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Keratin films for ocular surface reconstruction:

Evaluation of biocompatibility in an in-vivo model

Dissertation

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Summary

Background:

Human amniotic membrane (AM) is commonly used for the treatment of recurrent corneal epithelial defects, corneal ulcers and ocular surface reconstruction. Despite its many good properties like the promotion of epithelial growth and differentiation, the use of amniotic membrane exhibits certain disadvantages. These include low transparency, poor standardization because of intradonor and interdonor variability, the risk of transmitting diseases and the challenge to provide an unlimited supply. It is therefore necessary to develop a material which provides higher transparency and standardization, eliminates the risk of infection and further shows good biocompatibility and surgical feasibility. Keratin films (KF) are cysteine-rich structural proteins derived from a material such as human hair. They match several of these requirements demanded of a biomaterial and have been proposed for ocular surface reconstruction. The aim of this study was to evaluate the biocompatibility of keratin films in a rabbit model.

Material and Methods:

46 New Zealand White rabbits underwent a dissection of a corneal intrastromal pocket in which either amniotic membrane or keratin films of different compositions (KF100, KF90/10, KF80/20) were inserted and observed for 10 days or 4 weeks. Animals which underwent a dissection of a corneal intrastromal pocket but received no implant served as control group. Half of the animals received topical steroids while the other half was left without steroids. At the end of each follow-up, a clinical examination was performed to evaluate the parameters transparency, neovascularization and epithelial integrity. A histological examination was performed to evaluate the parameters inflammation, neovascularization and degradation. Keratin films prepared with different percentages of

aqueous and alkaline dialysate were used to evaluate a potential influence on their biocompatibility.

Results:

After 10 days follow-up the clinical results showed no significant differences in corneal transparency between the different keratin films. A slightly better transparency could be observed in the steroid treated eyes compared to the non-steroid treated eyes. No neovascularization was observed in eyes implanted with amniotic membrane or keratin film. The histological results were corresponding to the clinical results as they showed an increased inflammatory cell infiltration in the non-steroid group after 10 days.

After 4 weeks transparent corneas could be observed in all AM implanted eyes and in 3 out of 4 KF90/10 implanted eyes in both treatment groups. The histological results showed a tendency towards a higher inflammatory response in the steroid group compared to the non-steroid group. The inflammatory reaction in the non-steroid group seemed to decrease over time. This corresponds with a significant degradation of the keratin film thickness in this group compared to 10 days and also compared to the steroid group.

Conclusion:

In conclusion, keratin films show a good biocompatibility and transparency in vivo. They induce a minor host tissue response which can be controlled by the application of topical steroids. We assume that the medication with topical steroids slows down the inflammatory response as well as the degradation of the keratin film. A slower degradation of the keratin film under steroid medication might be beneficial for the tissue integration of the transplant and for the matrix regeneration depending on the ocular surface situation and the underlying cause.

Zusammenfassung

Hintergrund:

Amnionmembran (AM) wird üblicherweise zur Behandlung von persistierenden Defekten und Ulcera der Hornhautoberfläche, sowie zur Augenoberflächenrekonstruktion eingesetzt. Neben ihren positiven Eigenschaften, wie der Förderung des Wachstums und der Differenzierung von Epithelzellen, hat die Verwendung von Amnionmembran diverse Nachteile. Diese sind zum Beispiel eine geringe Transparenz, eine schlechte Standardisierung aufgrund der Variabilität der Präparate, ein Risiko für die Übertragung infektiöser Erkrankungen und eine begrenzte Verfügbarkeit. Daher ist es notwendig, ein biokompatibles Material zu entwickeln, welches eine bessere Transparenz bei gleich bleibender Qualität aufweist, kein Infektionsrisiko birgt und eine gute chirurgische Handhabbarkeit aufweist. Keratinfilme (KF) erfüllen viele dieser Anforderungen und stellen daher eine mögliche Alternative zur Amnionmembran dar. Das Ziel dieser Studie war es, die Biokompatibilität von Keratinfilmen in einem Kaninchenmodell zu evaluieren.

Material und Methoden:

Bei 46 Weißen Neuseeländern wurden 4 mm große Implantate, entweder Amnionmembran oder Keratinfilme mit unterschiedlichem Anteil von wässrigem und alkalischem Dialysat (KF100; KF90/10; KF80/20, z.B. enthält KF90/10 90 % wässriges und 10 % alkalisches Dialysat), in eine korneale intrastromale Tasche implantiert. Als Kontrollgruppe dienten Tiere, bei denen eine corneale intrastromale Tasche ohne eine Implantateinlage präpariert wurde. Die Tiere wurden für einen Zeitraum von entweder 10 Tagen oder 4 Wochen observiert. Die Hälfte der Tiere erhielt eine Medikation mit topischen Steroiden während die andere Hälfte der Tiere keine Steroide erhielt. Nach Beendigung der Observationszeit wurde eine klinische Evaluation mittels Spaltlampenuntersuchung vorgenommen, um Transparenz, Neovaskularisation und epitheliale Integrität der Implantate zu beurteilen. Danach wurden die Hornhäute zur

histologischen Aufbereitung und Beurteilung entnommen. Es wurden Keratinfilme mit unterschiedlichen Anteilen von wässrigem und alkalischem Dialysat getestet, um einen potentiellen Einfluss auf die Biokompatibilität zu beurteilen.

Ergebnisse:

Nach zehntägiger Observationszeit zeigte sich im klinischen Bild kein signifikanter Unterschied der Hornhauttransparenz zwischen den Keratinfilmen unterschiedlicher Zusammensetzung. Zudem zeigten sich keine Neovaskularisationen in Augen, welche mit Keratinfilmen oder Amnionmembran implantiert wurden. Es konnte eine etwas höhere Transparenz in den mit Steroiden behandelten Augen aufgezeigt werden, verglichen mit Augen ohne Steroidbehandlung. Die histologischen Ergebnisse korrelierten mit den klinischen Ergebnissen und zeigten eine stärkere Infiltration von Entzündungszellen in den Augen ohne Steroidbehandlung.

Nach 4 Wochen zeigten sich transparente Hornhäute in allen AM implantierten Augen, sowie in 3 von 4 KF90/10 implantierten Augen sowohl mit als auch ohne Steroidbehandlung. Die histologischen Ergebnisse zeigten eine Tendenz zur stärkeren Inflammation in der Gruppe der mit Steroiden behandelten Augen im Vergleich zu den Augen ohne Steroidbehandlung.

Die inflammatorische Reaktion war in der Gruppe der Augen ohne Steroidbehandlung nach 4 Wochen geringer im Vergleich zu 10 Tagen. Dies ging einher mit einem signifikanten Abbau der Keratinfilme in dieser Gruppe nach 4 Wochen verglichen zu 10 Tagen. Ebenso zeigte sich in der Histologie ein signifikanter Abbau der Keratinfilme in Augen ohne Steroidbehandlung verglichen zu den Augen mit Steroidbehandlung.

Schlussfolgerung:

Zusammenfassend kann gesagt werden, dass Keratinfilme eine gute Biokompatibilität und Transparenz in vivo aufweisen. Sie können

erfolgreich in die Kornea implantiert werden und induzieren eine milde inflammatorische Reaktion, welche sich durch die Gabe von topischen Steroiden kontrollieren lässt. Wir nehmen an, dass die Medikation mit topischen Steroiden die inflammatorische Reaktion und den Abbau der Keratinfilme verlangsamt.

Ein langsamer Abbau des Keratinfilms unter Medikation mit Steroiden kann für die Integration des Gewebes in die Augenoberfläche und die Regeneration von Vorteil sein. Weitere Experimente sind notwendig, um Langzeitwerte zur Biokompatibilität und Eignung von Keratinfilmen als Transplantat zur Augenoberflächenrekonstruktion zu erhalten.

Abbreviations

°C	degrees Celcius
%	percent
µM	micromole
AM	human amniotic membrane
ANOVA	analysis of variance
ARVO	Association for Research in Vision and Ophthalmology
ATPase	adenylpyrophosphatase
C	control
cm	centimeter
Da	dalton
DMEM	Dulbecco's modified Eagle's medium
EM	E- modulus
et al.	et alii, et aliae, et alia
g	gram
h	hour/hours
HE-staining	Haemalaun-Eosin staining
H_2O_2	dihydrogen dioxide
K^+	potassium ion
kg	kilogram
KF	keratin film/keratin films
LSCD	limbal stem cell deficiency
M	mole
min	minute/minutes
mL	milliliter
mM	millimole
mm	millimeter
MS	microsoft
Na^+	sodium ion
NaCl	sodium chloride
NaOH	sodium hydroxide
OCP	ocular cicatricial pemphigoid
OCT	optical coherence tomography
pH	potential of hydrogen
p.	page
pp.	pages

PDT	population doubling times
PBS	phosphate-buffered saline
PET	Polyethylene terephthalate
US	ultimate strength
SJS	Steven-Johnsen syndrome
SDS	sodium dodecyl sulfate
SD	standard deviation
x	magnification

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1. Introduction

1.1. The ocular surface

The ocular surface is composed of the cornea, the conjunctiva, the limbus and the tear film (Gipson, Joyce 2000, p. 612). The function of the ocular surface is to maintain optical clarity of the cornea by regulating the hydration of the cornea and conjunctiva and to protect the globe from mechanical, toxic, and infectious insults (Ahmad et al. 2010, p. 306; Tsai et al. 2011, p. 160).

1.1.1. The cornea

The cornea is the clear window at the front of the eye and an essential tissue for the eye to enable vision (Ahmad et al. 2010, p. 306; Lang 2008, p. 105).

It is a highly specialized tissue that refracts and transmits light to the lens and retina and resists adverse external forces such as dehydration, microbial invasion and trauma (Gipson, Joyce 2000, p. 612). The cornea has a diameter that ranges between 10 and 13 mm in adults (Lang 2008, p. 106). The corneal development begins in the second month of embryonic development. The epithelium is of ectodermal origin whereas the other layers are of mesenchymal origin (Lang 2008, p. 105).

1.1.1.1. Anatomy of the cornea

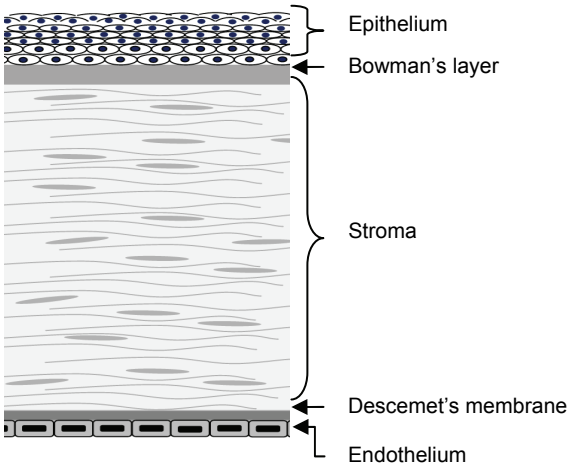


Fig. 1: Structure of the cornea

The cornea consists of five layers as seen in fig. 1. The epithelium is the outermost layer of the cornea and accounts for around 10 % of its thickness (50 to 52 µm) (Gipson, Joyce 2000, p. 612; Tsai et al. 2011, p. 160). It consists of a non-keratinized stratified squamous epithelium, which has five to seven cell layers (Tsai et al. 2011, p. 160). The innermost layer of the epithelium consists of columnar basal cells, which are attached to the basal membrane through hemidesmosomes (Kanski 2008, p. 254). 'Only the columnar basal cells are capable of the cell division required to replenish the continual desquamation of superficial cells from the anterior surface' (Tsai et al. 2011, p. 160). The columnar basal cells are covered by one to three layers of midepithelial cells termed wing cells. The outermost layer of the epithelium is formed by flat squamous cells termed squames (Gipson, Joyce 2000, p. 612). The apical cell membrane has microplicae and microvilli, which facilitate the maintenance of the tear film and mucous layer and hence provide a smooth and refractive surface of the cornea (Kanski 2008, p. 254). The stem cells of the epithelium are primarily found at the upper and lower limbus, probably in the palisades of Vogt, and are essential for the sustainment of the corneal epithelial cells (Kanski 2008, p. 254).

The Bowman's membrane is a thin acellular, superficial stromal layer of collagen fibrils (Tsai et al. 2011, p. 160). 'It is unable to regenerate and thus heals by scarring' (Tsai et al. 2011, p. 160).

'The corneal stroma is the connective tissue located between the epithelial basal lamina and Descemet's membrane' (Gipson, Joyce 2000 pp. 616–617). It composes up to 90 % of the cornea's thickness (Gipson, Joyce 2000, pp. 616–617). The stroma primarily consists of regularly arranged collagen fibrils of the types I, IV, V and VI as well as proteoglycan ground substance, mainly chondroitin sulfate and keratan sulfate (Tsai et al. 2011, p. 160; Kanski 2008, p. 254). 'Keratocytes are a resident population of modified fibroblasts involved in remodeling after injury' (Tsai et al. 2011, p. 160).

The Descemet's membrane is located between the posterior stroma and the endothelial cells (Gipson, Joyce 2000, pp. 618–619). It consists of a meshwork of collagen fibrils (Kanski 2008, p. 254).

The anterior banded zone is formed during fetal development whereas the posterior nonbanded zone is secreted by endothelial cells throughout the individual's lifetime (Kanski 2008, p. 254). Only the posterior layer of the Descemet's membrane can be regenerated after damage (Lang 2008, p. 106).

The endothelium is located at the posterior boundary layer of the cornea and consists of a monolayer of hexagonal cells, which only have a limited potential to regenerate in vivo (Gipson, Joyce 2000, p. 619; Kanski 2008, p. 254). The mean endothelial cell density in adults is 2500 cells/mm², with a yearly reduction in the number of cells by 0.6 % (Kanski 2008, p. 254). 'Cell loss with age is compensated by enlargement (polymegathism) and migration of neighboring cells' (Tsai et al. 2011, p. 160).

1.1.1.2. Physiology of the cornea

The cornea is unique among all other tissues of the body due to its properties of avascularity, transparency and its highly ordered structure (Gipson, Joyce 2000, p. 612). The main functions of the cornea include light refraction, which allows accurate focusing of light to produce a sharp image on the retina for subsequent visual perception, and acting as a barrier (Ahmad et al. 2010, p. 306). The cornea contributes 43 diopters to the total refraction power of the eye (Lang 2008, p. 105).

The epithelium of the cornea provides a barrier to fluid loss and pathogen entrance and resists abrasive pressure by cells tightly adhering to each other and to the underlying connective tissue stroma (Gipson, Joyce 2000, p. 612). Superficial cells of the epithelium possess surface microvilli and secrete abundant glycoproteins that form a rich glycocalyx (Tsubota et al. 2002, p. 7). These cell structures increase the surface area for oxygen diffusion into the avascular cornea and improve the surface hydrophilicity and tear film distribution (Tsubota et al. 2002, p. 7). 'The major functions of the stroma are to maintain the proper curvature of the cornea as the primary lens of the eye, to provide mechanical resistance to intraocular pressure, and to transmit light into the eye without significant absorbance' (Gipson, Joyce 2000, pp. 616–617). Stromal hydration is necessary for corneal transparency (Lang 2008, p. 107). A

relatively low level of stromal hydration is maintained by ionic pumps in the plasma membrane of enodethelial cells which pump Na^+ ions back out into the aqueous by active Na^+-K^+-ATPase (Tsai et al. 2011, pp. 160–161). Additionally, water may pass through hormonally mediated aquaporins (Tsai et al. 2011, pp. 160–161). Thus, the highly organized collagen lamellar structure is maintained (Gipson, Joyce 2000, pp. 616–617). The cornea counts as a bradytrophic tissue and thus regenerates slowly (Lang 2008, p. 106). Because of its avascularity the cornea relies on diffusion of metabolites and ions from the limbus and of nutrition on the exchanges from the aqueous humor and tear substance (Lang 2008, p. 106; Tsai et al. 2011, p. 161). The nerve supply is provided by the first division of the trigeminal nerve which forms stromal and subepithelial plexi, responsible for corneal sensation (Tsai et al. 2011, p. 161).

1.1.2. The limbus

The limbus functions as an anatomical transition zone between the cornea, conjunctiva and sclera and is believed to be the location of the epithelial stem cells of the cornea (Gipson, Joyce 2000, p. 623).

It is suggested that the adult limbal stem cell niche exists in the basal epithelium of the palisades of Vogt (Ahmad et al. 2010, p. 308). Limbal stem cells can differentiate down two pathways. Along one pathway the stem cells move centripetally and horizontally onto the cornea as they differentiate into transient amplifying cells which then constitute the basal layer of the cornea, proliferate and differentiate into postmitotic cells of the suprabasal corneal epithelium. ‘The second pathway results from vertical migration of the stem cells as they differentiate’ (Tsubota et al. 2002, p. 8). This process is believed to be important in establishing a barrier to separate the conjunctiva and cornea and prevent the cornea from conjunctival overgrowth (Tsubota et al. 2002, p. 8; Gipson, Joyce 2000, p. 623).

At the limbus, the stratified columnar conjunctival epithelium moves to the stratified squamous epithelium of the cornea, ‘and the vascular substantia propria of the conjunctival epithelium ends in a rich vascular limbal plexus’ (Tsubota et al. 2002, pp. 7–8). The latter is believed to provide nutrients and

oxygen to the mitotically active limbal stem cells (Tsubota et al. 2002, pp. 7–8). ‘In a severely injured cornea, in which the limbal and central epithelia are both absent, the neighboring conjunctival epithelial cells invade the corneal surface and the eye becomes covered with an abnormal conjunctiva’ (Koizumi et al. 2001, p. 1569).

1.1.3. The conjunctiva

‘The conjunctiva is the mucous membrane that covers the inner surfaces of the upper and lower lids and extends to the limbus on the surface of the globe’ (Gipson, Joyce 2000, pp. 624–625). Three regions of the conjunctiva can be distinguished: the palpebral or tarsal region, which is firmly adherent to the posterior lamella of the lid and contains the crypts of Henle and goblet cells (Tsai et al. 2011, p. 136); the forniceal region, which lines the upper and lower surfaces of the fornix, contains accessory lacrimal glands of Krause and Wolfring as well as goblet cells and is prone to swell easily due to its loose structure (Tsai et al. 2011, p. 136); and the bulbar region, which lines the surface of the sclera between the fornix and the limbus and contains glands of Manz as well as goblet cells (Tsai et al. 2011, p. 136; Gipson, Joyce 2000, p. 625). Radiary lammellae of the bulbar conjunctiva at the limbus form the palisades of Vogt (Kanski 2008, p. 254).

The conjunctiva consists of two different structural components: the epithelium and the stroma. The first is a non-keratinized epithelium and consists of about five layers (Kanski 2008, p. 220). The basal cuboidal cells develop into flattened polyhedrons. The epithelium has mucin-producing goblet cells intercalated. Inferonasal and in the forniceal conjunctiva goblet cells make up 5-10 % of the basal cells (Kanski 2008, p. 220).

The second is a highly vascularized connective tissue which is separated from the epithelium by a basement membrane (Schrader et al. 2009, p. 913). The stroma contains lymphoid tissue and accessory lacrimal glands (Tsai et al. 2011, p. 136).

The conjunctiva's main functions are provision of mucous for the tear film, protection of the ocular surface from pathogens and contribution to its immune defense (Schrader et al. 2009, p. 914; Gipson, Joyce 2000, pp. 624–625). 'The ducts of the lacrimal gland, accessory lacrimal glands, and meibomian glands enter the conjunctival epithelium and deliver their respective products to the tear film' (Gipson, Joyce 2000, pp. 624–625).

1.1.4. The tear film and the lacrimal apparatus

The preocular tear film contains water, cytokines, lipids, mucins, and tear-specific proteins like Lysozym, β-Lysin, Lactoferrin and immunoglobulin A which contribute to its antimicrobial character (Lang 2008, pp. 48-49).

The tear film consists of three layers: the lipid layer, the aqueous layer and the mucin layer. The lipid layer is the outermost layer and is secreted by the meibomian glands, sebaceous glands and sweat glands of the canthus. Its main function is to stabilize the tear film and to reduce evaporation (Tsai et al. 2011, p. 136; Lang 2008, p. 48). The aqueous layer is secreted by the lacrimal gland and by accessory lacrimal glands of Krause and Wolfring. It has cleaning and antimicrobial functions and provides for a refractive surface for optimal vision (Lang 2008, pp. 48-49; Tsai et al. 2011, p. 136). The innermost layer of the tear film is the mucin layer. It is mainly secreted by goblet cells of the conjunctiva and the lacrimal gland (Lang 2008, p. 49; Tsai et al. 2011, p. 136). With its hydrophilic character it contributes to the stabilization of the tear film (Lang 2008, p. 49).

The lacrimal apparatus consists of the following structures: the lacrimal gland, the superior and inferior lacrimal punctum, the lacrimal duct, the lacrimal sac and the nasolacrimal canal (Kanski 2008, p. 220; Lang 2008, pp. 47-49).

The lacrimal gland is located in the shallow lacrimal fossa of the superolateral orbit. In a healthy state it is neither visible nor palpable (Lang 2008, p. 47). The lacrimal gland is divided into two lobes by the tendon of the levator palpebrae superioris aponeurosis (Lang 2008, p. 48; Tsai et al. 2011, pp. 128-129). The gland is of serous type, but also contains mucopolysaccharide granules (Tsai et

al. 2011, pp. 128-129). Innervation of the lacrimal gland is carried out by the parasympathetic lacrimal nerve (Lang 2008, p. 48; Tsai et al. 2011, p. 128).

The tear drainage works as follows:

The musculus orbicularis oculi contracts in the direction from temporal to nasal (Lang 2008, p. 49). The tear fluid is thus transported to the medial angle of the lid and then drained into the upper and lower lacrimal punctae (Lang 2008, p. 49). These are located around 6 mm lateral to the medial canthus (Tsai et al. 2011, p. 128). The drainage takes place both passively, as drainage partially via the inferior punctuate and via the superior punctuate, and actively via suction (Tsai et al. 2011, p. 128). From there, an active lacrimal pump drives the tears through the superior and inferior canaliculi first into the lacrimal sac and then down the nasolacrimal duct (Tsai et al. 2011, p. 128; Lang 2008, p. 49). The superior and inferior canaliculi are about 10 mm long and fuse to form the common canaliculus, which enters the lateral side of the lacrimal sac (Tsai et al. 2011, p. 128).

The valve of Rosenmuller at the entry of the lacrimal sac prevents a reflux of tears (Tsai et al. 2011, p. 128). The lacrimal sac is around 12 mm in length and lies within the lacrimal fossa. The nasolacrimal duct is about 18 mm long. ‘The first 12 mm lies in the bony nasolacrimal canal and the last 6 mm within the mucous membrane of the lateral wall of the nose’ (Tsai et al. 2011, p. 128). ‘It opens into the inferior meatus via the ostium lacrimale just beneath the inferior turbinate’ (Tsai et al. 2011, p. 128). The exit from the nasolacrimal duct is partially closed by the valve of Hasner (Tsai et al. 2011, p. 128).

1.2. Ocular surface disorders

Origins of ocular surface disorders can be congenital, degenerative, nutrient, immunological (like ocular cicatricial pemphigoid (OCP) and Stevens-Johnson syndrome (SJS)), infectious, traumatic (like chemical or thermal burns) or iatrogenic (Ormerod et al. 1988, cited from: Schrader et al. 2009, p. 913). They can lead to severe conjunctival damage and scarring. ‘This can result in fornix shortening, symblepharon, and ankyloblepharon. Cicatrization of the fornices,

especially the inferior fornix, causes entropion and trichiasis which can persistently damage the ocular surface epithelia and result in recurrent erosions, ulcer formation and secondary bacterial infection' (Ormerod et al. 1988, cited from: Schrader et al. 2009, p. 913). Additionally, dry eye disease can occur due to ineffective blinking, reduction of the tear meniscus, loss of goblet cells, and keratinization of the ocular surface epithelia (Solomon et al. 2003, p.93). The prolonged inflammation causes a depletion of the limbal epithelial stem cell population which can result in limbal stem cell deficiency (LSCD) (Dua 1998, p. 1407; Schrader et al. 2009, p. 913).

'Limbal stem cell deficiency is the term given to a heterogeneous group of diseases characterized by excessive damage to the limbus resulting in an insufficient number of stem cells being available to replenish the constantly remodeling corneal epithelium' (Echevarria, Di Girolamo 2011, p. 261). It can be either hereditary, which is commonly associated with aniridia, or acquired (Echevarria, Di Girolamo 2011, p. 261). Acquired LSCD can develop through chemical or thermal injuries, contact-lens induced keratopathy, multiple surgeries, infections or therapeutic radiation (Levis, Daniels 2009, p. 593; Schwartz, Holland 2002, p. 131; Echevarria, Di Girolamo 2011, p. 261; Ahmad et al. 2010, p. 309). LSCD can also manifest through inflammatory diseases such as Stevens-Johnsen syndrome (SJS) and ocular cicatricial pemphigoid (OCP) (Echevarria, Di Girolamo 2011, p. 261; Ahmad et al. 2010, p. 309). The damage to the limbal epithelial stem cells can lead to the absence of an intact corneal epithelial layer (Tseng, 1996, cited from: Levis, Daniels 2009, p. 593). This can cause conjunctival epithelium to breach the limbal barrier and invade into the cornea, as well as neovascularization and chronic inflammation. Ultimately, this can lead to the formation of fibrovascular pannus tissue, increased glare sensitivity and loss of vision (Levis, Daniels 2009, p. 593; Meller et al. 2011, p. 246).

'Stevens-Johnson-Syndrome (erythema mulitforme major) is an acute inflammatory disease, predominantly affecing skin and mucosal membranes, including the ocular surface' (Notara et al. 2010, p. 190). The pathogenesis of SJS is thought to be related to an immunological hypersensitivity reaction to

drugs or microorganisms (Kawasaki et al. 2000, p. 1191). In the acute stage the entire ocular surface including the eyelids, conjunctiva and cornea can be involved (Tauber 2002, pp. 118–119). Pseudomembranous conjunctivitis can lead to rapid symplepharon formation due to inflammation. Toxic or infectious keratitis can lead to corneal perforation and endophthalmitis (Tauber 2002, pp. 118–119). In the chronic stage epitheliopathia with the formation of a fibrovascular pannus can develop, as well as subepithelial scarring and stromal neovascularization (Tauber 2002, pp. 118–119).

The OCP, also known as mucous membrane pemphigoid, is an immune-mediated disease that affects the ocular surface in approximately 80 % of the cases (Notara et al. 2010, p. 190). Chronic conjunctivitis and sub-epithelial fibrosis can lead to fornix shortening over time, symblepharon, eyelid deformation with entropion or ektropion and squamous metaplasia with keratinization of the ocular surface (Notara et al. 2010, p. 190; Tauber 2002, p. 114). This can result in limbal epithelial stem cell failure and corneal opacification (Notara et al. 2010, p. 190; Tauber 2002, p. 114).

Chemical burns to the ocular surface are a condition seen relatively frequently in ophthalmology (Meller et al. 2011, p. 246). Most chemical burns are known to occur in private households, as acidic and alkali compounds are frequently found in household chemicals such as cleaners (Schwartz, Holland 2002, p. 128; Kanski 2008, p. 891). Alkali burns occur twice as frequently as acidic burns, of which ammonia and lye cause the most severe injuries (Kanski 2008, p. 891; Kim, Khosla-Gupta 2002, p. 100). Alkali liquids cause saponification and disruption of fatty acids in cell membranes, leading to cellular necrosis and collagen fiber edema with subsequent thickening and shortening (Kim, Khosla-Gupta 2002, p. 100). Acids can cause cellular necrosis, protein denaturation and precipitation (Kim, Khosla-Gupta 2002, pp. 108–109). Severe thermal injuries can cause corneal necrosis and perforation (Kim, Khosla-Gupta 2002, p. 110). In the case of a loss of limbal epithelial stem cells due to chemical burns, the corneal epithelial defect is covered by a centripetal overgrowth of conjunctiva resulting in neovascularization and persistent corneal ulceration which can further lead to corneal opacification (Kanski 2008, p. 891).

Cornea-related blindness affects millions of people worldwide and accounts for 4 % of the global causes of blindness (Borrelli et al. 2013, p. 221; Pascolini, Mariotti 2012, p. 616). According to Pascolini et al. 285 million of the people in the world suffer from visual impairment, 39 million are blind and 246 million suffer from low vision (Borrelli et al. 2013, p. 221; Pascolini, Mariotti 2012, p. 616).

1.3. Ocular surface reconstruction

Ocular surface disorders require the reconstruction of the ocular surface. Persistent epithelial defects are the most important indications for ocular surface reconstruction (Meller et al. 2011, p. 243). Since medical therapy of ocular surface disorders often fails, microsurgical interventions such as penetrating or lamellar corneal transplantation are used to stabilize the ocular surface (Geerling, Seitz 2005, p. 612). Penetrating corneal transplantation is a form of keratoplasty which involves a full-thickness replacement of a button of corneal tissue, whereas lamellar corneal transplantation only involves a partial-thickness replacement of corneal tissue (Tsai et al. 2011, p. 205). Conventional corneal graft techniques have the downside that a graft in the central cornea does not restore the limbal stem cell population with the result of persistent epithelial defects and secondary infection (Kuckelkorn et al. 2001, p. 542). The additional transplantation of limbal epithelium, either autologous or allogenic, is therefore necessary when also the limbal region is affected.

However, autologous limbal epithelial stem cell transplantation requires a large limbal graft from a healthy eye and thus carries the risk of inducing ocular surface disease in the donor eye (Dua, Azuara-Blanco 2000, p. 277). This risk can be partly reduced by the transplantation of a sheet of expanded autologous epithelial cells from a very small biopsy (Feng et al. 2014, p. 543).

In patients with bilateral LSCD, the reconstruction of the cornea can be challenging because the transplantation of autologous limbal epithelium, as used to treat unilateral LSCD, is not an option due to the lack of appropriate ocular surface stem cells. Alternatively, transplantation of allogenic limbal epithelium as keratolimbal allograft from cadaverous tissue, or as conjunctival

limbal allograft from living related donors can be performed, but with limited success due to a high risk of rejection and side effects of a long-term systemic immunosuppressant treatment (Koizumi, Kinoshita 2010, p. 243; Borrelli et al. 2012, p. 30; Feng et al. 2014, p. 543).

Thus, patients with ocular surface disorders could benefit from a surgical reconstruction with the transplantation of either autologous or non-immunogenic material. Today, human amniotic membrane is commonly used for the reconstruction of the corneal epithelium (Rahman et al. 2009, p. 1954; Connon et al. 2010, p. 1057).

1.4. Amniotic membrane

The use of AM in ophthalmic surgery was first documented in 1940 by De Rötth. At that time, amniotic membrane was used for the reconstruction of conjunctival tissue loss and repair of symblepheron (De Rötth 1940, p. 525). In the 1990's, Kim et al. were the first to successfully transplant preserved amniotic membrane for corneal surface reconstruction in rabbits (Kim, Tseng 1995, p.474; Notara et al. 2010, p. 191).

As a transplant, amniotic membrane has gained significance because of its unique properties, such as the promotion of epithelial growth and differentiation (Park et al. 2008, p. 77).

It contains numerous basement membrane proteins and cytokines, such as collagen IV, Ln-1, Ln-5, fibronectin, and collagen VII, which can promote wound healing in the cornea (Reichl et al. 2011, p. 3375; Grueterich et al. 2003, p. 636). It has been shown to reduce inflammation by inhibiting the activity of pro-inflammatory cells and cytokines (Reichl et al. 2011, p. 3375). Additionally, amniotic membrane has the capability to suppress fibrosis and reduce inflammation (Thatte 2011, p. 71). Amniotic membrane is non-immunogenic and therefore does not require immunosuppression when used as a graft for transplantation (Grueterich et al. 2003, p. 635).

1.4.1. Structure of amniotic membrane

Amniotic membrane is the innermost layer of the placenta (Grueterich et al. 2003, p. 635). The thickness of the amniotic membrane depends on the distance from the placenta, measuring in average 95.3 µm in areas proximal to the placenta and 64.3 µm in areas distal to the placenta (Connon et al. 2010, p. 1059). The amniotic membrane consists of three layers: the epithelium, a thick basement membrane, and an avascular stroma (Grueterich et al. 2003, p. 635). 'The amniotic epithelium consists of a single layer of cuboidal cells that rests upon a thick basement membrane composed of reticular fibers' (Rodríguez-Ares et al. 2009, p. 398). The avascular stroma, the thickest layer, contains 'loose, fibrous connective tissue with occasional fibroblasts' (Rodríguez-Ares et al. 2009, p. 398).

1.4.2. Clinical use of amniotic membrane

The use of amniotic membrane as a biological substrate has improved the ability to treat ocular surface disorders in the past years (Thatte 2011, p. 71; Grueterich et al. 2003, p. 635). Some indications for AM transplantation are persistent epithelial defects, corneal ulcers, bullous keratopathy, infectious keratitis, SJS, melting ulcers, band keratopathy, and prevention of scarring following refractive keratectomy (Plummer et al. 2009, p. 19).

Amniotic membrane can also be used as a culture substrate for ex vivo expansion of epithelial cells to treat LSCD (Grueterich et al. 2003, p. 640; Meller et al. 2011, p. 245).

The transplantation of AM is also used for the reconstruction of the conjunctiva in cases of ocular surface neoplasia, pterygium surgery, chemical burns, cicatrizing conjunctivitis, symblepharon release, fornix formation, and lid reconstruction (Plummer et al. 2009, p. 19).

The amniotic membrane is obtained at the time of elective cesarean section after informed consent of the patient. It is stripped from the chorion by blunt dissection and sutured onto sterile sponge sheets (Thatte 2011, p. 68). The AM used for ophthalmic surgery can be either fresh or modified through a variety of

preservation methods such as lyophilization or cryopreservation in glycerol (Riau et al. 2010, p. 221). The latter method was introduced by Lee et al. in 1997 and has been employed by 95 % of the current literature on utilizing amniotic membrane for ocular surface reconstruction (Riau et al. 2010, p. 221; Lee, Tseng 1997, p. 305). The amniotic membrane is stored in glycerol that is usually mixed with DMEM in a ratio of 1:1 and then stored at -80°C for several months (Lee, Tseng 1997, p. 305). Lyophilization or freeze-drying is a preservation method that removes water from a tissue by sublimation (Riau et al. 2010, p. 221). Destructive chemical reactions that can lead to tissue alteration are inhibited due to the sublimation (Riau et al. 2010, p. 221). 'Lyophilized amniotic membrane can be stored at room temperature for a long period of time without deterioration and transportation is easy', which gives this method an advantage over cryopreservation (Riau et al. 2010, p. 221). However, as Rodríguez-Ares et al. reported, lyophilization of amniotic membrane causes a greater loss of protein than cryopreservation, which may affect the ease of handling and resistance when used as a transplant in ophthalmic surgery (Rodríguez-Ares et al. 2009, p. 399).

The amniotic membrane can be used either as cover for the cornea and conjunctiva (onlay) to provide mechanical protection for the healing superficial epithelium, or as a graft inserted into corneal defects (inlay), where it acts as basement membrane substitute and supports epithelial overgrowth (Plummer et al. 2009, p. 20; Reichl et al. 2011, p. 3375). Both techniques can also be combined (Reichl et al. 2011, p. 3375).

1.4.3. Disadvantages in the use of amniotic membrane for ocular surface reconstruction

Despite its frequent clinical use for ocular surface reconstruction, there are unfortunately a number of drawbacks associated with the use of amniotic membrane. As a donor tissue, amniotic membrane always brings along a donor-associated risk of transmitting viral agents such as human immunodeficiency virus, hepatitis B and C (Dua et al. 2010, p. 963). The use of AM as a transplant requires donor screening, which is costly and not necessarily feasible in the developing world (Borrelli et al. 2013, p. 221; Gicquel

et al. 2009, p. 1920; Schrader et al. 2009, p. 917). Due to being a donor tissue, with requirements such as elective cesarean section with informed consent from the pregnant woman, the amount of AM available is limited. Therefore, it can be challenging to provide a consistent supply of AM (Schrader et al. 2009, p. 917). Additionally, as cryopreservation seems to be a favored method of preservation, there is a subsequent and costly need for storage (Riau et al. 2010, p. 216).

A further disadvantage of amniotic membrane is a poor standardization due to intradonor and interdonor variations in the membrane and variations in the processing procedures (Ahmad et al. 2010, p. 311; Dua et al. 2010, p. 963; Grueterich et al. 2002, p. 70). Interdonor variations can be caused by variables such as age, race, duration of gestation or parity and gravidity of the donor (López-Valladares et al. 2010, p. 212). Intradonor variations can be caused by a variable thickness of the amniotic membrane depending on the location proximal or distal to the placenta (Connon et al. 2010, p. 1059).

The transparency of the AM is dependent on its thickness and thus on the location of the donor tissue (Connon et al. 2010, p. 1060). Different methods of preservation and sampling of AM can also influence its transparency and its refractive index (Connon et al. 2010, p. 1060; López-Valladares et al. 2010, p. 212; Rodríguez-Ares et al. 2009, p. 399). Transparency is a very important issue when dealing with ocular surface reconstruction and the low transparency of amniotic membrane limits postoperative vision if implanted into the visual axis (Ahmad et al. 2010, p. 311; Connon et al. 2010, p. 1057; Notara et al. 2010, p. 192; Letko et al. 2001, p. 663).

The above described limitations in the use of amniotic membrane strengthen the necessity for the development of additional biocompatible materials to reconstruct the ocular surface.

An optimal substitute would be a biocompatible material which allows the attachment, proliferation and maintenance of limbal epithelial stem cells (Borrelli et al. 2012, p. 30). A suitable carrier should also eliminate the risk of infection and provide for higher transparency. Further requirements to the material are

flexibility and high standardization, as well as good surgical feasibility (Borrelli et al. 2012, p. 30).

1.5. Alternative substrates for ocular surface reconstruction

In light of the drawbacks connected with the use of AM, there is an increasing need for an alternative material for ocular surface reconstruction.

The use of autologous oral mucosal epithelial cell transplantation has been proposed as an alternative in the treatment of bilateral LSCD, where recent options of allogenic limbal epithelial transplantation show a limited rate of success (Borrelli et al. 2012, p. 30; Liu et al. 2011, p. 739; Nakamura et al. 2003, p. 115). Also the use of direct transplantation of oral mucosal grafts to the limbal region in combination with an AM graft to reconstruct the cornea has recently been proposed (Liu et al. 2011, p. 739).

Selvam et al. demonstrated that transplanted oral epithelial sheets show a different morphological structure than the corneal epithelium (Selvam et al. 2006, p. 129).

Further work has been done in the study of silk fibroin, which is the primary structural protein of Bombyx mori silkworm cocoons. Lawrence et al. studied the use of silk film biomaterials as a scaffold for corneal fibroblast cultivation (Lawrence et al. 2009, p. 1308). Liu et al. showed that corneal epithelial cells adhered to silk fibroin and proliferated after plating (Liu et al. 2012, p. 4134). However, the cell growth on silk fibroin after 24 and 72 hours was not comparable to that on denuded amniotic membrane, which could be less advantageous for its future use in cell culturing (Liu et al. 2012, p. 4132). Furthermore, the production of silk film biomaterials is more cost effective than that of synthetic material (Levis, Daniels 2009, p. 595).

Some approaches have been made to employ cross-linked collagen gels as scaffolds for cell culture, since simple collagen hydrogels are inherently weak due to their water content, but major drawbacks include the cytotoxicity of the crosslinker (Levis, Daniels 2009, p. 595; Tachibana et al. 2002 p. 167; Chen et al. 2000, p. 274). The option of using collagen gels without cross-linking

treatment has been shown by Tachibana et al. to be disadvantageous for its use as a scaffold for cell cultivation, as these collagen gels were less resistant towards heat treatment than keratin films (Tachibana et al. 2002, p. 167).

A study by Fagerholm et al. showed that cross-linked collagen grafts without any epithelial line could be used for corneal reconstruction after keratoconus or central corneal scarring. The biosynthetic implants remained stably integrated and avascular for 24 months after surgery and a morphologically normal epithelium covered all the implants (Fagerholm et al. 2010, p. 6). Plastic compressed collagen-gels have also been tested for the cultivation of limbal epithelial cells, showing an increased cell density, cell-matrix and cell-cell attachment compared to uncompressed gels (Levis et al. 2010, p. 7726). On the downside, compressed collagen gels are not as strong and elastic as cross-linked collagen (Feng et al. 2014, p. 544).

Chitosan has also been proposed as a biocompatible matrix for cell cultivation either as pure Chitosan or as a Chitosan-Keratin mixture (He et al. 2009, p. 1465; Tanabe et al. 2002, p. 821). Tanabe et al. demonstrated that Chitosan films without the keratin component were too fragile, while Keratin-Chitosan composite films were stable (Tanabe et al. 2002, p. 822). To enhance the application of the Chitosan film on the curved ocular surface, Zhu et al. introduced a Gelatin-Chitosan composition (Zhu et al. 2006, p. 211). This study group also showed ‘that rabbit conjunctival epithelial cells cultured for 14 days on gelatin-chitosan hydrogel formed a confluent multilayered epithelium and maintained its conjunctival phenotype by means of CK4 expression’ (Feng et al. 2014, p. 545; Zhu et al. 2006, p. 213).

As a further biomaterial for ocular surface reconstruction, Fibrin gels have been proposed. Although the cultivation of limbal epithelial stem cells was shown to be successful, a major disadvantage is the fact that fibrin gels derive from fibrinogen and thrombin, which are harvested from plasma, and therefore could carry the risk of allogeneic rejection and transmission of diseases (Feng et al. 2014, p. 549).

Siloxane hydrogel contact lenses have been proposed by Di Girolamo et al. as a carrier for limbal epithelial stem cells and have already been tested in vivo (Di Girolamo et al. 2007, p. 460; Pellegrini et al. 1997, p. 992). In vitro results indicated that siloxane-hydrogel contact lenses seem to sustain proliferation and migration of human limbal tissue (Feng et al. 2014, p. 545; Girolamo et al. 2007, p. 462). Limitations of using therapeutic contact lenses are that epithelial layers may be lost on contact lens removal and that the epithelial phenotype may not be preserved on a contact lens (Di Girolamo et al. 2007, p. 463).

Other materials that were proposed as an alternative substrate for ocular surface reconstruction and as a carrier for cell cultivation are human anterior lens capsules, poly(lactide-co-glycolide)-scaffolds, polymethacrylate gels and hydroxyethylmethacylate hydrogels. Experiments on these substrates are still limited to in vitro experiments (Feng et al. 2014, p. 549).

Over the last decade keratin-based biomaterials have gained researchers' attention, in part because of new approaches to modify keratin and to overcome its water-insolubility (Reichl et al. 2011, p. 3376; Tachibana et al. 2002, p. 167; Verma et al. 2008, p. 11; Reichl 2009, p. 6854).

The earliest documentation of the use of keratin films for medical applications goes back to the 16th century (Rouse, Van Dyke 2010, p. 1001). In the early 20th century the primary challenge was to develop a method to extract keratins mainly from horns, hoofs and wool (Rouse, Van Dyke 2010, p. 1002). During the 1980's, collagen has become the most commonly used biomaterial in medical applications but the ambition to develop a new biomaterial led the focus of research back to keratin proteins (Rouse, Van Dyke 2010, p. 1003). In recent years, new developments in the use of keratin as a biomaterial have been followed with increasing interest, since Nakamura et al. have developed a new procedure to extract keratins from wool, feathers and hair called the Shindai method (Nakamura et al. 2002, p. 569).

1.6. Keratin films

Keratins are a group of cysteine-rich structural proteins formed in the epithelial cells of higher vertebrates. Keratins exhibit high mechanical strength due to a large number of disulfide bonds (Reichl et al. 2011, p. 3375–3376; Nakamura et al. 2002, p. 569). These proteins, along with others, are found in hard or filamentous structures such as hair, nails, horns, hooves and feathers (Reichl et al. 2011, pp. 3375–3376; Nakamura et al. 2002, p. 569). Keratins are present as unused protein-rich waste found in the food industry and at hair salons (Reichl 2009, p. 6854). Since hair is the only abundantly available material of human origin that can be obtained easily painlessly, keratin based substrates are always available and can provide unlimited supply (Verma et al. 2008, p. 8).

In contrast to keratoses, which are extracted using an oxidant, keratins are extracted using a reductant (Hill P 2010, p. 586). Due to this extraction method, keratins are less soluble in water, more stable at extremes of pH, and can be re-crosslinked through oxidative coupling of cysteine groups (Hill P 2010, p. 586). The biomaterials can persist in vivo for weeks to months (Hill P 2010, p. 591). Due to new methods for the use of keratin or modified keratin, this biomaterial has drawn attention as a substrate for tissue engineering in the field of regenerative medicine over the past years (Reichl et al. 2011, p. 3376).

Keratin films have been shown to support cell adhesion and to function as a scaffold for cell cultivation of animal cells such as mouse fibroblasts (Tachibana et al. 2002, p. 168; Yamauchi et al. 1998, p. 268). They have also been shown to be better for high density cell cultivation than collagens (Tachibana et al. 2002, p. 169; Yamauchi et al. 1998, p. 268). Among human hair proteins keratins have the cell adhesion amino acid sequence LDV which is recognized by the protein α4β1 of the integrin protein family found on the cell membranes (Verma et al. 2008, p. 8). This sequence makes the human hair protein surface cell adhesive (Verma et al. 2008, p. 8). Further investigation was done on the biocompatibility of keratins, and Fujii et al. showed that keratin from human hair and nails has inhibitory effects on the IgE-receptor stimulated histamine release from mast cells (Fujii et al. 2008, p. 2340).

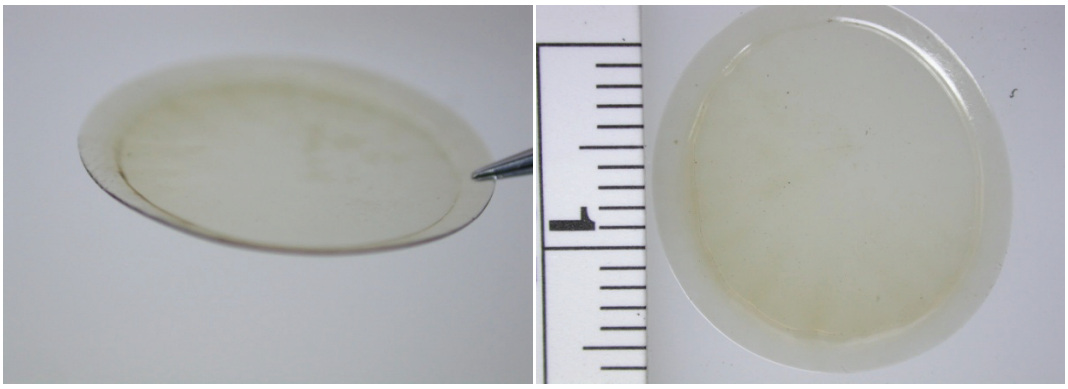


Fig. 2: Plasma sterilized dry keratin film

Keratin films are considerably more transparent than amniotic membrane. In in-vitro studies KF showed light transmission values ranging between 65 and 97 % at 400–700 nm (Borrelli et al. 2013, p. 225). Keratin films used in our experiments were plasma sterilized. In vitro experiments of Borrelli et al. indicated that plasma treatment was the best sterilization method for keratin films because it did not negatively impact light transmission, cell attachment or proliferation (Borrelli et al. 2013, p. 225). Other sterilization methods like ethanol-treatment slightly reduced the light transmission (Borrelli et al. 2013, p. 225).

Keratin films show different biomechanical properties depending on the ratio of aqueous to alkaline dialysate. In former observations keratin films of different ratios of aqueous to alkaline dialysate (e.g. KF90/10 consists of 90 % aqueous and 10 % alkaline dialysate) were examined, evaluating parameters like the tensile strength. The results showed that the E-modulus (EM) and ultimate strength (US) values decreased when the level of alkaline dialysate increased (Borrelli et al. 2013, p. 225). KF100 showed the highest values of tensile strength, and therefore seemed to be the material of choice. However, KF100 was too stiff to mold into shape when fitting to the corneal surface of a porcine eye (Borrelli et al. 2013, p. 227). In contrast, KF90/10 could easily fit the corneal curvature. KF90/10 was best applicable to the corneal surface, and the decreased tensile strength values were within acceptable ranges (Borrelli et al. 2013, p. 228). An example of a keratin film can be seen in fig. 2.

The surgical feasibility of keratin film is not statistically different from amniotic membrane. Keratin film can be sutured into a corneal defect using interrupted sutures (Borrelli et al. 2013, pp. 228–229). The sutures in keratin films show a higher tendency to be loose, which could lead to poor adaptation of the keratin film to the surface (Borrelli et al. 2013, p. 229). Due to this observation, a suture-less model of an intrastromal pocket was used in this experiment.

1.7. Purpose of this investigation

Biomaterials used for tissue engineering are traditionally non-reactive synthetic materials. Biocompatibility is generally governed by the interaction between the foreign material and the host's living tissue (Lai 2009, p. 3444). Hence, integration and immunological response still remains an issue.

Because many biomaterials currently in use have been tested through various methodological studies, using in vitro cell cultures first and in vivo animal models afterwards, our experiments on keratin films followed the same pathway, from in vitro to ex vivo experiments (He et al. 2009, p. 1463-1465, Stevens et al. 2002, p. 1353).

The aim of this study was to evaluate the corneal biocompatibility of a plasma sterilized keratin-based biopolymer film in an in vivo rabbit model. Since any material which could possibly be used for corneal epithelial reconstruction comes in contact with the corneal stroma, it is essential to test its general biocompatibility and verify its potential use before generating further concepts for its clinical application in ophthalmic surgery. Our experiment was designed to evaluate the biocompatibility of keratin films with different ratios of aqueous and alkaline dialysate (100, 90/10, 80/20) in a rabbit model to obtain answers to the following questions:

1. Do keratin films induce an inflammatory reaction once implanted into the corneal stroma?

2. Is the biocompatibility of keratin films comparable to that of amniotic membrane?

3. Does the different composition of the keratin films (100, 90/10, 80/20) influence the biocompatibility?

4. Do implanted keratin films degrade over time?

5. Does a topical medical treatment with steroids influence the inflammation and degradation?

2. Material and methods

2.1. Preparation of the keratin films

Keratin films were prepared according to a method previously reported by Reichl in 2009 (Reichl 2009, p. 6855). Human hair was obtained from a local hair salon, intensively washed with water containing 0.5 % SDS, rinsed with fresh water, and air-dried. In order to remove external lipids, the cleaned hair material was extracted for approximately 12 hours with n-hexane. The bulk hexane was removed and residual hexane was allowed to evaporate. The dry hair was pulverized using a ball mill (MM301 Retsch, Haan, Germany) (Reichl 2009, p. 6855).

Human hair keratin was then extracted according to the Shindai method (Nakamura et al. 2002, p. 569). The hair powder (20 g) was mixed with extraction medium, an aqueous solution (400 mL) containing 25 mM Tris, pH 8.5, 2.6 M thiourea, 5 M urea, and 5 % 2-mercapto-ethanol, at 50 °C for 72 h. The mixture was centrifuged at 4500 g for 15 min, and the supernatant was filtered using filter paper with a pore size of 2.5 mm. The filtrate (denoted as Shindai extract) was stored at 20 °C and thawed, if required. The Shindai extract was exhaustively dialyzed against demineralized water (100 mL extract in 5000 mL water) using an MWCO 6e8000 Da (Spectra/Por) cellulose membrane for 24 h at 20 °C. The procedure was repeated six times until no osmolarity or 2-mercaptoethanol (via Ellman's reagent) was detected in the dialysis fluid. The keratin dialysate (denoted as aqueous keratin dialysate) was centrifuged at 10,000 g for 30 min to remove coarse aggregates and immediately used to prepare the keratin films. During a second phase of dialysis, the same amount of Shindai extract was dialyzed against 0.25 M sodium hydroxide solution in the same manner as described above, at 4 °C. The 0.25 M NaOH dialysate was concentrated by means of ultrafiltration using Vivaspin® 20 concentrators with a MWCO of 5000 Da. The filtrates were discarded and the supernatant was diluted several times with 0.05 µM NaOH to generate the so-called alkaline keratin dialysate (Reichl et al. 2011, p. 3376).

Keratin film preparation was performed under aseptic conditions by mixing the aqueous keratin dialysate with portions of alkaline keratin dialysate (at ratios of 100, 90/10, 80/20, respectively; e.g., a KF90/10 consists of 90 % aqueous and 10 % alkaline keratin dialysate). Glycerol was added at a concentration of 1 % as a softening agent, and the mixture was then cast on hydrophobic-coated Polyethylene terephthalate (PET) sheets. The keratin samples were then allowed to dry overnight in air. A subsequent curing procedure involving dry heat was essential to obtain biomechanically stable films. The keratin films can differ in their mechanical properties, depending on the amount of glycerol added and on the curing temperature and duration (Reichl et al. 2011, p. 3377). The keratin films used in our experiments were cured at 110 °C for 2 h.

For additional quality control, and based on the results published by Reichl et al. in 2011 on the keratin film water absorption demonstrating that there is no considerable enlargement of KF80/20, 90/10 and 100 in the wet stage, we evaluated 12 extra samples of KF 90/10 at 100 °C cured for 2 h (Reichl et al. 2011, p. 3385). We determined the thickness of the dry film in the center and in the periphery on both sides; each measurement was performed in triplicate per keratin film. The same keratin films were then equilibrated in order to measure the film thickness in the wet state. For the equilibration they were intensively washed with a sterile solution containing antibiotics and antimycotics, replaced twice daily for seven days and finally left in DMEM medium for 6 hours. At the end of the procedure the films were embedded in Technovit to assess if the KF thickness was influenced by the histological preparation. All assessments for the dry and wet films were performed using a micrometer screw, and Cell F Software was used to measure the film thickness after embedding.

Before implantation in living animals, the keratin films were first plasma sterilized using H_2O_2 in a Sterrad® 100S plasma sterilizer (ASP, Irvine, California, USA) and then intensively washed with a sterile solution containing antibiotics and antimycotics (Borrelli et al. 2013, p. 223). This solution was replaced twice daily for seven days (Borrelli et al. 2013, p. 223). Finally, the keratin films were left in DMEM medium for 6 hours before use.

Keratin films used in our experiment were obtained from the research group of Stephan Reichl (Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstr. 1, Braunschweig 38106, Germany).

2.2. Preparation of the amniotic membrane

The method for securing human tissue was performed according to the Tenets of the Declaration of Helsinki. Samples of human amniotic membrane were obtained at the time of an elective caesarean section. Intact amnion was stripped from the chorion by blunt dissection and sutured onto 7.5 × 7.5 cm sterile sponge sheets (Katena, New Jersey, USA). All operations were performed under sterile conditions. The amniotic membranes were cryopreserved at -80 °C in a mixture of 50 % DMEM and 50 % glycerol (Lee, Tseng 1997, p. 305).

In order to compare the AM thickness with the KF implant thickness two freeze-thawed AM samples were measured in quadruplicate; unfortunately the micrometer-screw could not be used due to the tissue characteristics of the AM. For this reason the AM thickness was measured only after embedding.

2.3. Rabbit model

A rabbit model was used for our experiments because rabbit eyes are similar to the human eye in dimension and thickness and its anatomy and physiology is well-described. Contrary to animals like pigs or dogs, rabbits are easy to handle, economic in breeding and continually available.

All animals included in our study were female and weighed approximately 2 kg - 2.5 kg each. The animals were held in single rabbit cages of the size 80 cm x 50 cm x 60 cm (length x depth x height) with a room temperature of approximately 21 °C and a moisture of 50-70 % with a permanent air exchange of 15-20 times per hour. The litter was produced of wood.

The rabbits were fed once a day with ssniff® (ssniff Spezialdiäten GmbH, Soest) complete feeds for rabbits in the amount of 80 g per kg body weight. Additionally, hay was given once a day.

All the experiments were conducted in accordance with the ARVO statement for the use of animals in ophthalmic research and the institute had permission for keeping rabbits in state-of-the-art facilities within the central animal building (ZEET Düsseldorf). The animal experiments have been permitted by the local authorities (Registration number: 84-02.04.2011.A256).

2.4. Surgical procedure and follow-up

46 New Zealand White rabbits underwent a manual dissection of a corneal intrastromal pocket. 30 rabbits were followed up for a period of 10 days while the remaining 16 rabbits were followed up for 4 weeks.

All the surgeries were performed by the same surgeon in sterile conditions and with the aid of a surgical microscope (Carl Zeiss, Oberkochen, Germany). Before surgery all rabbits received intravenous injections of ketamine hydrochloride (25 mg/kg body weight) and xylazine hydrochloride (5 mg/kg body weight). The animals were placed in a laterally recumbent position for the surgery and only the right eye was operated on. When an adequate sedation was achieved, the fornices and the periocular area were rinsed with povidone-iodate 0.5 %, and the globe was prolapsed anterior to the upper and lower lid margin to stabilize the eye and to have easier access to the cornea. An incision of approximately 50 % stromal depth was made in the mid periphery of the cornea and a lamellar pocket was manually dissected in the center of the cornea with a crescent knife (see fig. 3 and 4). Then a KF100, KF90/10, KF80/20 or AM implant was punched out using a 4 mm biopsy punch (PFM Medical, Wankelstr. 60, Köln, Germany) and implanted into the pocket (see fig. 3 and 4). The wound was left unsutured. At the end of the surgery each animal received a siliconhydrogel contact lens and a third lid tarsorraphy using a 5.0 non-absorbable monofilament suture to secure the implant (see fig. 5).

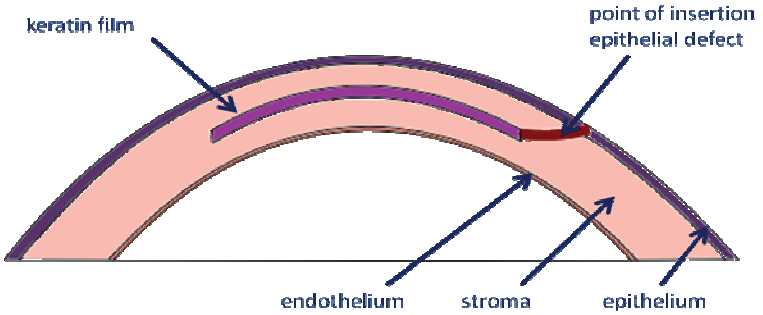


Fig. 3: Scheme of the intrastromal pocket and implanted KF, vertical dissection through the center of the cornea.

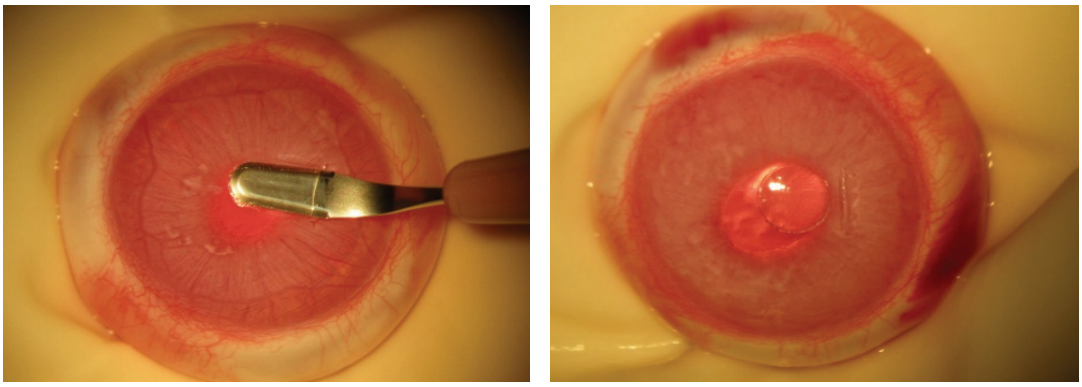


Fig. 4: Preparation of intrastromal pocket and implanted keratin film.

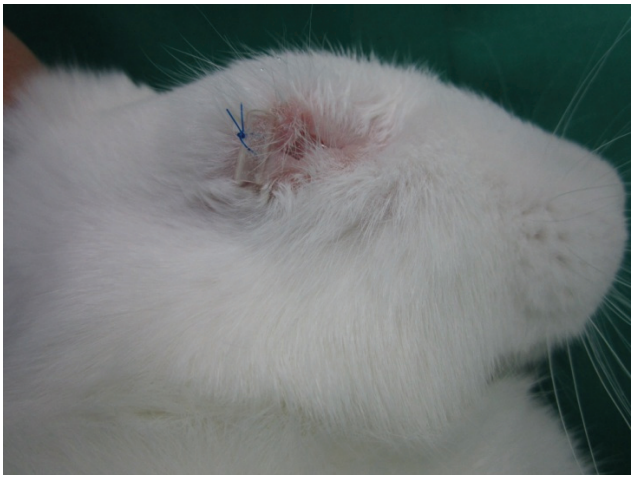


Fig. 5: Third lid tarsorraphy.

30 of the 46 animals were sacrificed 10 days after surgery, while the remaining 16 were followed up for 4 weeks. In the 10 days follow-up group, 18 of the 30 rabbits, 3 groups of 6 rabbits each, received a KF implant of a different composition of aqueous and alkaline dialysate (KF100, KF90/10, KF80/20). 6 of the 30 rabbits received an AM implant (gold-standard control), while the remaining 6 rabbits underwent a dissection of the intrastromal pocket without receiving an implant. These served as a negative control group (C). In each group (AM, KF or C) half of the animals received steroids (Dexamethason eye-drops) twice daily while the other half received no steroids but Ofloxacin eye-drops twice daily until the end of the follow up. Based on the results obtained at the end of the ten days follow-up, 16 rabbits received either a KF90/10 or AM implant and were followed up for a period of 4 weeks. In each group, KF90/10 or AM, half of the rabbits received steroids twice daily while the other half received no steroids but Ofloxacin eye-drops twice daily for the follow-up period of 4 weeks as seen in fig. 6.

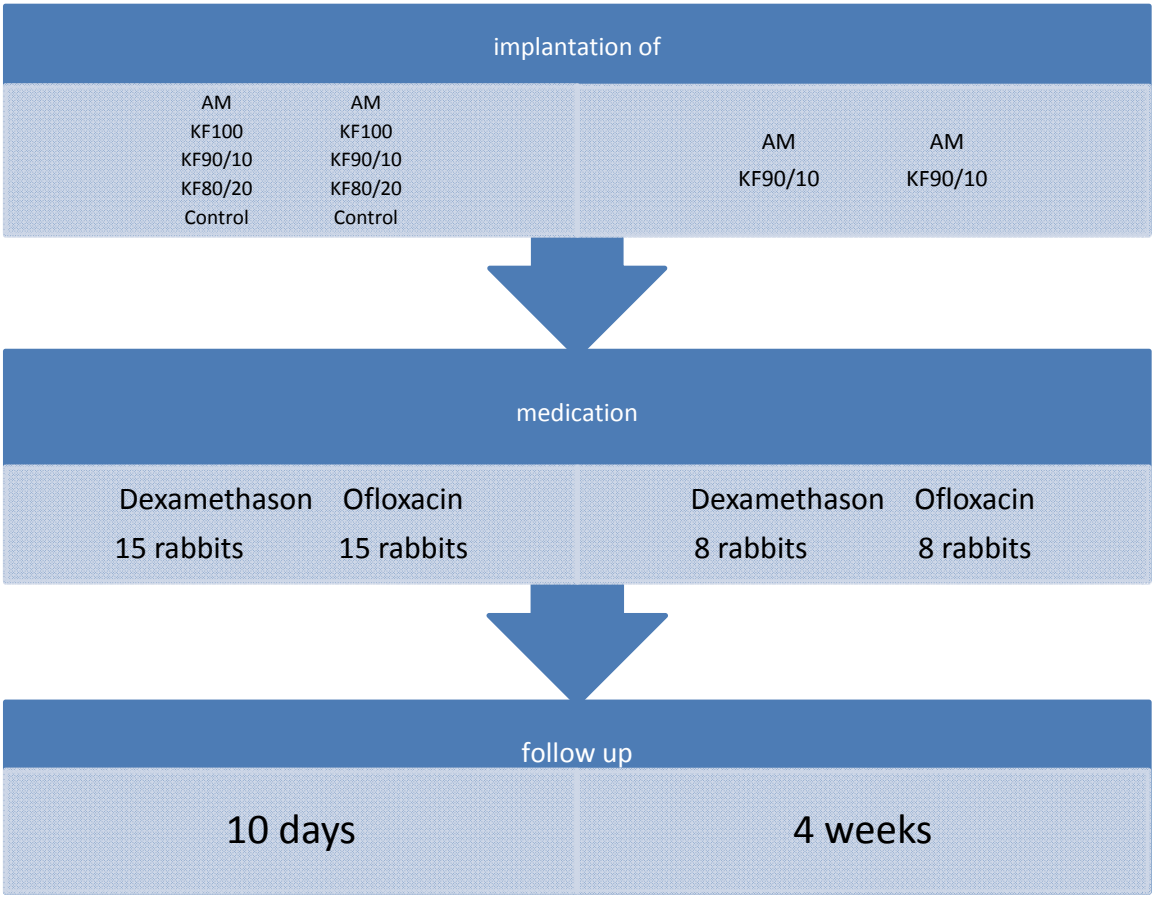


Fig. 6: Overview of implant groups, medical treatment and follow-up periods.

2.5. Post mortem assessment

2.5.1. Clinical examination

At the end of each follow-up period the animals were sacrificed. After opening the third lid tarsorraphy and removing the soft contact lens, photos of the anterior segment were taken using a digital camera (Canon IXUS 117HS) mounted on the microscope optic in order to obtain pictures with the same magnification (2.5 x and 4 x). Moreover a millimeter scale was included in the photo field to allow precise measurement of eventual corneal epithelial defect (see fig. 7). Fluorescein corneal staining was performed by instilling sodium fluorescein eye drops onto the ocular surface and washing out the extra dye with saline solution. The eyes were inspected and pictures were taken, as described above, by shining cobalt blue light on the ocular surface. Then a slit lamp biomicroscopy was performed, by the same observer, to evaluate three parameters according to a standardized grading score: (1) vascularisation of the cornea; (2) transparency; (3) fluorescein staining as seen in fig. 8 (Sitalakshmi et al. 2009, p. 410).

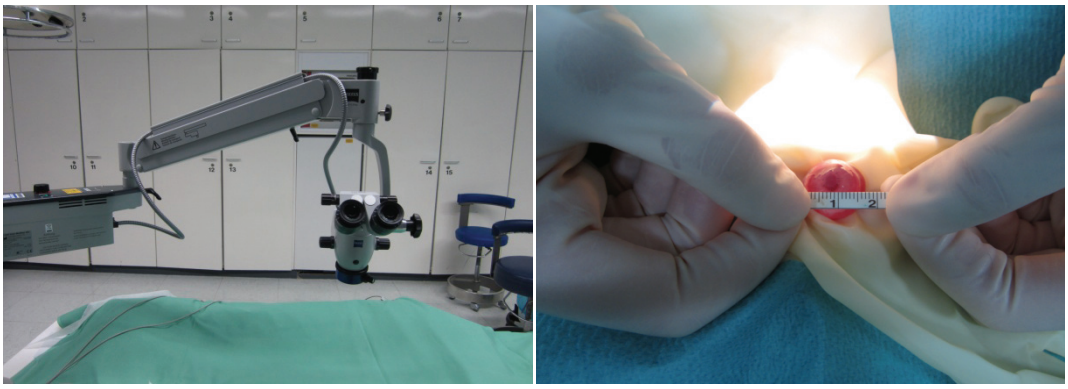


Fig. 7: Post-mortem assessment. Operation field with microscopic optic (left picture) and measuring eventual corneal epithelial defects with a millimeter scale.

Clinical criteria	
Corneal transparency	
0	No haze: clear cornea, iris details seen clearly
1	Mild haze: visible but iris details visible
2	Severe haze: iris details obscured
Neovascularization	
0	No vascularization beyond >4 mm in any area
1	Neovascularization of 4 mm or more in <3 clock hours
2	Neovascularization of 4 mm or more in >3 clock hours to <6 clock hours
3	Neovascularization of 4 mm or more in >6 clock hours to <9 clock hours
4	Neovascularization of 4 mm or more in >9 clock hours
Epithelial integrity (Fluorescein staining)	
0	Negative
1	Positive

Fig. 8: Clinical criteria recorded and corresponding scoring system used to evaluate the animals at the end of the follow up (Sitalakshmi et al. 2009, p. 410).

2.5.2. Histological examination

At the end of the follow up period, the corneoscleral button of each rabbit's right eye was excised at the limbus and processed for histology. The specimen was fixed in 4 % formalin in PBS pH 7.4 at 4 °C for 3-4 hours and then washed over night (12 hours) in 6.8 % sucrose solution at 4 °C. The specimen was washed in acetone until the solution was clear. Afterwards the cornea was dehydrated in 100 % acetone for at least 1 hour. The tissues were embedded according to the standard embedding protocol of Technovit 8100® (Heraeus Holding GmbH, Hanau, Germany). Therefore, infiltration solution A was prepared using 100 ml Technovit 8100® base liquid and 0.6 g Hardener 1. The tissues were infiltrated in Solution A for 6-10 hours at 4 °C. In order to standardize the histological examination and the implant thickness measurements the corneoscleral button was cut in half at the center of the implant and placed in two separate molds of the Histoform S® with the cutting edge facing down (see fig. 9). Infiltration solution B was prepared using 30 ml infiltration solution A and 1 ml Hardener 2. The tissues were immersed in infiltration solution B for 5 min at 4 °C.

Infiltration solution B was filled into the Histoform S® which was finally sealed with cover foil and stored on ice at 4 °C for 12 hours.

Fig. 9: Cutting of the cornea and placing into the histoform mold.

The embedded samples were mounted using the Technovit 3040® protocol. Before mounting, the cover foil was removed and histoblocs were placed on the Histoform S®. Then Technovit 3040® was prepared by mixing 2-3 parts of powder with 1 part of liquid. The Technovit 3040® was poured onto the histobloc. After 5-10 min the Technovit 3040® hardened and the samples were removed from the Histoform S® (see fig. 10).

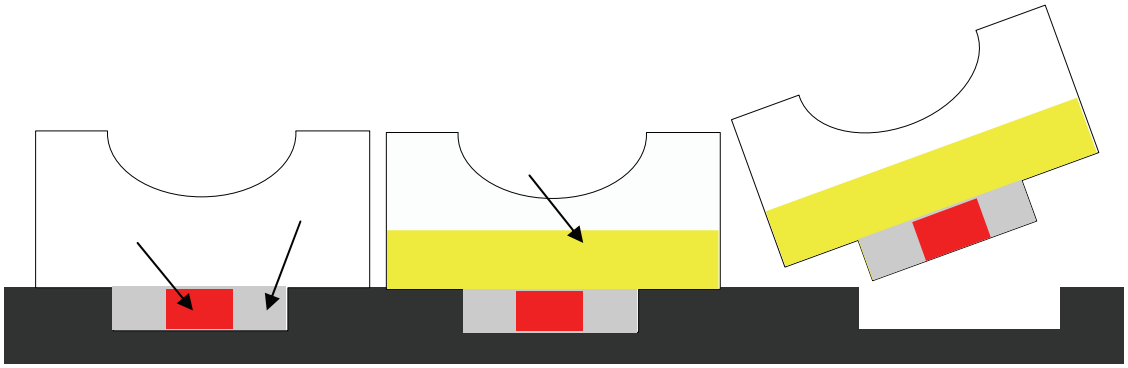


Fig. 10: Technovit mounting scheme adapted from Heraeus Kulzer showing from left to right: cornea sample (left arrow) embedded in Technovit (middle arrow) in the left mold; histobloc with poured in Technovit 3040® (right arrow) in the middle mold, and removal of the histobloc with the cornea sample in the right mold.

Histology sections of 4 µm thickness were cut using a microtome (Leica microtome RM 2255, Leica Biosystems GmbH, Nussloch, Germany).

The sections were stained using Hemalaun-Eosin staining (Haemalaunlösung nach Meyer, Carl Roth GmbH+ Co. KG, Karsruhe, Germany). Therefore, the sections were stained in Hemalaun for 15 min, then rinsed in tap water for 10 min and washed shortly in distilled water. The sections were then stained in Eosin (Eosin G, Carl Roth GmbH+ Co. KG, Karlsruhe, Germany) for 2-5 min.

Afterwards, the sections were dehydrated with 96 % Ethanol and then with 100 % Ethanol. After clarifying with Xylol the sections were covered with coverslips using Eukitt (Roti-Histokitt II, Carl Roth GmbH+ Co. KG, Karlsruhe, Germany) (Heraeous Kulzer GmbH 2012, p. 18).

Hematoxylin-Eosin-staining (HE-staining) stains basophil structures such as nuclei blue, while the rough endoplasmatic reticulum and azidophil structures such as cytoplasma and collagen fibers are stained red. This coloration enables one to distinguish between different tissue components such as nuclei and cytoplasma (Junqueira, Gratzl 2005, pp. 445–446). Additionally, keratin film stains blue and can thus be differentiated from the collagen-rich corneal stroma which stains red.

5 central slides of each cornea were examined by a blinded observer to assess the grade of inflammation and neovascularization according to a modified standardized grading score as seen in fig. 11 (Irvine, Kimura 1967, p. 193; Chang et al. 2000, p. 11833).

Histology criteria		
Grading score	**Inflammation**	**Neovascularization**
0	no inflammatory cells	no neovascularization
1	very few inflammatory cells	neovascularization in <25 % of the field
2	few inflammatory cells infiltrating the implant/ intrastromal pocket	neovascularization in 25-50 % of the field
3	many inflammatory cells infiltrating the implant surrounding stroma	neovascularization in 50-75 % of the field
4	massive inflammation extending over the whole depth of the cornea	neovascularization in >75 % of the field

Fig. 11: Histological grading score to evaluate the inflammation and neovascularization at the end of the follow up (Irvine, Kimura 1967, p. 193; Chang et al. 2000, p. 11833).

The implant-thickness after each follow-up period of 10 days and 4 weeks were measured with the use of the software Image J in 5 central slides and the mean value was calculated.

All experimental data were evaluated using MS Excel 2007 and Graph Pad Prism 6. The ANOVA test was used to test for a significant difference within the subgroups, while a t test with Bonferroni correction and a Kruskal-Wallis test were used to test for a significant difference between subgroups. Values of $P<0.05$ were considered statistically significant.

3. Results

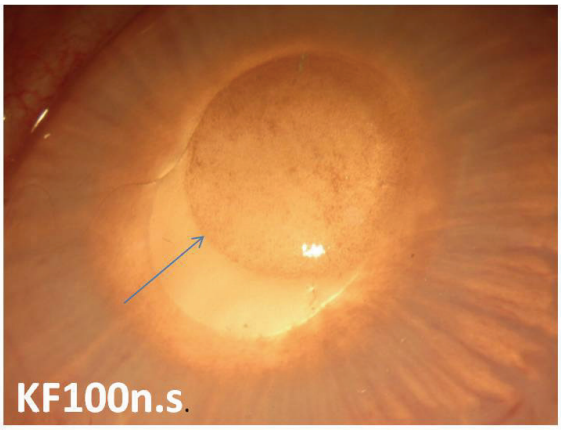
3.1. Clinical examination

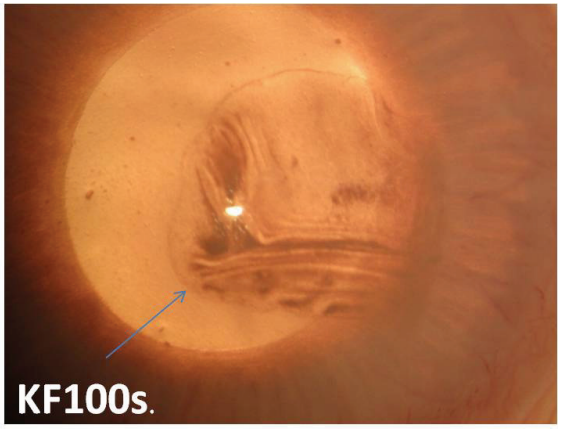
3.1.1. Corneal transparency, neovascularization and epithelial integrity after 10 days

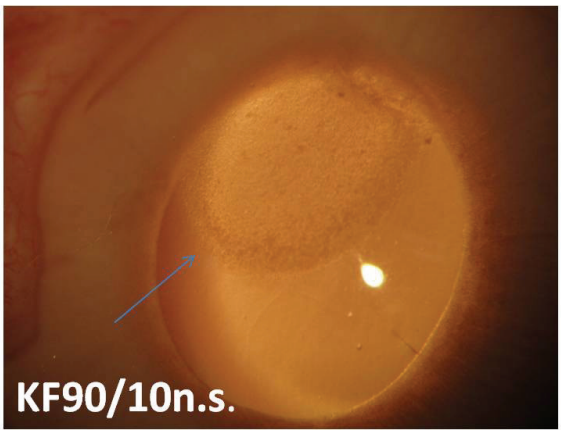
After a follow-up period of 10 days slit lamp examination revealed a higher number of transparent corneas in the steroid group than in the non-steroid group.

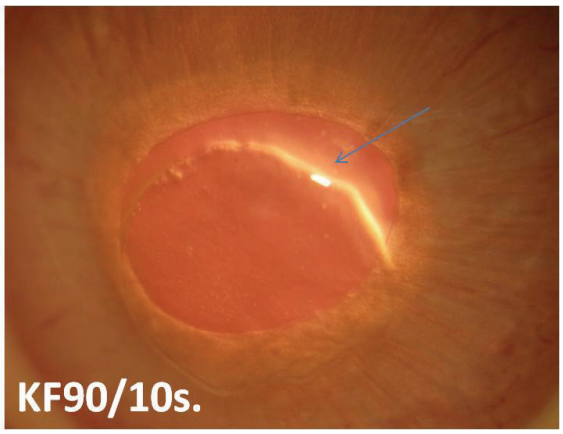
In the non-steroid group a transparent cornea (score 0) was observed in all control eyes and AM implanted eyes, as well as in 2 out of 3 eyes implanted with KF100. Mild corneal haze (score 1) was observed in 1 out of 3 eyes grafted with KF100. In the KF90/10 group 2 out of 3 eyes showed an almost transparent cornea, except for a paracentral haze, while the remaining eye showed a diffuse mild corneal haze (score 1). In the KF80/20 implanted eyes, 2 out of 3 corneas showed a mild haze (score 1) and 1 out of 3 eyes showed severe haze and corneal oedema (score 2). Corneal neovascularization was observed in none of the eyes belonging to this group. A small and round corneal epithelial defect was detected in only 1 out of 3 KF100 implanted eyes.

In the steroid group a transparent cornea (score 0) was observed in all of the control eyes, in 1 out of 3 eyes implanted with AM and KF100, in 2 out of 3 eyes implanted with KF90/10 and in all eyes implanted with KF80/20. Mild corneal haze (score 1) was described in the remaining eyes (2 out of 3 AM implanted eyes, 2 out of 3 KF100 implanted eyes and in 1 out of 3 KF90/10 implanted eyes). A slight temporosuperior neovascularization just overriding the limbus (score 1) was observed in 1 out of 3 eyes grafted with KF100. Corneal epithelial defects were found in none of the eyes within this group (see fig. 12).


KF100n.s.


KF100s.


KF90/10n.s.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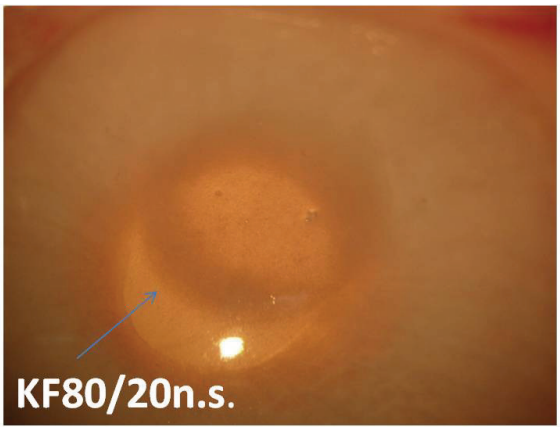
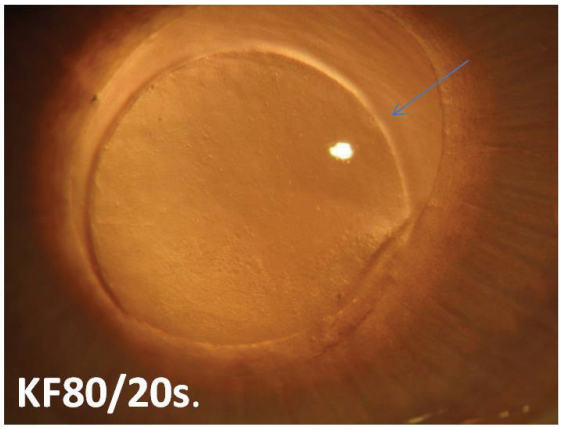
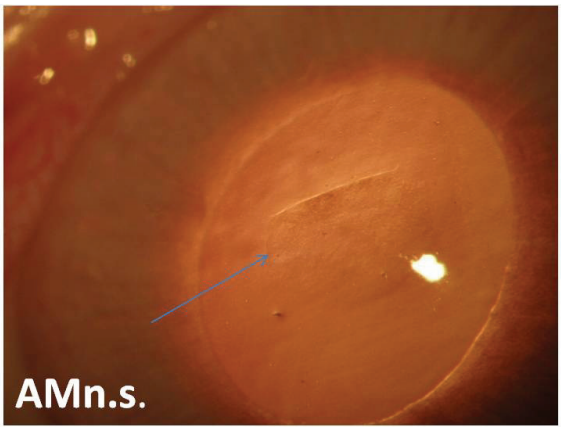
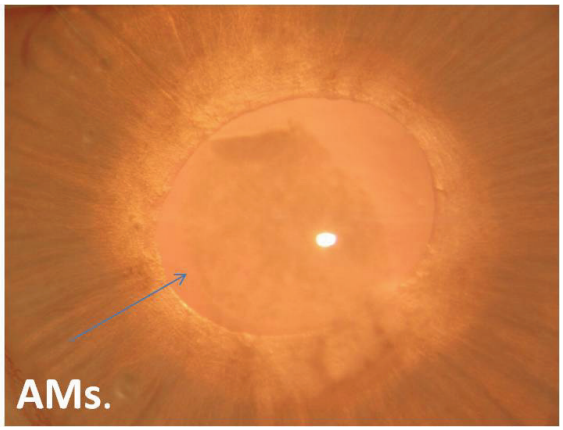

KF80/20n.s.


KF80/20s.


AMn.s.


AMs.

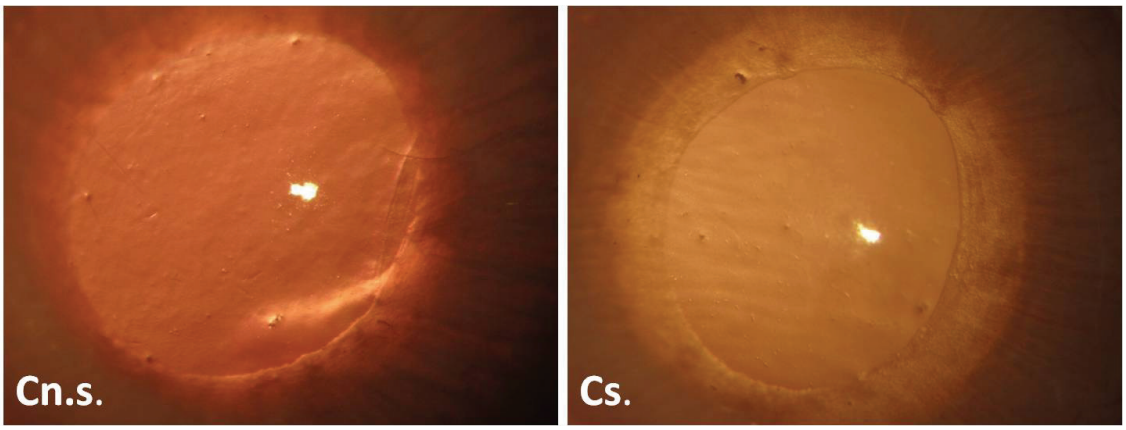


Fig. 12: 10 days follow up: clinical pictures of all KF implanted (KF100, 90/10, 80/20) eyes as well as AM implanted eyes (Gold-standard control) and negative controls (C) without implant treated without steroids (n.s.) and with steroids (s.). The arrow outlines the implant border. KF implanted corneas treated without steroids at this follow up point show haziness at the implant site (in particular KF80/20 show complete corneal oedema), corneas treated with steroids instead appear more transparent except for the KF100 implanted one where the implant shows folds. Both control groups show a clear cornea.

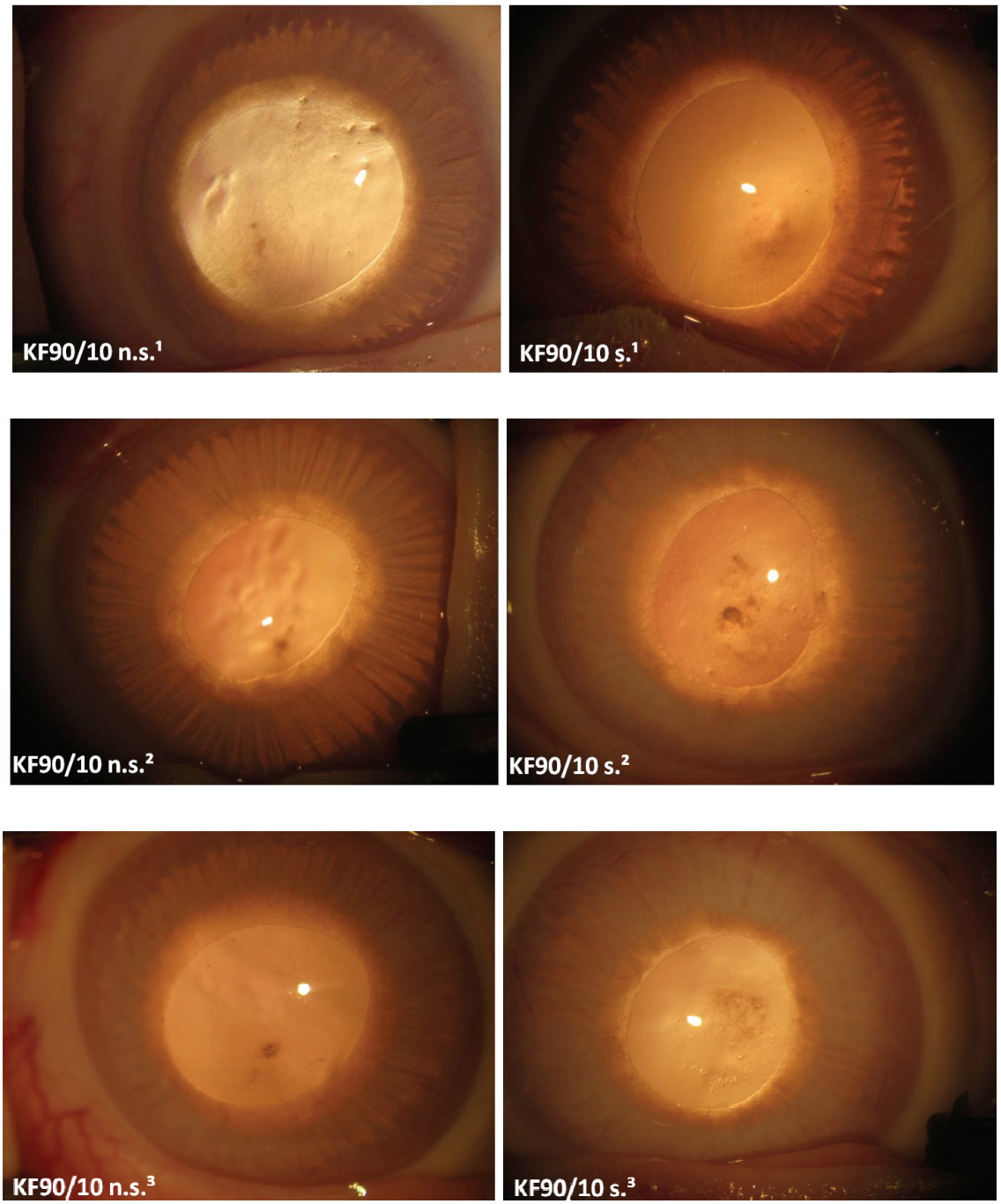
3.1.2. Corneal transparency, neovascularization and epithelial integrity after 4 weeks

The 4 weeks follow up series was performed based on the results obtained from the 10 days follow up series. Due to the lack of statistical significant difference between the 3 KF compositions concerning both the clinical and the histological results, only KF 90/10 was used in the 4 weeks follow up biocompatibility experiments, as it was also demonstrated in earlier studies to be superior in the surgical handling tests compared to the other keratin films (Borrelli et al. 2013, pp. 228–229). Furthermore our aim was to reduce the number of animals needed according to the principle of the 3 R's- replacement, reduction and refinement- defined by Russel et al. (Russell, Burch 1959, cited from Flecknell 2002, p. 73).

After a follow-up period of 4 weeks in the non-steroid group, slit lamp examination revealed a transparent cornea (score 0) in all eyes implanted with AM and in 3 out of 4 eyes implanted with KF90/10. Corneal neovascularization (score 1) and corneal epithelial defects (score 1) were observed in only 1 out of 4 eyes implanted with KF90/10 as seen in fig. 13.

In the steroid group a transparent cornea (score 0) was observed in all eyes implanted with AM and in 3 out of 4 eyes implanted with KF90/10. A mild

corneal haze with point shape opacification (score 1) was observed in the remaining examined eye as seen in fig. 13. Neither corneal neovascularization nor corneal epithelial defects were detected in any of the implanted eyes.



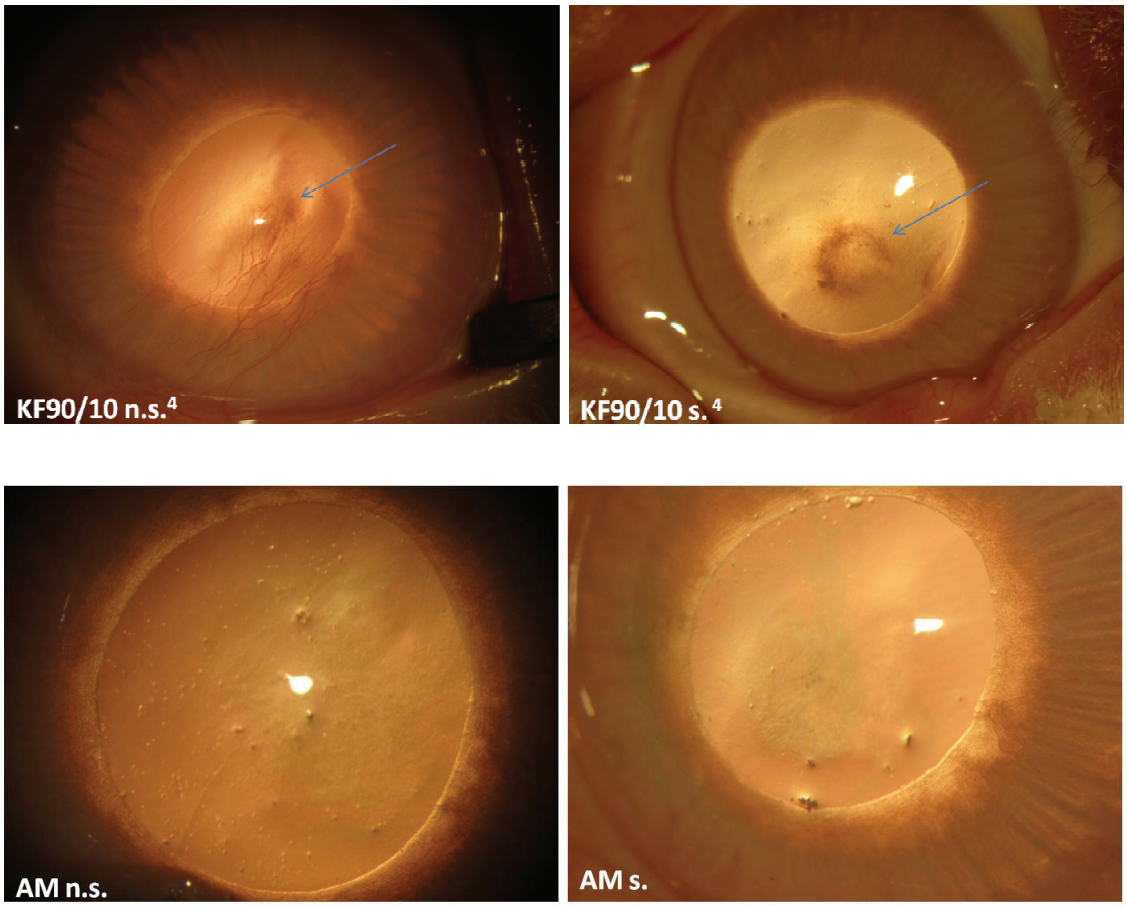


Fig. 13: Four weeks follow up: clinical pictures of the KF implanted eyes (KF 90/10) as well as AM implanted eyes treated without steroids (n.s.) and with steroids (s.). The corneas appear transparent in both groups; the implant margin is no longer recognisable. The arrow indicates in the non steroid treated eye (KF90/10 n.s.[4]) corneal neovascularization reaching the optical center and in the steroid treated eye (KF90/10 s.[4]) a paracentral haze.

3.2. Histological examination

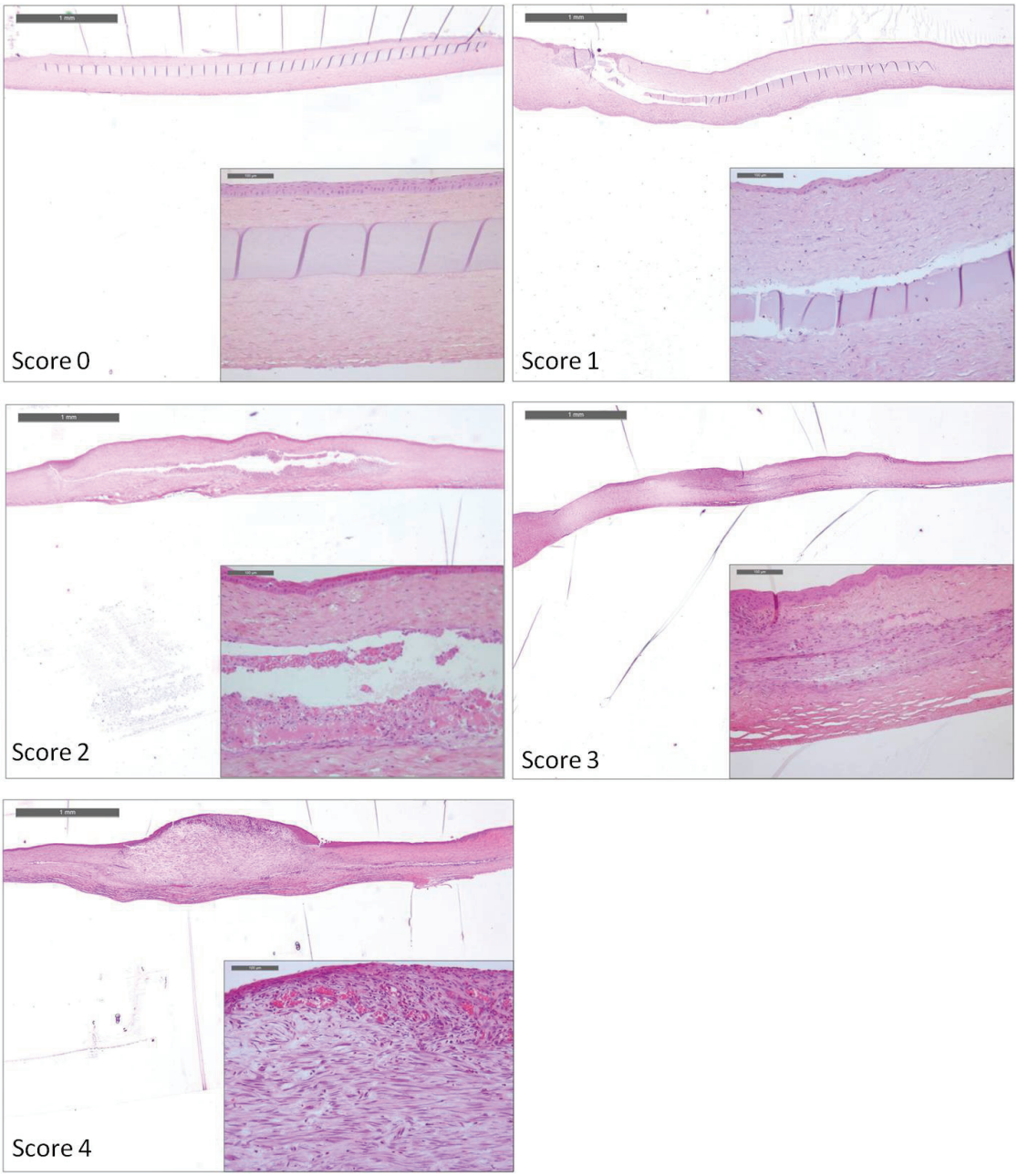


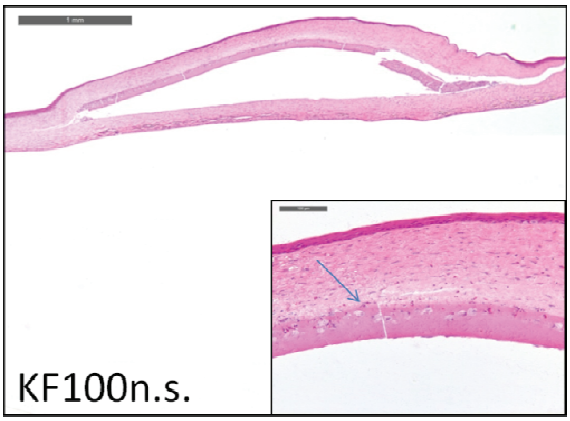
Fig. 14: Histologic pictures showing the Inflammation score: score 0: No inflammatory cells visible; score 1: Inflammatory cells visible, but very few in number and limited to implant or intrastomal pocket; score 2: More inflammatory cells infiltrating the implant/intrastromal pocket, but not the stroma; score 3: Many inflammatory cells infiltrating the implant surrounding stroma but not the whole depth of the cornea; score 4: Massive inflammation extending over the whole depth of the cornea.

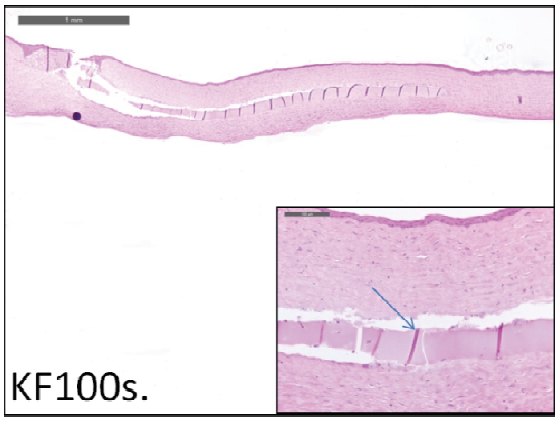
3.2.1. Inflammation and neovascularization after 10 days

After a follow-up period of 10 days the histological examination revealed in the non-steroid group very few eosinophilic and macrophage cells infiltrating the implant and intrastromal pocket (score 1) in all KF100 implanted eyes, as well as in 2 out of 3 eyes implanted with KF90/10 and in 2 out of 3 eyes implanted with KF80/20. The examination of the remaining KF90/10 implanted eye revealed a few eosinophilic and macrophage cells infiltrating the implant and the intrastromal pocket (score 2), while in 1 out of 3 eyes implanted with KF80/20 many inflammatory cells infiltrating the implant and the surrounding stroma (score 3) were detected as seen in fig. 15. Neovascularization was not observed in any of the KF or AM implanted eyes but only in 1 control eye.

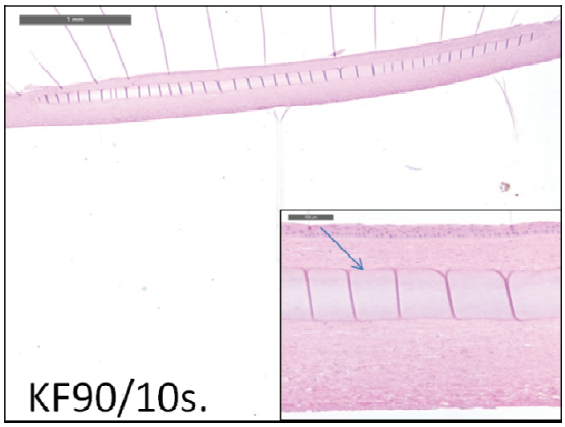
In the steroid group very few eosinophilic and macrophage cells (score 1) were observed in 1 out of 3 eyes implanted with KF100. All of the remaining eyes belonging to the steroid group showed an inflammation score of 0. None of the eyes in the steroid group revealed neovascularization (score 0) as seen in fig. 15 and 16.

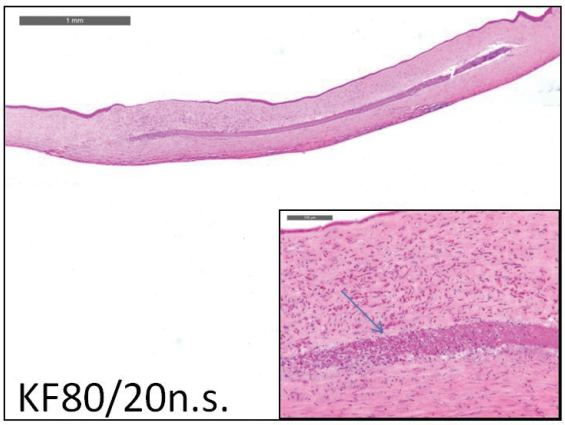
Comparing the different KF-compositions, no significant difference could be observed within the steroid group as well as within the non-steroid group as seen in fig. 17. Comparing the different medical treatment for each KF composition, a statistically significant lower inflammation score ($p \leq 0.05$) can be observed in steroid treated eyes compared to the non-steroid treated eyes for KF 90/10 and KF 80/20 as seen in fig. 18. For KF100 implanted eyes no statistically significant difference could be observed between the steroid treatment and non-steroid treatment.

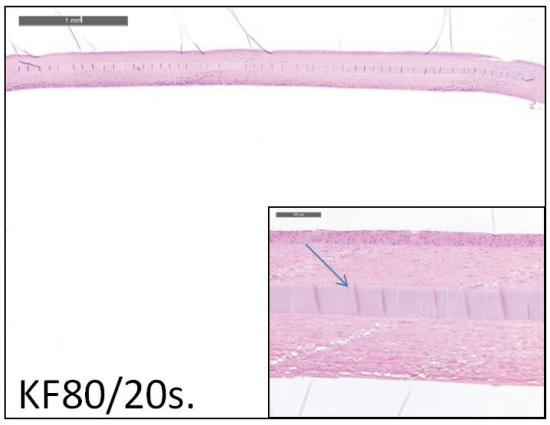

KF100n.s.

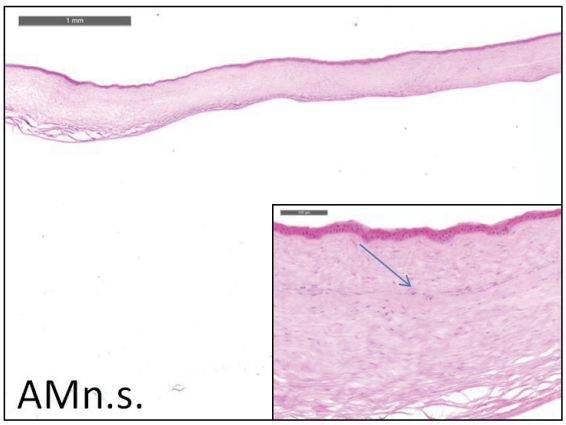

KF100s.

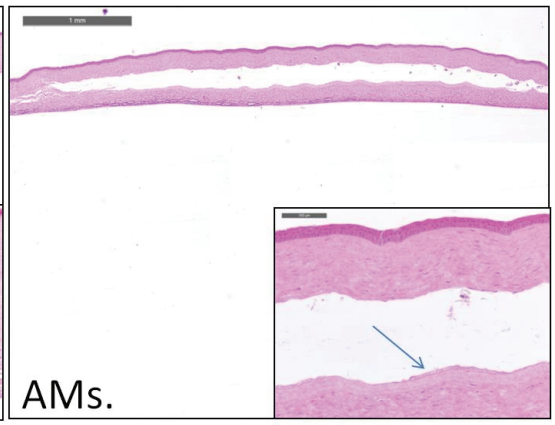

KF90/10n.s.


KF90/10s.


KF80/20n.s.


KF80/20s.


AMn.s.


AMs.

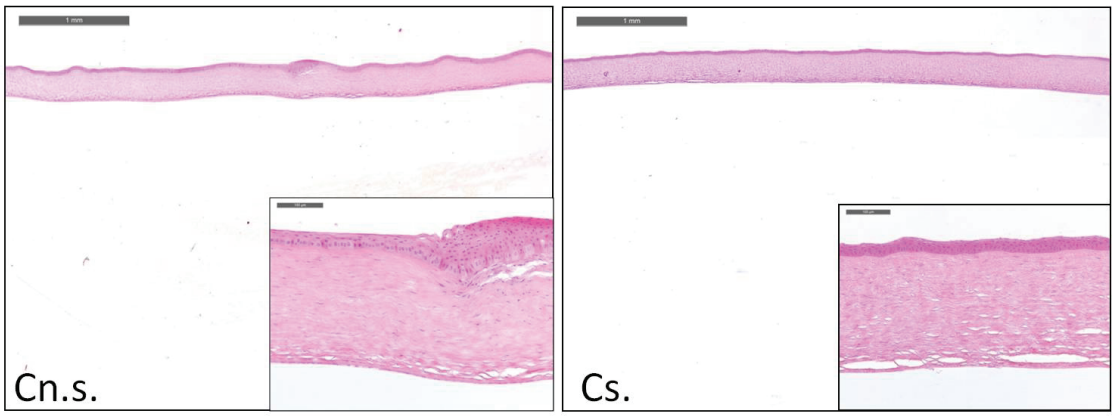


Fig. 15. Histologic pictures after 10 days follow-up of all KF (KF100, 90/10, 80/20) implanted groups as well as AM (as gold-standard control) and the negative control (C) without any implant treated without steroids (n.s.) and with steroids (s.). The arrow outlines the implant within the intrastromal-dissected pocket. The KF80/20n.s. eye shows a stronger inflammatory reaction of the corneal stroma (corresponding clinical picture in Fig. 12). Both control groups show no inflammatory reaction.

Inflammation and Neovascularisation 10 days follow-up																
ImplantGroups n=3	**KF100**			**KF90/10**			**KF80/20**			**AM**			**Control**			
Medication	**Non-steroid**			**Non-steroid**			**Non-steroid**			**Non-steroid**			**Non-steroid**			
InflammationScore	1	1	1	2	1	1	1	3	1	0	0	0	0	0	0	
mean	1,00			1,33			1,67			0,00			0,00			
±SD	0,00			0,58			1,15			0,00			0,00			
Neovascularisation	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
mean	0			0			0			0			0,33			
±SD	0			0			0			0			1			
Medication	**Steroid**			**Steroid**			**Steroid**			**Steroid**			**Steroid**			
InflammationScore	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
mean	0,33			0,00			0,00			0,00			0,00			
±SD	0,58			0,00			0,00			0,00			0,00			
Neovascularisation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
mean	0			0			0			0			0			
±SD	0			0			0			0			0			

Fig. 16: Histological results 10 days post-OP. This table shows the inflammation score and neovascularization score without steroid treatment and with steroid treatment after 10 days follow-up. Mean and ±SD are also shown.

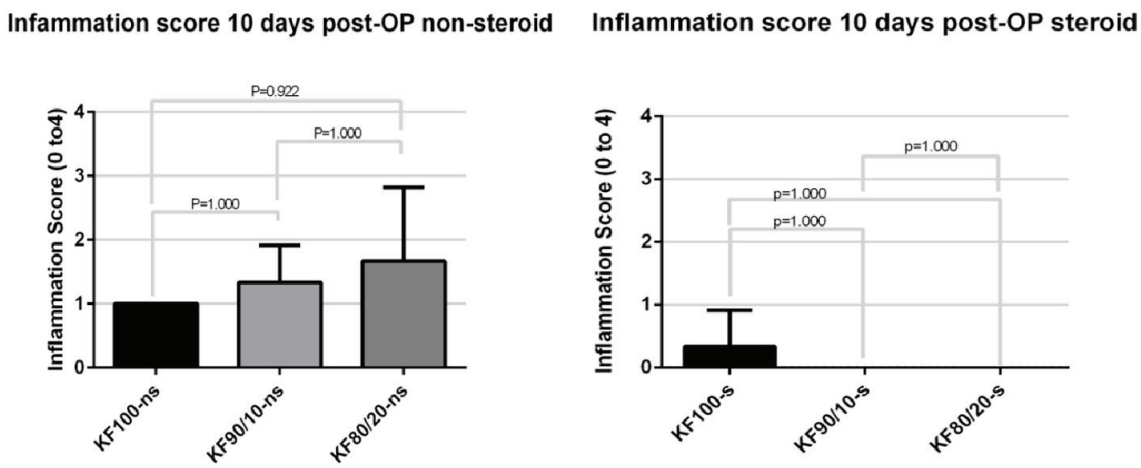


Fig. 17: Inflammation score of all KF groups 10 days post-OP. The graph shows a comparison of the different KF within the non-steroid (ns) and the steroid (s) group. No statistically significant difference can be seen between the different KF.

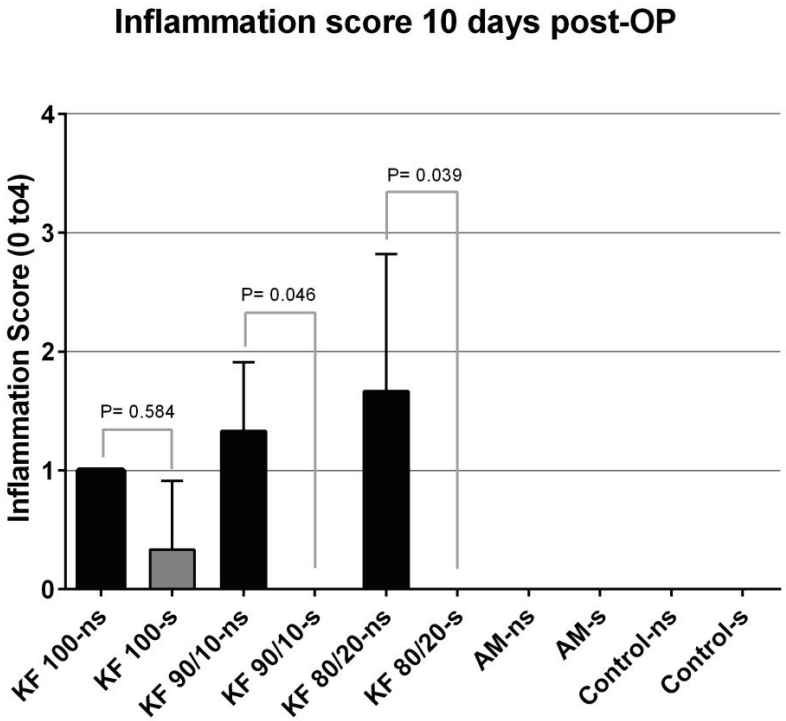


Fig. 18: Inflammation score at 10 days follow up. The graph shows all subgroups of keratin films (KF100, KF90/10 and KF80/20), amniotic membrane (AM) and control (C) both in the steroid group (s) and non-steroid group (ns). Bars show mean + SD.

3.2.2. Inflammation and neovascularization after 4 weeks

After a follow-up period of 4 weeks, no inflammation (score 0) was detected in 3 out of 4 eyes implanted with KF90/10 and AM in the non-steroid group.

Only 1 out of 4 eyes implanted with KF90/10 revealed many inflammatory cells infiltrating the implant and the surrounding stroma (score 3) as well as neovascularization (score 1) as seen in fig. 20. In 1 out of 4 eyes implanted with AM very few eosinophilic and macrophage cells (score 1) were detected.

In the steroid group, no inflammation (score 0) was detected in any of the eyes implanted with AM and in 1 out of 4 eyes implanted with KF90/10. In 1 out of 4 eyes implanted with KF90/10 very few inflammatory cells (score 1) were detected, whereas in 2 out of 4 eyes implanted with KF90/10 an inflammatory reaction of score 2 was detected (see fig. 19). Although no statistically significant difference was observed between the steroid and non-steroid groups for KF90/10 and for AM implanted eyes, in KF90/10 implanted and steroid treated eyes a tendency toward higher inflammatory reaction could be observed (see fig. 21 and 22). No neovascularization was observed in any eyes treated with steroids.

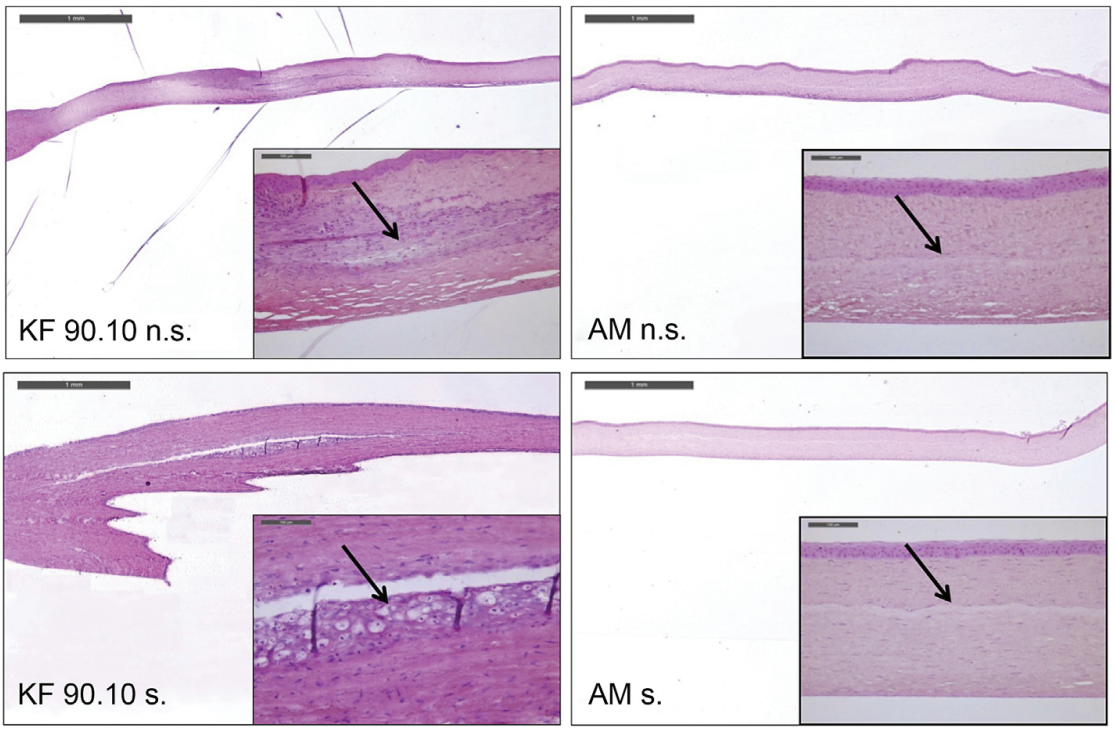


Fig. 19: Histology pictures of KF90/10 and AM implanted eyes after 4 weeks follow up treated without steroids (n.s.) and with steroids (s.). The arrow outlines the implant in the dissected intrastromal pocket. In the histology section of the KF90/10 (s.) the implant is easier to identify.

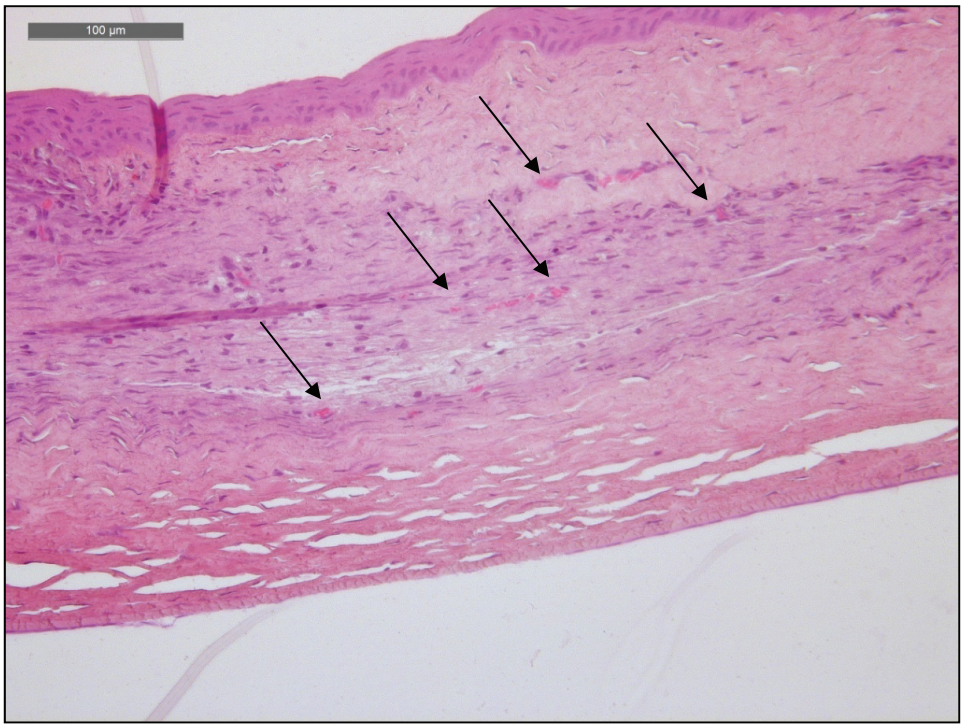


Fig. 20: 4 weeks follow-up. 1 out of 4 KF90/10 implanted eyes without steroid treatment showing an inflammation score of 3 and neovascularization score of 1. The arrows outline the neovascularization.

Inflammation and Neovascularisation 4 weeks follow-up																
ImplantGroups n=4	**KF90/10**								**AM**							
Medication	**Non-steroid**				**Steroid**				**Non-Steroid**				**Steroid**			
InflammationScore	0	0	3	0	0	2	1	2	1	0	0	0	0	0	0	0
mean	0,75				1,25				0,25				0,00			
±SD	1,50				0,96				0,50				0,00			
Neovascularisation	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
mean	0,25				0,00				0,00				0,00			
±SD	0,50				0,00				0,00				0,00			

Fig. 21: Histological results 4 weeks post-OP. This table shows the inflammation score and the neovascularization score without steroid treatment and with steroid treatment after 4 weeks follow-up. Mean and ±SD are also shown.

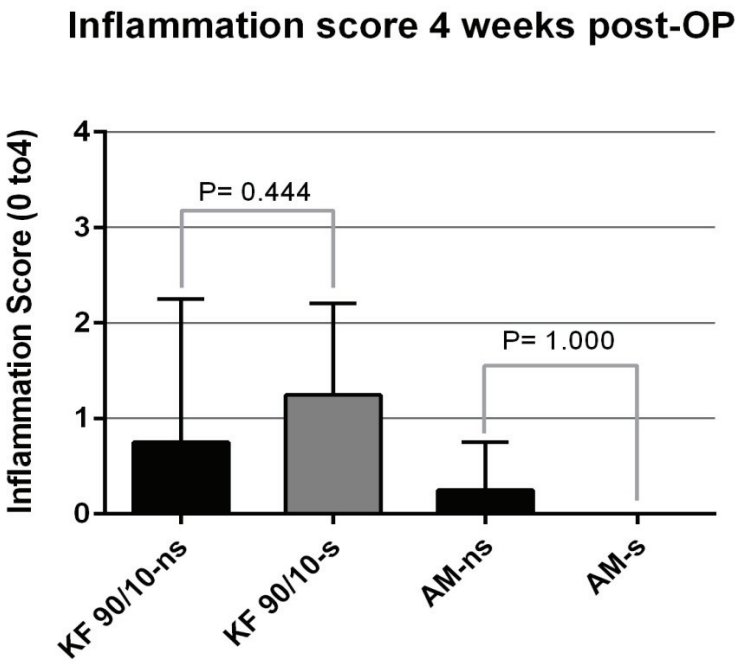


Fig. 22: Inflammation score at 4 weeks follow up. In the 4 weeks follow up the graph shows KF 90/10 and AM both in the non-steroid (ns) and steroid group (s). Bars show mean + SD.

3.2.3. Implant thickness

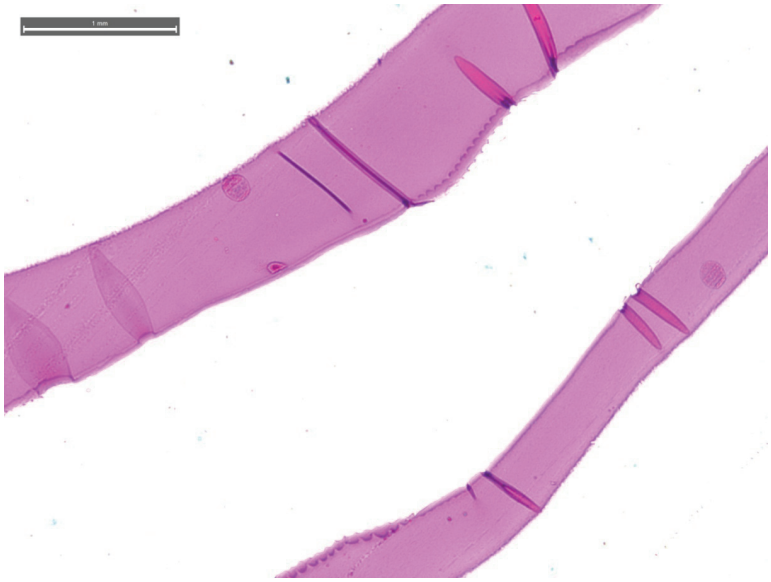


Fig. 23. Keratin film 90/10 embedded.

The implant thickness measurements performed before surgery showed good reproducibility for both KF and AM (see fig. 24 and 25). This allows the postulation that all of the implants preoperatively had equal thickness which could be measured and compared over the follow up period. Fig. 23 shows an example of a microscopic picture of an embedded keratin film.

Sample	Thickness center mean±SD	Thickness temporal side	Thickness nasal side
KF 90/10 dry	79±5µm	78±8µm	79±8µm
KF 90/10 wet	78±4µm	77±8µm	79±6µm
KF 90/10 Embedded	77±6µm	84±10µm	81±9µm
hAM Embedded	79±9µm		

Fig. 24: KF90/10 and AM implant thickness measured in the dry and wet state as well as after embedding (mean±SD). The KF were measured in the center and in the periphery. Due to tissue characteristics of the AM only the embedded AM could be measured.

After a follow-up of 10 days, measurements of the implant thickness revealed comparable results for the implant groups KF100, KF90/10 and KF80/20 with steroid treatment and without steroid treatment. The implant thickness of KF100 treated with steroids reveals higher values (mean of 87.56µm) than KF100 without steroid treatment (mean of 60.20µm). As demonstrated in fig. 25, there was no significant difference in the implant thickness when comparing the steroid treated eyes and the non-steroid treated eyes of all implant groups (KF100, KF90/10, KF80/20 and AM).

After a follow-up of 4 weeks, no statistically significant difference of the implant thickness could be observed between AM implanted eyes treated with steroids and without steroids ($p=1.00$).

The same result was observed when comparing the implant thickness of AM at 10 days and 4 weeks in eyes treated with and without steroids. A trend toward time-related implant thickness reduction could be observed. As fig. 25 shows, the implant thickness of KF90/10 was significantly reduced (87 % implant thickness reduction; $p<0.05$) in the non-steroid group compared to the steroid group (implant thickness reduction of 25 %). Moreover when the implant thickness of KF90/10 was evaluated after 10 days and 4 weeks, a statistically significant reduction ($p<0.05$) of the implant thickness of KF90/10 could be

observed in the non-steroid group after 4 weeks compared to 10 days. In the group of steroid treated eyes no statistically significant difference of the implant thickness was observed between 10 days and 4 weeks although a trend of a reduction of the implant thickness was registered. When comparing the preoperatively measured implant thickness of KF90/10 with the implant thickness after 10 days and 4 weeks, no statistically significant difference can be observed (see fig. 25).

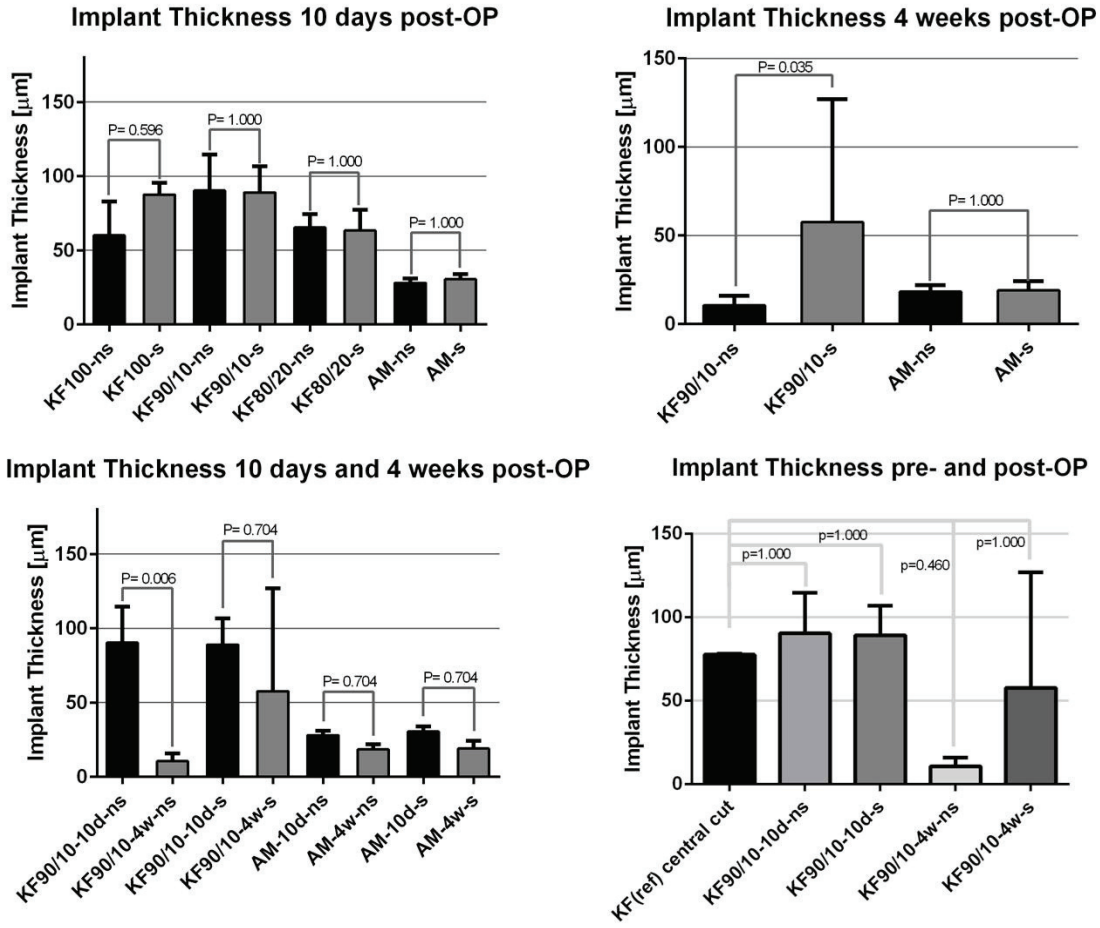


Fig. 25: Implant thickness in µm at 10 days and 4 weeks follow-up comparing the results in the steroid (s) and in the non-steroid (ns) groups for each implant (KF100, KF90/10, KF80/20, AM). The results are also compared to the preoperative thickness measurement of embedded KF90/10 (KF ref central cut). The bars show mean and SD. P<0.05 was considered statistically significant.

4. Discussion and Conclusion

4.1. Amniotic membrane for ocular surface reconstruction

Human amniotic membrane is commonly used for the reconstruction of the cornea (Rahman et al. 2009, p. 1954; Connon et al. 2010, p. 1057). It has been found to be an effective treatment for persistent epithelial defects with corneal ulceration, different forms of keratopathies, infectious keratitis and Stevens-Johnson syndrome (Plummer et al. 2009, p. 19). Amniotic membrane can also be used as a culture substrate for ex vivo expansion of corneal epithelial cells to treat limbal stem cell deficiency (Grueterich et al. 2003, p. 640; Meller et al. 2011, p. 245).

Despite a number of good properties, transplantation of amniotic membrane exhibits certain disadvantages which require the generation of an alternative substrate. The low transparency of amniotic membrane limits postoperative vision if implanted into the visual axis (Connon et al. 2010, p. 1057; Letko et al. 2001, p. 663). Poor standardization results in inconsistent quality of the tissue due to intradonor and interdonor variations which affect thickness and transparency of the AM (Ahmad et al. 2010, p. 311; Dua et al. 2010, p. 963). Interdonor variations can be caused by variables such as age, race, duration of gestation or parity and gravidity of the donor (López-Valladares et al. 2010, p. 212). Intradonor variations can be caused by a variable thickness of the AM depending on the location proximal or distal of the placenta (Connon et al. 2010, p. 1059). The transparency of the AM is dependent on its thickness and thus on the localization of the donor tissue (Connon et al. 2010, p. 1059). Preservation and sampling of AM can influence its transparency and its refractive index (Connon et al. 2010, p. 1060; López-Valladares et al. 2010, p. 212; Rodríguez-Ares et al. 2009, pp. 398–401).

Further limitations result from the fact that, despite strict regulations on clinical testing, the transplantation of AM is always associated with the risk of transmitting diseases (Dua et al. 2010, p. 96; Borrelli et al. 2013, p. 221).

This can especially be an issue in developing countries where the use of fresh unpreserved membrane is quite common (Borrelli et al. 2013, p. 221; Gicquel et al. 2009, p. 1920; Rahman et al. 2009, p. 1954). Compared to other materials, AM is less easy to handle during clinical procedure. It has a tendency of curling and wrinkling when lifted with the forceps or even tearing if not properly procured (Liu et al. 2012, p. 4133). A further major disadvantage of the clinical application of amniotic membrane as being a human donor tissue is the challenge to provide for a consistent supply (Schrader et al. 2009, p. 917).

To overcome some of the drawbacks in the use of AM, attempts have been made to modify AM and to optimize its qualities for ocular surface reconstruction with limited success (Borrelli et al. 2012, p. 31). The manufacturing and preservation process such as cryopreservation can alter its quality and even induce stromal oedema and a loss of proteins or reduce the viability of stromal fibroblasts (Rodríguez-Ares et al. 2009, pp. 398–401; Kruse et al. 2000, p. 72).

4.2. Requirements for an optimal biocompatible material

An optimal biocompatible material for ocular surface reconstruction should support the attachment, proliferation and maintenance of limbal epithelial stem cells and allow their ex-vivo expansion and subsequent delivery onto the cornea (Borrelli et al. 2012, p. 30). Further requirements are transparency and long term biocompatibility as well as surgical feasibility (Borrelli et al. 2012, p. 30; Reichl et al. 2011, p. 3376).

A biomaterial is distinguished from any other material by its ability to exist in a sustained long-term contact with tissues of the human body without causing an unacceptable degree of harm, achieved through chemical and biological inertness (Williams 2008, p. 2941). Intracorporally implanted materials provoke host reactions which include injury, blood–material interactions, provisional matrix formation, acute and chronic inflammation, development of granulation tissue and fibrous capsule development as well as foreign body reaction (Anderson et al. 2008, p. 86; Luttikhuizen et al. 2006, p. 1955).

The extent or degree of the inflammatory reaction (acute or chronic) is controlled by the extent of injury in the implantation procedure and the extent of provisional matrix formation, which follows the blood-material interactions in the very early process of implantation (Anderson et al. 2008, p. 87).

Acute inflammation is characterized by the presence of neutrophils, mast cell degranulation with histamine release and fibrinogen absorption, and a duration of less than a week (Anderson et al. 2008, p. 87). Chronic inflammation is characterized by the presence of monocytes, lymphocytes, a less uniform histological structure and a duration of about 2 weeks (Anderson et al. 2008, p. 87). A chronic inflammation with the presence of monocytes, macrophages and foreign body giant cells describes a foreign body reaction (Anderson et al. 2008, p. 87). A foreign body reaction is characterized by 3 distinct phases: onset, progression and resolution (Luttikhuizen et al. 2006, p. 1955). The onset of a foreign body reaction as a result of tissue injury initiates the wound healing process by increasing the blood vessel permeability, causing coagulation and the migration of neutrophils and macrophages, initiating the absorption of proteins and complement factors, and hydrolization of fibrinogen (Luttikhuizen et al. 2006, p. 1955). In the case of degradable materials, the reaction will become chronic until final degradation. In the case of non-degradable materials, the reaction continues until a capsule is formed around the implant, shielding it from the nonspecific immune system. Later, capsule shrinkage and damage to the implant can occur (Luttikhuizen et al. 2006, p. 1955). The progress of the foreign body reaction includes angiogenesis, leukocyte extravasation and migration, phagocytosis by macrophages and formation of giant cells, if the proteins are too large for phagocytosis. An ongoing inflammatory response can lead to extracellular matrix remodelling to maintain tissue integrity and to support cell adhesion. During the phase of resolution, formation of granulation tissue, identified by the presence of macrophages, fibroblasts, and neovascularization in the new healing tissue, as well as fibrous capsule formation can take place (Luttikhuizen et al. 2006, pp. 1956-1962; Anderson et al. 2008, p. 87).

Over the past decade the understanding of the interaction between biomaterials and the surrounding tissue has gained major importance in the ongoing research in tissue engineering. The mechanisms involved in this interaction have therefore been studied more extensively (Anderson et al. 2008, p. 87; Brown et al. 2012, pp. 3798-3800; Valentin et al. 2009, p. 1692; Fearing et al. 2014, pp. 3137-3143). Although biomaterials must provide the appropriate biomimetic environment for optimal material-cell interactions, they can also be 'used to direct desired cell behaviours such as orientation and migration to ensure that the appropriate cells migrate to or adhere to the implant' (Anderson et al. 2008, p. 96). The interaction between the biomaterial and macrophages plays a particularly important role in the outcome of an implant (Brown et al. 2012, p. 3799).

Badylak et al. recently examined the phenotype of macrophages at the site of injury after the implantation of biomaterials and found that M2 responses were associated with an organized, site-appropriate, tissue-remodelling outcome and an absence of persistent inflammation (Badylak et al. 2008, p. 1840). On the other hand, an M1 response was associated with long-term inflammation, and the formation of multinucleate giant cells and scar tissue (Badylak et al. 2008, p. 1840). In conclusion, the macrophage phenotype could be determinant in the host-tissue interaction and strategies for tissue engineering, which promote an M2 response, therefore could be favorable (Badylak et al. 2008, p. 1840; Valentin et al. 2009, p. 1692).

This has been confirmed by Fearing et al., who recently postulated that keratin biomaterials support an M2-dominated response and also down-regulate the differentiation toward an M1 phenotype at the site of injury (Fearing et al. 2014, p. 3142). This suggests that keratin biomaterials may offer an improved alternative to other biomaterials (Fearing et al. 2014, p. 3143).

4.3. Keratin films

Films and scaffolds based on human hair keratin match the requirements demanded of a biomaterial, and, due to a good biocompatibility, they have been proposed for cell culture and tissue engineering applications such as drug

delivery, tissue regeneration, and wound healing over the last decade (Reichl 2009, p. 6854; Verma et al. 2008, p. 11; Yamauchi et al. 1998, p. 270; Tachibana et al. 2002, p. 167; Sierpinski et al. 2008, p. 125; Srinivasan et al. 2010, p. 11; Saul et al. 2011, p. 544).

4.3.1. Biocompatibility and cell adhesion of keratin films

Keratin biomaterials, as published in the international literature, have good biocompatibility, support cell adhesion and function as a scaffold for cell cultivation (Guzman et al. 2011, p. 8216; Tachibana et al. 2002, p. 168; Yamauchi et al. 1998, p. 268; Reichl 2009, p. 6862; Tanabe et al. 2002, p. 821). Verma et al. proposed human hair proteins as a good material for tissue-engineering because of its human origin, biodegradability, and cytocompatibility, as well as its cell-cell and cell-matrix contacts (Verma et al. 2008, p. 11). Keratin biomaterials were also advocated as a promising material for dermal drug delivery (Srinivasan et al. 2010, p. 1).

4.3.2. Tissue healing properties of keratin films

Furthermore, keratin biomaterials reveal good tissue healing properties (Reichl et al. 2011, p. 3385; Pace et al. 2013, p. 5913; Shen et al. 2011, p. 9298; Peplow et al. 2009, p. 265; Sierpinski et al. 2008, p. 127; Rahmany et al. 2013, p. 2499) and a low degree of foreign body reaction in the host tissue (Shen et al. 2011, p. 9298). As Pace et al. showed, keratin biomaterials support the proliferation of Schwann cells and promote the clearance of myelin debris in the regeneration of peripheral nerves (Pace et al. 2013, p. 5913).

Keratin biomaterials have been shown to be beneficial as a wound dressing, since they show a faster rate of reepithelialization than the commonly used polyurethane wound dressings. Especially wounds like burns, pressure ulcers and diabetic ulcers, which create high expenses in the health system, could be treated at lower costs using keratin biomaterials (Pechter et al. 2012, p. 240).

4.3.3. Cell cultivation, transparency and biomechanical properties of keratin films

As Reichl et al. already demonstrated it is possible to prepare stable and transparent keratin films that represent a good growth substrate for corneal epithelial cells using a novel technology (Reichl et al. 2011, p. 3385). Cells on transferable keratin films exhibit population doubling times (PDT) and saturation density similar to those on AM (Reichl et al. 2011, p. 3385).

Keratin films are considerably more transparent than amniotic membrane. In previous examinations it has been shown that light transmission values of keratin films ranged from 65 % to 97 % at 400–700 nm, while for amniotic membrane light transmission values ranged from 60 % to 70 % at 400-700nm (Borrelli et al. 2013, p. 225).

The biomechanical properties of keratin films are comparable to those of amniotic membrane as the testing of tensile strength revealed comparable ultimate strength (US) and E-modulus (EM) values for KF and AM (Reichl et al. 2011, p. 3385). Moreover, ex vivo suture experiments conducted on enucleated porcine eyes showed that the keratin films could be sutured into a corneal defect by surgeons with variable experience, in a time that was clinically but not statistically different from amniotic membrane (Borrelli et al. 2013, p. 229).

4.3.4. Provision of keratin films

Furthermore, the possibility of an unlimited supply of keratin films for transplantation purposes opens new opportunities for corneal epithelial reconstruction. Since human hair is a material of human origin which can be obtained easily without any painful process, keratin films can be produced without the need of organ donation (Verma et al. 2008, p. 8). Keratins can also be obtained from protein-rich waste products; e.g. in hair salons approximately 300,000 tons of hair accumulate per year worldwide (Reichl 2009, p. 6854; Guzman et al. 2011, p. 8205). A consistent quality of the keratin film production can be ensured by the standardized extraction procedure using the Shindai method (Nakamura et al. 2002, p. 569).

4.4. Results of this experiment

The aim of this present study was to evaluate the in vivo biocompatibility of keratin films of different ratios (KF100, KF90/10, KF80/20) and to determine the interaction between tissue and implant in the corneal stroma. Clinical parameters such as corneal transparency, neovascularization and corneal epithelial defects as well as histologic evaluation of inflammatory reaction, neovascularization and the implant thickness were examined 10 days and 4 weeks after implantation of the keratin film to evaluate potential acute and chronic inflammatory reactions in the cornea. The results were compared to amniotic membrane implanted eyes and eyes without any implant, which served as a control group (C).

The results of previous biomechanical and ex-vivo experiments showed that depending on the percentage of alkaline keratin dialysate KF80/20 is too soft and too fragile for sutures and KF100 is too stiff to adapt to the corneal curvature (Borrelli et al. 2013, p. 227). Based on the results from a previous study by our group, and due to the results of the 10 days follow-up period, we decided to conduct the 4 weeks follow-up experiments using only KF90/10.

After a follow-up of 10 days the clinical results showed no significant differences in corneal transparency and neovascularization between the keratin films with different percentages of alkaline dialysate (100; 90/10 and 80/20).

Comparing the steroid treated eyes to the non-steroid treated eyes a slightly better transparency could be observed in steroid treated eyes (6 out of 9 transparent corneas in the steroid group) compared to the non-steroid treated eyes (4 out of 9 transparent corneas in the non-steroid group).

After 4 weeks follow-up, transparent corneas could be observed in all AM implanted eyes, in 3 out of 4 KF90/10 implanted eyes in the steroid group, and in the non-steroid group.

The histological results complement the clinical results of a reduced corneal transparency after 10 days in the non-steroid group, as they showed an increased inflammatory cell infiltration with an inflammation score of 1 to 3.

After a follow-up of 4 weeks the results show a tendency towards a higher inflammatory response in the steroid group compared to the non-steroid group, and the inflammation in the non-steroid group seems to decrease over the time period.

Based on our results summarized above, we can assume that the administration of topical steroids delays the degradation of the implant by suppressing the inflammatory reaction. As our results have demonstrated, very few inflammatory cells could be observed in the corneas of KF implanted and non-steroid treated eyes after 4 weeks, whereas more inflammatory cells in the KF implanted and steroid treated eyes could be observed, displaying a still ongoing inflammatory reaction.

This idea can be strengthened by the results of the measured implant thickness, which was significantly reduced ($p<0.05$) in the non-steroid group after 4 weeks compared to 10 days and also significantly reduced when compared to the steroid group after 4 weeks. Even though there is also a tendency towards a decrease of the implant thickness in the steroid group when comparing 4 weeks to 10 days, the decrease here is statistically not significant.

A suppression of the inflammatory reaction by topical steroids might also explain the light corneal haze observed in one of the KF implanted and steroid treated eyes after 4 weeks. In this context, the haziness could be caused by residuals of the keratin film, which is not yet degraded and therefore maintains a mild surrounding inflammatory process.

The overall mild inflammatory processes, as well as the good functional results with high transparency during the follow up, indicate that the keratin films induce a supportive inflammatory process. This is underlined by the fact that no giant cells can be observed at 10 days or 4 weeks follow-up. Because giant cells form by the fusing of macrophages, whenever proteins are too large for phagocytosis, we can assume that the absence of giant cells indicates a good degradability of the keratin films (Luttikhuizen et al. 2006, p. 1962; Anderson et al. 2008, p. 87). The formation of giant cells is associated with fibrous encapsulation and scar tissue formation (Luttikhuizen et al. 2006, p. 1962;

Anderson et al. 2008, p. 87). Since this is still a major obstacle to overcome in the development of appropriate biomaterials, some attempts have been made to produce porous biomaterials with the same composition of those, which elicit giant cell formation and encapsulation in their non-porous form (Brown et al. 2012, p. 3799).

Our results concerning in vivo biocompatibility of keratins are also in accordance with those published by other groups. Katoh et al. were able to show that fibroblast cells attached and proliferated well on keratin film, indicating that the film is biocompatible (Katoh et al. 2004, p. 2271). The good biocompatibility of keratin biomaterials was also demonstrated by Hill et al. who revealed the absence of foreign body cells on keratin biomaterials implanted subcutaneously in mice (Hill et al. 2010, p. 589).

Different statements can be found in the literature on the degradation time of keratin films. They can persist in vivo for weeks to months, depending on the method of production (Hill et al. 2010, p. 586). The degradation time of disulfide cross-linked keratin biomaterials implanted subcutaneously in mice is approximately 6 months (Hill et al. 2010, p. 591).

Valherie et al. used a rabbit model with subcutaneous implantation of the keratin film and found only minor signs of film degradation over two weeks (Valherie I. 1992, cited from: Borrelli et al. 2015, p. 119). This is in accordance with our results.

Although a rapid degeneration of the implant might be helpful to avoid a chronic foreign body reaction and scar tissue formation, a slow material degradation may be required to allow more time for the implant to integrate and for the host tissue to regenerate by foreign matrix production. A longer support by the matrix might be necessary, especially regarding the transplantation of epithelialized cell sheets, since transplanted cells could easily get lost if the implant broke down and degraded too early after transplantation.

In our in-vivo rabbit model we did not observe strong host tissue reactivity in terms of corneal oedema, neovascularization, or inflammation in the keratin film implanted eyes compared to amniotic membrane implanted eyes, especially

when treated with steroids. Nevertheless, when evaluating these results one has to take into account that the rabbit eye is generally more sensitive to biomaterials than the human eye (Wilhelmus 2001, p. 498). The rabbit cornea is thinner and has a heightened tissue response to minor surgical procedures, foreign materials, drugs, or chemicals (Wilhelmus 2001, p. 502). Moreover, the rabbit eye readily extrudes foreign material, limiting long term studies beyond a few months (Espana et al. 2011, p. 257).

4.5. Alternative substrates to keratin films for ocular surface reconstruction

Further work has been undertaken to develop alternative substrates to amniotic membrane such as silk fibroin, collagen-gels and chitosan films. Despite many indications for its application in biomedical use as a scaffold for cell cultivation, collagen has been shown to be less resistant to heat treatment than keratin films when not cross-linked (Tachibana et al. 2002, p. 167). Keratin films have also been shown to be better for high density cultivation of animal cells such as fibroblasts than collagen (Tachibana et al. 2002, p. 168). Chitosan films have been shown to support growth of mammalian cells and to reduce bacteria on the substrate's surface (Tanabe et al. 2002, p. 821). However, results of Tanabe et al. showed that chitosan films without any addition of keratin have the tendency to swell intensively even in neutral pH, resulting in fragile films after drying (Tanabe et al. 2002, p. 822). Silk fibroin films have been shown to be suitable for the cultivation of corneal fibroblasts and corneal epithelial cells. In-vitro studies of Liu et al. showed that the cell growth after 24 and 72 hours was not comparable to that on denuded amniotic membrane, which could be less advantageous for its use in cell culturing (Liu et al. 2012, p. 4132). Furthermore the production of biomaterial films from silk fibroin is more cost-effective than that of synthetic material (Levis, Daniels 2009, p. 595).

In summary, our investigation showed that keratin films show a good biocompatibility with minor host tissue reaction. The possibility to control inflammation by the administration of topical steroids allowing a slower

degradation process supports the idea that keratin films could serve as a scaffold for corneal epithelial cell growth in an in-vivo rabbit model.

4.6. Answers to the questions asked of this investigation

1. *Do keratin films induce an inflammatory reaction once implanted into the corneal stroma?*

   Keratin films implanted into the corneal stroma show a good biocompatibility and induce a minor host tissue reaction.

2. *Is the biocompatibility of keratin films comparable to that of amniotic membrane?*

   In comparison with amniotic membrane the implanted keratin film induces a mild inflammatory reaction which can be controlled by the administration of topical steroids. Taking its transparency and clinical outcome into account, keratin films can be proposed as an alternative substrate to amniotic membrane.

3. *Does the different composition of the keratin films (100, 90/10, 80/20) influence the biocompatibility?*

   Since there is no statistically significant difference between the different compositions of keratin films concerning the parameters measured, the composition of the keratin film does not seem to influence its biocompatibility. Nonetheless, KF90/10 is our film of choice because of its good biocompatibility results, biomechanical properties and surgical feasibility.

4. *Do implanted keratin films degrade over time?*

   Keratin films degrade over time. Implanted keratin film without steroid treatment was statistically significantly reduced after 4 weeks compared to 10 days.

5. *Does a topical medical treatment with steroids influence the inflammation and degradation?*

   Steroid medication seems to slow down the degradation process. After 4 weeks follow-up, the thickness of implanted keratin films without steroid treatment was statistically significantly reduced compared to the thickness of steroid treated keratin films.

4.7. Limitations of this experiment

In our investigation we were able to show significant differences in the measured parameters comparing the different time spans of 10 days and 4 weeks and also comparing the different medications. Nevertheless, group sizes of 3-4 are too small to draw meaningful conclusions, so in further experiments keratin films will have to be tested in bigger groups.

In this experiment keratin films were not sutured to the cornea. Despite former experiments testing the surgical feasibility of keratin films, the next step could be to test the keratin films surgical feasibility in an in-vivo model.

In future studies it might be beneficial to use optical coherence tomography in addition to the slit lamp observation, in order to give a more objective comparison of the corneal transparency of keratin film and amniotic membrane.

4.8. Conclusion

The results of our experiments reveal that keratin film based matrices can be implanted successfully into a corneal stromal pocket in vivo and show a good corneal biocompatibility with minor host tissue reaction. The administration of topical steroids seems to slow down the implant degradation which might be beneficial for certain applications such as transplantation of epithelial cell sheets. These results underline that keratin film is a promising biomaterial for future ocular surface reconstruction. However, further studies are necessary to evaluate the use of keratin films in an in-vivo model for ocular surface diseases.

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