,00	14,31	51,79
	800/P	2294	2292	0,08	20,81	45,22	***800/L***	2304	2236	2,98	14,78	46,21
average		**2478**	**2476**	**0,09**	**19,59**	**48,84**		**2523**	**2488**	**1,46**	**16,62**	**48,98**
±SD		425	424	0,09	2,79	6,49		363	381	2,40	2,39	9,33
21 days after lesion	***400/P***	1838	1838	0,00	18,50	48,44						
	401/L	2240	2240	0,00	20,80	45,13	***401/P***	1872	1354	27,69	21,79	49,32
	402/L	1781	1779	0,13	21,89	50,42						
	697/L	2193	2193	0,00	18,82	43,02	***697/P***	2238	2021	9,74	16,93	41,46
	700/P	2637	2637	0,00	22,57	52,12						
	727/L	2981	2981	0,00	19,62	52,69						
	728/P	2666	2666	0,01	19,45	43,81						
	768/L	3064	3062	0,04	17,01	47,80	***768/P***	2825	2779	1,65	17,62	41,25
	769/L	2037	2037	0,05	14,13	53,16	***769/P***	2546	2527	0,74	17,51	48,96
	771/L	2732	2731	0,03	17,55	44,27	***771/P***	3043	2978	2,12	16,30	52,63
	773/L	2349	2349	0,04	18,09	55,68						
	800/P	2150	2149	0,05	20,52	47,49						
average		**2389**	**2388**	**0,03**	**19,08**	**48,67**		**2505**	**2332**	**8,39**	**18,03**	**46,72**
±SD		424	424	0,04	2,30	4,16		465	654	11,38	2,17	5,11
28 days after lesion	***768/L***	2717	2717	0,00	19,18	48,31						
	769/L	2153	2153	0,02	19,35	46,28						
	771/L	2559	2558	0,05	21,28	51,69						
	773/L	2349	2349	0,04	18,09	55,68						
	800/P	1859	1859	0,00	19,22	35,51						
average		**2327**	**2327**	**0,02**	**19,42**	**47,49**						
±SD		636	635	0,02	5,26	14,59						

Figure 10: Phase contrast micrograph of the corneal endothelium 7 days after the induction of the lesion: an irregular cell mosaic (polymegatism and pleomorphism) is clearly visible in the pericentral area (A) and elongated cells are shifting centripetally to the site of the lesion (B). Scale bar represents 10 µm.

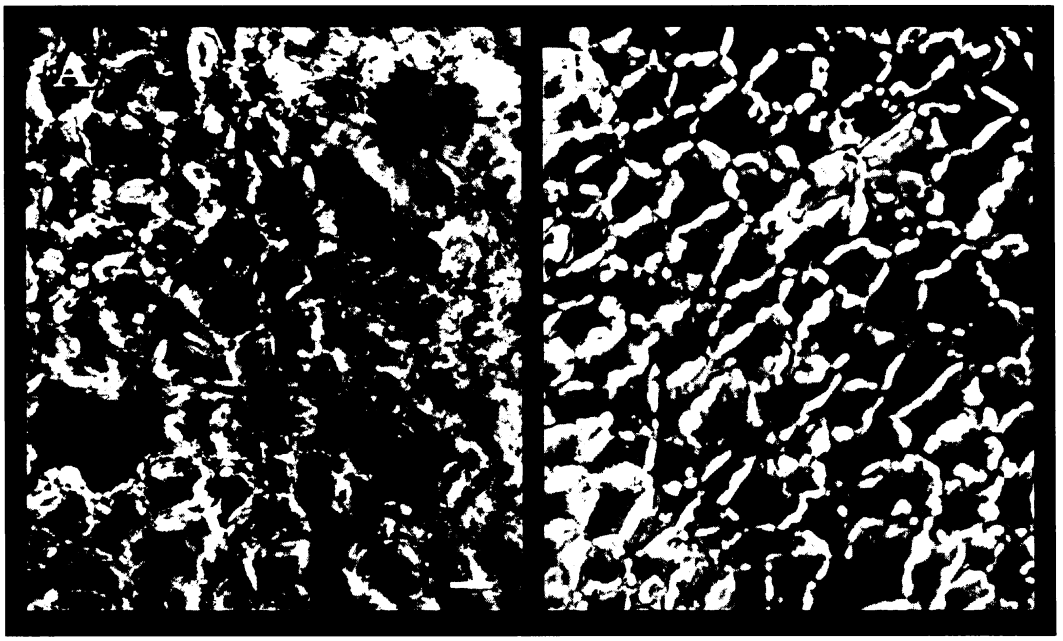


Figure 11: Phase contrast micrograph of the pericentral endothelium of fresh corneas and corneas stored in tissue culture (A-E) or hypothermic (F-J) conditions, phase contrast micrographs. Dead cells are stained with trypan blue. The corneal endothelium before lesioning (A, F), the corneal endothelium immediately after lesioning (B-G). A cornea stored in tissue culture medium for 7 days (C), 14 days (D), 21 days (E). (F-J) A cornea preserved in medium for hypothermic storage for 7 days (H), 14 days (I), 21 days (J). Scale bar represents 10 μm.

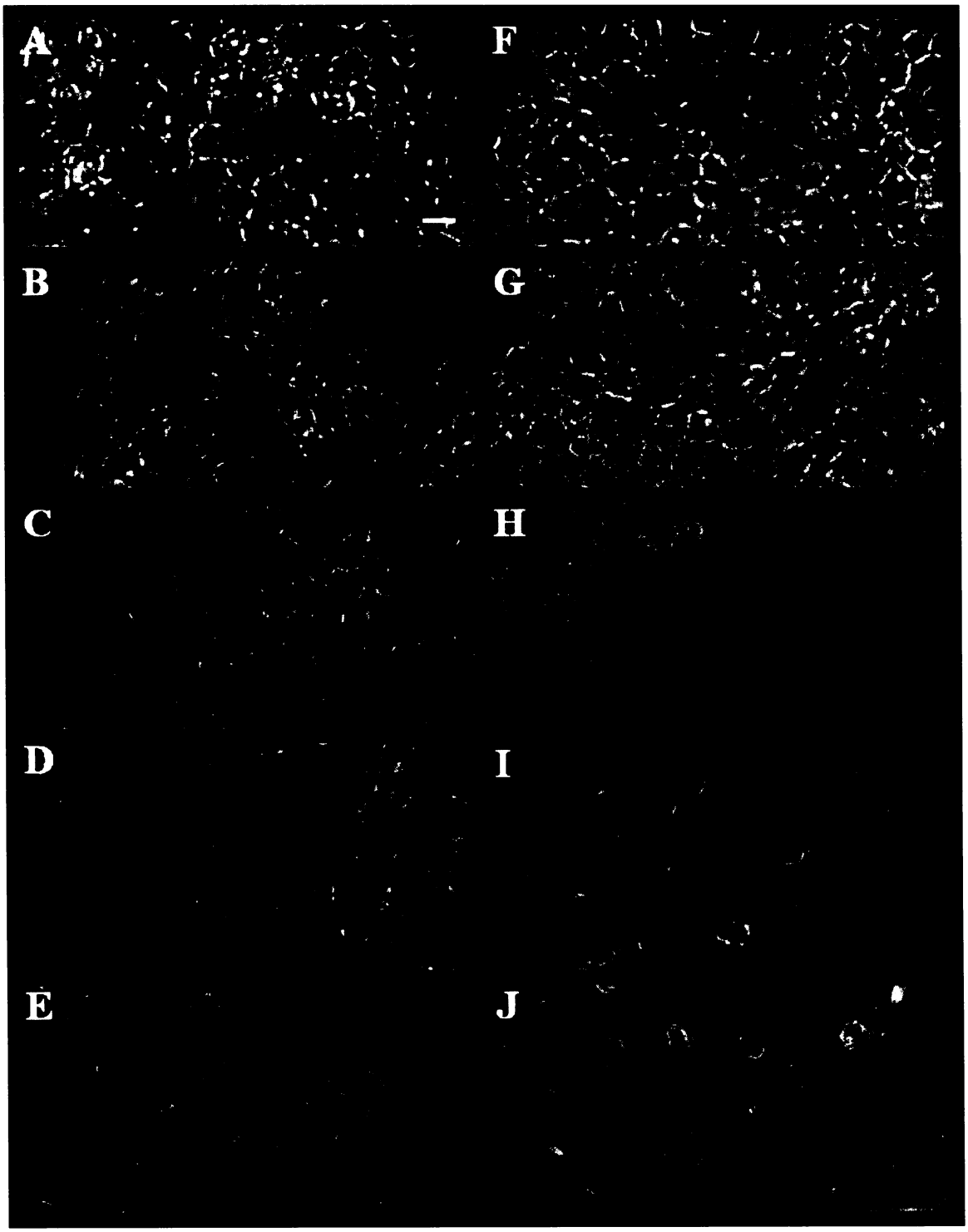


Figure 12: Reparative process of the corneal endothelium after a mechanically induced lesion in tissue culture (A-E) and hypothermic (F-J) storage medium, bright field micrographs. Dead cells are stained with trypan blue. The corneal endothelium before lesioning (A, F), the corneal endothelium immediately after lesioning (B-G). A cornea stored in tissue culture medium for 7 days (C), 14 days (D), 21 days (E). (F-J) A cornea stored in medium for hypothermic storage for 7 days (H), 14 days (I), 21 days (J). Scale bar represents 50 µm.

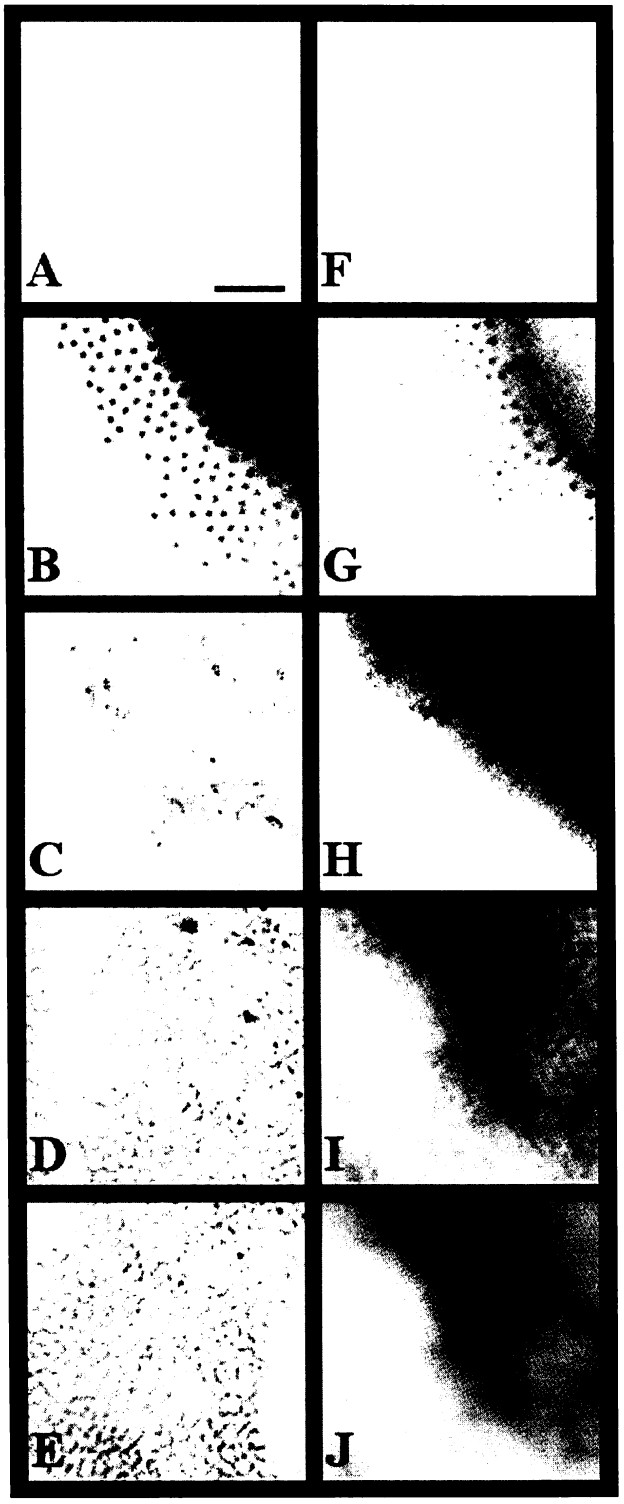


Table 9: Reparation rate of the corneal endothelium after mechanically induced damage. Lesion area and percentage of original lesion area. ± SD...standard deviation.

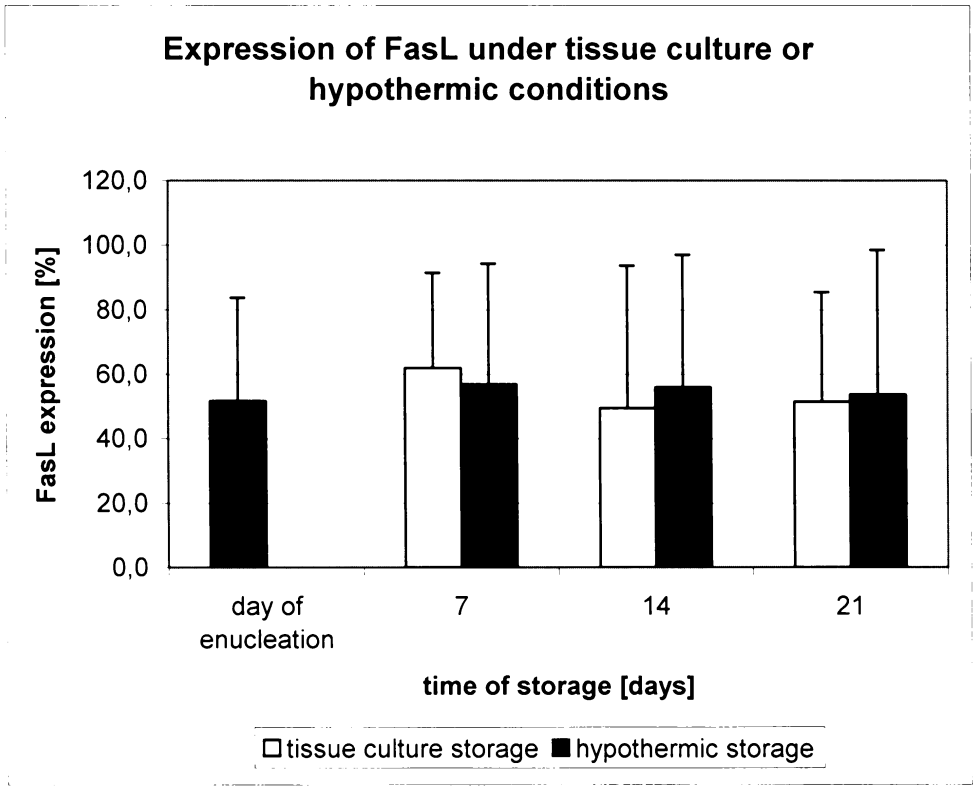
number of cornea	lesion area [mm2]	lesion area [%]	lesion area [mm2]	lesion area [%]	lesion area [mm2]	lesion area [%]	lesion area [mm2]	lesion area [%]	lesion area [mm2]	lesion area [%]	lesion area [mm2]	lesion area [%]
day of storage	0	0	1	1	2	2	3	3	4	4	5	5
812/P	1,25	100,0	1,05	84,0	0,68	54,4	0,10	8,0	0,00	0,0	0,00	0,0
843/P	1,08	100,0	0,99	91,7	0,50	46,3	0,34	31,5	0,00	0,0	0,00	0,0
843/L	1,18	100,0	0,95	80,5	0,44	37,3	0,03	2,5	0,00	0,0	0,00	0,0
848/P	1,25	100,0	1,15	92,0	0,60	48,0	0,38	30,4	0,04	3,2	0,00	0,0
848/L	1,03	100,0	0,98	95,1	0,44	42,7	0,01	1,0	0,00	0,0	0,00	0,0
851/P	1,03	100,0	0,73	70,9	0,42	40,8	0,02	1,9	0,00	0,0	0,00	0,0
851/L	1,17	100,0	0,84	71,8	0,37	31,6	0,00	0,0	0,00	0,0	0,00	0,0
average	**1,14**	**100,00**	**0,96**	**83,71**	**0,49**	**43,01**	**0,13**	**10,76**	**0,01**	**0,46**	**0,00**	**0,00**
± SD	0,10	0,00	0,14	9,83	0,11	7,45	0,16	14,02	0,02	1,21	0,00	0,00

5.2. FAS LIGAND EXPRESSION AND LOCALIZATION IN HUMAN CORNEAL ENDOTHELIUM

By comparing FasL mRNA levels to those of the β-actin housekeeping gene, FasL expression in the corneal endothelium was analyzed, depending on whether corneas were fresh or stored for 7, 14, or 21 days under tissue culture or hypothermic conditions (Fig.13). The percentage of FasL expression was calculated from three independent experiments performed in duplicate.

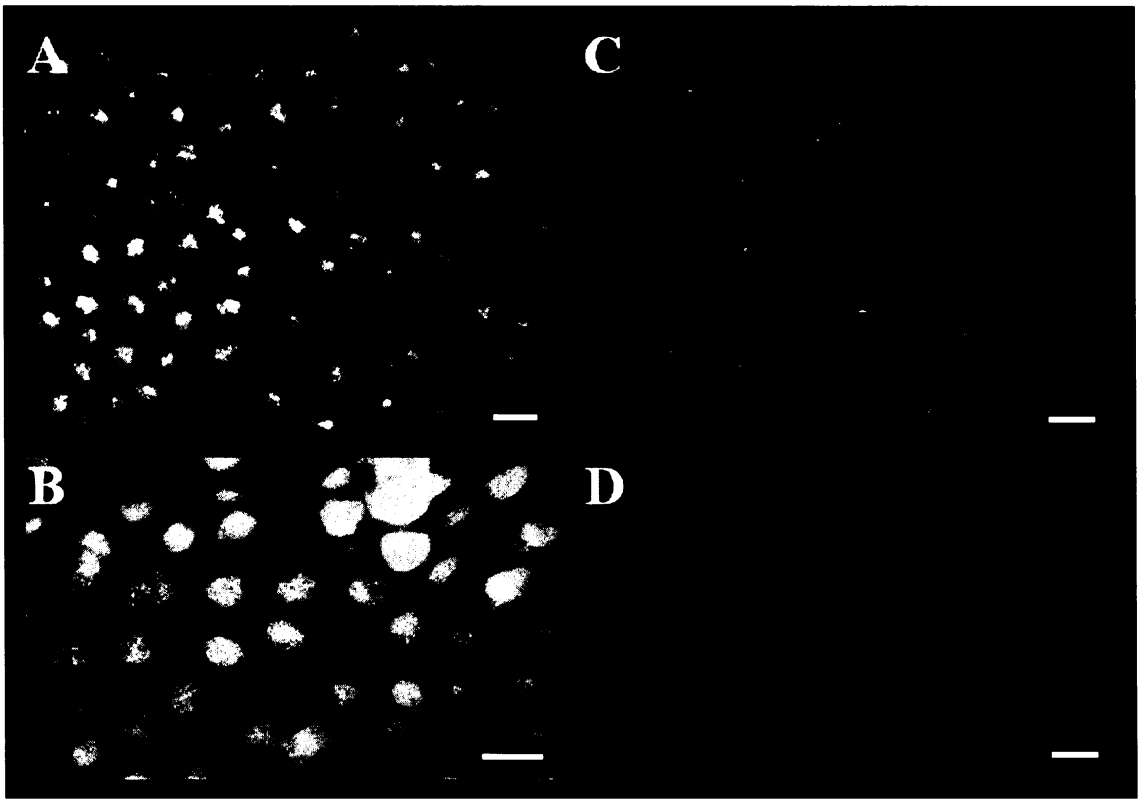
The average expression of FasL compared to the housekeeping gene did not differ significantly either between fresh or stored corneas or between the storage conditions, ranging from 49 to 62 %.

Figure 13: Expression of FasL of corneas stored under tissue culture or hypothermic conditions. Cumulative data from three independent experiments performed in duplicates.



FasL protein was detected at several localization in corneal endothelial cells. Positive staining was found on the plasma membrane, in the perinuclear area, and in the cell nucleus (Fig. 14).

Figure 14: FasL protein localization in the corneal endothelium. Positive staining for the plasma membrane (A), the perinuclear area (B), and the nucleus (C). Negative control (D). FasL is stained by FITC (green), nuclei are stained by propidium iodide (red). Scale bar represents 10 μm.



The mean percentage of FasL positive cells at different locations (plasma membrane, perinuclear area) in fresh corneas, as well as in corneas stored in tissue culture storage medium or hypothermic storage medium is presented in Tab. 10.

Table 10: The mean percentage of FasL positive cells and localisation of the signal in the corneal endothelium of fresh corneas and corneas stored under tissue culture or hypothermic conditions for 7, 14 or 21 days was determined for the plasma membrane and the nuclear and perinuclear areas.

day of storage	plasma membrane staining [%]		perinuclear staining [%]	
0	87		6	
	tissue culture conditions		Hypothermic conditions	
	plasma membrane staining [%]	perinuclear staining [%]	plasma membrane staining [%]	perinuclear staining [%]
7	85	51	59	27
14	70	0	42	41
21	67	0	41	14

FasL protein was detected in more than 90 % of endothelial cells of fresh corneas; the signal was present on the plasma membrane (85 % of cells), and in the perinuclear area (6 %).

Under tissue culture conditions, a small storage time-dependent decrease in plasma membrane staining could be seen, with 67 % of positive cells after 21 days of storage. In 50 % of endothelial cells a positive signal was present in the perinuclear area on day 7. No perinuclear positivity could be seen in the endothelium of corneas stored 14 or 21 days in tissue culture.

A pronounced decrease of FasL protein in the plasma membrane was observed under hypothermic conditions. Plasma membrane positivity of 59, 42, and 41 % of positive cells was found after 7, 14, and 21 days, respectively. The localisation of FasL in the perinuclear area was present for the whole experiment period, ranging from 14 to 41 % of positive cells.

Nuclear staining was found in 24 % of all corneal endothelial cells on average, the values ranged from 0 - 100 % positive.

5.3. NITRIC OXIDE DETECTION IN STORAGE MEDIUM

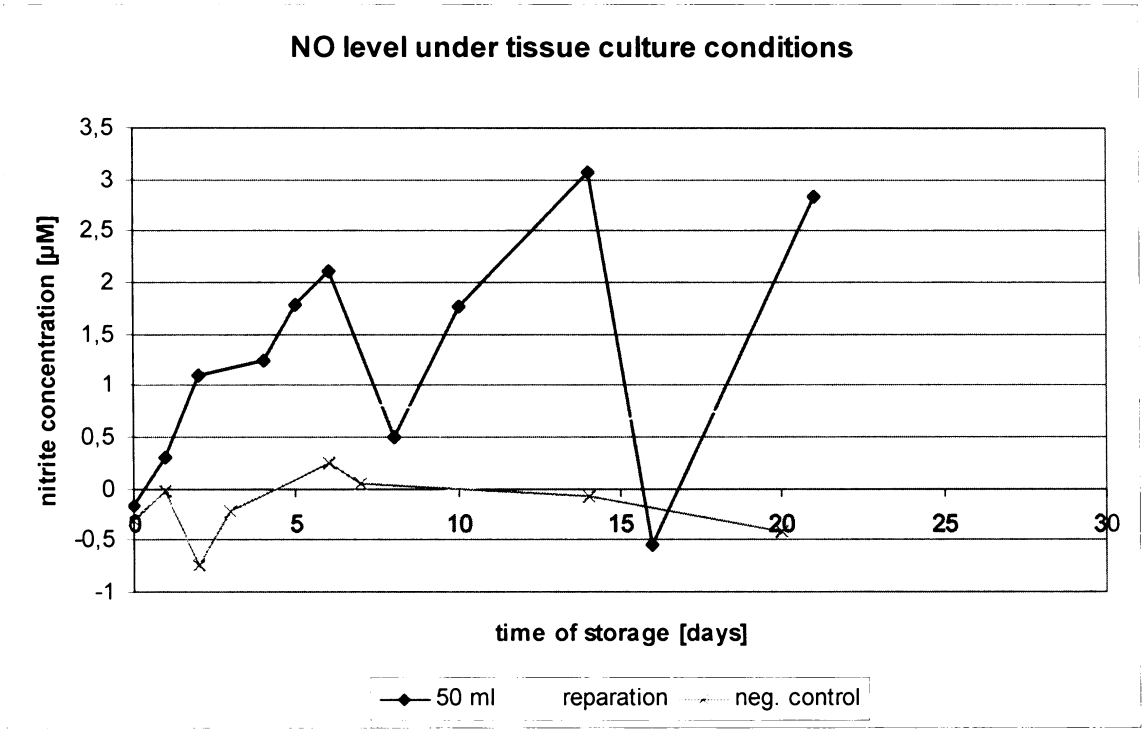
NO levels produced by corneas stored in tissue culture or hypothermic storage media were measured after different storage periods.

Corneas stored under tissue culture conditions in 50 ml of medium (normally used for the storage of corneas for grafting) showed an increase in nitrite concentration up to approximately 2, 3 and 3 μM at day 7, 14, and 21, respectively. After transfer to fresh medium, the nitrite concentration decreased to zero (Fig. 15).

Lower nitrite concentrations were calculated in corneas after endothelial damage, with a steady tendency about 0.8 μM.

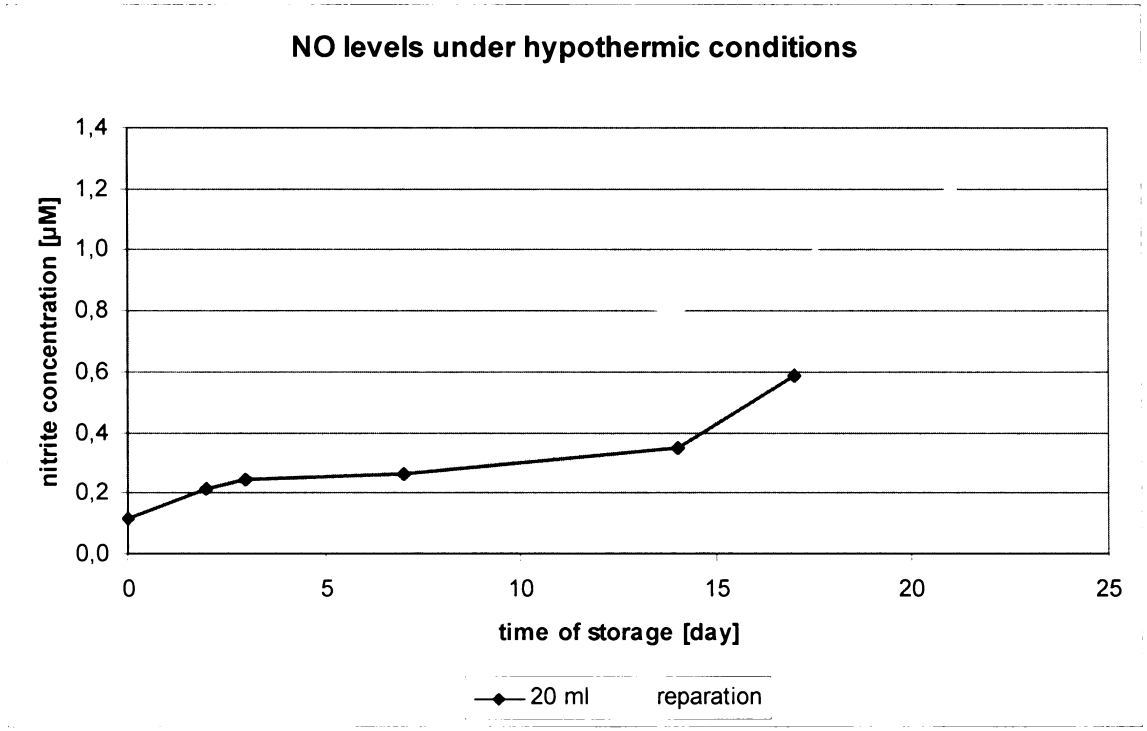
To exclude the possibility that phenol red pH indicator change could influence the colorimetric measurements, a tissue culture medium sample was included in the experiment, as a negative control. NO in the medium alone was not detected, indicating that phenol red does not interfere with the Griess reaction. As the detection limit of the Griess reaction in these conditions is about 0.7 μM, as indicated by standard ditution procedure (data not shown), the oscilations of calculated NO levels in tissue culture medium sample are not significant.

Figure 15: NO concentration produced by corneas in medium for tissue culture storage.



The production of NO by corneas stored in Optisol-GS medium slightly increased in a time dependent manner, reaching 0.6 μM on day 17. The nitrite concentration of corneas after endothelial damage was similar throughout the whole period, ranging from 0.8 to 1.2 μM (Fig. 16).

Figure 16: NO concentration produced by corneas in medium for hypothermic storage.



6. DISCUSSION

6.1. REPARATIVE CAPACITY OF CORNEAL ENDOTHELIUM UNDER HYPOTHERMIC OR TISSUE CULTURE CONDITIONS

This study examined the repair capacity of corneas that underwent a mechanical injury of the endothelium and the influence of the length and type of storage. A corneal endothelial lesion with a mean surface area of 1.14 mm^2 was completely restored within 5 days in tissue culture, while no repair was observed under hypothermic conditions.

Although the lesions of all the corneas healed relatively quickly, within 5 days of culturing, the ECD in the wounded central endothelium did not reach the lowest value of ECD (2000 cells/mm^2) that is considered acceptable for grafting (THURET *et al.* 2005) for up to 14 days after injury. This minimum density (2062 ± 417/mm2) was reached after 21 days storage in tissue culture medium only. The process of repair (increased ECD) continued throughout 21 days of culture (the central ECD reached 64, 70, and 78 % of original ECD values on day 7, 14, and 21 after injury, respectively).

Two opposite processes occurring in the corneal endothelium probably influence the ECD values. (1) the pericentral shift of the cells to the wounded area, which leads to an increased ECD in the wounded area, as well as to a decrease in the ECD in areas adjacent to the damage, (2) an overall decrease of ECD dependent on the length of storage. The storage of human corneas before grafting under either hypothermic or tissue culture conditions inevitably causes cell death in the endothelium, thus reducing ECD (FRUEH and BOHNKE 2000, MEANS *et al.* 1996).

A decrease in the mean ECD was observed in the pericentral area as well (reaching 90, 88, and 85 % of original values at day 7, 14 and 21 after injury, respectively). This 10–15 % decrease is slightly greater than the decrease seen in undamaged corneas stored under tissue culture conditions, in which 5 and 10 % of the original values were found after one and two weeks of storage (FRUEH and BOHNKE 2000).

On the other hand, a lesion to the endothelium of a cornea stored under hypothermic conditions does not change in either its extent or in the presence of dead cells at the injury site. Similarly, the mean ECD at locations adjacent to the wound is relatively stable, decreasing to 95, 93, and 90 % of original values after 7, 14, and 21 days of preservation, respectively. The cloudiness and edema of the cornea as well as the increase in the percentage of dead cells (1.5 and 8.4 % after 14 and 21 days of preservation, respectively) indicate a worsening of the corneal state from day 14 of preservation. Our experience has shown that the presence of dead cells before storage leads to a more pronounced decrease in ECD values within 14 days compared to corneas with no dead cells before storage (data not shown).

Although the presence of dead cells as a restrictive factor for corneal storage or grafting is not commonly accepted, several banks require no more than 5-10 % of dead cells at the second (before grafting) assessment in order to release a cornea for grafting (EEBA Directory 2007). The assessment of dead cells is performed by most banks that prefer tissue culture storage.

In contrast, under hypothermic storage (used regularly in banks associated under EBAA) the percentage of dead cells is not calculated because the staining of dead cells could not be perform in the closed system in which the corneas are kept from excision up to grafting (CHU 2000). The impossibility of an assessment of dead cells under hypothermic preservation conditions, where the replacement of dead cells is blocked probably due to an inhibition of cell metabolism at low temperature, increases the risk of transplanting corneas with high numbers of dead cells.

The reparative capacity of the culture medium should be effective the same way as its ability to improve of corneal endothelium when hypothermic storage was prolonged to the upper temporal limit for this procedure, and corneas were later used for transplantation after spending some time in tissue culture (CAMPOSAMPIERO *et al.* 2003).

In our experiments, the size of the lesion that had to be repaired negatively influenced the ECD. However, in cases in which dead cells are located in one or more smaller areas or are diffusely present in the endothelium, the shifting and enlargement of cells to repair such damage may lead to a relatively smaller decline in ECD.

The stimulative effect of tissue culture on the repair of endothelial cells is supported by the proliferative capacity (expressed by the presence of proliferative cell nuclear antigen) detected in the peripheral corneal endothelium after cornea storage in tissue culture, but not observed before culturing (GAN and FAGERHOLM 1998).

We have shown here that corneas with a relatively high percentage of DC together with a high ECD can be stored for grafting. The quality of corneas with a high incidence of dead cells has to be checked by a second assessment, the results of which will indicate whether the cornea is suitable for grafting or not.

6.2. COMPARISON OF FAS LIGAND EXPRESSION AND LOCALIZATION IN FRESH AND STORED CORNEAS

The detection of FasL expression was performed on endothelial cells of fresh corneas and corneas stored under tissue culture or hypothermic conditions.

The main difficulty with the quantification of FasL expression is due to the low number of cells obtained from one cornea using impression cytology. Within our experimental work we had to solve a number of technical problems associated with the small amount of tissue.

Two RNA isolation methods were attempted; (1) isolation using TRI Reagent resulted in lower RNA purity and subsequently leading to non-specific product amplification. (2) isolation using the NucleoSpin RNAII kit led to more pure RNA, but the amount of RNA harvested by this technique was insufficient, so the first technique was preferred.

For the detection of the amplification product, both agarose electrophoresis and real-time PCR were used, and many optimization procedures had to be performed, including designing of 4 pairs of primers. Due to the small amount of RNA available and possible DNA contamination, specific intron-spanning primers combined with TaqMan probe detection were finally used.

The epression of the FasL gene compared tothat of housekeeping gene for the most positive sample was accepted as 100 % expression in each experiment, and the other samples were compared with this reference sample.

The expression did not change significantly after the corneal storage under either tissue culture or hypothermic conditions for different time periods. This persistent FasL expression during corneal storage and consequently soon after grafting may markedly help corneal survival shortly after grafting.

To confirm the data obtained by real-time RT-PCR and to localize the FasL protein, immunohistochemical detection was performed on human corneal endothelial cells obtained by the impression cytology method. The impression cytology of corneal endothelium was introduced by our laboratory with the aim of obtaining intact endothelial cell layer. Compared to the the evaluation of corneal tissue sections, this

method enables a more detailed observation of the cellular structures, including the morphology of the cell nuclei and cell-to-cell junctions.

The localization of FasL protein in fresh corneas and corneas stored in tissue culture or hypothermic medium for different time periods was compared. FasL localization was observed on the plasma membrane, in the perinuclear area and in the nuclear area.

The intense signal for FasL protein was observed in about 90 % of control endothelial cells obtained from fresh corneas, mostly localized on the plasma membrane. This localisation in plasma membrane is consistent with the findings of STUART *et al.* (1997) who detected FasL in healthy human corneal sections.

Besides this distribution, FasL was found in perinuclear area (6 % of endothelial cells). Similar perinuclear positivity in corneal endothelial cells was observed on corneal sections of patients with conjunctival melanoma by WILSON *et al.* (1996).

At day 7 of tissue culture storage, the percentage of plasma membrane-positive cells remained almost the same, but there was an abrupt increase in perinuclear staining (from 6 to 51 % of positive cells). On the other hand, a slight decrease (to about 70 %) could be observed after 14 and 21 days of storage, but no FasL-positivity was found in the nuclear area.

The perinuclear localization of FasL on day 7 of tissue culture could be explained as the reaction of the cornea to the transfer of fresh tissue into preservative medium. This is in agreement with the fact that no perinuclear localization was found at day 14 or 21 of tissue culture storage, indicating that the FasL distribution in corneal endothelial cells is the same under physiological and storage conditions.

The presence of FasL protein during tissue culture storage and in corneas prepared for grafting may improve the survival of the corneal graft immediately after keratoplasty, because FasL kills infiltrating lymphocytes of the recipient. The significance of FasL in preventing graft rejection was proven by experiments with FasL-blockage which demonstated that FasL-deficient corneas underwent immunological rejection quickly after grafting (HORI *et al.* 2000). However, other changes in FasL expression and localization can occur after grafting and there is the possibility that FasL expression on the plasma membrane can reappear after keratoplasty is performed.

On the other hand, a strong decrease in the number of FasL-positive cells was observed under hypothermic storage conditions. At day 7, 14, and 21, a time-dependent decrease of plasma membrane-positivity (up to 40 %) was found, compared to fresh corneas, while perinuclear positivity could be seen at all time points (ranging from 14 to 27 %).

The total number of FasL-positive endothelial cells in corneas stored in hypothermic medium appears to remain constant, but there was a difference in their localization (with a higher percentage of positive cells seen in the area adjacent to the nucleus). These observations could be explained by the inhibition of FasL transport from the perinuclear area (probably the endoplasmic reticulum) to the plasma membrane under cell metabolism-inhibiting conditions.

A signal that appeared to be nuclear was seen in approximately 24 % of cells (ranging from 0 to 100 %) independently of storage time and conditions. However, studies to distinguish between nuclear or cytoplasmic localization (for example, by confocal microscopy) have not yet been performed. No data concerning nuclear FasL-localization are known from the literature.

6.3. CONCENTRATION OF NITRIC OXIDE, GENERATED BY CORNEAL CELLS UNDER STORAGE CONDITIONS

The higher concentrations of NO in storage medium than in aqueous humour could negatively influence corneal cell vitality, especially the vitality of the unproliferating endothelium, whose quality is essential for graft survival. We believed that an increase in NO concentration would occur mainly in tissue culture storage medium, where the corneas are stored at 31 °C. An increase in NO concentation in medium for hypothermic storage (4 – 8 °C) was though to be unlikely. The results obtained have confirmed our presumption. The increase in NO concentration was observed in a time- and storage-dependent manner.

Under tissue culture storage conditions, there was a gradual increase over 7 days when the cornea was maintained in unchanged storage medium. Subsequently, the original medium was removed and fresh medium added. The level of NO in the fresh medium then increased again from zero until day 14 when the medium was changed again, followed by a similar increase over the subsequent week-long time period. The maximum NO level (expressed as nitrite and nitrate concentration) was found after 14 days of storage (3.1 μM). This level should not be toxic to the corneal endothelial cells, because it is even lower than the normal NO concentration in the aqueous humour (KIM *et al.* 2002). No similar experiments with NO concentration measurements have been performed to confirm or refute our data.

In medium for hypothermic storage, a slight increase in NO concentration was observed up to 0.6 μM on day 17. These results can be compared to the experiments of JENG *et al.* (2002), who measured the level of NO in Optisol-GS storage medium as well. Nitrite concentration on day 17 ranged from about 1.5 to 2.5 in their study. Nitrite concentrations measured by our laboratory were slighly lower, but still consistent with the results obtained by JENG *et al.* (2002).

The level of NO produced in hypothermic storage medium (20 ml) increased in a time dependent manner and was lower than NO concentration in tissue culture media as well as in the aqueous humor, a physiological conditions for the corneal endothelium.

The increase in nitrite and nitrate levels in corneal storage media over time suggest that NO is constitutively realesed by corneas during storage before transplantation. Our results show that the level of NO produced in corneas stored under both storage methods, is lower than the NO concentration necessary to induce cytotoxic damage to the corneal endothelium.

The concentration of NO in tissue culture storage medium is higher than that in medium for hypothermic storage despite the fact that the corneas are stored in 50 ml of tissue culture medium, whereas in Optisol-GS, a 20-ml volume is used. This observation is likely to be due to the metabolic activity of corneal cells under tissue culture conditions and the inhibition of cell metabolism in hypothermy.

7. CONCLUSION

The aims of this study were to examine the behaviour of corneas under tissue culture or hypothermic condtitions, to characterize the repair capacity of human corneal endothelium and its rate, as well as to examine the other factors essential for the maintaining of corneal quality during storage and after grafting, namely Fas ligand expression and presence in the corneal endothelium and nitrite oxide level in the storage medium.

Our results showed that the repair process occurs only during tissue culture storage, where a lesion area of 1 mm^2 was fully repaired within 5 days; however, the endothelial cell density in the wounded area continued to increase up to 3 weeks post-injury. These results indicate that corneas with the presence of dead cells or endothelial defects should be stored under tissue culture conditions. The repair kinetics enable us to estimate the storage times that are sufficient for repairing larger defects of the corneal endothelium.

We discovered that Fas ligand is maintained on the plasma membrane for at least 3 weeks of tissue culture storage. Fas ligand is expressed in the endothelium of corneas stored under hypothermic conditions, as well, but partial inhibition of protein transport to the plasma membrane occurs. Fas ligand protein was found on the plasma membrane and perinuclear area

We determined the level of nitric oxide in corneas stored under tissue culture or hypothermic conditions and found that these concentrations are lower that those that could cause cytotoxic effects, especially in the endothelial cells.

The results of this work have practical consequences that can be useful for further improving of tissue quality for keratoplasty and increasing our understanding of the behaviour of corneas stored under different conditions and for different lengths of time.

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ORGAN CULTURE, BUT NOT HYPOTHERMIC STORAGE, FACILITATES THE REPAIR OF THE CORNEAL ENDOTHELIUM FOLLOWING MECHANICAL DAMAGE.

Jana Nejepinska[1], Kristyna Juklova[1] and Katerina Jirsova[1,2]

1 Laboratory of the Biology and Pathology of the Eye, 1st Faculty of Medicine, Charles University, Prague, Czech Republic

2 Ocular Organ Bank, General Teaching Hospital, Prague, Czech Republic

Corresponding author:

Katerina Jirsova, PhD

Laboratory and Ocular Tissue Bank, Department of Ophthalmology, VFN, U nemocnice 2, 128 08, Prague, Czech Republic,

E-mail: katerina.jirsova@vfn.cz

Tel: +420 224 963 008, Fax: +420 224 962 338,

Abstract:

Purpose: To evaluate the reparative capacity of the mechanically injured endothelium of corneas stored under organ culture (OC) or hypothermic conditions.

Methods: The central endothelium of 24 paired human corneas with similar endothelial parameters was damaged to create an endothelial lesion about 1.0 mm^2. One cornea from each pair was stored under OC (E-MEM) and one under hypothermic conditions (Optisol-GS).

The endothelial cell density (ECD), coefficient of varation, hexagonality and the percentage of dead cells were assessed using a Lucia computer analysis system before and after damage and on days 7, 14, 21 and 28 of storage.

Results: The mean ECD before damage was 2765 ± 349 and 2834 ± 381/mm^2 in the central and pericentral areas, respectively. Immediately after the damage, a denuded Descemet's membrane with dead cells was observed at the injured area. After 7 days storage under OC conditions, almost no dead cells were observed at the place of injury. A non-significant worsening of the qualitative parameters (polymegatism and pleomorphism) was found. After 14 days ECD was 1933 ± 354/mm^2 and 2478 ± 425/mm^2 centrally and pericentrally, respectively. Similar values were found after 21 and 28 days of storage. A lesion with remnant dead cells was observed after 7, 14 and 21 days of hypothermic storage; from day 14 the corneas became cloudy and were in poor condition.

Conclusion: The reparative capacity of the cornea is maintained under OC but not under hypothermic conditions. The quality of corneas containing dead endothelial cells can be improved during OC storage, which is therefore "the method of choice" for storing corneas with dead cells.

Introduction

There are two distinct approaches that are commonly used for storing corneas for subsequent grafting: hypothermic and organ culture (OC) storage (Maas-Reijs et al. 1997).

Storage under hypothermic conditions is the method of choice in the United States. Of the European eye banks associated with the European Eye Bank Association (EEBA), 57 % use only the organ culture (OC) method, 17 % preserve corneas only under hypothermic conditions, while 26 % use both methods (EEBA 2007).

Under hypothermic storage, the cornea is generally maintained in a commercially available medium (Optisol-GS, Eusol-C, Likorol at 4–8 °C. All these media have very similar components: nutrients, energy-rich molecules, antibiotics, antimycotics and osmotically active substances (chondroitin sulphate, dextran) to maintain physiologic thickness and clarity of the tissue. The cornea is maintained in medium in a closed system from preparation to grafting, including during corneal assessment using slit-lamp and specular microscopy (Chu 2000). The aim of this approach is to minimize or even inhibit cell metabolism in order to preserve the original condition of the cornea as long as possible. Although the maximum recommended storage time is 14 days, it is well known that shorter storage periods are usually used for corneas intended for grafting, so that they remain in optimal condition. In the United States as well as in Europe, the average storage time is 4 days (Wilhelmus et al. 1995; EEBA 2007).

Corneal storage under OC conditions is more technically difficult and time-consuming. The cornea is maintained at 31-37 °C in media based on minimal essential medium with 2% foetal calf serum (Pels et al. 1999). Media are prepared by the bank staff or are purchased (CorneaMax, Eurobio, les Ulis, France; Inosol, Opsia, France) from commercial sources.

The main aim of OC is to support cellular (especially endothelial) metabolism and viability so as to maintain the cornea under conditions similar to physiological ones. OC corneas can be stored up to 35 days, which enables the creation of better transplantation schedules and allows matching between donor cornea and recipient and the performance of microbiological controls (Armitage & Easty 1997). A few days before transplantation, the cornea is transferred into transport medium, in which osmotically active substances (usually 5% dextran) leads to deswelling of the cornea. Then a second assessment is performed to detect potential latent endothelial cell damage and to determine the condition of the cornea shortly before transplantation.

An intact functional endothelium with high endothelial cell density (ECD) is critical for long-term tissue survival (Bourne 2001). It is well known that the proliferative capacity of

the corneal endothelium is inhibited soon after birth (Laule 1987). The endothelial cells are arrested in the G_1-phase of the cell cycle due to contact inhibition, the antiproliferative activity of TGFβ 2 in the aqueous humor and an age-related decrease in sensitivity to mitogens (Joyce et al. 1996; Joyce et al. 2002; Chen et al. 1999; Senoo & Joyce 2000; Joyce et al. 2004). ECD decreases from 6000 cells per mm^2 at birth to 3500 at age 4–5 (Nucci et al. 1990). Later, ECD decreases with age at a rate of approximately 0.6 % per year (Murphy et al. 1984; Bourne et al. 1997); thus, at age 85, the endothelium has 2300 cells/mm2 (Yee et al.1985). The lowest permissible ECD generally accepted for corneas intended for grafting is between 2000 – 2300 cells/mm^2 (Pels et al. 1999; Thuret et al. 2005). When ECD decreases to 300 – 500 cells/mm2, the endothelial monolayer can be compromised, resulting in stromal edema, corneal clouding and loss of visual acuity. Currently, there is no medical treatment available for a decrease in ECD below this critical threshold other than corneal transplantation (Joyce et al. 2004).

The aim of this study was to compare the extent and speed of repair of the mechanically damaged endothelium of corneas stored under OC or hypothermic conditions. When dead cells are removed from the endothelium, cell-free areas are repaired by a shift of the surrounding endothelial cells and by their enlargement (Hoppenreijs et al. 1994). The assessment of dead endothelial cells is one of the crucial aspects of corneal evaluation. The incorrect assessment of dead cells as viable cells in the corneal endothelium may lead to falsely elevated ECD values. The number of dead cells is difficult to evaluate in a closed hypothermic system, because no staining of dead cells is usually performed and morphological evaluation can only be performed over a relatively small area using specular microscopy. In an open system in which the cornea is prepared under sterile conditions in a laminar hood, trypan blue staining of dead cells is usually performed and the percentage of dead cells is calculated using a computer analysis system (Deb-Joardar et al. 2007). The percentage of dead cells in corneas intended for grafting should not exceed 5 % (EEBA 2007). Corneal ECD decreases during both OC and hypothermic storage (Pels & Schuchard 1983; Komuro et al. 1999). The cell loss may not exceed a maximum of 20 % during 30 days of storage because an abnormal cell loss of more than 20 % may indicate latent damage to the endothelial cells which was not visible before culturing (Pels & Schuchard 1983).

Materials and methods

Repair of corneas under OC and hypothermic conditions

The use of human corneas adhered to the tenets of the Declaration of Helsinki. Twelve corneal pairs unsuitable for transplantation (reasons other than endothelial quantity or quality) were used for the study. The whole bulbi were obtained from donors (mean age 67 ± 12 years, range 47-81 years) less than 24 hours after death. After decontamination, the eye bulbi were examined by slit lamp, than corneoscleral rings were obtained by trephination under sterile conditions in a biohazard hood. Corneas with a calculated endothelial cell density (ECD) per mm^2, percentage of dead cells (DC), endothelial cell density of live cells (ECDA) per mm^2, coefficient of variation of cell area (CV) and percentage of hexagonal cells (6A), were used.

Only corneas with an ECDA higher than 2000 cells/mm^2 and with similar endothelial parameters between paired corneas were used: ECD differed no more than 10 %, the percentage of DC did not differ more than 1.6 %. The number of Descemet's folds did not differ by more than three.

The assessment was performed after the corneal endothelium was treated with 0.12 % trypan blue in PBS and 0.9 % sucrose using light microscopy (Olympus, CKX41, Tokyo, Japan) and Olympus C-3040 camera, (Olympus, Tokyo, Japan). The ECD, CV and 6A were assessed from 1 central (0.3 mm^2) and 4 non-overlapping pericentral phase contrast photographs (magnification 20x). The ECD of dead cells was assessed from bright field photographs of the same parts of the cornea at a magnification of 100x to include a larger area for assessing dead cells and the percentage of dead cells was calculated. All photographs were processed by a semi-automated Lucia computer analysis system to obtain average values of the ECD, ECDA percentage of DC, CV and 6A separately in the central and pericentral parts of the cornea.

After the initial assessment, the central part of each cornea was mechanically damaged using a special metal rod. The flat end of the rod (diameter: surface: 0.79 mm^2) was gently pressed for 15 seconds onto the central part of the endothelial surface to induce injury. Subsequently, the damaged corneas were treated with trypan blue and sucrose and photographs were taken again. The size of the wounds varied between corneas from 0.95-1.25 mm^2 (mean 1.14 mm^2).

Two more corneas were used to confirm the induction of corneal damage: immediately after the injury, the endothelium was stained with a trypan blue solution and alizarin red to visualize the denuded Descemet's membrane and any remaining dead cells (Taylor & Hunt 1981).

Subsequently, one cornea of each pair was randomly assigned to hypothermic conditions (Optisol-GS, Bausch&Lomb Inc, Rochester, NY, USA) and stored at 4 °C. The second cornea was cultured at 31 °C in E-MEM (Minimum Essential Medium with Earle's salts), L-Glutamine, 25mM Hepes (AppliChem, GmbH, Germany), NaHCO3 (2,24 G/L), an antibiotic-antimycotic solution of penicillin G (100U/ml), streptomycin sulphate (100µg/ml), and amfotericin B (Fungizone), and 2 % Foetal Bovine Serum both from Invitrogen/Gibco, Glasgow, UK.

Each cornea was assessed again on day 7, 14, 21 and 28 (OC stored corneas only) after approximately 12-hours deswelling in OC medium containing 5% dextran at room temperature.

Speed of repair of corneas stored under OC conditions

Seven additional corneas were mechanically damaged (as described above), and the surface of the injury was examined in bright field photographs (magnification of 100x) taken after trypan blue staining. The same procedure was performed 1, 2, 3, 4 5, and 6 days after damage. Blue spots containing no or dead cells, indicating the extent of injury, were measured using a Lucia computer analysis system.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The quantitative variables were compared by a paired student t test in the case of normal distribution and otherwise by a Wilcoxon nonparametric test. Statistical significance was considered to be a p-value of 0.05 or less. Data analysis was carried out using STATISTICA software.

Results

Repair of corneas under OC and hypothermic conditions

The mean ECD of corneas subsequently stored under OC was 2765 ± 349 and 2834 ± 381/mm^{2}in the central and pericentral areas, respectively. The mean ECD in the central area before damage was 2677 ± 404 and 2761 ± 389/mm^2 in the pericentral area of corneas later stored under hypothermic conditions. The mean percentage of dead cells of all examined corneas was 0.45 ± 0.52 % (ranged from 0 – 1.84 %).

Immediately after inducing an injury of the central cornea, a bluish denuded Descemet's membrane with remnant dead endothelial cells was observed, with little difference between corneas.

As was confirmed on two corneal pairs, the extent of damage was complete over all the surface that was touched by the flat end of the metal rod. No live endothelial cells remained at the site of injury, **Fig. 1**.

In the region adjacent to the wound (pericentral area), the mean ECD was not changed significantly. In this area the percentage of dead cells increased to 2.08 or 2.58 % in corneas destined for storage under OC or hypothermic conditions, respectively. In some of the corneas, bluish spots of different shapes, containing no dead cells, were observed in the areas adjacent to the wound.

The mean values of ECD (± SD), percentage of dead cells, CV, and 6A in the central and pericentral endothelium of fresh corneas and ones stored under OC or hypothermic conditions for different time periods are presented in **Table 1** and **Table 2**.

The presence of cell debris indicated the location of lesions in the central endothelium, observed using bright field microscopy after 7 days storage in OC. No or few dead cells were present in such areas nor in adjacent areas. The original damaged area was fully repaired by enlarged cells of irregular shape: ECD in this central part was significantly decreased ($1762 \pm 267/mm^2$) as was 6A (45.4 ± 7.6), while CV increased (19.6 ± 3.6), **Fig. 2a**. A number of elongated cells were observed shifting centripetally to the site of the former lesion, **Fig. 2b.** The endothelial morphology of fresh and stored corneas is shown in **Fig. 3.**

Seven days of storage does not lead to repair in corneas stored under hypothermic conditions. The bluish-colored wounds of the corneal endothelium, indicating the presence of dead cells, were clearly visible, with no changes in their extent compared to their state immediately after the injury. The area adjacent to the wound was cloudy, but the endothelial cell mosaic was relatively unchanged compared to that before and just after damage. One hundred per cent and 0.7 % of DC were present centrally and pericentrally, respectively. ECD in the pericentral area was $2635 \pm 388/mm^2$.

After 14 days, no signs (presence of dead cells or cell debris) of the injury was observed in OC maintained corneas. The locations of the original wounds were identifiable only by the presence of larger and irregularly shaped cells. ECD was 1933 ± 354 in the central and $2478 \pm 425/mm^2$ in the pericentral area. The percentage of dead cells did not exceed 0.1 %.

Twenty-one days of OC storage led to complete repair. The mean ECD was slightly increased in the central endothelium ($2162 \pm 417/mm^2$) and decreased pericentrally ($2389 \pm 424/mm^2$). A similar condition of the corneas could be observed 28 days after wounding.

The corneas stored under hypothermic conditions became cloudy; the pericentral ECD could be assessed in 10 corneas on day 14 (2523 ± 363/mm^2) and 5 corneas on day 21 (2505 ± 465/mm^2). The extent of the central injury was unchanged compared to the previous assessments, with blue spots indicating the endothelial lesion. In addition to this damage, 1.5 and 8.3 % of dead cells were detected pericentraly on days 14 and 21.

Speed of repair of corneas stored under OC conditions

The mean surface of damaged area was 1.14 ± 0.1 mm^2 (range 1.03–1.25 mm^2) immediately after the injury. The extent of the injury was significantly decreased up to 84, 43, and 11 % of original values after 24, 48 and 72 hours, respectively, **Table 3**. The spot with dead cells was observed in one cornea (0.04 mm^2, i.e 3.2 % of the original injury) after 4 days in OC. No dead cells were present at the place of former wound indicating by rest of cell debris at 6 other corneas. The centra were fully repaired by enlarged cells of irregular morphology.

Discussion

This study examined the repair capacity of corneas that underwent a mechanical injury of the endothelium and the influence of the length and type of storage. A corneal endothelial lesion with a mean surface area of 1.14 mm^2 was completely restored within 5 days in OC, while no repair was observed under hypothermic conditions.

Although the lesions of all the corneas healed relatively quickly -within 5 days of culturing- the ECD in the wounded central endothelium did not reach the lowest value of ECD (2000 cells/mm2) that is considered acceptable for grafting (Thuret et al. 2005) for up to 14 days after injury. This minimum density (2062 ± 417/mm2) was reached after 21 days storage in OC medium only. The process of repair (increased ECD) continued throughout 21 days of culture (the central ECD reached 64, 70, and 78 % of original ECD values on day 7, 14, and 21 after injury).

Two opposite processes occurring in the corneal endothelium probably influence the ECD values: first: the pericentral shift of the cells to the wounded area, which leads to an increased ECD in the wounded area as well as to a decrease in the ECD in areas adjacent to the damage, and second, an overall decrease of ECD dependent on the length of storage. The storage of human corneas before grafting under either hypothermic or OC conditions inevitably causes cell death in the endothelium, thus reducing ECD (Frueh & Bohnke 2000; Means et al. 1996).

A decrease in the mean ECD was observed in the pericentral area as well (reaching 90, 88, and 85 % of original values at day 7, 14 and 21 after injury). This 10 – 15 % decrease is slightly greater than that decrease seen in undamaged corneas stored under OC conditions, in which 5 and 10 % of the original values were found after one and two weeks of storage (Frueh & Bohnke 2000).

On the other hand, a lesion to the endothelium of a cornea stored under hypothermic conditions does not change in either its extent or in the presence of dead cells at the injury site. Similarly, the mean ECD at locations adjacent to the wound is relatively stable, decreasing to 95, 93, and 90 % of original values after 7, 14, and 21 days of preservation. The cloudiness and edema of the cornea as well as the increase in the percentage of dead cells (1.5 and 8.4 % after 14 and 21 days of preservation) indicate a worsening of the corneal state from day 14 of preservation. Our experience has shown that the presence of dead cells before storage leads to a more pronounced decrease in ECD values within 14 days compared to corneas with no dead cells before storage (data not shown).

Although the presence of dead cells as a restrictive factor for corneal storage or grafting is not commonly accepted, several banks require that there be no more than 5 – 10 % of dead cells at the second (before grafting) assessment in order to release a cornea for grafting (EEBA 2007). The assessment of dead cells is performed by most banks that prefer OC storage.

In contrast, under hypothermic storage (used regularly in banks associated under EBAA, the Eye Bank Association of America) the percentage of dead cells is not calculated because the staining of dead cells could not be perform in the closed system in which the corneas are kept from excision up to grafting (Bourne 1997). The impossibility of an assessment of dead cells under hypothermic preservation conditions, where the replacement of dead cells is blocked probably due to an inhibition of cell metabolism at low temperature, increases the risk of transplanting corneas with high numbers of dead cells.

The reparative capacity of the culture medium should be effective the same way as its ability to improve of corneal endothelium when hypotermic storage was prolonged to the upper temporal limit for this procedure, and corneas were later used for transplantation after spending some time in OC (Camposampiero et al. 2003).

In our experiments the size of the lesion that had to be repaired negatively influenced the ECD. However, in cases in which dead cells are located in one or more smaller areas or are diffusely present in the endothelium, the shifting and enlargement of cells to repair such damage may lead to a relatively smaller decline in ECD.

The human cornea has an increased ECD in the pericentral and peripheral regions of cornea compared with the central region. Over a lifetime the central ECD decreases by about 0.6 % per year in (Laule 1978). Therefore the peripheral endothelium may be the area of continual cellular supply used to maintain the central ECD at a relatively constant level during lifetime (Amann et al. 2003).

The stimulative effect of OC on the repair of endothelial cells is supported by the proliferative capacity (expressed by the presence of proliferative cell nuclear antigen) detected in the peripheral corneal endothelium after cornea storage in OC, but not observed before culturing (Gan et al. 1998).

We have shown here that corneas with a relatively high percentage of DC together with a high ECD can be stored for grafting. The quality of corneas with a high incidence of dead cells has to be checked by a second assessment, the results of which will indicate whether the cornea is suitable for grafting or not.

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TABLE 1: The quantitative and qualitative endothelial parameters of the central (injured) and pericentral (adjacent to the injury) endothelium of fresh corneas and corneas stored under organ culture (OC) or hypothermic conditions.
Standard deviation ± SD.

storage (days)	ECD [mm^2]	ECDA [mm^2]	% DC	CV	6A
			organ culture		
before storage	2764,9 ± 348,8	2756,2 ± 345,5	0,3 ± 0,5	17,1 ± 3,6	57,7 ± 6,8
aftter lesion	1762,1 ± 266,7	1755,0 ± 264,9	0,4 ± 0,5	19,6 ± 3,6	45,4 ± 7,6
14	1933,0 ± 354,1	1928,3 ± 349,3	0,2 ± 0,2	20,1 ± 3,0	48,0 ± 10,6
21	2162,0 ± 417,0	2159,4 ± 416,9	0,1 ± 0,3	20,1 ± 3,4	45,4 ± 6,4
28	1889,1 ± 595,4	1878,8 ± 593,2	0,5 ± 0,5	20,1 ± 5,9	49,8 ± 14,3
			hypothermic storage		
before storage	2677,0 ± 403,7	2661,6 ± 408,2	0,6 ± 0,9	18,2 ± 4,4	50,8 ± 11,4

TABLE 2: The quantitative and qualitative endothelial parameters of pericentral (adjacent to the injury) endothelium of the fresh corneas and that ones stored under organ culture (OC) or hypothermic conditions. Standard deviation ± SD.

storage (days)	ECD [mm^2]	ECDA [mm^2]	% DC	CV	6A
		organ culture			
before	2834,0 ± 381,2	2818,1 ± 376,4	0,6 ± 0,6	16,8 ± 2,8	53,8 ± 5,1
aftter lesion	2803,3 ± 406,8	2737,7 ± 467,4	2,6 ± 5,5	17,6 ± 2,6	53,5 ± 7,5
7	2521,9 ± 366,3	2520,4 ± 365,6	0,1 ± 0,1	19,2 ± 1,5	45,0 ± 5,8
14	2478,2 ± 424,9	2475,9 ± 424,2	0,1 ± 0,1	19,6 ± 2,8	48,8 ± 6,5
21	2389,0 ± 424,0	2388,5 ± 424,2	0,0 ± 0,0	19,1 ± 2,3	48,7 ± 4,2
28	2327,4 ± 635,6	2327,1 ± 635,3	0,0 ± 0,0	19,4 ± 5,3	47,5 ± 14,6
		hypothermic storage			
before storage	2760,5 ± 388,7	2751,9 ± 394,9	0,4 ± 0,5	17,2 ± 2,0	52,7 ± 8,1
aftter lesion	2795,3 ± 417,8	2777,3 ± 431,3	0,7± 0,9	17,5 ± 1,9	54,5 ± 7,6
7	2635,0 ± 388,2	2617,6 ± 388,5	0,7 ± 0,6	18,0 ± 2,2	46,6 ± 9,2
14	2522,8 ± 363,5	2488,3 ± 381,2	1,5 ± 2,4	16,6 ± 2,4	49,0 ± 9,3
21	2504,9 ± 465,0	2331,6 ± 653,7	8,4 ± 11,4	18,0 ± 2,2	46,7 ± 5,1

TABLE 3: The extension of the endothelial lesion of corneas assessed at different time periods. *p < 0.05, **p < 0.01, ***p < 0.001. Standard deviation ± SD.

Extension of the lesion at different time periods						
day	immediately after lesion	1	2	3	4	5
mean ± SD	1.14 ± 0.1	0.96 ± 0.14	0.49 ± 0.11	0.13 ± 0.16	0.01 ± 0.02	0.00 ± 0.00
p		0.004027**	0.000001***	0.000001***	0.000001***	0.000000***

FIGURE 1: Bright field micrograph of the corneal endothelium immediately after the induction of the lesion. The extent of the damage was complete over all of the injured surface. Alizarin red S staining. Scale bar represents 50 μm.

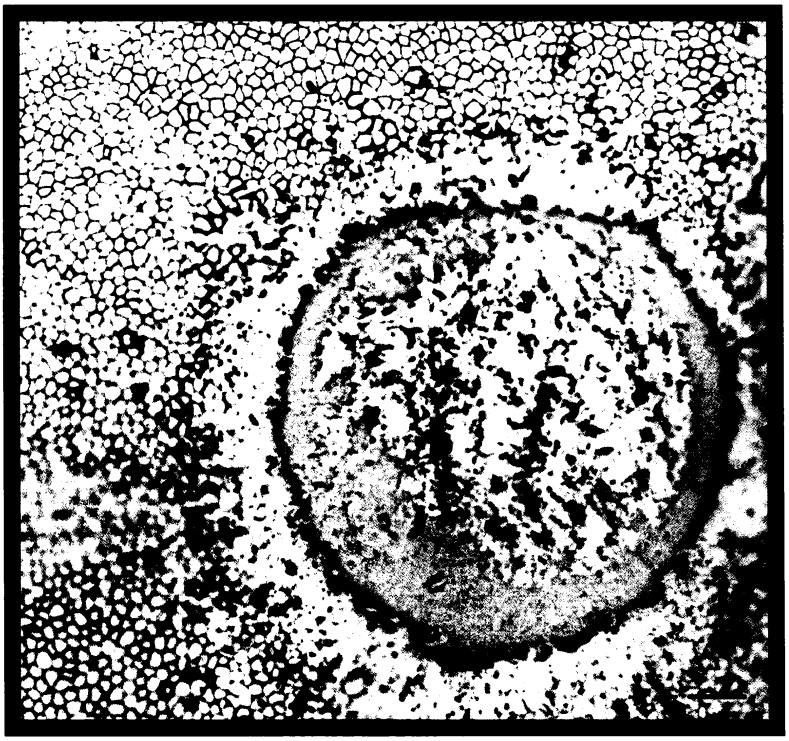

FIGURE 2: Phase contrast micrograph of the corneal endothelium 7 days after the induction of the lesion: irregular cell mosaic. Polymegatism and pleomorphism are clearly visible in the central as well as the pericentral areas (A) and elongated cells are shifting centripetally to the site of the lesion (B). Scale bar represents 10 µm.

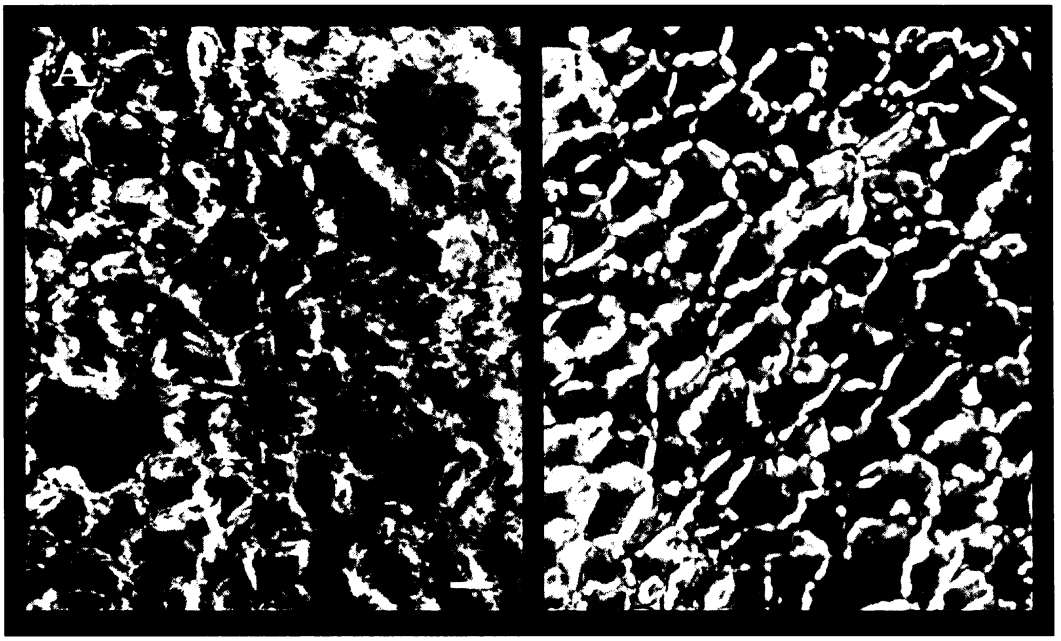

FIGURE 3: Phase contrast micrograph of the pericentral endothelium of fresh corneas and corneas stored under OC (OC) or hypothermic conditions (HYPO) immediately (A), 7 (B), 14 (C), and 21 (D) days after the induction of the lesion. Scale bar represents 10 μm. The bright field microscopy picture demonstrate the central part of the endothelium. Dead cells are stained by trypan blue. Scale bar represents 50 μm.