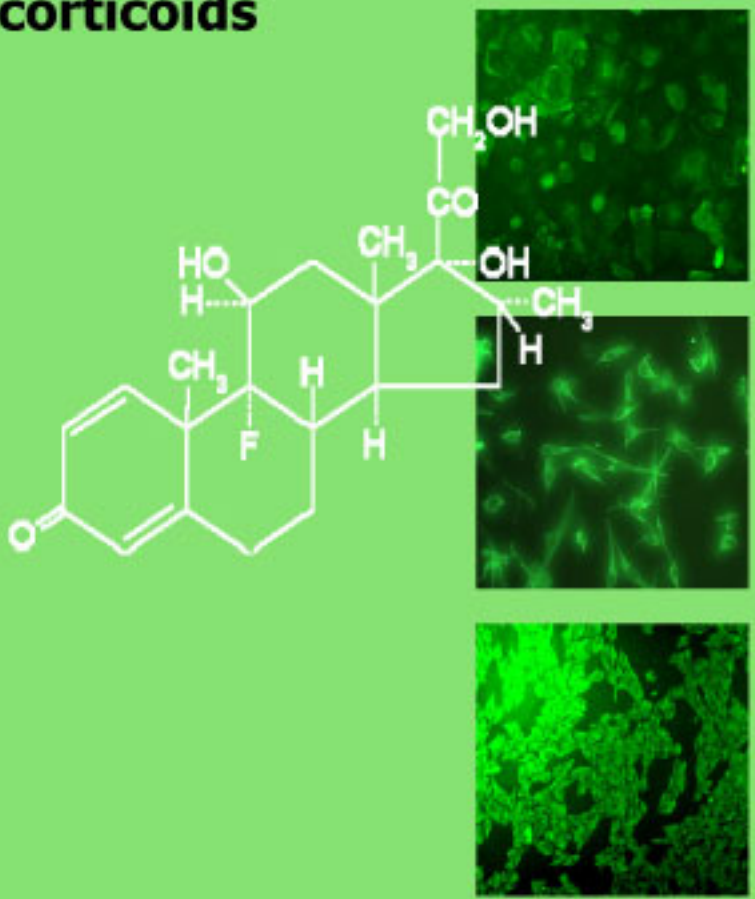
ANKE WERNER

Establishing a three-dimensional culture of canine corneal cells for *in vitro* studies on the effects of glucocorticoids



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University of Veterinary Medicine Hannover

Establishing a three-dimensional culture of canine corneal cells for *in vitro* studies on the effects of glucocorticoids

THESIS

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Holzhacken ist deshalb so beliebt,
weil man bei dieser Tätigkeit den Erfolg sofort sieht.

Albert Einstein (1879 – 1955)

Meinen Eltern
in Liebe und Dankbarkeit
gewidmet

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List of abbreviations

A	ampere
approx.	approximately
aq. bidest.	aqua bidestillata
aq. dest.	aqua destillata
AP	alkaline phosphatase
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumine
CD	cluster of differentiation
CO_2	carbon dioxide
COX	cyclooxygenase
DEPC	diethylpyrocarbonat
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
DNAse	desoxyribonuclease
E. coli	*Escherichia coli*
EDTA	ethylenediamin tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immuno sorbent assay
et al.	et alii
FCS	fetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
g	gram
GAPDH	glycerinaldehyd-3-phosphat-dehydrogenase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HAT	histone acetyltransferase

HDAC	histone deacetylase
HE	haemalum eosin (staining)
HRP	horse raddish peroxidase
hsp-90	heat shock protein-90
IFNγ	interferon γ
IL	interleukine
IU	international unit
kDa	kilodalton
l	liter
LPS	lipopolysaccharide
m	milli
μ	micro
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	methyltetrazolium
n	number of experiments
NF-κB	nuclear factor-κB
p	pico
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
RT-PCR	reverse transcriptase polimerase chain reaction
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV	simian virus
Tab.	table
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
UV	ultra violet

TM	trademark
♂	male
♀	female

1 Introduction

As the first part of the refractive system in the eye, corneal transparency is vital for vision. Ocular disease conditions affecting the cornea can result in the loss of corneal transparency and thus greatly impair vision in companion animals. Inflammations as well as traumatic lesions of the cornea are common causes for such a loss of transparency.

Most ophthalmic drugs used in veterinary ophthalmology have been approved for the use in humans but not in animals. Therefore, their use in humans, including dosing intervals, is often directly transferred to companion animals without formal testing. The successful use of those drugs in human ophthalmology as well as morphological similarities between the species leads to a certain degree of confidence regarding their use in veterinary ophthalmology. Nevertheless, direct correlations cannot be made between species due to interspecies differences (HENDRIX et al. 2002). So far, drug effects on the cornea were only studied on single cell cultures, and the effects were measured using morphological parameters (cell size and shape). In the present study, the inflammatory reaction is also considered as well, thus closer resembling the situation in the inflamed eye.

Recently, studies have been conducted to reconstruct the cornea in different species (bovine, porcine, rabbit, and human) using separately cultured corneal cells (i.e. epithelial cells, stromal keratocytes, and endothelial cells) which were reassembled in cell culture step by step into a three-dimensional cornea culture (termed cornea equivalent) (MINAMI et al. 1993; ZIESKE et al. 1994; PARNIGOTTO et al. 1998; FERBER 1999; GERMAIN et al. 1999; GRIFFITH et al. 1999; SCHNEIDER et al. 1999; TEGTMEYER et al. 2001; REICHL 2003; REICHL et al. 2004; TEGTMEYER et al. 2004; ALAMINOS et al. 2006). So far, a model for the canine cornea has not been described. The use of a cornea equivalent could be a valuable tool in pharmacological investigations. The advantage of such a system is the possibility for intercellular communication and interaction between the three corneal cell types, which is likely to be influential on inflammatory reactions and the efficacy of ophthalmic drugs. Also, establishing such an *in vitro* model is beneficial with regard to ethical considerations. By using cultured corneal cells, it can be expected that less donor animals are needed compared to experiments conducted on excised canine corneas.

2 Literature review

2.1 Bulbus oculi

The eye is made up of the eye bulb (bulbus oculi) with different aiding and protective structures (e.g. blood vessels, nerves, muscles, fat, eye lids and lacrimal glands), the optic nerves (nn. optici), the optic tract and the visual cortex. The eye ball is nearly spherical. The walls consist of three concentrical layers: the sclera, the uvea with the vascular tunic and the retina. These embrace the large inner transparent media of the eye: the lens, the vitreous body and the communicating chambers of the eye containing the aqueous humor (Fig. 1).

The outer coat of the eye ball wall (tunica externa bulbi) is a fibrous tunic, which consists of the opaque white sclera in the posterior segment and the smaller transparent cornea in the anterior segment. Both structures interconnect at the limbus. The sclera is composed of collagen fibers and fibroblasts. The collagen fibers differ in size and shape and run in different directions in different parts of the globe (SLATTER 2001a). They have to withstand the intraocular pressure of 19 +/- 8 mmHg (GELATT and MACKAY 1998) and the tension of the ocular muscles.

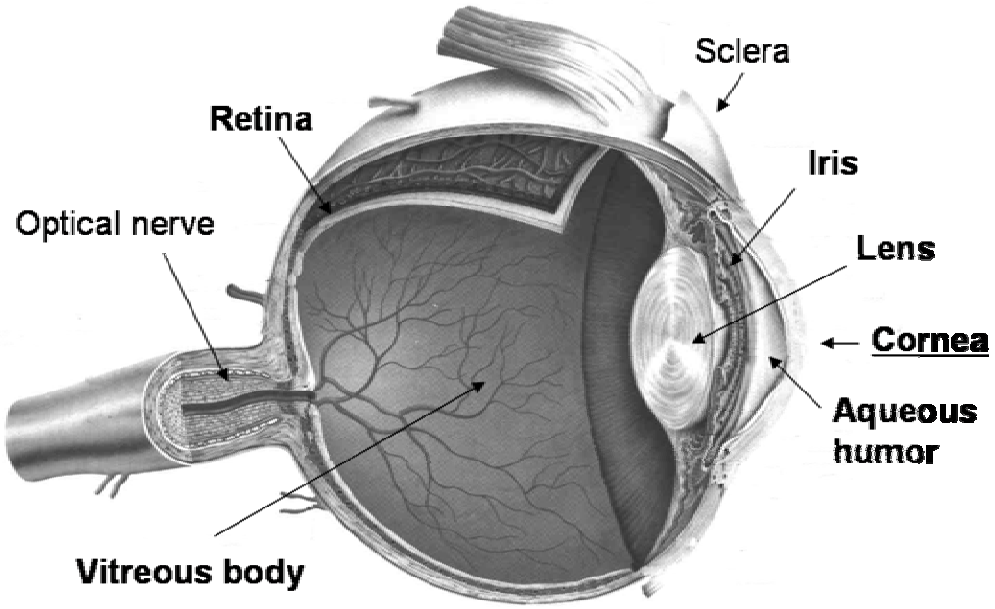


Fig. 1 Bulbus oculi; schematic illustration highlighting the major structures of the eye (source: OFTALNET (2007))

2.2 Cornea

The transparent cornea separates the anterior chamber from the surrounding environment, therefore serving as a protective barrier. The cornea is the most powerful optical refracting surface in the eye and is characterized by its transparency. The corneal transparency is based on a lack of blood vessels (CURSIEFEN et al. 2006), a lack of cells and pigment as well as on the control of its water content, a smooth optical surface provided by the precorneal tear film and a regular, highly organized arrangement of collagen fibrils (SLATTER 2001a).
The corneal thickness varies among species and shows a mean thickness of 0.56 - 0.62 mm in the dog (STAPLETON and PEIFFER 1979; GILGER et al. 1991). Contrary results exist whether the canine cornea is thicker peripherally or in the center. Female corneas are thinner and thickness generally increases with age (GWIN et al. 1982; GILGER et al. 1991; SLATTER 2001a; GELATT 2007). The cornea is made up of 3 main layers: the epithelium with its basement membrane (in some species prominent, considered a separate layer and called Bowman-membrane), the stroma, the Descemet's membrane (basement membrane of the endothelium) and the endothelium (Fig. 2).

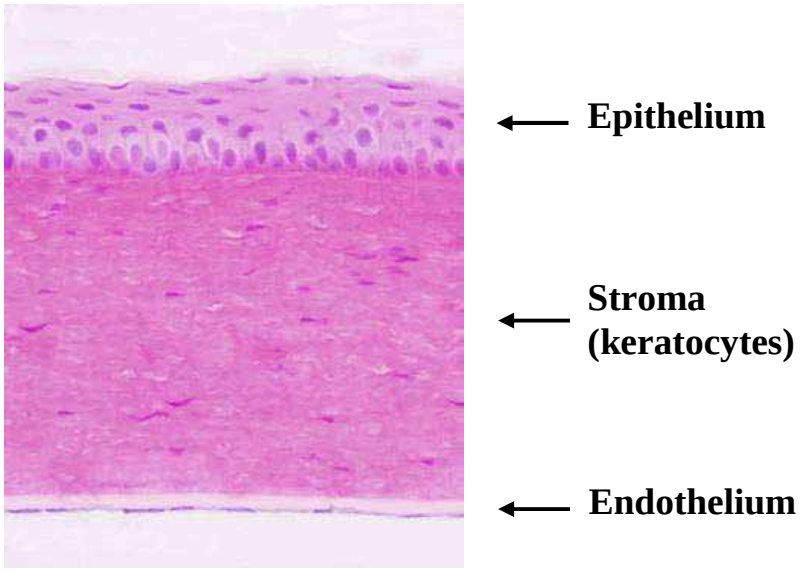


Fig. 2 Transverse section through the cornea; HE stain of a rat cornea (source: THE UNIVERSITY OF WESTERN AUSTRALIA (2007))

The outmost layer is the corneal epithelium, a simple, squamous, and non-keratinized epithelium with the basic pattern of basement membrane, basal epithelial cells, wing cells,

and squamous surface cells as depicted in Fig. 3 (SLATTER 2001a). The epithelium is approximately 50 µm thick, contributing 10 % to the total corneal thickness. The epithelium consists of a different number of layers, depending on the species (EHLERS 1970; NISHIDA and KRACHMER 1997). The apical surface of the corneal epithelium is covered by a thin precorneal tear film which is anchored by small villous projections of the surface cells. The canine cornea does not have a prominent Bowman's membrane, nevertheless the basement membrane functions as part of the diffusion barrier that the cornea resembles and thus hinders the influx of water into the next layer, the stroma.

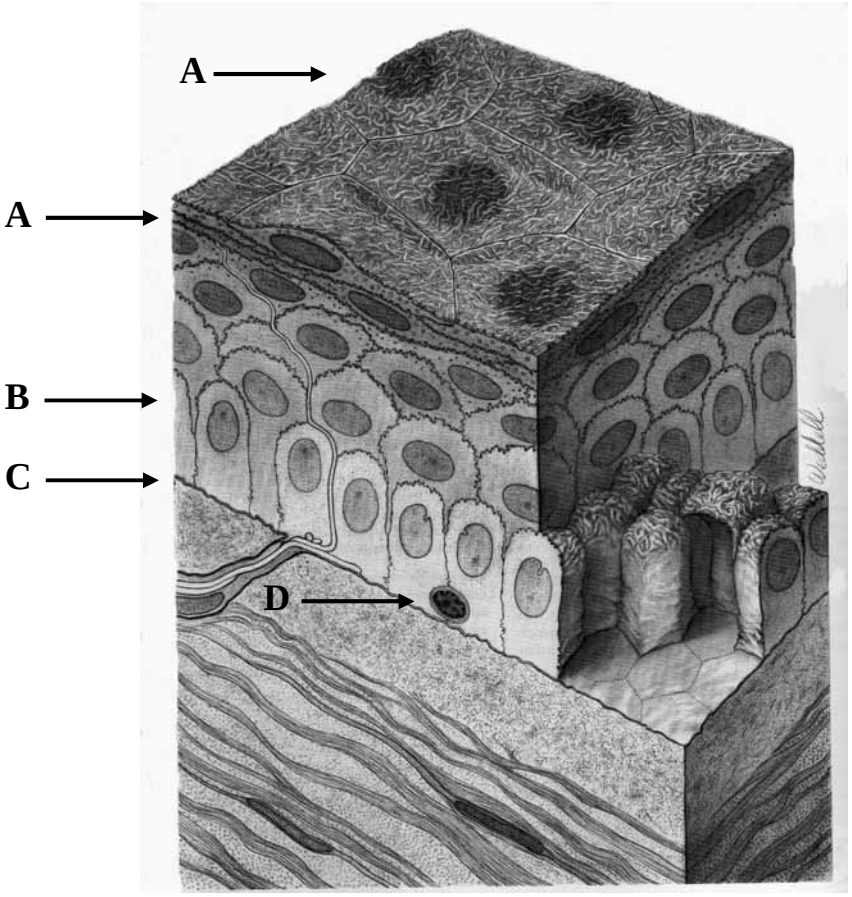


Fig. 3 Cornea epithelium. **A** = flattened apical epithelial cells with microplicae and microvilli, **B** = columnar basal epithelial cells, **C** = basement membrane, **D** = lymphocyte (SLATTER 2001a).

The stroma is composed of stromal cells, collagen and a large amount of ground substance (approximately 90 %). This layer constitutes approximately 90 % of the total corneal substance. The collagen fibrils are arranged in parallel and form interlacing lamellae with a

slight variation between the superficial and the deep layer as illustrated in Fig. 4 (FREUND et al. 1995). The spindle shaped stromal cells are fibroblasts which are also referred to as keratocytes. They built a network within the collagen lamellae. Since the stroma does not contain any blood vessels, nutrients reach the cells by means of diffusion from the peripheral arteries, the tear film and the aqueous humor (LIEBICH and LIEBICH 2004).

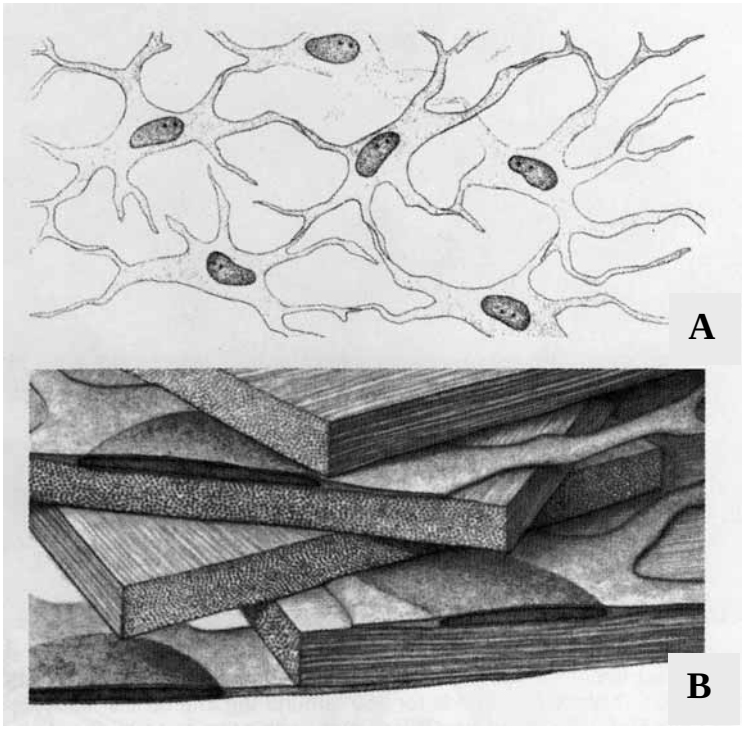


Fig. 4 Cornea stroma. **A** = fibroblasts lying between the stromal lamellae. The cells are thin and flat, with long processes that are in contact with other fibroblasts of the same plane. **B** = lamellae. The lamellae consist of collagen fibrils, which are oriented in parallel to each other. Successive lamellae are oriented at an angle to each other, with fibroblasts in between the planes (SLATTER 2001a).

The Descemet's membrane is the basement membrane of the endothelium and is laid down through life, increasing in thickness with age (SLATTER 2001a). It is located between the stromal layer and the endothelium. It consists of a mesh construction of collagen fibrils which are connected by microfilaments (KANSKI et al. 1987).

The innermost layer of the cornea consists of a single cell layer of epithelial cells called endothelium. The cells are hexagonal in shape with a diameter of 15 to 20 µm and a mean density of 3,000 cells/mm² in dogs (STAPLETON and PEIFFER 1979; BEFANIS et al. 1981).

Corneal transparency is vital for vision. The clarity is mostly due to stromal transparency. The crucial factors are the precise organization of the collagen fibrils (which eliminates destructive interference by scattered light), the relatively low water content and the absence of blood vessels and pigmentation (SLATTER 2001a; MARTIN 2005b; GELATT 2007). Both epithelium and endothelium are involved in removal of water from the stroma, an important aspect in retaining this transparency. This process is energy-dependent and is mostly controlled by the endothelial cells. Nevertheless, both cell types contain large amounts of Na+-/K+-activated ATPase, which is associated with the sodium pump to eliminate water from the stroma against the intraocular pressure gradient (SLATTER 2001a).

In most ocular tissues, the regenerative capacity is very low because most cells are in a post mitotic state and thus these tissues are susceptible to scarring. The adult corneal endothelium has practically no regenerative capacity in most species, although the dog might be an exception (BEFANIS et al. 1981; PEIFFER et al. 1981; RODRIGUES et al. 2006). The corneal stroma has been reported to have poor regenerative capacity as well (MARTIN 2005a). Furthermore, neither the Bowman membrane nor the Descemet's membrane are able to regenerate in humans, but a regenerative potential has been demonstrated for the dog (BEFANIS et al. 1981).

In these optically sensitive tissues, the type of repair phenomena that might return other organs to acceptable function often does not restore (and might even worsen) ocular function (SLATTER 2001b). This can be seen, for example, after injury in deep stromal layers. In this case, granulation tissue will form within the stroma, but although the fibroblasts will become oriented parallel to the epithelial surface with time, such granulation tissue will never completely disappear. It is claimed that injured corneal stroma will thus never be remodeled with the extremely precise architecture required to ensure perfect corneal clarity (MARTIN 2005a).

2.3 Corneal cell culture

2.3.1 Primary corneal cells

The culture of corneal cells started in 1914 with the isolation and culture of endothelial cells (NAGANO 1914). STOCKER et al. (1958) were the first to isolate and separately culture the three major cells of the rabbit cornea. Since then the corneal cells have been isolated and cultured by various authors (BURSTEIN and KLYCE 1977; CHAN and HASCHKE 1982; ELDOR et al. 1983; HALABAN and ALFANO 1984; COOK et al. 1987; NISHIDA et al. 1988; PISTSOV et al. 1988; ENGELMANN and BOHNKE 1989; HE and MCCULLEY 1991; BEDNARZ et al. 1996b; KOMAI-HORI et al. 1996; BORDERIE et al. 1998; BEDNARZ et al. 2001; TUNGSIRIPAT et al. 2004). For the canine cornea the culture of epithelial cells for pharmacological studies has recently been described (HENDRIX et al. 2002).

Since the corneal epithelial cells are poor in proliferation and difficult to passage as a primary culture, different authors have worked on improving the culture of these cells. Initially an explant technique was used to isolate the cells (HE and MCCULLEY 1991) which has been improved recently by using a special mini-microtome designed by WEBB et al. (2003). But studies by different authors have pointed out the superiority of a single cell culture over the explant technique (EBATO et al. 1988; KIM et al. 2004; ZHANG et al. 2004). Additionally, the origin of the epithelial cells, that is whether central, peripheral or limbal cells are taken into culture, is influential on the culture success. The limbal region seems to contain the highly proliferative stem cells that are easiest to culture and passage successfully (THOFT and FRIEND 1983; EGGLI et al. 1989; LAVKER et al. 1991; LAUWERYNS et al. 1993b; TUNGSIRIPAT et al. 2004).

For the culture of primary corneal cells, similar basic culture media were used (DMEM, Ham's 12 medium, MEM and medium 199). For the culture of the epithelial cells, mainly DMEM and Ham's 12 medium or a combination of both was used.

All culture media were supplemented with fetal calf serum in different concentrations. For endothelial and especially epithelial cell culture mostly in humans, but also in other species like the rabbit, further growth stimulating supplements like EGF and cholera toxin were added

to the culture media (EBATO et al. 1988; PANCHOLI et al. 1998; WEBB et al. 2003; KIM et al. 2004; TUNGSIRIPAT et al. 2004; ZHANG et al. 2004; MELLER et al. 2005; LI et al. 2006).

2.3.2 Immortalization of corneal cells

To further improve the cell yield for the corneal cells and especially increase the amount of passages possible, different authors have established protocols for the long-term cultivation (ENGELMANN et al. 1988; PISTSOV et al. 1988; TAMARIZ et al. 2007) or immortalization of corneal cells (ARAKI et al. 1993; KAHN et al. 1993; SHARIF et al. 1998; BEDNARZ et al. 2000). Since the stromal keratocytes are comparably unproblematic to culture even as primary cells, most of the work regarding long-term cultivation and immortalization has concentrated on endothelial cells and epithelial cells. Human endothelial cells have been cultured for extended periods using selective culture media (ENGELMANN et al. 1988; PISTSOV et al. 1988) and immortalized with electroporation (BEDNARZ et al. 2000). Human epithelial cells have also been immortalized by KAHN et al. (1993) and SHARIF et al. (1998) by transfection of the primary cells with simian virus 40 (SV 40), Adeno 12-SV40 hybrid virus or plasmid RSV-T (which is a SV40 ori-construct containing the SV40 early genes and the Rous sarcoma virus long-terminal repeat). Cell lines have also been established for rabbit corneal epithelial cells: ARAKI et al. (1993) have transfected rabbit epithelial cells with SV 40, whereas TAMARIZ et al. (2007) recently described the establishing of a spontaneous cell line by serial transfer of the same cell type.
The advantage of these cell lines is their increased capability to proliferate, thus overcoming the problems of limited passaging compared to the primary cells. Nevertheless, this advantage results in negative effects like an altered functionality of the cells, mostly apparent in a disturbed differentiation.

2.4 Tissue culture

In the past years the cell culture has evolved with the construction of organotypical tissue equivalents. These equivalents are based on the knowledge that different cell types have an

influence on their respective growth and differentiation, and result in a closer resemblance of the *in vivo* situation than a single cell culture. Their use has been very versatile.
The first organotypical cocultures were performed with skin-cells. Initially the two major cell populations of the skin (i.e. keratinocytes and fibroblasts) were cocultured and such primitive skin equivalents have been successfully developed for murine and human tissue and were very useful in dermatological research (BELL et al. 1991; PONEC 2002). More advanced systems include a gel-biomatrix consisting of collagen or other matrix proteins, in which the fibroblasts are embedded, resulting in a three-dimensional culture system of the skin. Very recently such a model has been established with canine tissue (SERRA et al. 2007). These organotypic skin equivalents have been used in studies of skin biology and physiology, toxicity testing, in general studies on clinical, biological and pharmacological applications and as skin substitutes for wound closure (DAMOUR et al. 1998; SORENSEN 1998; SERRA et al. 2007).

2.4.1 Cornea

The construction of three-dimensional cornea equivalents started with the perception that corneal epithelial cells grown on a collagen substrate proliferated better and expressed more specific corneal parameters (FRIEND et al. 1982; HE and MCCULLEY 1991). Using a fibroblast containing collagen-layer improved the cell culture of corneal cells further towards a closer resemblance with the *in vivo* situation (PARNIGOTTO et al. 1998; GERMAIN et al. 1999). The first completely reconstructed cornea equivalent containing all three cell types was described by MINAMI et al. (1993). They used bovine cells and developed a culture system which involved the culture at the air-liquid-interface to promote epithelial cell differentiation. The endothelial cells were separated from the other two cell types by a nitrocellulose membrane. ZIESKE et al. (1994) described a cornea equivalent built of rabbit epithelium and fibroblasts as well as endothelium from an immortalized mouse cell-line. They showed that a culture on the air-liquid-interface and a close integration of the endothelium resembled most the morphologic and biochemical parameters of the model compared to those *in vivo*. SCHNEIDER et al. (1999) constructed a three-dimensional corneal equivalent from fetal porcine cornea for toxicological *in vitro* studies. An equivalent using adult porcine corneal cells was described by REICHL et al. (2003). A complete human cornea equivalent has been

constructed using immortalized cell lines (GRIFFITH et al. 1999) and a combination of immortalized endothelial and epithelial cells with primary fibroblasts (REICHL 2003). The reconstruction of the entire bovine (TEGTMEYER 2000; TEGTMEYER et al. 2004) and a partial as well as a full rabbit cornea (LU et al. 1996; ALAMINOS et al. 2006) have also been described.

2.5 Corneal disease

2.5.1 Inflammation in the eye

The eye seems reluctant to become involved in inflammatory disease, which seems sensible: the visual function of the eye is easily disturbed and is sometimes destroyed by minor degrees of inflammation that would be considered inconsequential in most other tissues. The blood-eye barrier, the protective presence of eyelids, a tear film, and a bony orbit as well as a carefully regulated system of intraocular immune tolerance all seem designed to spare the globe from the need to participate in inflammatory responses. Because the eye is a closed, fluid-filled sphere, it seems likely that inflammatory mediators are not as easily dissipated or inactivated as they are in other tissues. One manifestation of this closed-system concept is that ocular inflammatory disease is almost always diffuse (SLATTER 2001b).

Inflammation in the eye does not include reactions unique to this structure. But many of the familiar general inflammatory events are sometimes altered in their expression by factors related to unique ocular anatomy and physiology (MAGONE and WHITCUP 1999). Such factors include an unusually strong interdependence between the structural and functional elements of the eye, with intolerance for even minor imperfections. For example, corneal disease can lead to uveal inflammation, which in turn can cause lenticular changes. Another example is edema, a manifestation of acute inflammation, which is the result of increased vascular permeability and movement of low-protein fluid from within the vascular component to the extravascular tissues. Edema in the cornea results in decreased corneal clarity; therefore, even minor degrees of disruption by edema, that in almost any other tissue would be insignificant and even imperceptible, are significant in the eye (LAURELL and ZETTERSTROM 2000; MARTIN 2005a)

The consequences of an inflammatory reaction in the eye are similar to that of other structures: a cellular infiltration of the respective tissues or compartments, accompanied by neovascularization and a disruption of the barriers with the systemic blood supply (REGNIER and GELATT 1999).

2.5.2 Significance of corneal disease

Because inflammation is fundamentally a vascular event, the avascular cornea cannot undergo true inflammation until it has acquired blood vessels by ingrowths from the limbus. The very acute manifestations of corneal “inflammation” following injury (neutrophilia and corneal edema) are in fact passive events related to corneal ulceration, so that neutrophils and fluid are attracted from the adjacent tear film. The first genuine inflammation reaction to corneal injury occurs in the nearest available vascular bed, ordinarily that of the limbus (SLATTER 2001b).

As described above, corneal transparency is vital for vision and a complexly regulated system is involved in securing this clarity. Inflammatory reactions connected with edema, but also the formation of scar tissue following an inflammation, compromises this clarity and is thus detrimental for vision.

2.5.3 Immune reactions in the eye

The eye is an organ which exhibits immune reactions different to other parts in the body, which are termed immune privilege. Immune privilege describes the experimentally defined phenomenon that certain tissues and organs fail to obey the rules of transplantation immunology. Foreign tissue grafts placed in immune privileged sites (e.g. eye, brain, testis, pregnant uterus) enjoy extended, often indefinite, survival, whereas similar grafts placed at other sites of the body (like the skin) are rapidly rejected. Similarly, grafts derived from those immune privileged sites experience extended, often indefinite, survival when placed at conventional body sites (STREILEIN 2003a). Immune privilege is actively acquired and maintained and the immune system participates in active regulation of the immune responses in privileged sites (STREILEIN and STEIN-STREILEIN 2000). Such immune privilege effects adaptive as well as innate immunity and involves local as well as systemic features that contribute to the phenomenon (STREILEIN and STEIN-STREILEIN 2000). The local features in regard to the eye as a site of immune privilege are anatomical features, like a strict blood-ocular barrier and the relative absence of lymphatic drainage from the eye. Furthermore the cornea is devoid of expression of MHC class II molecules and the endothelium is deficient

in expression of MHC class I molecules, which combined renders this tissue rather invisible to T cells since those require MHC expression to recognize antigens (STREILEIN and STEIN-STREILEIN 2000). In addition, cells at the privileged site express surface molecules (e.g. complement inhibitors, CD95 ligand) and secrete factors (e.g. transforming growth factor beta (TGF-β)), that suppress local expression of both innate and adaptive immune responses (STREILEIN 2003a). The presence of active TGF-β in normal aqueous humor has a profound inhibitory effect on lymphocyte (particularly T-cell) proliferation and also on the action of such potent effectors of cell-mediated immunity as interleukin-2 and -4 (SLATTER 2001b).

Immune privilege is thought to reflect an evolutionary adaptation to protect vital structures from damage by inflammatory responses directed against pathogens, with an emphasis on specialized tissues and those incapable of regeneration (HONG and VAN KAER 1999; STREILEIN 2003b). In the eye, immune privilege ensures visibility as most inflammatory reactions are accompanied by impairment or even loss of vision.

The most common cause of inflammatory ocular diseases is microbial infection. These microbes include viruses, bacteria, protozoa, fungi, and parasites. Bacteria synthesize and release specific exotoxins, which initiate inflammation, or endotoxins, which are associated with their cell walls (MARTIN 2005a). One example for such an endotoxin is lipopolysaccharide (LPS, Fig. 5), which is part of the bacterial membrane of gram-negative bacteria. It is generally the most potent immunostimulant among bacterial cell-wall components (AKIRA et al. 2006). A lipid portion of LPS termed “lipid A” is responsible for most of the pathogenic phenomena associated with Gram-negative bacterial infection (AKIRA et al. 2006). LPS liberated from Gram-negative bacteria associates with LPS binding protein in the blood stream, and then binds to CD14 on the cell surface of phagocytes. LPS finally interacts with a toll-like receptor (TLR), initiating an inflammatory reaction in the host (POLTORAK et al. 1998; SHIMAZU et al. 1999).

Different bacteria produce structurally different LPS molecules varying in their phosphate patterns, number of acrylic chains, and fatty acid composition (AKIRA et al. 2006).

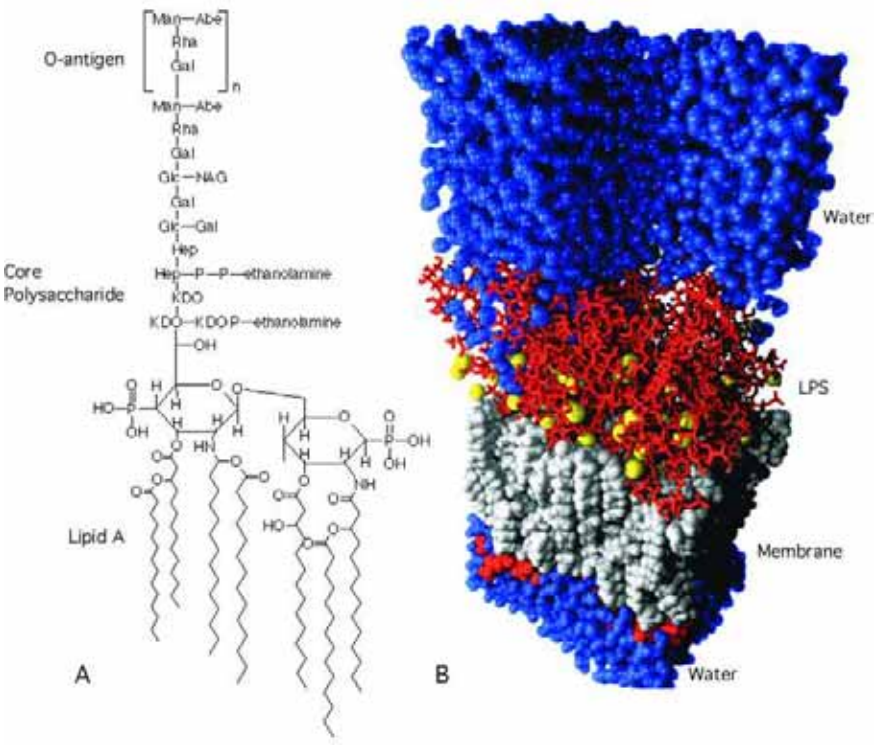


Fig. 5 Chemical and three-dimensional structure of lipopolysaccharides (LPS) (source: UNIVERSITY OF WISCONSIN-MADISON (2007)).

Corrosive chemicals (acids, alkalis, oxidizing agents) provoke inflammation through direct tissue damage. The eye is susceptible and sensitive to damage by a variety of corrosive chemicals, damage which often first manifests as corneal damage (MARTIN 2005a). One example of a corrosive chemical is sodium dodecyl sulfate (SDS, also referred to as sodium lauryl sulfate (SLS), Fig. 6). SDS is an anionic detergent and widely used in cosmetics, pharmaceuticals, household products and as food constituents (NEUMÜLLER 1985).

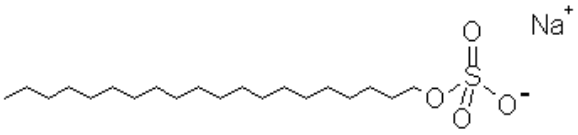


Fig. 6 Chemical structure of sodium dodecyl sulfate (SDS).

Since this substance is frequently an ingredient in soaps and shampoos, most effects of SDS have been studied in regard to the skin. When used regularly or in high concentrations detergents can lead to changes in the skin ranging from mild irritation to severe damage.

In humans, the effects of SDS are dose-, and time-dependent with a high interindividual variance (PETERS et al. 2006). Topical application of SDS onto the skin usually exhibits irritating effects at concentrations between 1 and 20 %, ranging from slight dryness or scaliness to erythema with histologically manifested epidermal cell necrosis, parakeratosis, hyperplasia and toxic damage (FARTASCH 1997; MÜLLER-DECKER et al. 1998). Low concentrations can cause molecular changes before the onset of erythema, measured as an increased transepidermal water loss (LE et al. 1997). These changes are associated with damage in nucleated cells and the lamellar body lipids. The relative amounts of the different lipids are changed as mainly free fatty acids and cholesterol are removed from the lower portion of the stratum corneum (FULMER and KRAMER 1986; FROEBE et al. 1990) while the upper portion of the stratum corneum displays intact lipid layers (FARTASCH 1997). Generally, the skin reaction is associated with increase of proinflammatory mediators like eicosanoids (including PGE_2), and IL-1α (MÜLLER-DECKER et al. 1998). Human skin is even more sensitive to SDS when the substance is applied intradermally. In this case a concentration of 0.1 % and 0.5 % SDS leads to a significant increase in local blood flow, widespread reddening, pain, and a 20 to 30-fold increase in PGE_2 levels (FAIRWEATHER et al. 2004).
Other species are also sensitive to SDS irritation. Rabbits show erythema and further skin damage when topically treated with SDS in high concentrations over a prolonged time. A topical application of 10 % SDS for 1 hour onto the skin of a bovine udder leads to a 30 % decrease in mitochondrial activity (PITTERMANN et al. 1995).

SDS is also cause for ocular irritation. The local, reversible reaction generally originates from corneal and conjunctival cells upon contact with the irritant. XU et al. (2000) conducted studies using the cornea, where low concentrations (0.1 – 0.3 %) of SDS cause mild disruption of epithelial cell tight junctions. With increasing concentrations (up to 15 %) the tight junctions are increasingly destroyed, allowing a penetration of molecules into the epithelium and the underlying stroma, and occurrence of morphological alterations in corneal structure. Similarly to an increased concentration applied for a constant period of time, an elongation of the contact time of a low concentration of SDS (1 %) increased the irritation reaction of the tissue.

2.5.4 Mode of action of LPS via the Toll-like receptor

The bacterial endotoxin LPS described in chapter 2.5.3 has a very specific way of triggering an inflammatory reaction in the organism it invades. This endotoxin, like other microbial components, mediates its pro-inflammatory effects through binding to toll-like receptors (TLRs).

Innate immunity is the first line of host defense that is responsible for the immediate and rapid immune response to microbial challenge and has in the past been considered non-specific (CHANG et al. 2006). Recently it has been recognized, that this type of immune response is not completely nonspecific but is able to discriminate between self and a variety of pathogens (AKIRA et al. 2006).The innate immune system recognizes microorganism with a variety of pattern recognition receptors (PRRs) (AKIRA et al. 2006; CHANG et al. 2006). PRRs can be divided into a group of receptors that is expressed on the cell surface (including phagocytic C-type lectin receptors and TLRs), into a group of intracellularly expressed receptors (like nucleotide oligomerization domain (NOD) proteins) and finally the secreted recognition molecules (such as mannan binding lectins) (CHANG et al. 2006). A common characteristic of the PRRs is their germline encoded, nonclonal expression on all cells of a given type regardless of their immunologic memories. All PRRs recognize microbial components, called pathogen-associated molecular patterns (PAMPs). These PAMPs are microbial structures, not limited to pathogens (therefore sometimes referred to as MAMPs (microorganism-associated molecular patterns)), which are essential for the survival of the microorganisms and therefore difficult for those to alter (AKIRA et al. 2006; CHANG et al. 2006; KAISHO and AKIRA 2006).

TLRs are a family of phylogenetically conserved PRRs, with a unique ligand specificity for each TLR (AKIRA et al. 2006; CHANG et al. 2006). To date at least 10 members of the TLR family have been identified in mammals (KAISHO and AKIRA 2006). A summary of the human TLRs and their known ligands is listed in Tab. 1. Structurally, TLRs are type I transmembrane proteins with leucine rich repeats in the extracellular domain for ligand recognition, and Toll/Il-1 receptor (TIR) domain in the cytoplasmic portion for intracellular signaling (TAKEDA et al. 2003; AKIRA et al. 2006; CHANG et al. 2006). They are expressed on the one hand on immune cells that are most likely to first encounter microbes,

such as neutrophils, monocytes, macrophages, and dendritic cells, but also on epithelial cells at host/environment interfaces, including the skin, gastrointestinal tract, respiratory tract, the urogenital tract and the eye (BACKHED and HORNEF 2003; TAKEDA et al. 2003; CHANG et al. 2006). The expression of TLRs is not static, but is influenced by pathogens, various cytokines, and environmental stress (AKIRA et al. 2006). They can be expressed intra- (TLRs 3, 7, 8, 9) and extracellularly (TLRs 1, 2, 4, 5, 6).

Ligand recognition by the TLRs leads to a dimerisation of the receptors which triggers the activation of signaling pathways. These signals originate from the cytoplasmatic TIR domain, and lead to the activation of the transcription factor NF-κB through which the expression of pro-inflammatory genes such as TNFα, IL-1 and IL-12 is induced (TAKEDA et al. 2003; TAKEDA and AKIRA 2005). The binding of the ligand leads to a conformational change of the receptor, which is a requirement for one of five adaptor molecules (for example myeloid differentiation factor 88 (MyD88)) needed for the TIR domain activation. Depending on the adaptor molecule used, different pathways are triggered, lending an explanation for the phenomenon that differential responses are mediated by distinct TLR ligands (AKIRA et al. 2006).

It has been shown previously, that specific PAMPs which stimulate different TLRs, induce distinct patterns of cytokines resulting in a Th1/Th2 polarization that is most appropriate for the pathogen (CHANG et al. 2006). Although stimulation of TLRs leads rather to a Th1 differentiation accompanied with the production of INFγ to resolve bacterial and viral infections, a Th2 differentiation with increased IL-4 and IL-13 production as a response to helminth infection or allergic reactions is also possible (AKIRA et al. 2006; KAISHO and AKIRA 2006).

TLRs expressed on professional antigen presenting cells are a critical link between innate and adaptive immunity as they are important in both triggering and modulating the activation of the adaptive immune response (IWASAKI and MEDZHITOV 2004; MAZZONI and SEGAL 2004; SPORRI and REIS E SOUSA 2005).

Tab. 1 Human Toll-like receptors (TLRs) and their known ligands (CHANG et al. 2006).

TLR	Principle exogenous ligand(s)
TLR2	Lipoproteins/lipopeptides (various pathogens)
	Peptidoglycan and lipoteichoic ascid (Gram positive bacteria)
	Zymosan (fungi)
TLR3	Double stranded RNA (viruses)
TLR4	LPS (Gram negative bacteria)
	Bacterial HSP60
	Respiratory syncytial virus coat protein
TLR5	Flagellin (flagellated bacteria)
TLR7	Imidazoquinolone antiviral drug
TLR8	Single stranded RNA (viruses)
	Imidazoquinolone antiviral drug
TLR9	Unmethylated CpG motifs of bacterial DNA
TLR10	Unknown

TLRs in the structure of the eye

LPS is recognized by the TLR4 (SONG et al. 2001), which has been detected in human ocular tissue. In the cornea the TLR4 has been demonstrated in epithelial cells and keratocytes but not in endothelial cells (SONG et al. 2001; JOHNSON et al. 2005; KUMAGAI et al. 2005). The mRNA and in part also the protein expression for other TLRs (e.g. TLR2, 3, 5, 6, 7, 9, 10) have additionally been described for corneal epithelium and fibroblasts (CHANG et al. 2006).

CHANG et al. (2006) also provide a helpful summary of the TLR4 in other ocular structures (e.g. conjunctiva, uvea, retina and sclera) and the detection of other TLRs in cultured retinal pigment epithelial cells.

2.6 Glucocorticoids

2.6.1 Structure and synthesis

Corticosteroids are 21-carbon steroid hormones composed of four rings (JOHNSON 1996; ITO et al. 2006) that are synthesized in the adrenal cortex from cholesterol. Corticosteroids are divided into two groups: the glucocorticoids (with their main effect exerted on glucose, protein and calcium metabolism and anti-inflammatory action (UNGEMACH 2006)) and the mineral corticoids (with their main effect on regulation of electrolyte and fluid balance (UNGEMACH 2006)).
Glucocorticoids are thought to freely diffuse from the circulation into the cells across the cell membrane. Within the organism they have versatile effects. The main pharmacological properties of this class of substances are anti-inflammatory, immunosuppressive, anti-exudative, anti-allergic and anti-toxic effects (FLOWER et al. 1989).

Dexamethasone

Dexamethasone was synthesized through selective modification of the endogenous glucocorticoid cortisol. The inclusion of a double bond between C_1 und C_2, and the addition of a CH_3-group at the position of C_{16} changed the properties regarding effectiveness, mineralocorticoid effect, therapeutic window and pharmacokinetics compared to cortisol (Fig. 7).
Dexamethasone is a long acting glucocorticoid with a 30-fold increase in effect in comparison to cortisol (OETTEL et al. 1996). The undesirable mineralocorticoid effects are excluded and the effect on the gluconeogenesis and anti-inflammatory properties increased 25-fold compared to cortisol (OETTEL et al. 1996). Dexamethasone is often included in ophthalmic drugs.

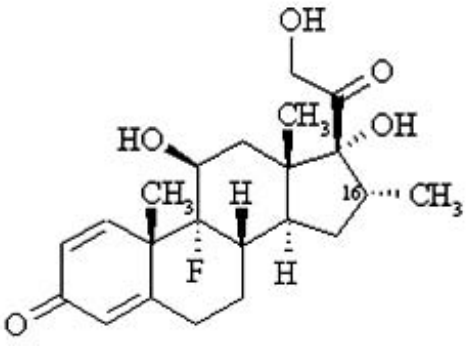


Fig. 7 Chemical structure of the glucocortiocoid dexamethasone.

2.6.2 Mode of action of glucocorticoids

Different modes of action have been described to illustrate the effects glucocorticoids exert within the organism. They can be roughly divided in genomic versus non-genomic effects. The genomic effects are either mediated directly through the cytosolic glucocorticoid receptor (GR) or indirectly by activating transcription factors. The non-genomic actions are thought to act through a distinct membrane receptor (CHEN and QIU 1999; NORMAN et al. 2004), thus accounting for some very rapid effects of glucocorticoids (HAYASHI et al. 2004).

Glucocorticoids exert their direct genomic effects by binding to a cytoplasmic GR that has several functional domains, including a ligand binding domain, a DNA binding domain, and two domains that are involved in the transactivation of genes once binding to DNA has occurred via association with other proteins (like activation function- 1 and -2) (KUMAR and THOMPSON 1999; HAYASHI et al. 2004; KUMAR and THOMPSON 2005). The GR is a simple polypeptide chain with a molecular weight of 90 kDa. This slightly unsymmetrical protein is postulated to be phosphorylated (BAXTER et al. 1989). The inactivated GR is bound to a protein complex that includes two subunits of the heat shock protein hsp-90. These act as molecular chaperones, preventing the nuclear localization of the unoccupied GR (KARIN 1998; WU et al. 2004). Once the glucocorticoid binds to its receptor, the hsp-90 dissociate, and a conformational change of the receptor structure allows its nuclear localization, its binding as a dimer to glucocorticoid response elements (GRE), and its

interaction with coactivator complexes (KARIN 1998; HAYASHI et al. 2004; KUMAR and THOMPSON 2005). Glucocorticoids produce their effect on responsive cells by activating the GR to either directly or indirectly regulate the transcription of target genes (HAYASHI et al. 2004) as described in Fig. 8.

The direct regulation, which includes the interaction of GR with GRE, classically leads to an increase in gene transcription (termed *trans*-activation), but negative GRE sites have also been described where binding of the GR leads to gene suppression (*cis*-repression) (DOSTERT and HEINZEL 2004; BARNES 2006). There are few well documented examples of negative GREs, some of these are relevant to corticosteroid side effects, for example genes regulating the hypothalamic – pituitary axis, the bone metabolism and skin structures (BARNES 2006).

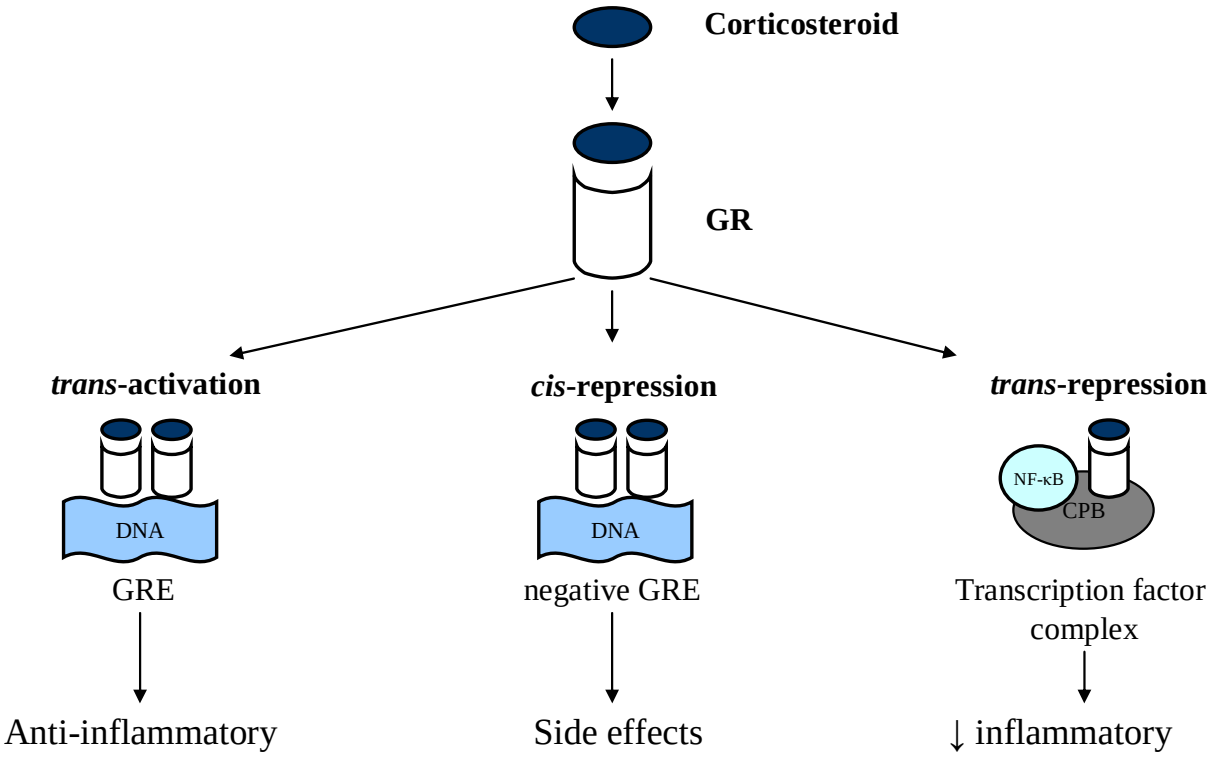


Fig. 8 Genomic effects of glucocorticoids. CPB = CREB-binding protein, NF-κB = nuclear factor- κB, GRE = glucocorticoid-response elements in the promoter region of steroid-sensitive genes. Adapted from BARNES (2006).

The number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with other

transcription factors and coactivators (ADCOCK et al. 2004). These effects exerted through transcription factors usually lead to a decreased transcription of genes, mostly those responsible for the synthesis of multiple inflammatory proteins and is termed *trans*-repression (BARNES 2006). Examples of glucocorticoid-sensitive genes are listed in Tab. 2.
High doses of glucocorticoids usually lead to the binding of a glucocorticoid-receptor dimer to the GRE in the promoter region of steroid-sensitive genes and a subsequent activation of genes with anti-inflammatory effects, including annexin-1 (lipocortin-1), Il-10 and the inhibitor of NF-κB, IκB-α.
Lipocortin in turn inhibits the enzyme phospholipase A2 (FLOWER 1988), leading to a decreased liberation of arachidonic acid. The result is a decrease in inflammatory mediators of the prostaglandin and leukotriene type.

The main anti-inflammatory effects of glucocorticoids are not by activation of genes with anti-inflammatory effects, as described above, but through suppression of genes that encode for synthesis of inflammatory proteins (BARNES 2006) as indicated in Tab. 2. It was originally believed that this effect was exerted through binding to negative GRE sites, but it has recently been proposed, that glucocorticoids rather inhibit the effects of pro-inflammatory transcription factors (like AP-1 and NF-κB), that regulate the expression of genes that code for many inflammatory proteins. Thus they influence the synthesis of cytokines, inflammatory enzymes, adhesion molecules and inflammatory receptors (BARNES and ADCOCK 1998).

Activated GRs have been shown to interact functionally with other activated transcription factors, either through protein–protein binding or downstream of the binding of pro-inflammatory transcription factors to DNA and their effects on chromatin structure and histone acetylation (BARNES 2006). Thus activated GRs may bind to CBP or other coactivators directly to exert their effects on histone acetylation/deacetylation (ITO et al. 2000; ADCOCK et al. 2004). The inhibition of histone acetyltransferase (HAT) or induction of histone deacetylases (HDACs) leads to the winding of the DNA around core histones and thereby a repression of inflammatory genes (ITO et al. 2000; ADCOCK et al. 2004; BARNES 2006).
It is still not known, why corticosteroids selectively switch off inflammatory genes without exerting an effect on genes that regulate proliferation or metabolism. It was postulated by

BARNES et al. (2006) that GRs only bind to coactivators that are activated by pro-inflammatory transcription factors, like NF-κB and AP-1, but the mechanism of a specific recognition remains unknown.

Tab. 2 Selected glucocorticoid-sensitive genes. (Modified according to ADCOCK (2004) and BARNES (2006))

decreased transcription	**increased transcription**
chemokines - IL-8, RANTES	Lipocortin-1/annexin-1 (phospholipase A_2 inhibitor)
cytokines - IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18, TNFα	β_2-adrenoceptor
inducible enzymes - COX2, cytoplasmic phospholipase A_2	IκB-α (inhibitor of NF-κB)

Although most of the actions of glucocorticoids are mediated through influencing transcription, it is increasingly recognized that they may also affect protein synthesis by reducing the stability of mRNA so that less protein is synthesized (BARNES 2006; ITO et al. 2006). Corticosteroids may have inhibitory effects on the proteins that stabilize mRNA, leading to a more rapid breakdown, resulting in a reduced expression of inflammatory protein (BERGMANN et al. 2000).

Non genomic effects are usually achieved with very high concentrations of glucocorticoids. One effect is the membrane stabilizing properties which leads to a reduced degranulation and liberation of pro-inflammatory mediators (especially histamine) from mast cells and the reduced release of lysosomal enzymes from basophil and neutrophil granulocytes. (BRINCKERHOFF et al. 1980; MUNCK et al. 1984).

2.6.3 Use of glucocorticoids in veterinary ophthalmology

In concentrations above the physiological range, glucocorticoids suppress all stages of an inflammatory reaction. These anti-inflammatory and immunosuppressive effects have made the glucocorticoids a powerful tool in preventing scarring, maintaining transparency, and treating the immune-mediated inflammations of some forms of keratitis, uveitis, conjunctivitis, scleritis/episcleritis, and corneal transplants. Glucocorticoids do not eliminate noxious stimuli but appear to only modify the response to noxious stimuli. Glucocorticoids at therapeutic doses have some action on every facet of the immune response (SLATTER 2001c). The following lists the anti-inflammatory effects of therapeutic doses of glucocorticoids on ocular structures: They - block permeability of capillary endothelium, prevent intracellular edema, inhibit migration of neutrophils by decreasing vascular permeability and vasoconstriction, reduce neutrophil adherence, inhibit ingestion of bacteria and release of proteolytic enzymes by neutrophils and macrophages, prevent antibody production of B-lymphocytes before humoral digestion, suppress lymphokines from stimulated T-lymphocytes, interfere with complement sub fractions, inhibit histamine synthesis and counteract histamine vascular effects, decrease fibroblastic proliferation and collagen deposition, possibly stabilize lysosomal membranes and possibly affect prostaglandin synthesis (SLATTER 2001c).

This broad spectrum of effects leads to a similarly versatile list of indications for the use of glucocorticoids in veterinary ophthalmology. The most important indications with some examples are summarized in Tab. 3.

Tab. 3 Indications for the use of glucocorticoids in the eye. Listing in accordance with SLATTER (2001c) and MARTIN (2005c).

Indications	Examples
sterile immune-mediated ocular diseases	seasonal allergic conjunctivitis, drug and contact allergies, lens-induced uveitis, chronic immune-mediated keratoconjunctivitis syndrome, pannus, atopic conjunctivitis, VKH
nonpyogenic inflammation	episcleritis
traumatic conditions resulting in severe inflammation	prolapse of the globe, contusion with hyphema,
postoperative immunosuppression	corneal transplants, cataract extraction
reduction of postoperative swelling and inflammation after cryosurgery	cyclocryotherapy, cryoepilation for distichiasis
ocular infections with significant destructive immune-mediated inflammation	feline infectious peritonitis (FIP) associated uveitis → therapy with systemic infectious agents is almost always limited to topical glucocorticoid therapy
reduction of neovascularization, pigmentation and scarring in the cornea (provided it is fluorescein negative)	

2.6.4 Penetration of drugs through the cornea

To penetrate into the eye after topical administration, most drugs will mainly penetrate through the cornea, and to lesser degree through the conjuctival-scleral route, as most drugs have the adequate properties for corneal absorption (DOANE et al. 1978; DAVIES 2000). The above described layers of the cornea (see chapter 2.2) represent distinct barriers to absorption. An aqueous phase (stroma) is surrounded by two lipid layers (epithelium and endothelium). The penetration through the epithelium can either be on a transcellular or a paracellular route (GRASS and ROBINSON 1984; GRASS et al. 1988). Since the superficial cells are sealed off by annular tight junctions, this form of penetration is reserved only for very small and hydrophilic molecules. Since the epithelium is lipophilic, penetration is dependent on the oil/water partition coefficient and allows only lipophilic molecules to penetrate (GRASS and ROBINSON 1984; MAURICE 1984). The epithelium is therefore the rate-limiting barrier for highly polar drugs. The stroma in contrast has 78 % water content and thus allows free passage of compounds with high aqueous solubility, acting as a barrier to lipophilic molecules (HEGEMAN et al. 1984). Since the endothelium is only one cell layer thick and interconnected with junctional gaps, this layer does not act as a strong barrier (BARTLETT et al. 1984; HEGEMAN et al. 1984; PRAUSNITZ and NOONAN 1998).
In order for drugs to penetrate the cornea, they must have intermediate solubility characteristics to penetrate both epithelium and stroma (GELATT 2007). For moderately lipophilic drugs like dexamethasone, the epithelium contributes 50 % to the total resistance, whereas the stroma and endothelium each contribute 25 % (LEE 1985). The relative amount of contribution of the epithelium increases with increased hydrophilic properties of the drugs (GELATT 2007).

2.6.5 Local side effects and complications

When used according to indication, short term glucocorticoid therapy, even in high doses, is mostly without side effects. Long-term systemic, and in a few cases even local treatment (ROBERTS et al. 1984), can lead to systemic side effects, which resemble Morbus Cushing. Nevertheless, the local anti-inflammatory and immunosuppressive effects of glucocorticoids can have negative side effects.

Glucocorticoids activate collagenases, which, in the presence of corneal ulcerations and abrasions, may induce a progressive ulceration (BROWN et al. 1970; MARTIN 1971). A progression of corneal ulceration due to augmentation of matrix metallo proteinases (MMPs) is also possible. Since glucocorticoids inhibit cell proliferation, they will also interfere with wound healing (BOURCIER et al. 1999; LU et al. 2004).

The immunosuppression induced by glucocorticoids will worsen a bacterial infection if not covered by appropriate antibiotics. Previously clinically unapparent viral and fungal infections may be worsened to clinically manifest forms. Therefore, the use of glucocorticoids in infectious inflammations is in principle contraindicated. In the cornea, band keratopathy or superficial calcification may occur with topical therapy using phosphate salts of the drug. In human medicine the most important local side-effects of glucocorticoids are the development of subcapsular cataracts and ocular hypertension. These side-effects usually do not occur in animal patients (REGNIER and GELATT 1999), although a mean increase in intraocular pressure of 5 mm Hg in glaucomatous beagles has been described (GELATT and MACKAY 1998; MARTIN 2005c).

2.6.6 Systemic side-effects after local application

Generally, glucocorticoid side effects are limited to the local site of the eye. Nevertheless, it has been shown that topically applied glucocorticoids (like prednisolone) lead to systemic side effect like iatrogenic Cushing's syndrome and adrenal suppression (REGNIER et al. 1982; ROBERTS et al. 1984; GLAZE et al. 1988; MURPHY 1990; MARTIN 2005c). The occurrence of such side-effects is both dose and duration dependent and they are in principle less common than after systemical application of the drug. A brief overview of the systemic side effects glucocorticoids can exert in general is listed in Tab. 4 .

Tab. 4 Systemic side-effects of glucocorticoids (UNGEMACH 2006).

mineralocorticoid	
sodium retention with edema	hypokalemia
glucocorticoid	
ACTH-suppression	decreased glucose tolerance
atrophy of the adrenal cortex	diabetic effect
immunosuppression	polyphagia, polyuria/polydipsia
gastric ulcers	decreased seizure threshold
retarded wound healing	hepatopathy (dogs)
skin atrophy	thrombosis
arthropathy	hypertension
osteoporosis	teratogenicity
muscle atrophy	initiation of abortion (dog)
growth retardation	initiation of parturition (cattle, sheep)
laminitis	decreased lactation (cattle)
Cushing's syndrome	

2.7 Working hypothesis

As the first part of the refractive system in the eye, the corneal transparency is vital for vision. Ocular disease conditions affecting the cornea can result in the loss of corneal transparency and thus greatly impair vision in humans and companion animals. Inflammations as well as traumatic lesions of the cornea are common causes for such a loss of transparency.

Most ophthalmic drugs used in veterinary ophthalmology have been approved for the use in humans but not in animals. Therefore, their use in humans, including dosing intervals, is often directly transferred to companion animals without formal testing. The successful use of those drugs in human ophthalmology as well as morphological similarities between the species leads to a certain degree of confidence regarding their use in veterinary ophthalmology. Nevertheless, direct correlations of tolerance as well as pharmacokinetic and therapeutic effects should not be made between species due to interspecies differences (HENDRIX et al. 2002).

Glucocorticoids are widely used in human and veterinary ophthalmology as described in the literature review. Since glucocorticoids exert the majority of their effects through binding to the GR, it is essential to detect these structures in order to gain a better understanding of their effects. The receptor has been detected in different ocular structures for various species (WEINSTEIN et al. 1982; SOUTHREN et al. 1983; WILSON et al. 1993, 1994; STOKES et al. 2000; BESSONOVA 2006). So far the GR has not been described for canine corneal tissues.

The penetration of topically applied glucocorticoids, including dexamethasone, into the eye has been studied for different species, including the dog (LEIBOWITZ et al. 1978; MIDELFART et al. 1999; REICHENBECKER 2002; KAISER 2003; CIVIALE et al. 2004). Few studies have been conducted to measure effects of glucocorticoids on canine corneal cells. MARTIN et al. (1971) showed that methyl prednisolone inhibits corneal healing. HENDRIX et al. (2002) could show *in vitro* that dexamethasone, hydrocortisone and prednisolone affect the morphology and migration of epithelial cells in a dose-dependent manner.

Based on this knowledge, the aim of this study was the detection of the GR in the canine cornea and the investigation of the effect of dexamethasone on the three major cell types. So

far, drug effects on the cornea were only studied on single cell cultures and the effects measured using morphological parameters (cell size and shape). In the present study, the inflammatory reaction was also taken into consideration thus creating a situation with closer resemblance of the situation in the inflamed eye.

Going by previous studies with skin tissue (SERRA et al. 2007), recently studies have been conducted to reconstruct the cornea. Such a three-dimensional equivalent using two (GERMAIN et al. 1999; CIVIALE et al. 2004), or all three of the corneal cell types (MINAMI et al. 1993; GRIFFITH et al. 1999; SCHNEIDER et al. 1999; TEGTMEYER 2000; TEGTMEYER et al. 2001; REICHL 2003; REICHL et al. 2004) allows cellular interactions and thus creates a situation closer to that *in vivo*. Starting with a full reconstruction of the bovine cornea (MINAMI et al. 1993), cornea equivalents for porcine (SCHNEIDER et al. 1999; REICHL 2003), rabbit (ZIESKE et al. 1994; ALAMINOS et al. 2006), bovine (PARNIGOTTO et al. 1998; TEGTMEYER et al. 2001; TEGTMEYER et al. 2004) and human (FERBER 1999; GERMAIN et al. 1999; GRIFFITH et al. 1999; REICHL et al. 2004) tissue have been established for different applications as described above. So far, no model has been described for the canine cornea.

The use of a cornea equivalent could be a valuable tool in pharmacological investigations. The advantage of such a system is the possibility for intercellular communication and interaction between the epithelial cells, keratocytes and endothelial cells, which is likely to be influential on inflammatory reactions and the efficacy of dexamethasone. Also, establishing such an *in vitro* model is significant in regard to ethical considerations. By using cultured corneal cells, it is expected that less donor animals are needed compared to experiments conducted on excised canine corneas.

In summary, the goals of this study were:

1. establishing an isolation- and culture-protocol for the primary culture of canine corneal cells (i.e. epithelial cells, keratocytes and endothelial cells)
2. establishing a three-dimensional canine cornea equivalent
3. investigating the GR in the single cells and the cornea equivalent
4. conducting inflammatory experiments with the single cells and the cornea equivalent
5. describing the dexamethasone effect in regard to the inflammatory reaction in canine corneal cells and the cornea equivalent

3 Materials and Methods

3.1 Experimental setting

An overview of the experimental setting for this study is given in Tab. 5.

Tab. 5 Experimental setting

number	experiments	methods
I	isolation and cultivation of canine corneal cells	cell culture
II	characterization of primary canine corneal cells	MTT, Western Blot, immunocytochemistry
III	detection of the GR in canine corneal cells	RT-PCR
IV	influence of LPS and dexamethasone on the PGE_2 production in the single cells	ELISA
V	influence of SDS and dexamethasone on the PGE_2 production in the single cells	ELISA
VI	expression of Il-1β, Il-8, TNFα, COX-2, GR	RT-PCR
VII	construction of a cornea equivalent	cell culture
VIII	characterization of the cornea equivalent	histology, immunohistology
IX	detection of the GR in the cornea equivalent	immunohistology
X	functional testing of the cornea equivalent	ELISA

3.2 Materials

All purchased equipment and materials are listed in the following chapters.

3.2.1 Cell culture reagents

3.2.1.1 Culture media

The basic culture medium used for the primary canine endothelial cells and keratocytes as well as for the proliferation phase of the cornea equivalents was Dulbecco's modified Eagle's Medium (**DMEM**) (PAA, Pasching, Germany). All ingredients of this basic culture medium are listed in Tab. 6.

Tab. 6 Listing of the ingredients of the DMEM basic medium [mg/ml].

Calcium Chloride anhydrous	200.00	L-Serine	42.00
Ferric(III)-Nitrate • $9H_2O$	0.10	L-Threonine	95.00
Potassium Chloride	400.00	L-Tryptophan	16.00
Magnesium Sulphate anhydrous	97.70	L-Tyrosine	72.00
Sodium Chloride	6400.00	L-Valine	94.00
Sodium Dihydrogen Phosphate • $9H_2O$	125.00		
Sodium Hydrogen Carbonate	3700.00	D-Calcium-Pantothenate	4.00
		Choline Chloride	4.00
L-Arginine • HCl	84.00	Folic Acid	4.00
L-Cystine	48.00	Myo-Inositol	7.20
L-Glutamine	584.00	Nicotinamide	4.00
Glycine	30.00	Pyridoxal • HCl	4.00
L-Histidine • HCl • H_2O	42.00	Riboflavin	0.40
L-Isoleucine	105.00	Thiamine • HCl	4.00
L-Leucine	105.00		
L-Lysine • HCl	146.00	D-Glucose anhydrous	1000.00
L-Methionine	30.00	Phenol Red	15.00
L-Phenylalanine	66.00	Sodium Pyruvate	110.00

The basic culture medium used for the primary canine epithelial cells was **Williams E** medium (Sigma-Aldrich, Taufkirchen, Germany). All ingredients of this basic culture medium are listed in Tab. 7.

Tab. 7 Listing of the ingredients of the Williams E basic medium [mg/ml].

Calcium Chloride • $2H_2O$	265.00	L-Threonine	40.00
$CuSO_4$ • $5H_2O$	0.0001	L-Tryptophan	10.00
Ferric(III)-Nitrate • $9H_2O$	0.0001	L-Tyrosine • 2Na • dehydrate	50.45
Potassium Chloride	400.00	L-Valine	50.00
Magnesium Sulphate anhydrous	97.70		
Magnesium Chloride • $4H_2O$	0.0001	Ascorbic Acid • Na	2.27
Potassium Chloride	400.00	D-Biotin	0.50
Sodium Hydrogen Carbonate	2200.00	Calciferol	0.10
Sodium Chloride	6800.00	Choline Chloride	1.50
Di-Sodium Dihydrogen Phosphate anhydrous	122.00	Folic Acid	1.00
Zinc Sulphate • $7H_2O$	0.0002	Glutathione, reduced	0.05
		Myo-Inositol	2.00
L-Alanine	90.00	Menadione Sodium Bisulfite	0.01
L-Arginine	50.00	Niacinamide	1.00
L-Asparagine • H_2O	20.00	D-Pantothenic Acid • ½ Ca	1.00
L-Aspartic Acid	30.00	Pyridoxal • HCl	1.00
L-Cysteine	40.00	Retinol Acetate	0.10
L-Cystine	20.00	Riboflavin	0.10
L-Glutamic Acid	44.50	Thiamine • HCl	1.00
Glycine	50.00	(+)-α-Tocopherol Phosphate • 2Na	0.01
L-Histidine	15.00	Vitamin B12	0.20
L-Isoleucine	50.00		
L-Leucine	75.00	D-Glucose	2000.00
L-Lysine • HCl	87.46	Methyl Linoleate	0.03
L-Methionine	15.00	Phenol Red • Na	10.70
L-Phenylalanine	25.00	Pyruvic Acid • Na	25.00
L-Proline	30.00		

The basic culture medium used for the RCE cells, some experiments with primary canine epithelial cells and the differentiation phase of the cornea equivalents was **DMEM: Ham12** medium (1:1) (PAA, Pasching, Germany). All ingredients of this basic culture medium are listed in Tab. 8.

Tab. 8 Listing of the ingredients of the DMEM: Ham12 basic medium [mg/ml].

Calcium Chloride anhydrous	116.60	L-Proline	17.25
Ferric(III)-Nitrate • $9H_2O$	0.05	L-Serine	26.25
Ferric(III)-Sulphate • $7H_2O$	0.417	L-Threonine	53.45
Potassium Chloride	311.80	L-Tryptophan	9.02
Cupric(II)-Sulphate • $5H_2O$	0.0013	L-Tyrosine	38.70
Magnesium Chloride • $6H_2O$	61.20	L-Valine	52.85
Magnesium Sulphate anhydrous	48.84		
Sodium Chloride	6996.00	D(+)-Biotin	0.0035
Sodium Dihydrogen Phosphate• H_2O	62.50	D-Calcium-Pantothenate	2.24
Di-Sodium Dihydrogen Phosphate anhydrous	71.02	Choline Chloride	8.98
Zinc Sulphate • $7H_2O$	0.432	Folic Acid	2.65
Sodium Hydrogen Carbonate	1200.00	Myo-Inositol	12.60
		Nicotinamide	2.02
L-Alanine	4.45	Pyridoxal • HCl	2.00
L-Arginine • HCl	147.50	Pyridoxine • HCl	0.031
L-Asparagine • H_2O	7.50	Riboflavin	0.219
L-Aspartic Acid	6.65	Thiamine • HCl	2.17
L-Cystine • HCl • H_2O	31.29	Thymidine	0.365
L-Cysteine • 2HCl	17.56	Vitamin B12	0.68
L-Glutamic Acid	7.35		
L-Glutamine	365.00	D-Glucose anhydrous	3151.00
Glycine	18.75	Hypoxanthine	2.10
L-Histidine • HCl • H_2O	31.48	DL-68-Lipoic Acid	0.105
L-Isoleucine	54.47	Linoleic Acid	0.042
L-Leucine	59.05	Phenol Red	8.10
L-Lysine • HCl	91.25	Putrescine • 2HCl	0.081
L-Methionine	17.24	Sodium Pyruvate	110.00
L-Phenylalanine	35.48		

The basic culture media were substituted for different culture purposes. In chapter 4.3 the culture media used are described by numbers according to the following list:

1) DMEM regular

DMEM basic culture medium
10 % fetal calf serum
100 IU/ml penicillin
100 µg/ml streptomycin
0.25 µg/ml amphotericin B

2) DMEM + EGF

DMEM basic culture medium
10 % fetal calf serum
100 IU/ml penicillin
100 µg/ml streptomycin
0.25 µg/ml amphotericin B
10 ng/ml epidermal growth factor

3) Williams E for epithelial cells

Williams E basic culture medium
10 % fetal calf serum
2 mmol/l glutamine
100 IU/ml penicillin
100 µg/ml streptomycin
10 ng/ml epidermal growth factor

4) DMEM: Ham12 for epithelial cells

DMEM: Ham12 basic culture medium
10 % fetal calf serum
5 µg/ml insulin
4 mmol/l glutamine
0.4 µg/ml hydrocortisone
0.18 mmol/l adenine
0.1 µg/ml colera toxin
100 IU/ml penicillin
100 µg/ml streptomycin
10 ng/ml epidermal growth factor

5) RCE culture medium

DMEM: Ham12 basic culture medium
15 % fetal calf serum
2 mmol/l glutamine
5 µg/ml insulin
10 ng/ml epidermal growth factor
0.1 µg/ml cholera toxin
0.5 % DMSO
100 IU/ml penicillin
100 µg/ml streptomycin

6) Equivalent medium for differentiation

DMEM: Ham12 basic culture medium
2 % fetal calf serum
5 µg/ml insulin
24.3 µg/ml adenine
6.1 µg/ml ethanolamine
14.1 µg/ml phosphoethanolamine
100 IU/ml penicillin
100 µg/ml streptomycin
0.25 µg/ml amphotericin B

7) Cryomedium for primary canine corneal cells

DMEM basic culture medium
20 % FCS
100 IU/ml penicillin
100 µg/ml streptomycin
0.25 µg/ml amphotericin B
10 % glycerol

8) Cryomedium for RCE cells

DMEM: Ham12 basic culture medium
20 % fetal calf serum
2 mmol/l glutamine
5 µg/ml insulin
10 ng/ml epidermal growth factor
0.1 µg/ml cholera toxin
10 % DMSO

3.2.1.2 Substances

Fetal calf serum	Biochrom AG, Berlin, Germany
EDTA (Versen) 1 %	Biochrom AG, Berlin, Germany
Trysin/EDTA (0.05 %/0.02 %)	Biochrom AG, Berlin, Germany
Dispase II	Gibco Invitrogen Corporation, Auckland, New Zealand
L-Glutamine	Sigma-Aldrich, Steinheim, Germany/ PAA Laboratories GmbH, Pasching, Germany
EGF	Biochrom AG, Berlin, Germany
Penicillin	PAA Laboratories GmbH, Pasching, Germany
Streptomycin	PAA Laboratories GmbH, Pasching, Germany
Amphotericin B	PAA Laboratories GmbH, Pasching, Germany
Insulin	Sigma-Aldrich, Steinheim, Germany
Cholera Toxin	Sigma-Aldrich, Steinheim, Germany
Adenine	Sigma-Aldrich, Steinheim, Germany
Ethanolamine	Biochrom AG, Berlin, Germany
Phosphoethanolamine	Biochrom AG, Berlin, Germany

Glycerol	Merck, Darmstadt, Germany
DMSO	Sigma-Aldrich, Steinheim, Germany
Dexamethason	Sigma-Aldrich, Steinheim, Germany
LPS (E.coli, 0111:B4)	Sigma-Aldrich, Steinheim, Germany
SDS	Sigma-Aldrich, Steinheim, Germany
Collagen (type I, rat tail)	Roche Diagnostics GmbH, Mannheim, Germany
Minimum Essential Medium	PAA Laboratories GmbH, Pasching, Germany
Sodium bicarbonate	Merck, Darmstadt, Germany
Acetic Acid	AppliChem GmbH, Darmstadt, Germany

3.2.1.3 Cell count and viability	
Trypan Blue	Sigma, St. Louis, USA
Celltiter 96® Aqueous One Solution Cell Proliferation	Promega, Mannheim, Germany

3.2.1.4 Measurement of prostaglandine	
Prostaglandin E_2-Immunoassay	R&D Systems, Minneapolis, USA

3.2.1.5 Disposable materials	
6-well Thin Certs^{TM} (TC)-plate	Greiner BIO-ONE GmbH, Frickenhausen, Germany
Insert for 6-well TC-plate, transparent, 3.0 µm pores	Greiner BIO-ONE GmbH, Frickenhausen, Germany
12-well TC-plate	Greiner BIO-ONE GmbH, Frickenhausen, Germany
96-well flat bottom tissue culture plate	Greiner BIO-ONE GmbH, Frickenhausen, Germany

25 cm² tissue culture flask, 50 ml	Greiner BIO-ONE GmbH, Frickenhausen, Germany
Sterile cell scraper	TPP, Omnilab, Mettmenstetten, Germany
Scalpel blade	Bayha, Tuttlingen, Germany
Syringes (2, 5, 10, 20 ml; Omnifix®)	B. Braun, Melsungen, Germany
Minisart® filter unit	Millipore, Carrigtwohill, Ireland
Terumo® needle (0.9 x 40 mm/ 0.6 x 25 mm)	Terumo Europe, Leuven, Belgium
Cryovials (1 ml, Cryo.s)	Greiner BIO-ONE GmbH, Frickenhausen, Germany

3.2.2 Cell culture equipment

Incubator	
- CO_2-auto-zero	Heraeus-Kulzer, Hanau, Germany
- CO_2 water-jacketed incubator , Nuaire US Auto Flow	Zapf Instruments, Sarstedt, Germany
Centrifuge 5804R	Eppendorf , Hamburg, Germany
Sterile work bench	
- Heraeus LaminAir HLP 2472	Heraeus-Kulzer, Hanau, Germany
- Heraeus LaminAir 2448	Heraeus-Kulzer, Hanau, Germany
Phase contrast microscope (Axiovert 25)	Zeiss, Oberkochen, Germany
Canon PowerShot A70	Canon Deutschland GmbH, Krefeld, Germany
Egg cup	IKEA, Germany

3.2.3 Reagents for RT-PCR

3.2.3.1 RNA isolation	
peqGold TriFast	peqlab, Erlangen, Germany

RNAse-free DNAse	Promega, Madison, USA
10x RQ1 Reaction Buffer	Promega, Madison, USA
Chloroform	Merck, Darmstadt, Germany
2-Propanol (Isopropanol)	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
DEPC (Diethylpyrocarbonate)	Roth, Karlsruhe, Germany

3.2.3.2 RT-PCR	
Superscript™ One-Step RT-PCR with Platinum® Taq	Invitrogen, Karlsruhe, Germany
RNAse-Inhibitor	Roche Applied Biosystems, Mannheim, Germany
Agarose	Invitrogen, Karlsruhe, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
DNA ladder (100 bp)	Invitrogen, Karlsruhe, Germany
primers	
- TLR 4	APARA-BIOSCIENCES, Denzlingen, Germany
- GR, GAPDH, IL-1β, IL-8, COX-2	Hermann Synthetische Biomoleküle, Denzlingen, Germany

3.2.4 RT-PCR equipment

Thermocycler (personal cycler)	Biometra, Göttingen, Germany
Electrophoresis unit	
- Agagel Mini	Biometra, Göttingen, Germany
- PS304 (power supply)	Gibco BRL, Karlsruhe, Germany
UV-Transilluminator	Biometra, Göttingen, Germany

Polaroid Gel Cam (GH 10 with 0.8 x electrophoresis hood)	Polaroid, UK
Centrifuge 5415C	Eppendorf, Hamburg, Germany

3.2.5 Reagents and materials for SDS-PAGE and western blotting

Bromphenol blue	Merck, Darmstadt, Germany
DL-Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, Germany
Acrylamide	Roth, Karlsruhe, Germany
Ammoniumperoxiddisulfate	Merck, Darmstadt, Germany
Temed (N,N,N',N'-Tetramethylethylenediamine)	Sigma-Aldrich, Steinheim, Germany
Tween 20 (Polyoxyethylenesorbitan Monolaurate)	Sigma-Aldrich, Steinheim, Germany
Glycine	Sigma-Aldrich, Steinheim, Germany
BSA	Sigma-Aldrich, Steinheim, Germany
SDS	Sigma-Aldrich, Steinheim, Germany
TRIS-HCl	Merck, Darmstadt, Germany
TRIS-Base	Merck, Darmstadt, Germany
EDTA	Sigma-Aldrich, Steinheim, Germany
Blocking solution	Amersham cell proliferation ELISA system, Amersham Biosciences, Freiburg, Germany
Ponceau S	Sigma-Aldrich, Steinheim, Germany
Molecular weight standard (Dual Color)	Bio-Rad laboratories GmbH, München, Germany
Primary antibodies	
- anti-cytokeratin (pan-antibody clone PCK-26)	Sigma-Aldrich, Steinheim, Germany
- anti-vimentin (clone V-9)	Sigma-Aldrich, Steinheim, Germany
- anti-ß-actin-IgG (mouse, clone AC-15)	Sigma-Aldrich, Steinheim, Germany

- anti-glucocorticoid-receptor	abcam, Cambridge, UK
Secondary antibody	
- AP.-conjugated Anti-mouse-IgG (Sheep)	Chemicon International, Hofheim, Germany
BCIP/NBT-substrate	Sigma-Aldrich, Steinheim, Germany

3.2.6 Equipment for western blotting

Multiple Gradient Caster (P1-CST)	Pequlab Biotechnologie GmbH, Erlangen, Germany
Electrophoresis unit	
- 2303 Multidrive XL	LKB, Bromma, Germany
- 2117 Multiphor II	LKB, Bromma, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Hybrid kiln	GFL, Hannover Vinnhorst, Germany

3.2.7 Reagents and materials for histology and immunohistology

Methanol	Labscan Limited, Dublin, Ireland
Acetone	Sigma-Aldrich, Seelze, Germany
Ethanol	Sigma-Aldrich, Seelze, Germany
Formaldehyde solution	Merck, Darmstadt, Germany
Triton-X	Serva, Heidelberg, Germany
Xylol	Riedel-de Häen, Seelze, Germany
Paraffin	Paraplast, Sherwood, UK
Lumox Slide Flask	In Vitro Systems & Services GmbH, Göttingen, Germany
flexiPERM slides	In Vitro Systems & Services GmbH, Göttingen, Germany

Superior slides	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Adhesive slides (HistoBond®)	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Methacrylat mixture (Technovit 7100)	Heraeus-Kulzer, Hanau, Germany
Eosin Y	Merck, Darmstadt, Germany
100 % acetic acid	AppliChem GmbH, Darmstadt, Germany
Toluidine blue	Niepötter Labortechnik, Bürstadt, Germany
Acetic Acid	AppliChem GmbH, Darmstadt, Germany
Primary antibodies	
- anti-cytokeratin (pan-antibody clone PCK-26)	Sigma-Aldrich, Steinheim, Germany
- anti-vimentin (clone V-9)	Sigma-Aldrich, Steinheim, Germany
- anti-GR (rabbit polyclonal)	Lab Vision, Fremont, USA
Secondary antibodies	
- F(ab)2 Goat anti-mouse IgG:FITC	Serotec GmbH, Düsseldorf, Germany
- Cy2-conjugated anti-mouse antibody	AffiniPure, Jackson Immuno Research, Soham/Cambridgeshire, UK
- Dako Envision™ + System	Dako Corporation, Carpinteria, USA
Eukitt Mounting Medium	Elecron Microscopy Sciences, Hatfield, USA
Depex Mounting Medium	Elecron Microscopy Sciences, Fort Washington, USA
Entellan Mounting Medium	Merck, Darmstadt, Germany

3.2.8 Equipment for histology and immunohistology

Tissue Block Systems (TBS 88) (Dispenser Unit)	Medite, Burgdorf, Germany
Stretching Table (OTS 30)	Medite, Burgdorf, Germany
Tissue Flotation Bath (TFB 45)	Medite, Burgdorf, Germany
Rotational microtome	Jung, Germany

Autocut-microtom	Reichert-Jung, Germany
Axioscope	Zeiss, Oberkochen, Germany
Axiocam	Zeiss, Oberkochen, Germany
Software KS 400 3.0	Kontron Systems, München, Germany
Software AXO VISION 4.6	Zeiss, Oberkochen, Germany

3.2.9 Analytical equipment

MRX Microplate-Reader	Dynatech Laboratories, Denkendorf, Germany
Centrifuges	
- 5403	Eppendorf, Hamburg, Germany
- 5415L	Eppendorf, Hamburg, Germany
Orbital Shaker Reax 2000	Heidolph, Kehlheim, Germany
pH-meter (pH 320)	WTW, Weilheim, Germany
Scale SBA 53	Scaltec, Heiligenstadt, Germany
Precision Scale ALS 120-4	Kern & Sohn GmbH, Balingen, Germany

3.2.10 Solutions

PBS (0.01 mol/l; pH 7.4)

NaCl	137 mmol/l	Merck, Darmstadt, Germany
KCl	2.7 mmol/l	Merck, Darmstadt, Germany
$Na_2HPO_4 \cdot 2H_2O$	6.5 mmol/l	Merck, Darmstadt, Germany
KH_2PO_4	1.5 mol/l	Merck, Darmstadt, Germany

TBE-Buffer (10x)

Tris	1 mol/l
Boric acid	0.9 mol/l
EDTA	0.01 mol/l
Aq. dest.	

TBS (pH 7.6)

NaCl	0.15 mol/l
TRIS-HCl	0.04 mol/l
TRIS Base	0.01 mol/l
Aq. bidest.	ad 1 l

adjust pH to 7.6 using 1N NaOH or HCl

RIPA-Buffer

TRIS-HCl (pH 8)	50 mmol/l
NaCl	150 mmol/l
Nonident-P40	1 %
Sodium Deoxycholate	0.5 %
SDS	0.1 %

Bouin-solution according to Böck (1989)

saturated picric acid	15 parts
37 % formaldehyde	5 parts
100 % acetic acid	1 part

3.3 Methods

3.3.1 Cell Culture

3.3.1.1 Animals

All canine eyes used for the isolation of the corneal cells were from dogs recently euthanized for reasons not related to this study. The initial studies to establish the isolation protocol and the dose finding studies were conducted on eyes from dogs that had to be euthanized due to traumatic or health reasons not related to this study. These dogs were received from the Clinic for Small Animals at the University of Veterinary Medicine Hannover (Foundation) and were of different breeds, age and gender.

For the main experiments, including the optimizing of the epithelial cell culture, the stimulation and treatment of the cells and the construction of the cornea equivalent, eyes from beagle dogs euthanized during a study at the Institute for Parasitology, University of Veterinary Medicine Hannover (Foundation) were used. The study conducted did not involve the eyes and all dogs were healthy at the time of death. The sex distribution was even (♀: 21 /♂: 26) and the mean age was 19 weeks (Min: 14.5, max: 24.5).

All dogs were euthanized on average less than one hour prior to enucleation of the bulbi oculi (maximum time between euthanasia and enucleation: 3 h). Only macroscopically healthy eyes were used and the excised bulbi stored in sterile PBS containing 200 IU/ml penicillin, 200 µg/ml streptomycin and 0.5 µg/ml amphotericin B at 4 °C for a maximum of 24 hours before the isolation was conducted. Cells were isolated according to Reichl (2003) with some modification. A schematic overview of the isolation protocol is given in Fig. 9.

3.3.1.2 Isolation of canine corneal cells

For the isolation of the corneal cells, the bulbi were rinsed with sterile PBS and the corneas excised with a 1 – 2 mm scleral rim under sterile conditions. The corneas were then placed epithelial side down in a sterilized porcelain egg cup.

The **endothelial cells** were enzymatically detached using 50 – 75 µl of a 0.05 % trypsin/0.02 % EDTA-solution for 7 min at 37 °C, 5 % CO_2. Care was taken to only cover the endothelial cells but not the stroma.

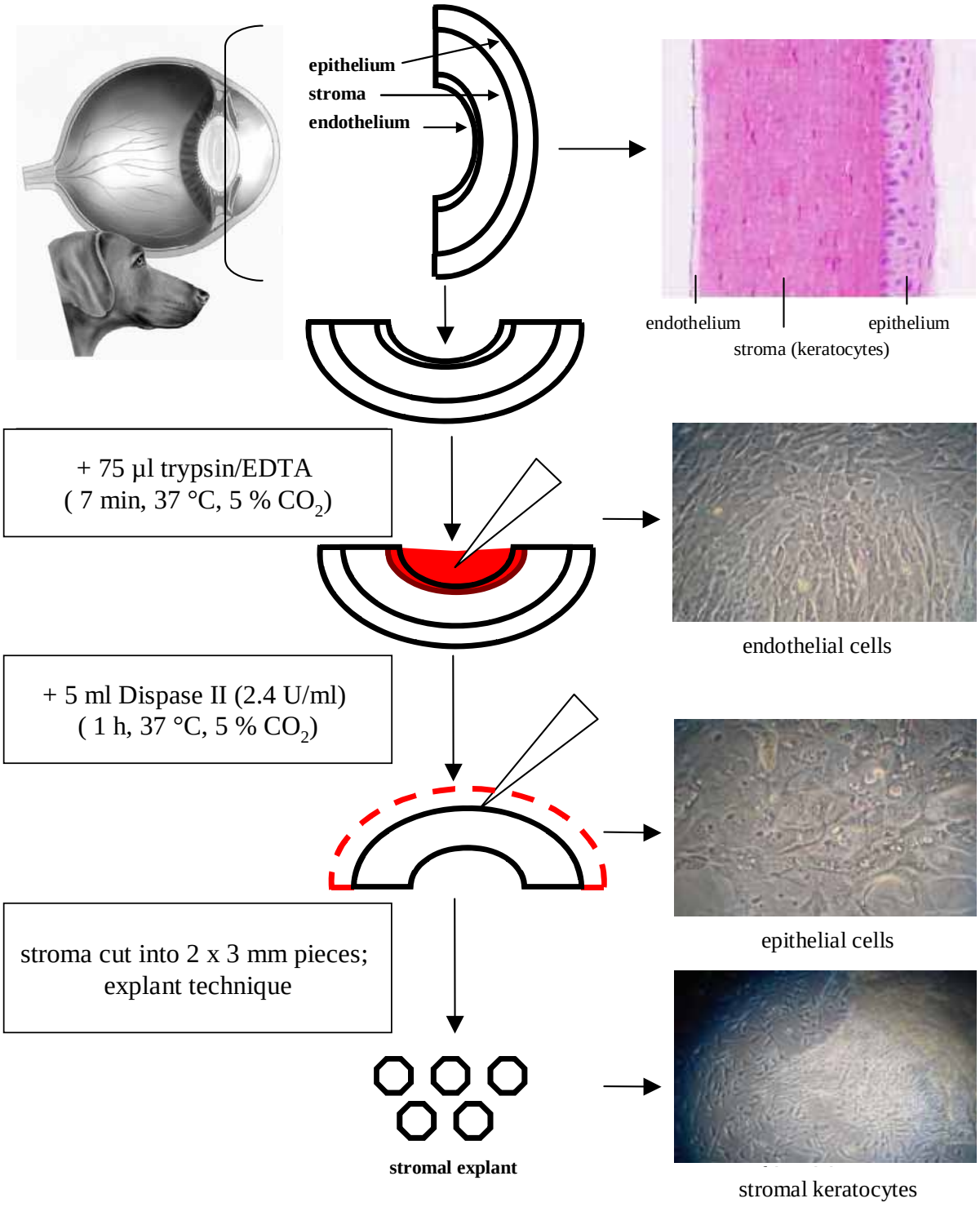


Fig. 9 Schematic illustration of the isolation of the three major cell types of the canine cornea

The cells were carefully scraped off the stromal layer using a sterile cell scraper and plated in one well of a 6-well plate with 2 ml medium 2. Remaining endothelium was removed using a scalpel blade and sterile PBS.

Next the corneal tissue was incubated epithelial side down in one well of the 6-well plate for 1 h at 37 °C, 5 % CO_2 with 2 ml Dispase II to selectively detach the basal cells of the epithelium. The **epithelial cells** were carefully scraped off and rinsed off the cornea, centrifuged (10 min at 300 x g, 4 °C) and seeded at 400,000 - 600,000 cells/well in collagen coated wells (1.5 mg/ml collagen, 40 µl per well) of a 6-well plate. Different culture media were tested (medium 2 – 4). Remaining epithelial cells were again removed using a scalpel blade and sterile PBS.

Finally the stromal **keratocytes** were isolated. The rest of the corneal tissue (the stromal layer) was cut into approximately 2 x 3 mm pieces. 4 – 6 tissue explants per well were placed into a 6-well culture plate and incubated without medium for 30 – 45 min at 37 °C, 5 % CO_2 for adherence. Then enough medium 1 was added to barely cover the explants (1 – 1.5 ml) and incubation continued. The addition of a larger volume of culture medium led to the detachment of the explants and a reduced cell yield.

3.3.1.3 Culture of canine corneal cells

All cells were cultured under standard conditions (37 °C, 5 % CO_2 in a humidified atmosphere). Culture medium was changed every 2 – 3 days, and the cells were washed with 1 ml sterile phosphate buffered saline (PBS) per well before addition of fresh medium. Endothelial and epithelial cells were passaged after 4 and 8 days, respectively.

For detachment of the endothelial cells 1 % EDTA-solution was added for approximately 40 s at room temperature, removed and replaced by 0.05 % trypsin/0.02 % EDTA-solution for 2 – 3 min at 37 °C, 5 % CO_2 until the cells were detached and separated. DMEM containing 10 % FCS was added to inactivate the trypsin and the cells were centrifuged for 10 min at 300 x g, 4 °C. The supernatant was discarded and the cells were seeded in fresh medium 2 at a mean density of 20,000 cells/cm² into 25 cm² culture flasks.

The epithelial cells were passaged similarly except that the EDTA-solution was added for 3 min at room temperature and trypsin/EDTA solution was incubated for 9 min at 37 °C, 5 % CO_2. The cells were seeded at a mean density of 20,000 cells/cm² into 25 cm² culture flasks.

The culture flasks were collagen-coated (1.5 mg/ml collagen, 50 µl per flask). Culture media 2 – 4 were tested for their influence on proliferation, morphology and suitability for continuous passage of the epithelial cells.

The keratocytes grew out of the explants after 3 – 5 days. After 10 days of culture the tissue explants were removed, the cells were carefully washed with sterile PBS and passaged into 25 cm² culture flasks. 1 % EDTA-solution was added for 2 min at room temperature, removed and 0.05 % trypsin/0.02 % EDTA-solution was added for 5 min at 37 °C, 5 % CO_2 until cells were detached and separated. DMEM containing 10 % FCS was added to inactivate the trypsin and after centrifugation (10 min at 300 x g, 4 °C) the cells were seeded at a density of 10,000 – 20,000 cells/cm² using culture medium 1.

For the experiments primary corneal cells were used at passage 2 to 4.

3.3.1.4 Culture of the immortalized rabbit corneal epithelial (RCE) cells

The rabbit corneal epithelial cells (RCE, ECACC, Reference number: 95081046, Sailsbury, UK) are cells that were immortalized by infection with the simian virus 40 (SV 40) (ARAKI et al. 1993). The cells were cultured in medium 5 according to the supplier's protocol. Cells were cultured in 25 cm² culture flasks and passaged when reaching 70 – 80 % confluence. To transfer the cells to new culture flasks, the culture medium was removed and the cells rinsed twice with 2 ml sterile PBS (to remove the FCS). Next, 1 ml of 0.25 % Trypsin/EDTA solution was added and evenly distributed to cover all cells. Surplus Trypsin/EDTA solution was discarded and the cells incubated for 7 - 10 min at 37 °C, 5 % CO_2 until all cells were detached and separated. At least 1 ml of fresh culture medium was added and the cells sub cultured in a ratio of 1:4. For the experiments RCE cells were used at passage 9 to 14.

3.3.1.5 Cryoconservation

Primary canine corneal cells as well as RCE cells were cryoconserved and stored in liquid nitrogen (-196 °C) to establish a cell pool for later use.

The **primary cells** were detached as described in chapter 3.3.1.3. After centrifugation, a cryoprotective medium was added (medium 7) and the cells were transferred to cryovials at a density of 2,000,000 – 3,000,000 cells/ml. The vials were then placed at – 20 °C for 1 – 2 h, followed by 12 – 24 h at – 80 °C and finally transferred to liquid nitrogen.

Cells were thawed quickly by placing the cryovials in a 37 °C water bath until only a frozen piece of 2 mm remained. Care was taken that the water did not reach the cap to avoid contamination. The vials were wiped with 70 % ethanol and quickly placed under the sterile bench. Next, the cells were carefully resuspended, transferred to a culture flask containing pre-warmed culture medium and directly placed in an incubator.

The **RCE cells** were frozen and thawed according to the supplier's protocol. Therefore, the cells were detached as described above (chapter 3.3.1.4). After detachment, they were directly resuspended in 1 ml of the cryoprotective culture medium (medium 8) and transferred to a cryovial (without centrifugation). The vials were placed in a polystyrene box at – 80 °C for 24 h and then transferred to liquid nitrogen. To thaw the cells, the cryovials were placed at room temperature for 1 min and then warmed in a 37 °C water bath until completely thawed. Care was taken that the water did not reach the cap to avoid contamination. The vials were wiped with 70 % ethanol and quickly placed under the sterile bench. The cells were carefully resuspended, transferred to a culture flask containing pre-warmed culture medium and directly placed in an incubator.

3.3.1.6 Cell count, viability and proliferation

To determine the cell count, cells were stained with trypan blue and counted using a Neubauer Chamber. 50 µl of a cell suspension were mixed with 100 µl trypan blue suspension (40 mg trypan blue in 10 ml aq. dest.) and 10 µl of this suspension placed in the counting chamber. The cell count was determined in four quadrants with a 100-fold magnification under the microscope. Trypan blue enables a differentiation of viable and dead cells. All cells incorporate the stain, but only viable cells are able to eliminate the blue stain. When viewed under the microscope, dead cells have a blue cytoplasm and nucleus, whereas viable cells are not stained. The total cell count can be calculated with the following formula:

$$Cells_{absolute} = \frac{Cells_{counted}}{4} \times 3 \times 10{,}000$$

$Cells_{counted}$: number of cells in 4 quadrants

$Cells_{absolute}$: absolute cell count in 1 ml of culture medium

Cell viability and proliferation for each cell type was evaluated using a modified MTT test according to the manufacturer's protocol. Through enzymatic activities of viable cells the reduction equivalent of the tetrazolium salt is formed. Thus, viable cells reduce the yellow, water soluble inner salt MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-terazolium] to the brown, water soluble (490 nm absorbing) formazan compound. Phenazine ethosulfate (PES) serves as an electron coupling reagent.

To obtain a growth curve, 5,000 cells/well were plated in a 96-well plate and measured in intervals of 24 h over the course of seven (to eight) days. The reagent solution was diluted 1:5 with the culture medium used for the respective cell type and 100 µl of this solution added to each well. Measurement of the extinction with a wave length of 490 nm was performed after one hour of incubation at 37 °C, 5 % CO_2.

3.3.1.7 Cells cultured for immunocytochemistry

For immunocytochemistry staining, cells were cultured with one of the following three methods. Cells were either grown in 96-well plates to confluence, washed with PBS and fixed with cold 50 % methanol/50 % acetone for 5 min. Alternatively, cells were seeded in lumox slide flasks with 2.5 ml of culture medium and grown to confluence. Then the cells were washed with PBS, the top part of the flask removed and the cells fixed as described above. The third method was the culture on adhesive slides using flexiPERM slides until confluence was reached. For this technique, the sterilized flexiPERM slides were placed on adhesive slides and 500 µl cells in culture medium carefully pipetted into each "well". Each slide was placed in a petri dish and incubated for 24 – 48 h until confluence was reached. Slides were then further processed as described for the other methods.

Staining was either performed directly in the 96-well plate or using wet chambers for the slides.

3.3.1.8 Construction of the canine cornea equivalent

Cornea equivalents were constructed using 6-well culture plates with inserts (thin certs^{TM}, 3 µm pore size) according to REICHL (2003). The inserts are plastic cylinders with a porous bottom membrane that are equipped with a hanging construction to place them in the centre of

a well of a 6-well plate as shown in Fig. 10. (Fluid-) Content of either the bottom well or the insert can only interact through the 3 µm pores of the bottom membrane. This construction allows cells in the insert to be cultured either in a submerged culture or a culture at the air-liquid interface. For the latter, the culture medium can be lowered to the level of the insert bottom, leading to a nutritional supply solely based on diffusion through the bottom membrane.

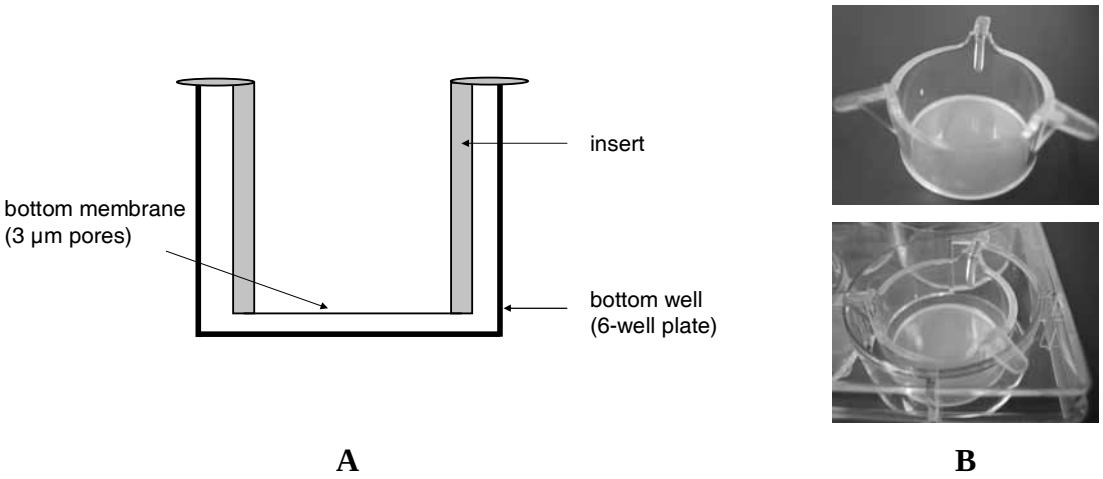


Fig. 10 Membrane insert for a 6 – well plate used for the construction of the cornea equivalents. **A** = schematic illustration of a 6-well with the insert, **B** = pictures of the insert.

A schematic illustration of the culture protocol is given in Fig. 11. The insert membranes were coated with 20 µl of 1.5 mg rat tail collagen each to allow for better attachment of the endothelial cells, dried and rinsed with sterile PBS or culture medium prior to construction of the equivalents. First, 200,000 endothelial cells per well were seeded onto the insert membrane and grown to confluence in culture medium 2 over the course of one week. The cells were grown in a submerged culture, thus the bottom well contained 2.5 ml and the insert well 1 ml of culture medium.

Next, 200,000 keratocytes were seeded on top of the endothelial layer in a collagen gel matrix. For the collagen gel one part MEM (10X), one part Hepes (10X), one part bicarbonate (10X) and one part cell suspension (containing 200,000 cells per equivalent) were carefully mixed with three parts 0.2 % sterile acetic acid. Finally, three parts of a cold 3.3 mg/ml collagen solution were added and mixed carefully (final collagen concentration: 1.5 mg/ml). 500 µl of this cell suspension were added to each insert and the culture plates immediately

placed in the incubator. Within 45 min the gelification of the collagen layer was completed and the same amount of culture medium was added as described for the endothelial cell layer. The equivalents were cultured for one week during which viable keratocytes led to a contraction of the collagen gel to one third of its initial size.

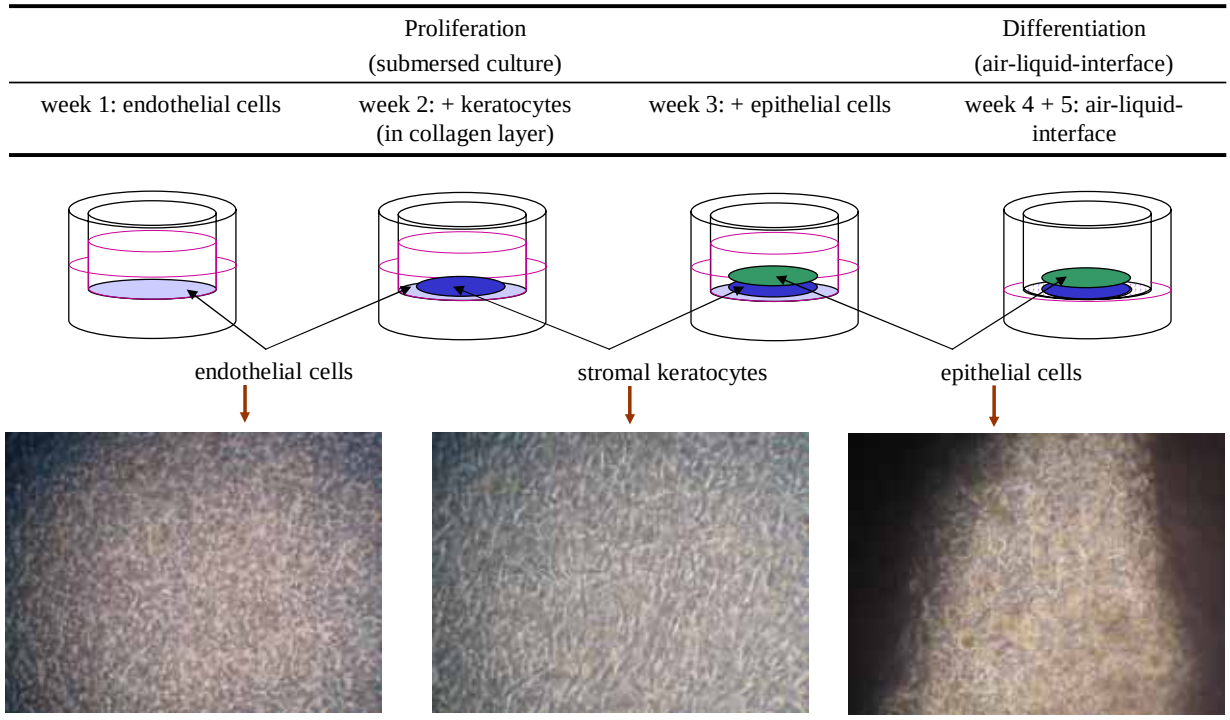


Fig. 11 Schematic illustration of the construction of the canine cornea equivalent over the course of five weeks.

Finally, epithelial cells were seeded onto the contracted collagen layer in 50 - 60 µl culture medium per insert at a density of 300,000 cells. 2.5 ml of culture medium 2 were added to the bottom well and the equivalents returned to the incubator for one hour to allow for attachment of the epithelial cells. Then 1 ml of culture medium 3 was carefully added onto the epithelial cell layer and these cells also cultured to confluence over the course of one week. The equivalents were then raised to the air-liquid-interface to allow for differentiation of the epithelial cells. During these final 2 weeks the culture medium in the insert was omitted and the medium level in the bottom well lowered to the level of the insert membrane by adding only 1 ml culture medium/well. The culture medium was changed to medium 6.

For comparison, cornea equivalents were additionally constructed with RCE cells instead of primary canine epithelial cells. RCE cells were handled in the same way the primary canine epithelial cells were handled during the construction of the cornea equivalent.

During the entire period of the construction of the cornea equivalents the culture medium was changed three times each week: the used culture medium was carefully removed without touching the insert membrane with the pipette tip in order to avoid detachment of the membrane from the insert walls. Before adding fresh culture medium, both sides of the insert membrane were carefully rinsed with sterile PBS and the inserts transferred to new, sterile 6-well plates. Throughout the culture period of 5 weeks, the growth of the cells and a possible bacterial or fungal contamination were controlled by phase contrast microscopy. Only equivalents with little bacterial and no fungal contamination were kept in culture.

For the cornea equivalents only primary cells of passages 2 – 4 were used, RCE cells were used at passages 9 – 14. Cell viability and proliferation of the cells prior to the construction of the cornea equivalent were evaluated using a modified MTT test as described in chapter 3.3.1.6.

3.3.1.9 Corneal cells and cornea equivalents cultured for the inflammation model

The single cell cultures were seeded in 96-well plates and grown to confluence. The stimulating agent with or without the dexamethasone treatment was added in 100 µl of culture medium and the cells were incubated at 37 °C, 5 % CO_2.

The cornea equivalents were cultured over the course of 5 weeks as described above. The substances for the stimulation and the treatment of the equivalents were dissolved in 50 µl of culture medium and carefully added onto the epithelial layer of the equivalents. The PGE_2 concentration at the end of each experiment was measured in the culture medium beneath the equivalents.

3.3.1.10 Measurement of PGE_2 in the culture medium

The PGE_2 concentration in the culture medium supernatant was measured with a competitive enzyme immunoassay. This assay is based on the competition between PGE_2 in the sample and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 tracer) for a limited amount of

PGE_2 monoclonal antibody. Because the concentration of the PGE_2 tracer is held constant while the concentration of PGE_2 in the samples varies, the amount of PGE_2 tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of PGE_2 in the sample. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well of the assay kit. The plate is washed to remove any unbound reagents and then the substrate to AChE is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PGE_2 tracer bound to the well, which is inversely proportional to the amount of free PGE_2 present in the sample during the incubation. A standard curve was performed with each ELISA assay. The percentage of binding was used to establish calibration curves. The measurements for the extinction of each sample were put in relation to these curves and the PGE_2 concentration calculated.

The samples were usually measured in duplicates and the following controls included in each assay: blank, total activity, non-specific binding and maximum binding.

3.3.1.11 Measurement of TNFα in the culture medium

The TNFα concentration in the culture medium supernatant was measured similarly to the PGE_2 concentration with an enzyme immunoassay. Briefly, the wells of the assay were pre-incubated with a monoclonal anti-canine TNFα antibody, which binds any TNFα of the sample. The bound TNFα is detected using a biotinylated anti-canine TNFα antibody and TMB-substrate, leading to a color reaction which can be measured spectrophotometrically at 450 nm. The intensity of the color is proportional to the concentration of TNFα in the sample.

3.3.2 Verification of canine corneal cells

The corneal cell type was verified using a monoclonal mouse anti-vimentin antibody (clone V-9) to detect endothelial cells and keratocytes and a monoclonal mouse anti-cytokeratin antibody (pan-antibody clone PCK-26) to detect epithelial cells.

Vimentin is one of the five major groups of intermediate filaments with a molecular weight of 58 kDa (BOHN et al. 1992). The antibody is used to localize tissue of mesenchymal origin, thus staining endothelial cells and fibroblasts. Cytokeratins are another group of intermediate filaments, comprising of at least 29 different proteins, which are characteristic for epithelial cells. The anti-cytokeratin antibody used recognizes an epitope located on the type II cytokeratins 1, 5, 6 and 8 which are members of the neutral-to-base subfamily. Those four cytokeratin peptides are expressed on differentiated, stratified, simple and hyperproliferative epithelial cells, respectively, therefore the antibody will detect undifferentiated as well as differentiated cells (MOLL et al. 2007).

All three cell types were always incubated with both antibodies, therefore epithelial cells incubated with anti-vimentin served as negative control as well as endothelial cells and keratocytes incubated with anti-cytokeratin. For the immunocytochemistry, samples incubated without the primary antibody also served as negative controls.

3.3.2.1 Immunocytochemistry

All three cell types were verified by indirect immunofluorescence staining. The monoclonal mouse anti-vimentin antibody was used in a 1:100 dilution and the monoclonal mouse anti-cytokeratin antibody was used in a 1:300 dilution. For staining, cells were grown in 96-well plates to confluence, washed with PBS and fixed with cold 50 % methanol/50 % acetone for 5 min. Plates were usually processed for staining immediately. Cells cultured on slides (lumox slide flask or slides with flexiPERM) were also washed with PBS and fixed with cold 50 % methanol/50 % acetone for 5 min. Slides were either stained immediately or stored in sterile PBS at 4 °C until further processing. When using 96-well plates all following reagents were added at 100 µl/well, when using slides, 100 µl of the antibodies were added per slide while the washing was done with 1 ml/slide. The primary and secondary antibodies were diluted in PBS containing 1 % BSA and 0.25 % Triton-X. During the entire process of the

staining, drying of the wells and slides was strictly avoided. Staining of the cells was performed according to the following steps:

1. washing with PBS (5 min, max. 2x)
2. incubation with blocking/antigen-retrieval solution (PBS containing 1 % BSA and 0.25 % Triton-X) for 30 min at room temperature
3. incubation with the primary antibody for 1 h at 37 °C
4. washing with PBS (5 min, max. 2x)
5. incubation with the fluorescence-labeled secondary antibody for 30 min at 37 °C in the dark
6. washing with PBS (5 min, max. 2x)

In the wells of the culture plate 100 µl PBS were left and the cells evaluated and photographed immediately or stored at 4 °C in the dark until evaluation. The slides were mounted in Entellan before microscopical evaluation using the software KS 400.

3.3.2.2 Western Blot analysis

Protein isolation

Cultured corneal cells (second to fifth passage) were washed in sterile PBS and lyzed using peqGold TriFast. Proteins were extracted from the phenol phase that resulted from the precipitation of the DNA with 100 % ethanol (incubation for 10 min followed by centrifugation for 5 min at 4 °C and 12,000 x g). The proteins were then precipitated through the addition of isopropanol for 10 min and subsequently centrifuged for 10 min at 4 °C and 12,000 x g. Next, the protein pellet was washed three times with 0.3 mol/l guanidinhydrochlorid in 95 % ethanol (incubation for 20 min at room temperature followed by centrifugation for 5 min at 4 °C and 7,500 x g) and once with 100 % ethanol (incubation for 20 min at room temperature, centrifugation for 5 min at 4 °C and 7,500 x g). Finally the proteins were resuspended in 1 % SDS and stored at –20°C until the analysis was performed.
Alternatively, corneal cells were lyzed with RIPA buffer. In this case cells were detached from the culture flasks with trypsin/EDTA solution, centrifuged for 10 min at 4 °C and

300 x g, and the supernatant discarded. The cell pellet was resuspended in 2 ml ice-cold RIPA-buffer and the samples incubated on ice for 30 min with gentle agitation. Next, the samples were centrifuged for 10 min at 4 °C at 120 x g. Placed on ice, the supernatant containing the proteins was carefully transferred to a new reaction tube and either stored at – 80 °C or directly processed for western blot analysis.

SDS-PAGE

The proteins were separated according to their molecular weight using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples and molecular weight marker were prepared by boiling in reduction-buffer containing dithiothreitol (DTT) for 5 min. This led to a cleavage of disulfide bonds and subsequently to a stretching of the proteins which improved the electrophoretic separation.

A gradient gel consisting of a 3.9 % polyacrylamide "collection gel" and a 10.8 % polyacrylamide separation gel was prepared, placed in the electrophoresis unit and covered by electrode buffer. Next, the aliquots were loaded onto the polyacrylamide gel and the proteins were separated by SDS-PAGE at 15 °C and 200 mV for 2 h.

Staining of the gel as a control for the electrophoresis was performed according to BRAUN (2002). The composition of the solutions used for the electrophoresis and SDS-PAGE have been previously described (BRAUN 2002).

Western Blot

After electrophoresis, the gel was transferred to nitrocellulose transfer membranes in a semi-dry system. The transfer was conducted between graphite electrodes for two hours at 0.8 mA/cm².

Color development of the nitrocellulose membrane

To reduce background staining, the membranes were incubated over night at 4 °C with blocking solution containing 0.05 % Tween 20, followed by three washes (1 x 10 min, 2 x 5 min) or an additional staining with Ponceau S to validate the transfer of the proteins onto

the membrane as described in Fig. 12. Next, the membrane was incubated with the primary antibody for one hour at room temperature with gentle agitation. Dilutions of the primary antibodies were 1:200 for anti-vimentin and 1:300 for anti-cytokeratin. Visualization was performed using an AP-conjugated goat anti-mouse IgG (diluted 1:2500) and SigmaFast BCIP/NBT substrate to develop a color reaction.

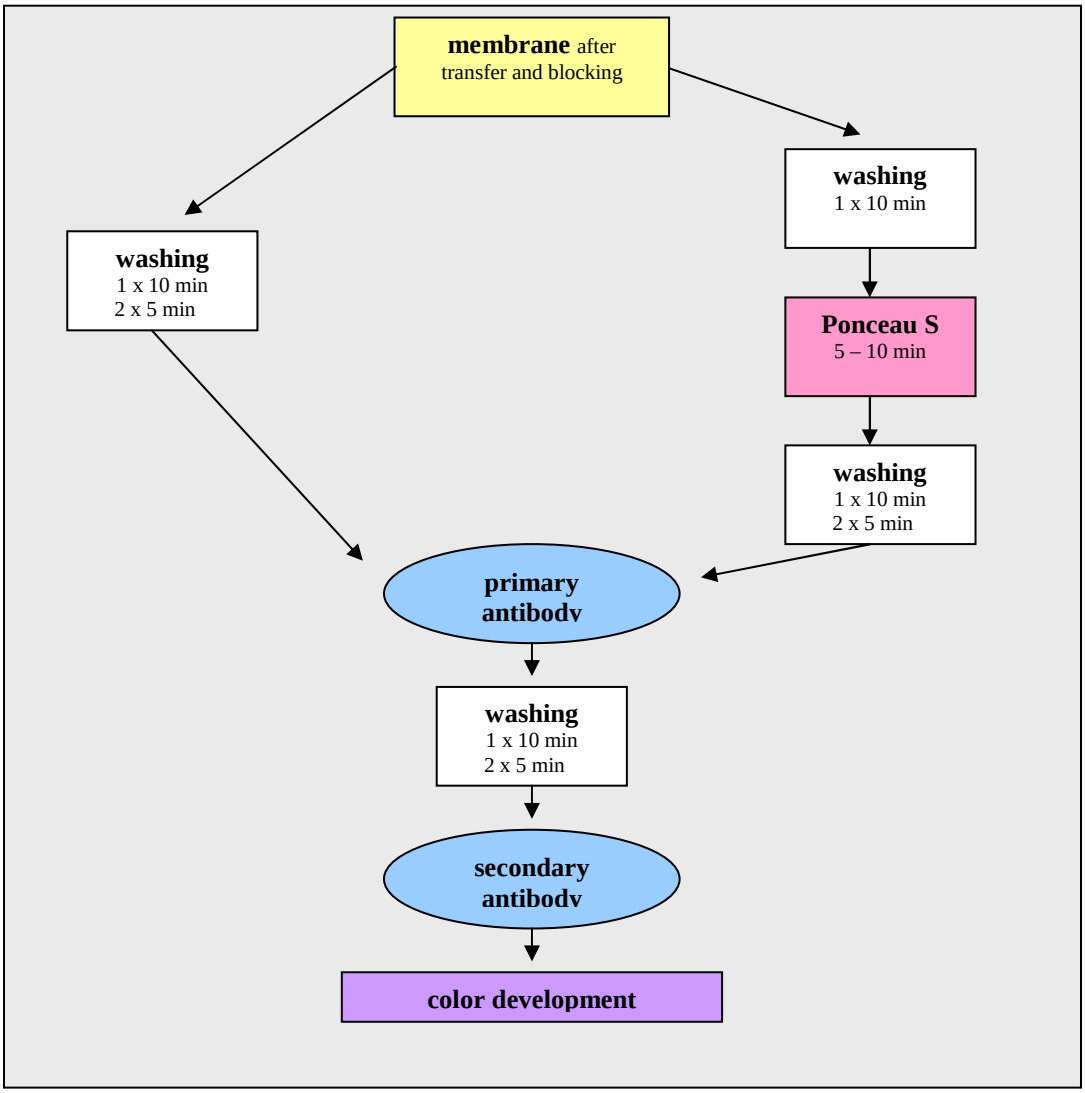


Fig. 12 Schematic illustration of the staining of the western blot membranes.

3.3.3 Histology and immunohistology

3.3.3.1 Fixation

The tissue for histological and immunohistological examination was fixed in Bouin-solution according to BÖCK (1989) for a minimum of one week. The cornea equivalents were first rinsed in sterile PBS and then fixed for 2 weeks. The yellow color was removed by repeatedly washing the equivalents in 70 % ethanol (containing a few drops 32 % NH_3) and the cleared equivalents were then placed in 80 % ethanol until processed for histology.

3.3.3.2 Histological analysis

Embedding

Paraffin-embedding: The reference tissues as well as some of the cornea equivalents (n = 15) were embedded in paraffin. Cornea equivalents were first embedded in agarose and cut out from the insert membrane with an agarose block. Those were transferred to 80 % ethanol over night. Next, all samples were processed for 2 h each in 96 % ethanol and isopropanol, followed by 2 x 2 h in xylol and paraffin (60 °C, overnight and 2 x 4 h the following day) and finally embedded in paraffin. 5 µm-thick transverse sections were made using a rotational microtome and transferred to regular slides for haemalum and eosin (HE-) and toluidine blue staining and adhesive slides for immunohistochemistry. Prior to staining the slides were deparaffinized with decreasing concentrations of alcohol (2 x 10 min xylol, 2 min each in isopropanol, 96 % , 80 % and 70 % ethanol).

Technovit-embedding: The other cornea equivalents (n = 15) were embedded in technovit, a structure conserving method to avoid disintegration of the fragile equivalents during the embedding process.

Samples were dehydrated in increasing concentrations of ethanol (90 %, 96 % and 100 %; each 3 x for 20 – 40 min) and embedded in the water soluble methacrylat mixture Technovit 7100 in teflon forms according to the manufacturer's protocol. 2 µm-thick transverse sections were made using an autocut-microtom and transferred to regular slides. For each equivalent at least three sections were stained in a standard fashion with toluidine blue, some samples were also stained with HE.

Staining

Haemalum-eosin (HE) stain

Following deparaffinization of the paraffin-embedded slides (2 x 10 min xylol, 2 min each in isopropanol, 96 %, 80 % and 70 % ethanol) all slides were rinsed in aq. dest. and, depending on the embedding material used, stained as described in Tab. 9.

Tab. 9 Protocol for HE staining on technovit- and paraffin-embedded material.

Technovit	Paraffin
• 1 h haemalum (Delafield) • rinsing in 0.1 % HCl in 70 % ethanol • rinsing for 15 min with tab water • 5 min 1 % eosin in 96 % ethanol (containing acetic acid) • rinsing briefly in 70 % ethanol • rinsing briefly in 80 % ethanol • rinsing 2 x in absolute ethanol • air drying of slides • mounting of the slides with depex	• 10 min haemalum (Delafield) • rinsing in 0.1 % HCl in aq. dest. • rinsing for 15 min with tab water • 5 min 1 % eosin in aq. dest. (containing acetic acid) • rinsing briefly in 70 % ethanol • rinsing briefly in 80 % ethanol • rinsing briefly in 96 % ethanol • 2 min isopropanol • 2 x 5 min xylol • mounting of the slides with eukitt

Toluidine blue staining

Again, paraffin-embedded slides were first deparaffinized, technovit-embedded slides stained without pre-treatment. The protocol for the toluidine blue staining is given in Tab. 10.

Tab. 10 Protocol for staining with toluidine blue on technovit- and paraffin-embedded material.

Technovit	Paraffin
• 1 min toluidin blue • rinsing in aq. dest. • 3 x rinsing in 80 % ethanol (until stain is removed from the technovit) • 2 x rinsing in absolute ethanol • air drying of the slides • mounting of the slides with eukitt	• 20 sec toluidin blue • rinsing in aq. dest. • 3 x rinsing in 80 % ethanol (until stain is removed from the technovit) • 2 x rinsing in 96 % ethanol • 2 min in isopropanol • 2 x 5 min xylol • mounting of the slides with eukitt

3.3.3.3 Immunohistology

Selected equivalents (n = 7) were additionally studied immunohistologically. The primary antibodies used were a polyclonal rabbit anti-GR antibody (dilution 1:100), anti-vimentin (dilution 1:400) and anti-cytokeratin (dilution 1:1000). For the secondary antibody and color detection the Dako Envision™ + System was used according to the manufacturer's protocol. The staining was conducted as follows:

- 2 x 10 min xylol
- 3 min isopropanol
- 3 min 100 % ethanol
- 30 min 80 % ethanol containing H_2O_2
- 3 min 70 % ethanol
- rinsing in PBS
- 30 min antigen retrieval with TEC-buffer (97 – 99 °C)
- 20 min goat-serum (1:5 in PBS)
- primary antibody in PBS containing 1 % BSA, 4 °C over night
- 3 x 5 min PBS

- secondary antibody for 45 min at room temperature
- 3 x 5 min PBS
- 5 min DAB-substrate
- 1 x 5 min PBS
- 2 x 10 min PBS

The slides were immediately dehydrated with increasing concentrations of alcohol (2 min each in 70 %, 80 %, absolute ethanol and isopropanol) and xylol (2 x 5 min). Still moist, each slide was mounted using eukitt mounting medium.
During the entire procedure of the immunohistological staining, the slides were kept moist at all times. This was ensured by the use of wet chambers and the rapid handling of samples.

3.3.3.4 Reference tissue and controls

Different reference tissues were used for histology and immunohistology. Bouin-fixed and paraffin-embedded canine cornea was used for histological comparison with the cornea equivalents. Formalin-fixed canine cornea and Bouin-fixed canine uterus (both paraffin-embedded) were used as control tissues for the immunohistology; the uterus served as positive control for the GR staining, the cornea tissue served as positive control for the vimentin and cytokeratin staining. Cornea equivalents with omitted primary antibody served as negative control for all antibodies.

3.3.3.5 Evaluation of the samples

After complete drying of the mounting medium, all slides were evaluated and photographed using an axioscope and axiocam with the software AXO VISION 4.6.

3.3.4 RT-PCR

3.3.4.1 RNA Isolation

The primary cultures were washed with sterile PBS and lyzed with 1.5 ml peqGold TriFast per 25 cm² culture flask. The addition of 0.3 ml chloroform, followed by 10 min incubation and 5 min centrifugation (4 °C, 12,000 x g), led to the separation of RNA, DNA and proteins into three distinct phases. The RNA containing upper, clear phase was transferred to a new reaction tube. The RNA was precipitated through the addition of 0.75 ml isopropanol and incubated 10 min at room temperature. After centrifugation (10 min, 4 °C, 12,000 x g), the RNA-pellet was washed twice with 75 % ethanol.

To ensure DNA free samples, all samples underwent a DNAse digestion: the RNA pellet was resuspended in 30 µl DEPC-treated water and 5 µl RQ1 RNAse-free DNAse to which 4 µl 10x RQ1 reaction buffer were added. This mixture was incubated for 30 min at 37 °C with mild agitation. Finally, the TriFast RNA isolation was repeated, the RNA pellet resuspended in 40 µl of DEPC-treated water and stored at – 20 °C until analysis was performed.

3.3.4.2 Amount of RNA

To verify the amount of RNA in each sample, a test gel was run. 2 µl sample, 2.5 µl loading-buffer (200 µl Ficoll 50 %, 80 µl EDTA [0.5 mol/l, pH 8.0], 40 µl SDS 10 %, 10 µl BPB 2.5 %, 40 µl TBE-buffer) and 15.5 µl DEPC-treated water were gently mixed, separated on a ethidium bromide containing 1.5 % agarose gel with 105 V and 240 mA. Visualization of the bands for qualitative analysis was performed with an UV-Transilluminator.

3.3.4.3 Primer

For RT-PCR different primers were used (Tab. 11). All primer pairs were tested for their optimal annealing temperature and number of cycle-repeats prior to the main experiments. Information about primer sequences, expected size of the PCR-product as well as the settings for annealing temperature and number of cycles are summarized in Tab. 11.

Tab. 11 Primers used with their characterization regarding sequence, product size, annealing temperature and optimal number of cycles for the PCR-reaction.

primer		sequence	product size (bp)	annealing temp. ° C	number of cycles
gapdh	3’	TC TTC TGA CAC CTA CCG GGG	229	59	26
	5’	GCC AAA AGG GTC ATC ATC TC			
GR	3’	GGT CGG TCT TGA CCG TTG CGA	511	59	27
	5’	TAC GCA GGG TAT GAC AGC TC			
COX-2	3’	AG ACT TCA CCC AGT CCT ACA	388	58	35
	5’	CAG GTC CTC GCT TAT GAT CT			
IL-8	3’	CG TTG TCG GTC GAA CCT TCA	172	56	30
	5’	GGC CAC TGT CAA TCA CTC TC			
IL-1β	3’	G CTT TAC GGA GTC TGA GAA CAA TGT	225	58	35
	5’	GCA CCA GGT ATT TGT GGC TTA TGT			
TNFα	3’	CG ATG ACC GAA CAG TGA ACC	274	56	30
	5’	TCT TGA TGG CAG AGA GRA CG			
TLR 4	3’	CG TGT CAG GAA GGT CCT CTC	179	56	30
	5’	CCG TTG CCA TCT GAG ATT TT			

3.3.4.4 RT-PCR

RT-PCR analysis was performed using Superscript™ One-Step RT-PCR with Platinum® Taq and a thermocycler. A master-mix was prepared on ice as described in Tab. 12 and 1 µl of sample RNA added to 49 µl of that master-mix per PCR-reaction tube. The samples were thoroughly mixed and immediately placed in the pre-warmed thermocycler.

Tab. 12 Master-mix for the RT-PCR reaction.

reaction components	final concentration	volume per reaction (µl)
DEPC water	--	20.7
forward primer	0.1 nmol	1
backward primer	0.1 nmol	1
RNAse inhibitor	8 U	0.4
One-step *Taq* DNA polymerase		0.9
2X reaction mix	1X	25
final volume		49

The settings for the RT-PCR were the following: reverse transcription (30 min, 50 °C), initial activation (15 min, 94 °C), 3-step cycling (denaturation (15 s, 94 °C), annealing (1 min) and extension (1 min, 72 °C)) and final extension (5 min, 72 °C). Detailed information on the number of cycles and annealing temperature for the different primers used are listed in Tab. 11. The amplificates were separated on an ethidium bromide containing 1.5 % agarose gel. Visualization of the bands for qualitative and semi-quantitative analysis was performed with an UV-Transilluminator and gels photographed immediately with a Polaroid camera..

Semi-quantitative analysis*:* To measure the effects of LPS-stimulation and dexamethasone treatment on the cells, a semi-quantitative RT-PCR was performed. Therefore, the RNA content of all studied samples was first equalized in a PCR reaction with the housekeeping gene gapdh. The amount of sample used for the PCR reaction was increased or decreased, depending on the size of the band produced with the gapdh, until all samples produced bands of an equal size. To ensure a constant reaction volume of 50 µl, the volume of DEPC-water was changed accordingly for each sample.

Finally all samples were run with the primers for IL-1β, IL-8, TNFα, COX-2 and GR. Since the same amount of RNA was used for all samples, the size of the band could be used as a semi-quantitative measure for the degree of activation of the respective genes.

3.3.4.5 Controls

For each RT-PCR reaction DEPC water without RNA served as negative control. To ensure DNA free samples, all samples underwent a DNAse digestion, and a PCR omitting the reverse transcription step was run.

3.3.5 *In vitro* inflammation model for canine corneal cells and cornea equivalents

The following experiments were conducted with single cell cultures of endothelial cells, keratocytes, epithelial cells and RCE cells as well as with the cornea equivalents.
The single cell cultures were seeded in 96-well plates and grown to confluence (as described in chapter 3.3.1.9). The stimulating agents and/or the dexamethasone treatment were added in 100 µl of culture medium.
The cornea equivalents were cultured over the course of 5 weeks as described above (chapter 3.3.1.8). The substances for the stimulation and the treatment of the equivalents were dissolved in 50 µl of culture medium and carefully added onto the epithelial layer of the equivalents. The PGE_2 concentration at the end of each experiment was measured in the culture medium beneath the equivalents.

3.3.5.1 Stimulation with LPS

Corneal cells and equivalents were stimulated with LPS (E.coli, strain 0111:B4) to provoke an inflammatory-like reaction.
Initially, dose-finding studies were performed to elucidate the optimal LPS concentration and stimulation time. The task was to determine the lowest concentration of LPS that led to a marked increase in PGE_2. LPS concentrations of 1, 10, 30 and 100 µg/ml were tested over the course of 48 h. The optimal concentration was found to be 100 µg/ml for the single cells and 10 µg/ml for the equivalents with a stimulation time of 24 h. These conditions were applied for all further experiments.
Dexamethasone was added simultaneously to the LPS stimulated cells at concentrations of 0.01 µg/ml and 1 µg/ml. Cells treated with LPS alone served as positive controls (C_{LPS}), cells treated with pure culture medium served as negative controls (C_0). After 24 hours of incubation the supernatant was collected and PGE_2 concentration determined using an ELISA kit according to the manufacturer's protocol as described in chapter 3.3.1.10.
To study the effects of stimulation and dexamethasone treatment on gene expression, the primary canine corneal cells were seeded in 48-well plates and experiments repeated under the above described conditions. After the collection of the cell culture supernatant for PGE_2-measurments, the cells were rinsed with sterile PBS and lyzed using peqGold TriFast. Cell

lysates were stored at – 80 °C until RNA isolation was performed as described in chapter 3.3.4.1.

3.3.5.2 Irritation with SDS

Corneal cells and equivalents were irritated with 0.001 % SDS. This concentration was chosen on the basis of a study conducted by ZORN-KRUPPA et al. (2004) who found this concentration to be the highest concentration without negative affects on cell viability. Nevertheless, dose finding studies were performed on the primary canine corneal cells using 0.01 %, 0.005 % and 0.001 % SDS. Also a viability study was performed with keratocytes using 0.5 %, 0.25 %, 0.05 %, 0.025 %, 0.02 %, 0.01 %, and 0.001 % SDS.

Again, to measure the effects of dexamethasone, stimulated cells and equivalents were treated with 0.01 µg/ml and 1 µg/ml dexamethasone, respectively. Keratocytes, endothelial cells and RCE cells were pre-treated with dexamethasone for 2 h. Epithelial cells were simultaneously treated with SDS and dexamethasone. After one hour of incubation the SDS was removed, the cells or equivalents rinsed with sterile PBS and fresh culture medium with or without dexamethasone added for another 23 h. The cell culture supernatant was collected and the PGE_2 concentration measured.

3.3.5.3 Stimulation with IFNγ and TNFα

Epithelial cells were also stimulated using 10 µg/ml and 100 µg/ml of IFNγ as well as 100 µg/ml and 200 µg/ml TNFα (canine). Again, the effect of the stimulation was measured in the culture medium supernatant after 24 h of incubation through the detection of PGE_2. To test whether dexamethasone has an effect on stimulation with theses two substances, epithelial cells stimulated with the higher concentration of both stimuli were also simultaneously treated with 1 µg/ml dexamethasone.

3.3.5.4 Viability testing

To evaluate the viability of the single cells after the stimulation, and thus ensure that the PGE_2 concentration in the culture medium is not due to cell death, a modified MTT-test was performed at the end of each experiment as described in chapter 3.3.1.6.
This viability test was also performed with a limited amount of cornea equivalents (n = 9). Most equivalents were not formally tested after the experiments, as that would have interfered with the histological and immunohistological examinations. Instead, the viability of the equivalents was judged microscopically (cell morphology, contraction of collagen layer) before and after the inflammation experiments.

3.3.6 Statistical analysis

For the canine corneal cells, the main experiments with LPS were conducted on cells from 5 – 8 different dogs for each cell type. The experiments were performed on at least two different days. The main SDS experiments were repeated at least twice and for each cell type, an average of cells of 5 different dogs was used. Stimulation of RCE cells was conducted on two distinct passages with n = 3. PGE_2-ELISA samples were usually measured in duplicates.
The LPS stimulation of the cornea equivalents was conducted on a mean of 6 equivalents per stimulus-/treatment-group. The SDS-irritation of the equivalents was only conducted on equivalents constructed with RCE cells and two equivalents per stimulus were tested. To enable better comparison of the experiments using the canine equivalents, results of the PGE_2 concentration are expressed in percent, normalized to the negative control (C_0 = 100 %).
For statistical calculation of differences in the PGE_2-production in the single cells, a Dunn's test was performed after t-test revealed significant differences. For the cornea equivalents One-Way ANOVA was performed with a Bonferroni post-test. In both tests a p-value < 0.05 was considered significant.
Statistical calculations were performed with GraphPad Prism® (version 4.01, GraphPad Software Inc., Sandiego, USA).

4 Results

4.1 Cell culture of corneal cells

4.1.1 Isolation of canine corneal cells

Canine corneal cells were enzymatically and mechanically isolated and taken into culture. The three cell types were morphologically distinct when viewed under the phase contrast microscope (Fig. 13 **A**, **C**, **D**). Each cell type exhibited the typical morphological growth characteristics: the endothelial cells grew as small, hexagonal cells, the fibroblasts showed a spindle shaped morphology and the epithelial cells grew in a cobblestone-like pattern.
The purity of each cell population was ensured by the specific isolation method and the typical morphological appearance of the cells, respectively. Only cells that were considered to be monocultures were kept in culture to establish a single cell pool.

4.1.2 Culture of corneal cells

Endothelial cells were passaged for the first time after 4 days, epithelial cells and fibroblasts after 8 and 10 days, respectively. Thereafter, cells could be passaged every three to five days with an average number of 4 - 6 passages without changes in cell morphology (this was demonstrated with the example of the keratocytes in Fig. 14).
RCE cells were cultured according to the supplier's recommendations, and grew in an epithelial cell-like appearance (Fig. 15), were highly proliferative and could readily be passaged numerous times without changes in morphology.

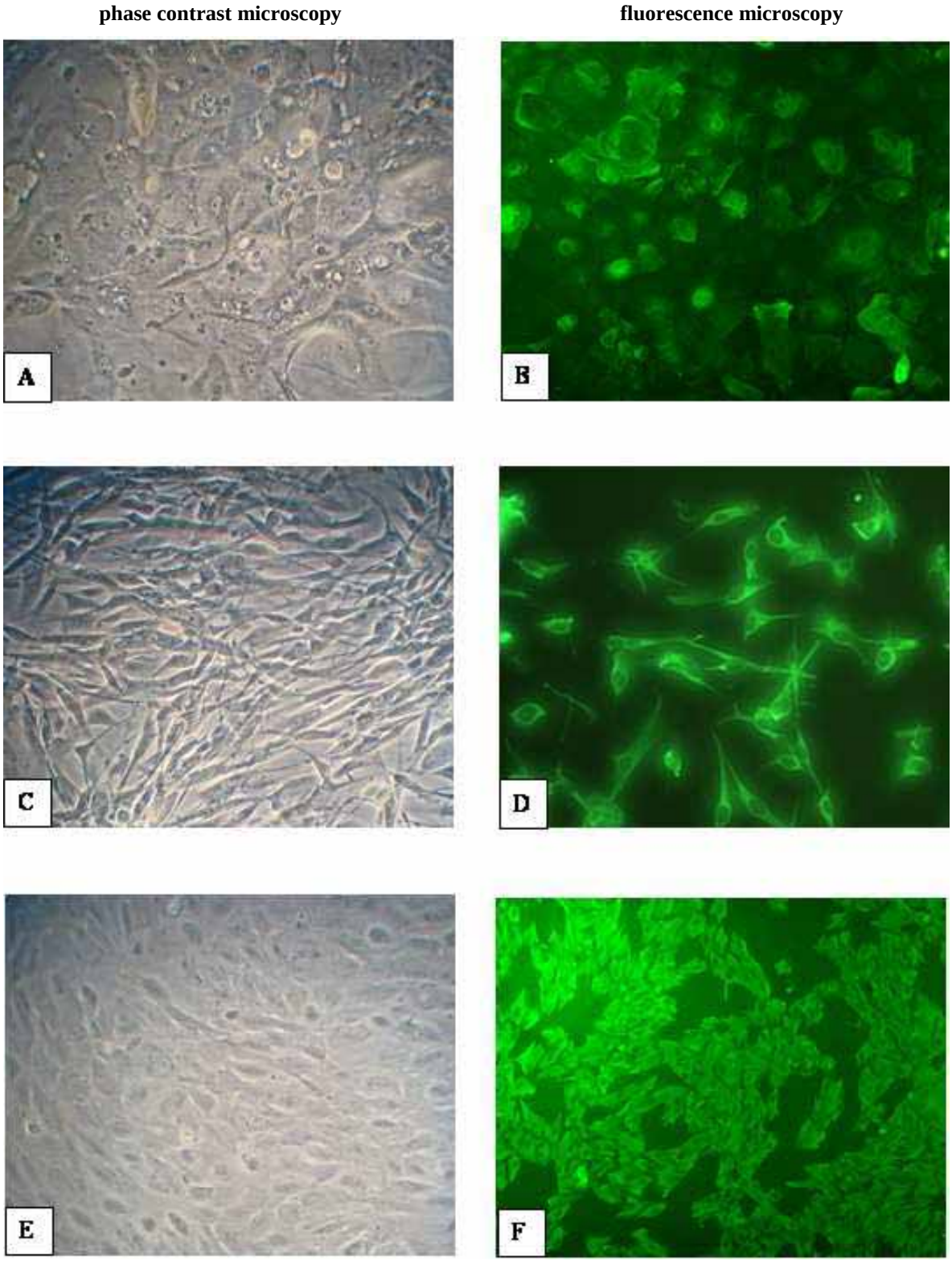


Fig. 13 Verification of the canine corneal cells: phase contrast microscopy (A, C, E) and fluorescence microscopy of immunocytological stains (B, D, F). Epithelial cells (A, B) have a cobblestone-like growth and stain for cytokeratin (B), whereas the keratocytes (C, D) show a typical spindle-like morphology and are vimentin positive (D). Endothelial cells (E, F) exhibit a heterologous, polygonal structure and are vimentin positive (F).

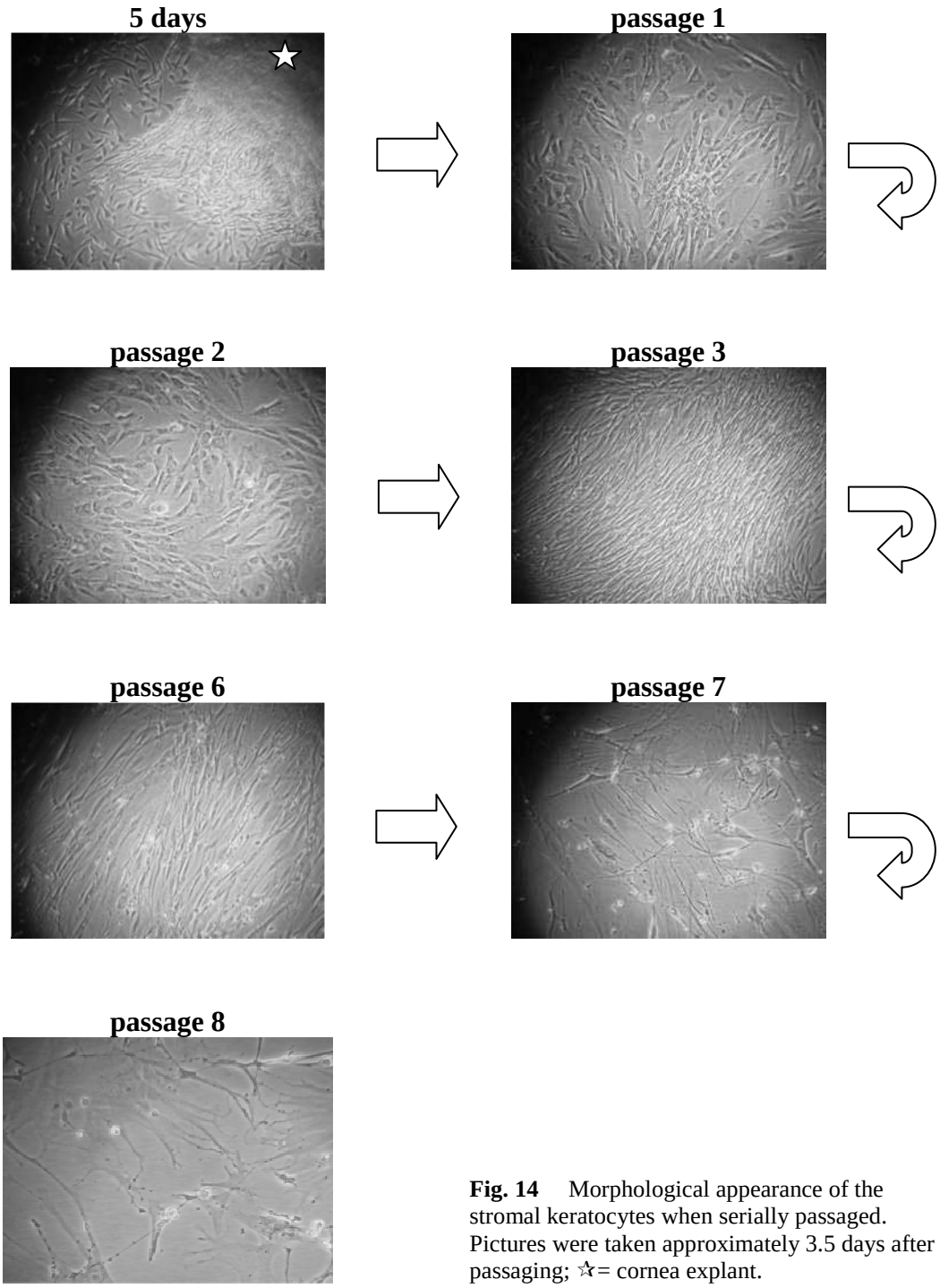
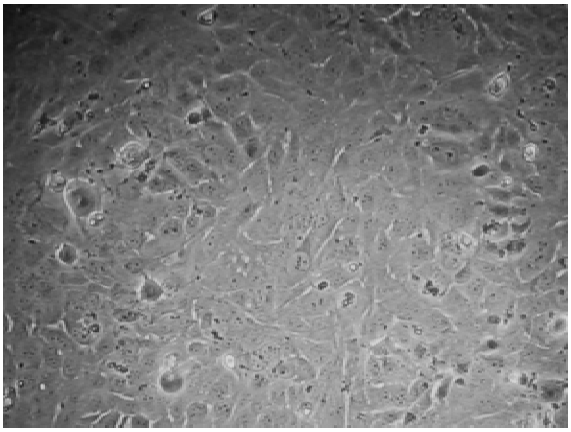


Fig. 14 Morphological appearance of the stromal keratocytes when serially passaged. Pictures were taken approximately 3.5 days after passaging; ☆= cornea explant.

canine epithelial cells



RCE cells

Fig. 15 Morphologic comparison of primary canine corneal epithelial cells (Williams E culture medium) and RCE cells. Phase contrast microscopy of confluent cultures.

4.1.3 Cell count, viability and proliferation

The initial absolute cell count was only determined for epithelial cells. Endothelial cells and keratocytes were cultured in 6-well plates until confluent and then passaged and the first cell count determined at passage 1. When looking at absolute numbers of the cell count of the different cell types between isolation and passage 3, it was noted, that endothelial cells and keratocytes are highly proliferative, thus resulting in increasing numbers of absolute cell counts with each passage (Fig. 16). The keratocytes were only kept in culture for two passages and then cryoconserved due to high proliferation and thus high absolute cell counts. The epithelial cells (Fig. 16, culture medium: DMEM + EGF) were difficult to passage as can be seen by the decreasing mean value of absolute cells with a high variation for the first passage. These difficulties manifested as a reluctance of the cells to separate and detach from the culture flask, which led to increased trypsin times with a negative influence on cell viability.

When studied over the course of 144 h, the three primary corneal cell types showed different growth characteristics (Fig. 17 and Fig. 18). The proliferation of endothelial cells (Fig. 17 and Fig. 18, **3**) and keratocytes (Fig. 17 and Fig. 18, **2**) followed a (typical) sigmoid pattern. It was noted, that the overall proliferation of keratocytes was higher than that of the endothelial cells. Contrarily, the epithelial cells (Fig. 17 and Fig. 18, **1**) showed only a slight increase in proliferation over the course of seven days. This “steady state” growth rate combined with difficulties in passaging led to an overall decrease in absolute cell numbers (Fig. 16). To establish a cell pool for later use, endothelial cells and keratocytes were cryoconserved and stored at – 196 °C. Upon thawing, the cells had an overall decreased rate of proliferation, which is especially noticeable in the keratocytes (Fig. 17 and Fig. 18). In contrast to the growth curves of the fresh cells, endothelial cells and keratocytes now show a similar rate of proliferation (Fig. 17 (thawed cells)). Nevertheless, both cell types retain their sigmoid pattern of growth.

The data used for the illustration of the above results is listed in the appendix.

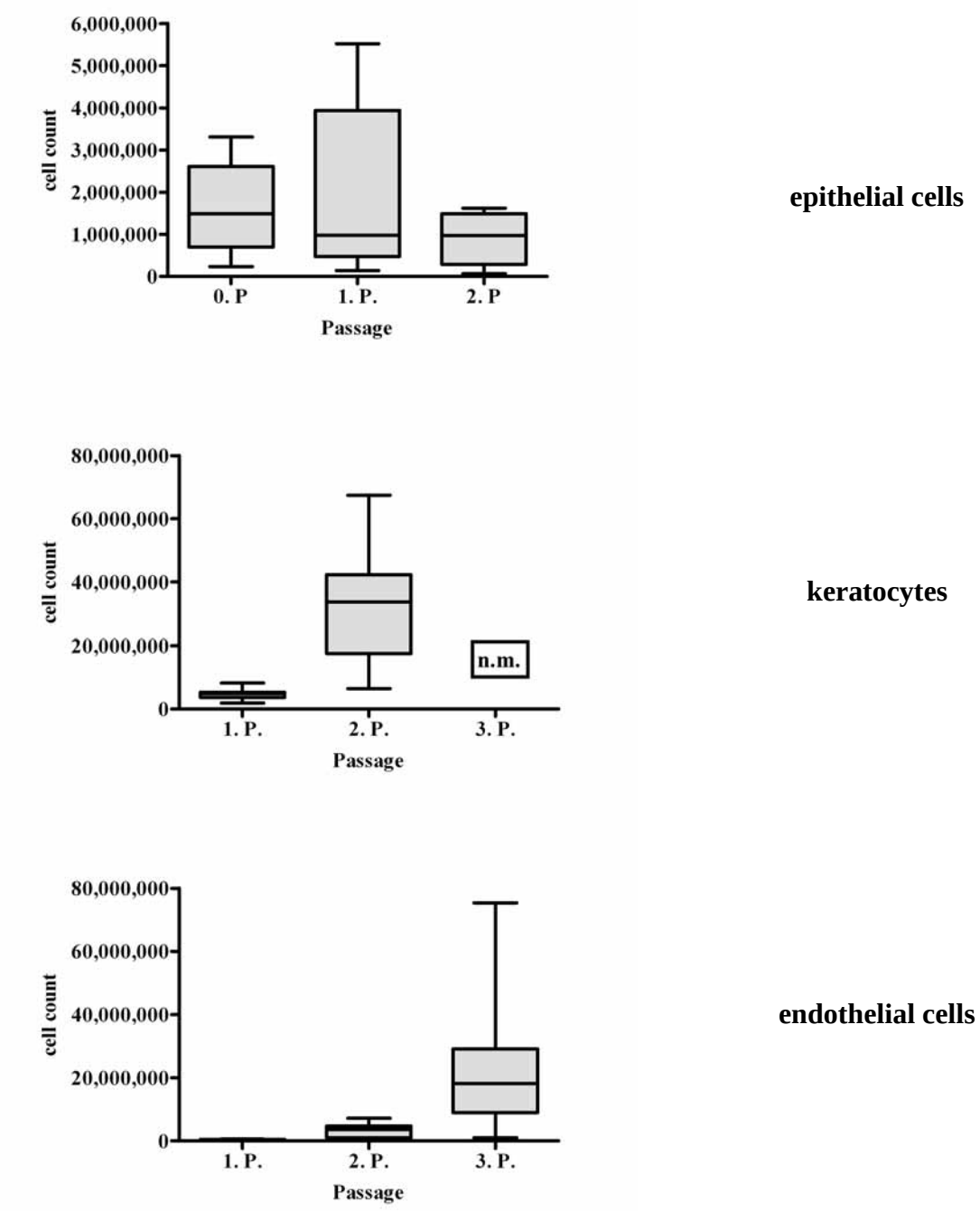
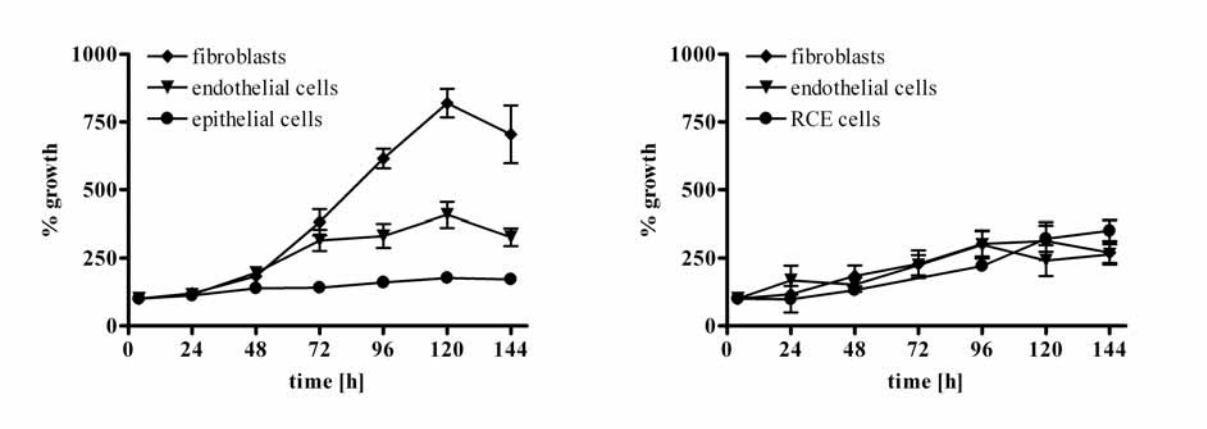


Fig. 16 Cell counts for the three primary corneal cell types between isolation and passage 3. Epithelial cells (n = 6) are difficult to passage with a high proliferation, whereas keratocytes (n = 19) and endothelial cells (n = 16) are highly proliferative over the 2 – 3 passages studied. Cell count depicts the absolute number of cell that were isolated and cultured from the corneas. Initial isolation cell count was only determined for epithelial cells. Seeding density as described in chapter 3.3.1.2.; Box-plot (median with range); n.m. = not measured.



fresh cells **thawed cells**

Fig. 17 Growth curves for the three primary canine corneal cells and the RCE cells using a modified MTT-test over the course of 144 h. Cell growth is expressed in percentage compared to 4 hour-control (= 100 %); mean and SD, n = 5 – 6 dogs (with 6 measures each) per cell type, RCE measured for two distinct passages (p13 and p20).

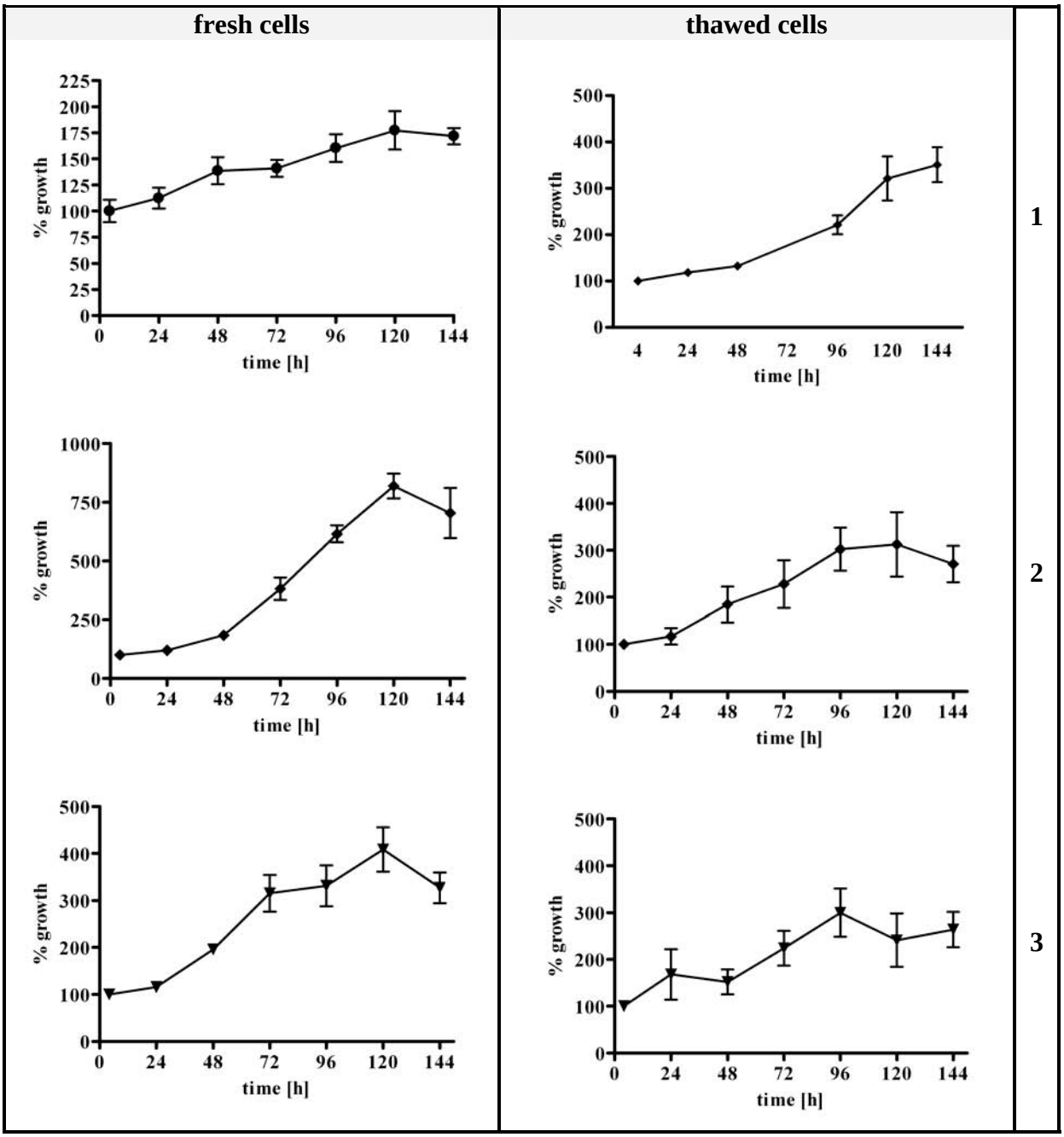


Fig. 18 Growth curves for the three primary canine corneal cells and the RCE cells using a modified MTT-test over the course of 144 h. Cell growth is expressed in percentage compared to 4 hour-control (= 100 %); mean and SD, n = 5 – 6 dogs (with 6 measures each) per cell type, RCE measured for two distinct passages (p13 and p20). **1** = canine epithelial cells (fresh) and RCE cells (thawed), **2** = keratocytes, **3** = endothelial cells.

4.1.4 Epithelial cell growth

Epithelial cells showed the lowest proliferation rate of the three cell types and were the most difficult to culture beyond 4 passages. To elucidate the influence of the culture medium on the cell morphology, proliferation and ability to serial passaging, different media were tested. The influence on the cell morphology is depicted in Fig. 20, the influence on cell proliferation is shown in Fig. 19. DMEM with EGF (medium 2) was the initial medium tested. The cells grew in islands and were rather slow in reaching confluence, but showed a homologous morphology of cobblestone-like cells. Cells cultured with DMEM: Ham12 as described by most authors for human epithelial cells (BOURCIER et al. 2000; KIM et al. 2004) also grew initially in islands. Here, cells exhibited a more heterologous morphology (mostly small, pentagonal cells, mixed with occasionally larger, vacuolic cells). Cells cultured with this culture medium were the most difficult to passage, as cells were reluctant to detach from the culture plates and/or flasks. Finally, Williams E medium was tested. Cell appearance was comparable to those cultured with DMEM + EGF, but showed a higher proliferation (between 48 and 120 hours) and had the best passaging-characteristics. Therefore, this culture medium was chosen for the final experiments and used for most cornea equivalents.

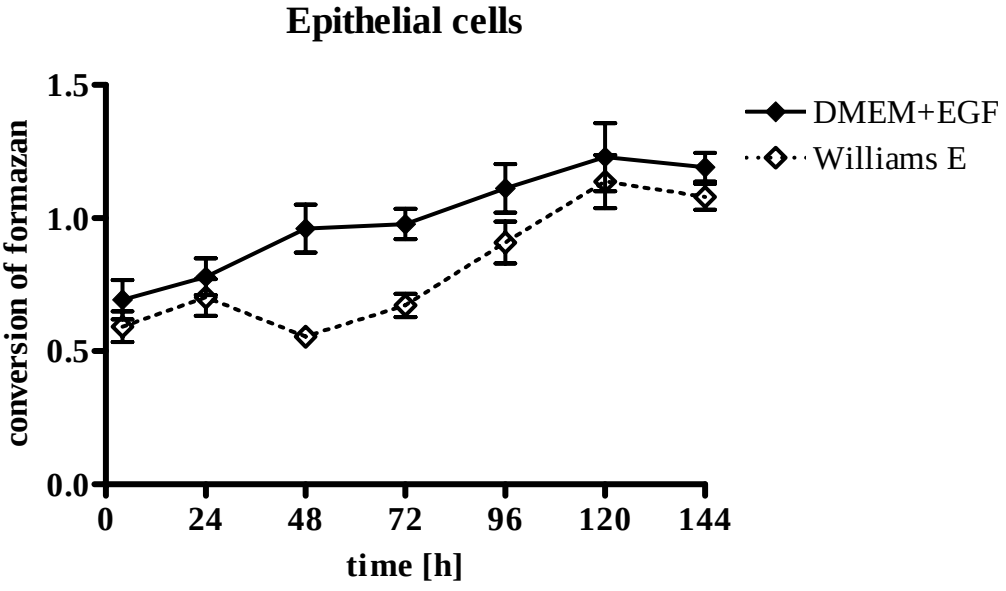


Fig. 19 Influence of the culture medium on the proliferation of canine epithelial cells over the course of 144 h using a modified MTT-test.

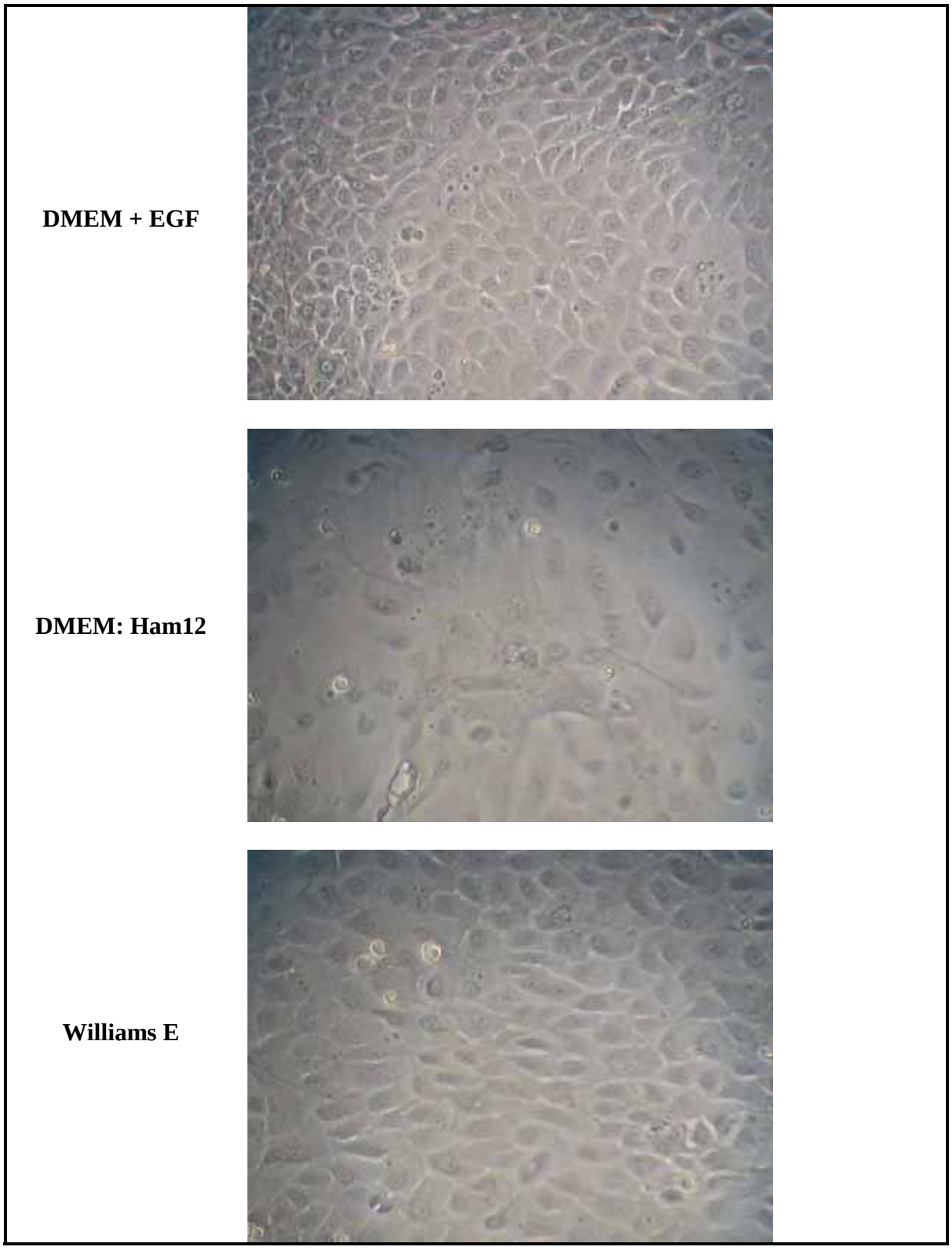


Fig. 20 Influence of the culture medium on the morphology of primary canine corneal epithelial cells.

4.2 Verification of corneal cells

4.2.1 Immunocytochemistry

Immunocytochemistry was performed to verify the morphological distinction of the three cell types. Anti-vimentin was used for staining of the endothelial cells (Fig. 13, **F**) and keratocytes (Fig. 13, **D**). Adding anti-cytokeratin to these cells did not result in staining of the cells. Incubation of anti-cytokeratin with epithelial cells resulted in staining (Fig. 13, **B**) whereas the isotype control with the anti-vimentin antibody was negative.

4.2.2 Western Blot

Using western blot analysis, the vimentin protein was visualized in endothelial cells and fibroblasts at the specific molecular weight of 58 kD (Fig. 21, **2 B/C**). Epithelial cells did not contain vimentin protein (Fig. 21, **2 A**) but the cell type specific cytokeratin protein, which has a specific molecular weight between 52 and 68 kD (Fig. 21, **1 A**). Endothelial cells and fibroblasts lack this protein (Fig. 21, **1 B/C**).

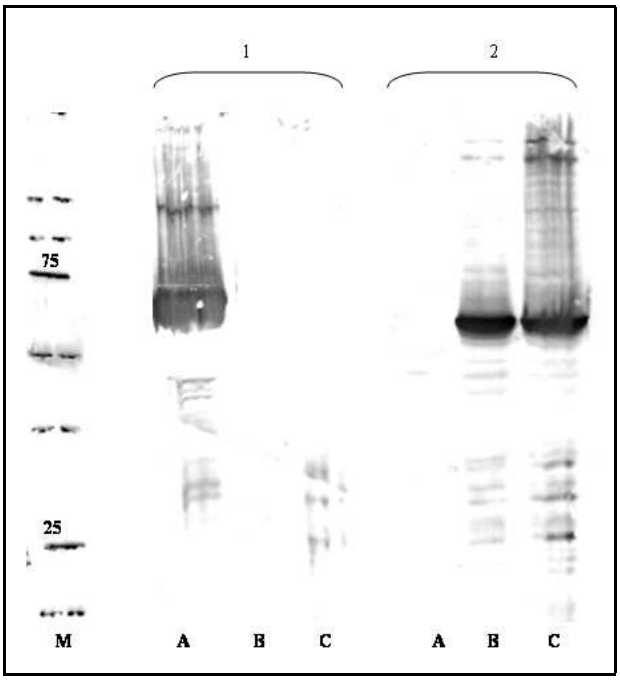


Fig. 21 Representative western blot analysis of the three cell types using anti-cytokeratin (**1**) and anti-vimentin (**2**) antibodies. Epithelial cells (**A**) are cytokeratin positive and vimentin negative. Endothelial cells (**B**) and keratocytes (**C**) are vimentin positive and cytokeratin negative. M = molecular weight marker (kDa).

4.3 Cornea equivalent

4.3.1 Culture

A schematic illustration of the construction of the canine cornea equivalent is given in Fig. 11. Each cell layer reached confluence after one week of incubation as shown in Fig. 11 (**A – C**), allowing for the addition of the next cell layer. After the addition of the epithelial cells it was increasingly difficult to view the underlying layers microscopically due to the opacity of the stromal layer. Cornea equivalents were routinely macro- and microscopically checked for bacterial and fungal contamination and only equivalents without fungal contamination were kept in culture over the period of 5 weeks.

At the end of the 5-weeks culture period, most of the equivalents were highly contracted to less then a fourth of the original size (Fig. 22).

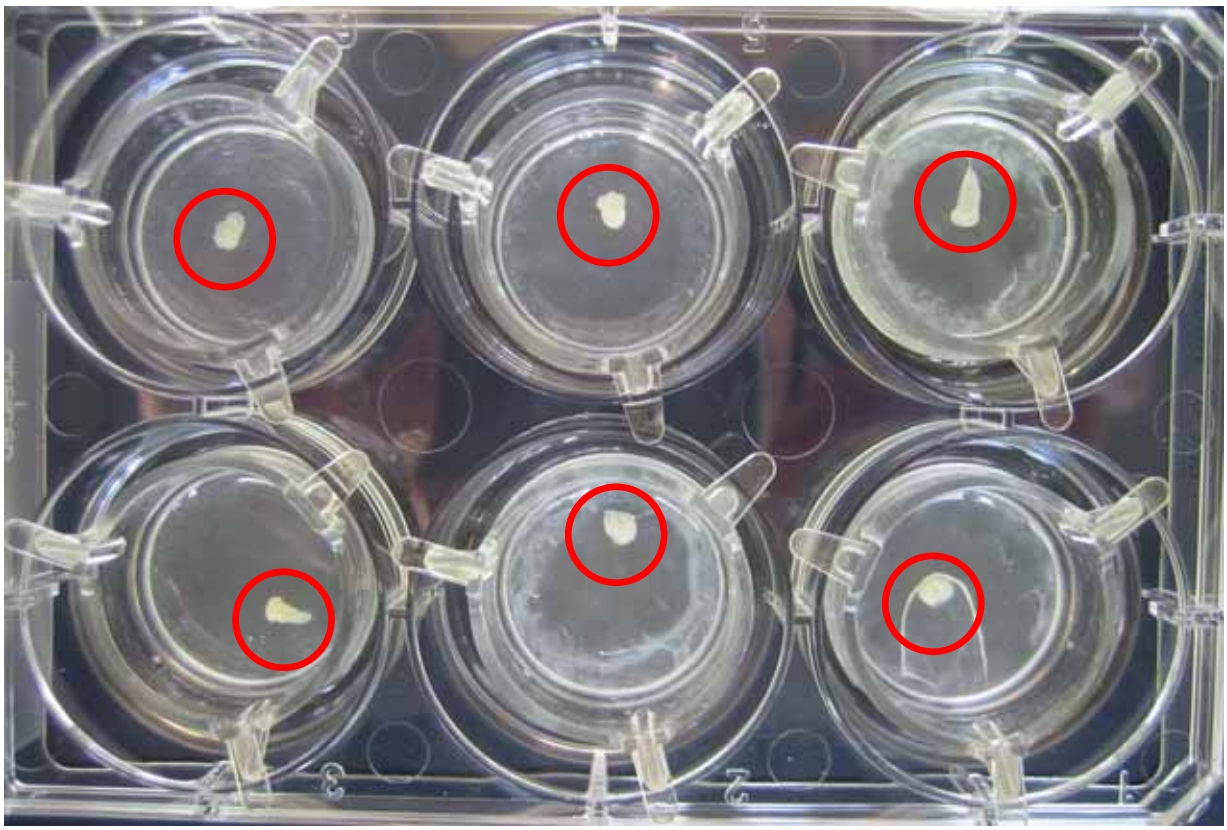


Fig. 22 Picture of the canine cornea equivalents at the end of the 5-week culture period. The red circles highlight the contracted equivalents.

4.3.2 Histology

Histology was performed on a random selection of cornea equivalents to study their cellular structure as shown in Fig. 23 and Fig. 24. The equivalents consist of a monolayer of endothelium (Fig. 23 and Fig. 24 **D**; sometimes not visible in histological pictures because of the adherence of these cells to the insert membrane), followed by a comparatively thick layer of keratocytes in the collagen matrix (Fig. 23 and Fig. 24 **C**). In some parts of the equivalents, these cells are aligned in parallel to the surrounding layers, but there are also areas present, where the keratocytes are irregularly distributed. On the outside a multilayered epithelium is present (Fig. 23 and Fig. 24 **B**). There is a morphological difference between the primary canine epithelial cells (Fig. 23 **B**) and the rabbit epithelial cell line (Fig. 24 **B**) as the latter are generally smaller, rounder and rather homogenous compared to the canine cells, which developed into two layers: columnar basal and flattened superficial cells.

Besides the described structure of the equivalents, a few variations were observed. A high activity of keratocytes led to a marked contraction of the collagen gel matrix in some of the equivalents, which occasionally resulted in a folding of the stromal layer over the epithelium.

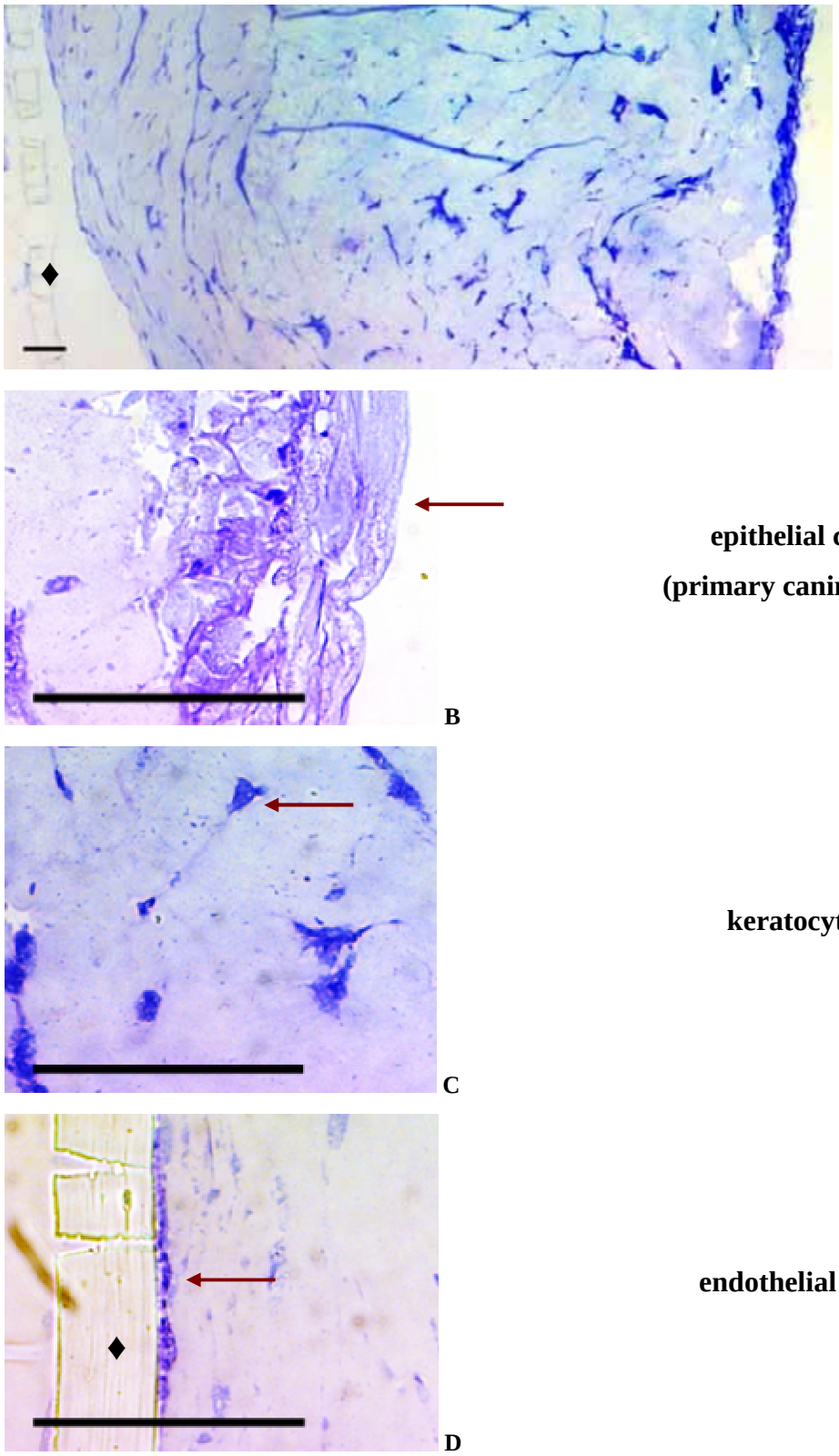


Fig. 23 Transverse section through a cornea equivalent (**A**, 100 x) and close up of the three different cell types (**B – D**, 400 x), toluidine blue stain; epithelial cells are primary canine cells; arrows point to the cells of interest, ♦ = insert membrane; bar = 50 µm .

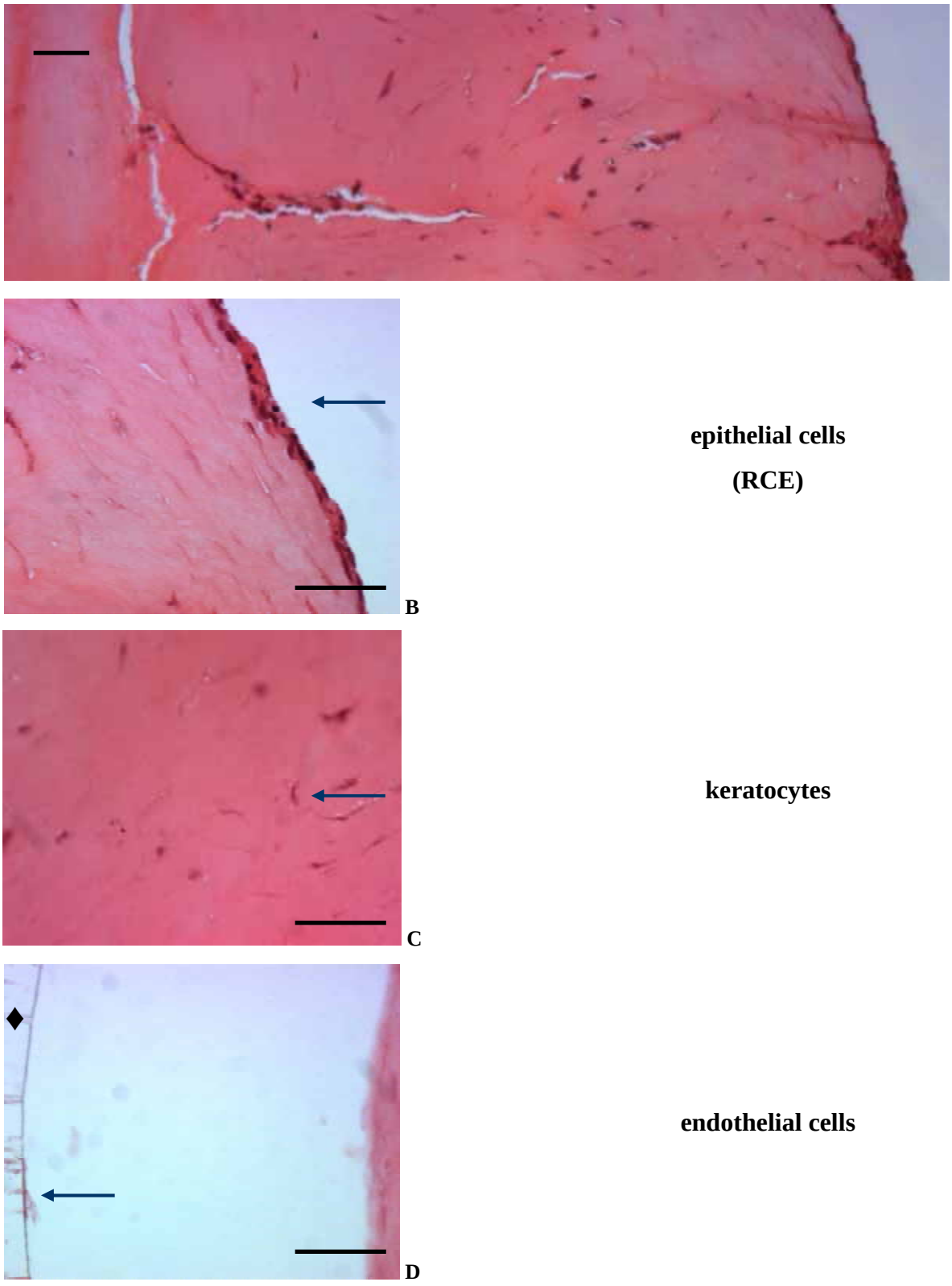


Fig. 24 Transverse section through a cornea equivalent (**A**, 100 x) and close up of the three different cell types (**B – D**, 400 x), H&E stain; epithelial layer consists of RCE cells. Arrows point to the cells of interest, ♦ = insert membrane; bar = 50 µm.

4.3.3 Immunohistochemistry

Selected equivalents were additionally studied using immunohistochemistry as demonstrated in Fig. 25 and Fig. 26. When looking at the canine epithelial cells in the equivalents (Fig. 25, left column), it can be noted, that the cytokeratin antibody stains some cells rather prominently (**3**), while the rest of this layer is stained diffusely. Anti-Vimentin leads to a weaker, only diffuse staining of the epithelium (**2**). In contrast to this result, the equivalents constructed with RCE cells are only weakly positive for cytokeratin in the epithelium layer (Fig. 25, right column, **3**). Instead, the anti-vimentin stains not only keratocytes and endothelial cells as expected (Fig. 26, left column, **2**), but also epithelial cells (Fig. 25 and Fig. 26, right column, **2**).

Positive and negative controls were performed for all three antibodies. Representative pictures of the control samples are given in Tab. 26 (appendix). The results for the staining with the anti-GR antibody to detect the glucocorticoid receptor are described in chapters 4.4.2 and 4.4.3.

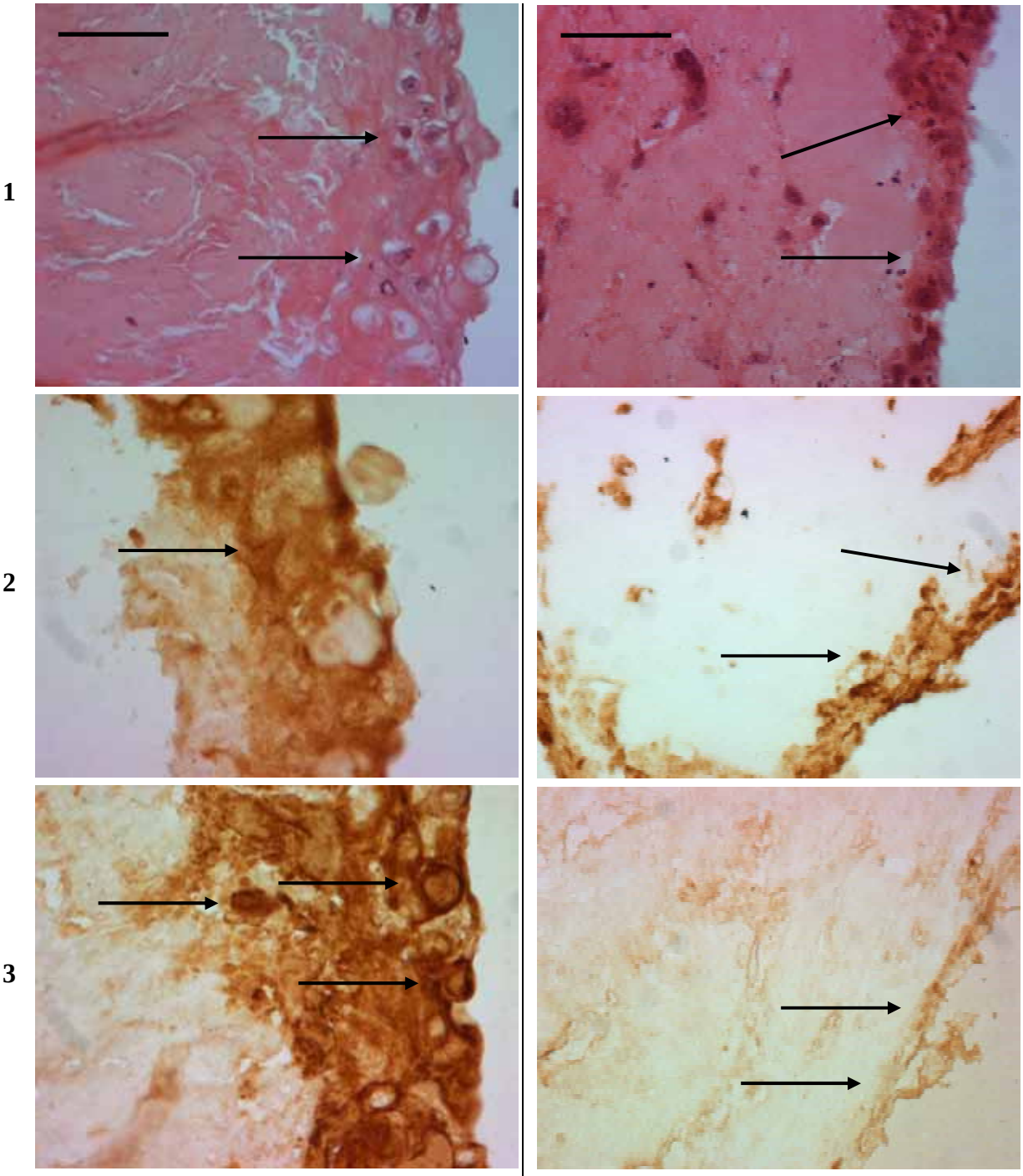


Fig. 25 Cornea equivalents stained with HE (**1**), anti-vimentin (**2**) and anti-cytokeratin (**3**).Close up of the epithelium with underlying stroma. Left column: primary canine epithelial cells; right column: RCE cells; magnification = 400 x; bar = 50 µm. Arrows point to the cells of interest as indicated at the bottom of each column.

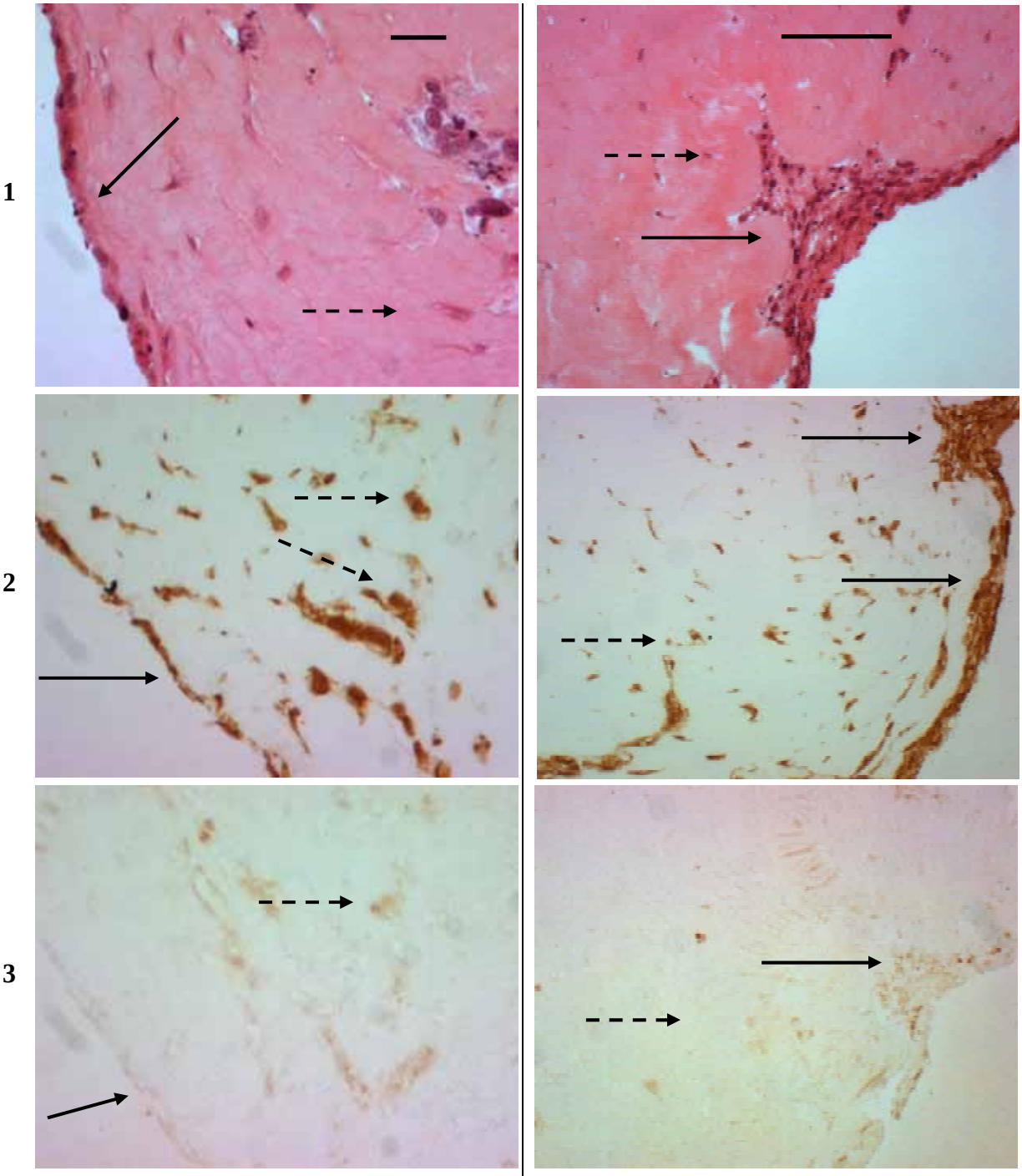


Fig. 26 Cornea equivalents stained with HE (**1**), anti-vimentin (**2**) and anti-cytokeratin (**3**). magnification: left column = 400 x, emphasis on endothelial cells and stromal layer, right column = 200 x, emphasis on epithelial cells and stromal layer of the same equivalent; bar = 50 µm. Arrows point to the cells of interest as indicated at the bottom of each column (short, dotted arrows always point to keratocytes).

4.4 Detection of the GR

4.4.1 GR mRNA in single cells

Using RT-PCR the glucocorticoid receptor was expressed in all three canine corneal cell types, seen as a 511 base pair product (Fig. 27 to Fig. 30).

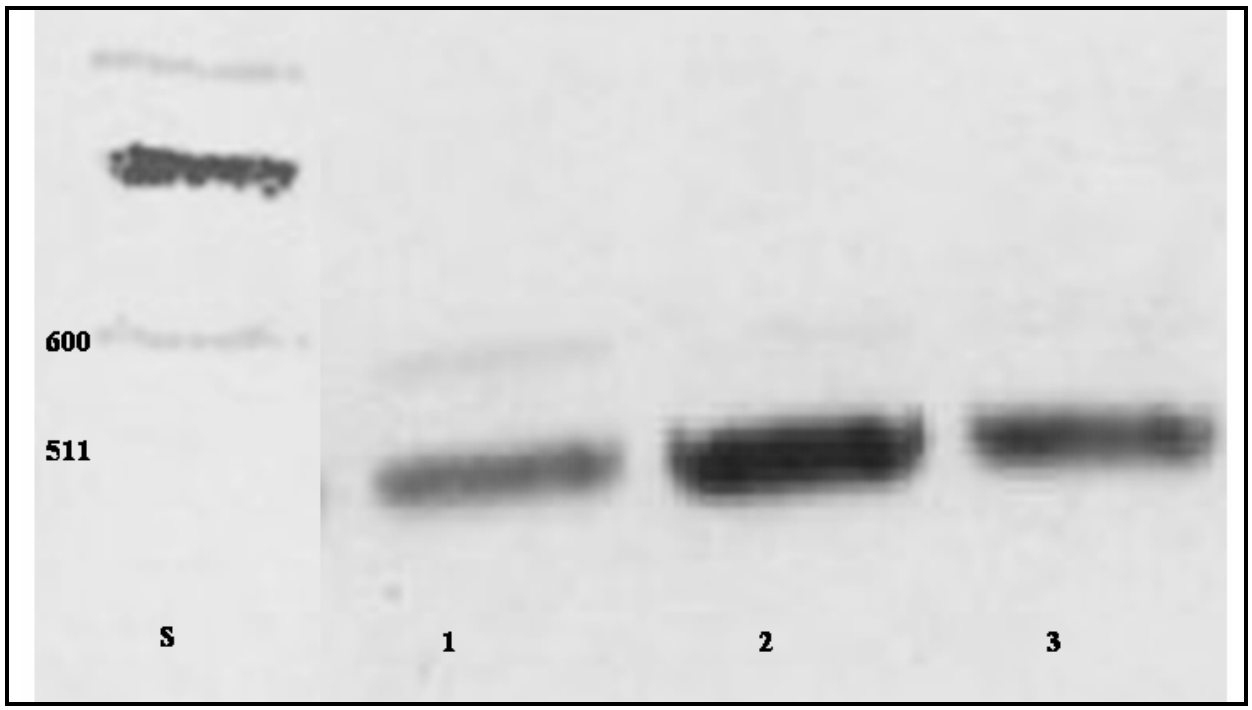
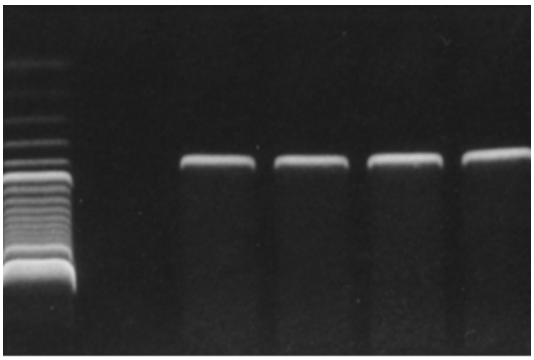


Fig. 27 RT-PCR of cultured epithelial cells (**1**), keratocytes (**2**) and endothelial cells (**3**) using a GR primer. All three cell types express the mRNA for the GR as a 511 bp product; S = DNA ladder (bp).

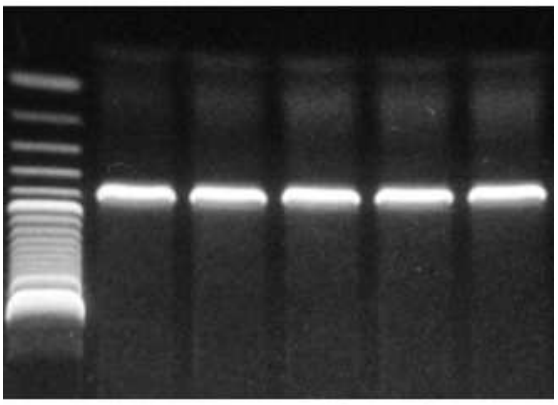
4.4.2 GR protein in corneal cells

Western Blot was performed on all three corneal cell types to detect the GR protein. Unfortunately, no suitable primary antibody was available to detect the GR in canine tissue. Different lysis-buffers were used (TriFast and RIPA) and different detection systems were tested (AP and HRP-conjugated secondary antibodies) to exclude methodical errors. Nevertheless, the attempts of the detection had to be discontinued and the results of the preliminary studies were omitted.



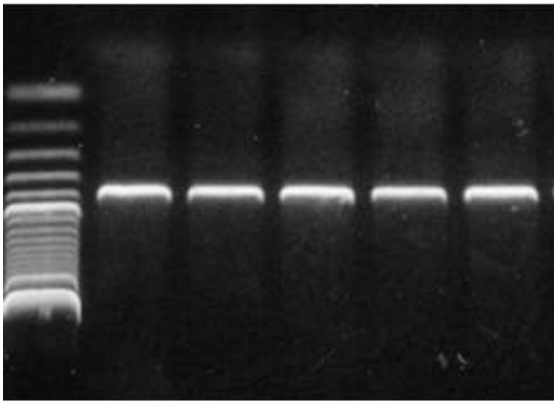
epithelial cells

Fig. 28 Dexa. [µg/ml]



keratocytes

Fig. 29 Dexa. [µg/ml]



endothelial cells

Fig. 30 Dexa. [µg/ml]

Fig. 28 - Fig. 30 GR mRNA expression in unstimulated, stimulated and stimulated + dexamethasone treated cells (representative gels). All cells express the GR regardless of the environmental situation. S = DNA ladder (bp; upper brigh band = 600bp, lower bright band = 1200 bp); C_0 = unstimulated control; C_{LPS} = stimulated control; 1, 0.1, 0.01 = LPS-stimulated treatment group (indicating the respective dexamethasone concentration in µg/ml).

4.4.3 GR protein in cornea equivalents

As shown for the mRNA of three different corneal cell types in the single cell culture in chapter 4.4.1, the GR in its nuclear localization can be detected on the protein level in all three cell types of the cornea equivalents (Fig. 31).

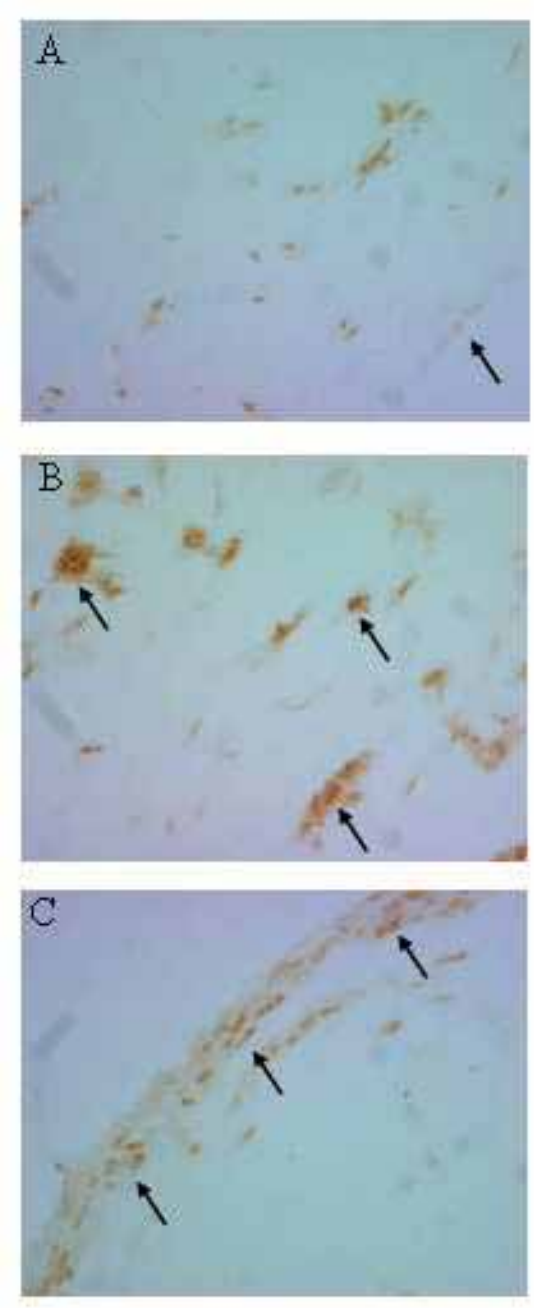


Fig. 31 GR expression in cornea equivalents on cross sections (400 x, representative sections). Immunohistochemistry using a polyclonal antibody. Arrows point to nuclear localization of the GR in endothelial cells (**A**), keratocytes (**B**) and epithelial cells (**C**).

4.5 *In vitro* inflammation model

In the following chapter the results for the experiments with the *in vitro* inflammation model are presented. The graphs and figures are based on the data listed in the appendix.

4.5.1 Stimulation of single cells

Viability study with SDS

To investigate the influence of SDS on the cell viability, corneal keratocytes were stimulated with increasing concentrations of SDS (0.001 – 0.5 %) and the viability measured using a modified MTT-test. The result is shown in Fig. 32. Only the highest concentrations (0.25 and 0.5 %) of SDS lead to a statistically significant reduction of formazan production. Nevertheless, a reduction is already noticeable at a concentration of 0.02 %. To ensure that the effect on the PGE_2 production is not due to cell death, only the low concentrations of SDS were used to determine the optimal stimulation in regard to PGE_2 production.

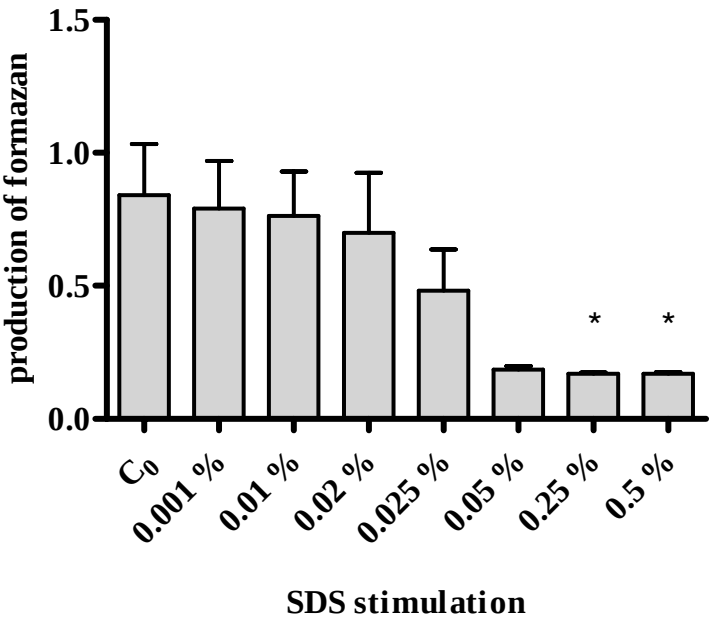


Fig. 32 Influence of different concentrations of SDS on the viability of canine corneal keratocytes. Mean and SD, * $p < 0.01$ (ANOVA with Dunn's post-test; n = 5; 6 measures each).

As demonstrated in Fig. 33, the lowest concentration of SDS that was used (0.001%) led to a marked increase of PGE_2. Therefore, this concentration was used for the following experiments.

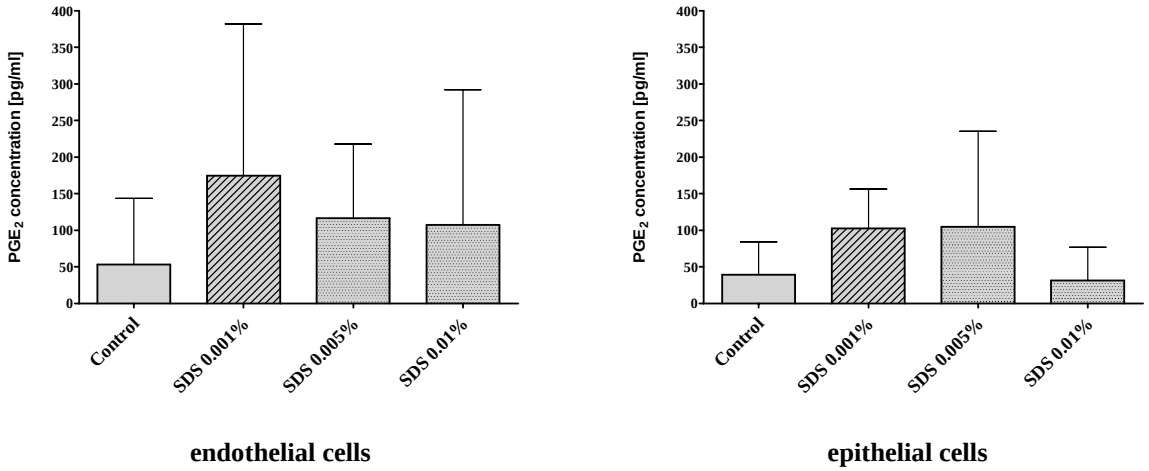
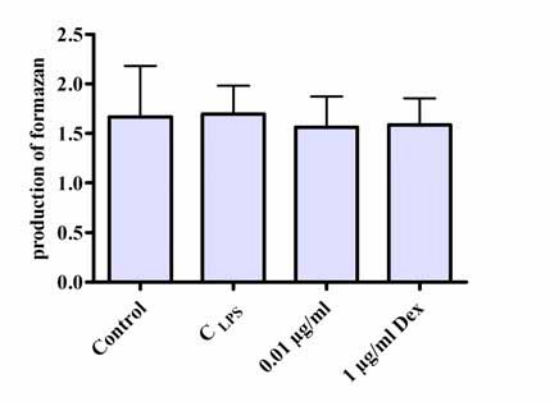


Fig. 33 PGE_2 concentration [pg/ml] after stimulation with three different concentrations of SDS. Mean and SD (epithelial cells n = 6, endothelial cells n = 3).

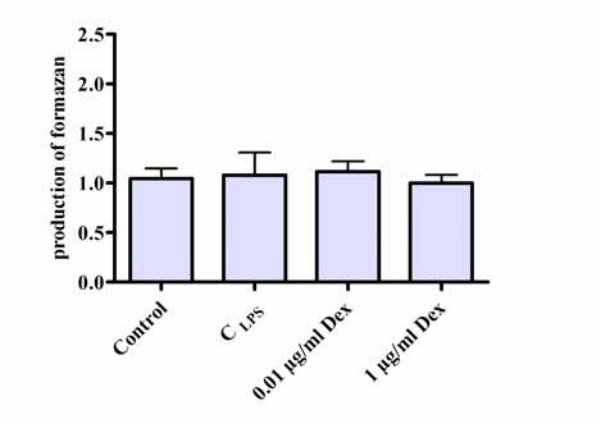
Viability testing after stimulation

Examination of the cell viability after each experiment by conversion of MTT showed no differences in the treatment groups (stimulated vs. treated) compared to the control cells. Fig. 34 to Fig. 36 give the results for LPS-stimulated cells, Fig. 37 and Fig. 38 give the results for SDS-stimulated cells. The viability of SDS-stimulated epithelial cells was only verified microscopically since cells were needed for gene expression studies (due to limited amounts of fresh cells). The morphology of the stimulated and treated cells was similar to that of the negative control, indicating that the viability was not affected by the experimental setting.



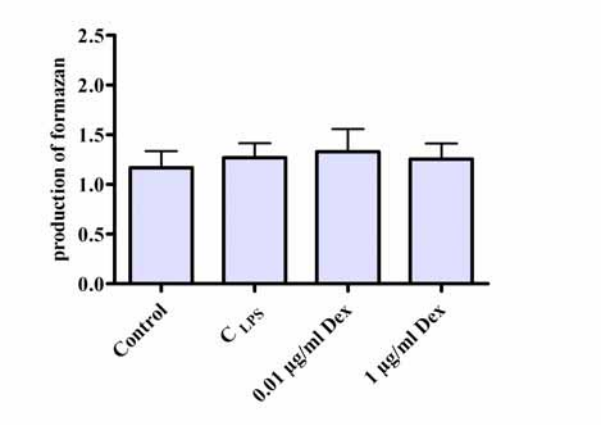
epithelial cells

Fig. 34



keratocytes

Fig. 35



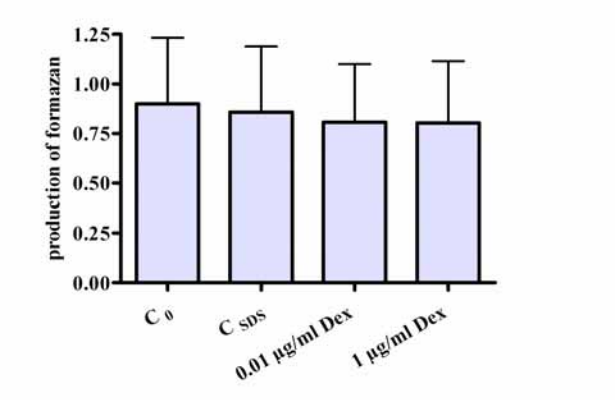
endothelial cells

Fig. 36

Fig. 34 - Fig. 36 Viability testing for all three major corneal cell types using a modified MTT-test after the stimulation with LPS with or without dexamethasone treatment. Mean and SD (n = 5 – 7 per cell type; mean of 6 values per n) .

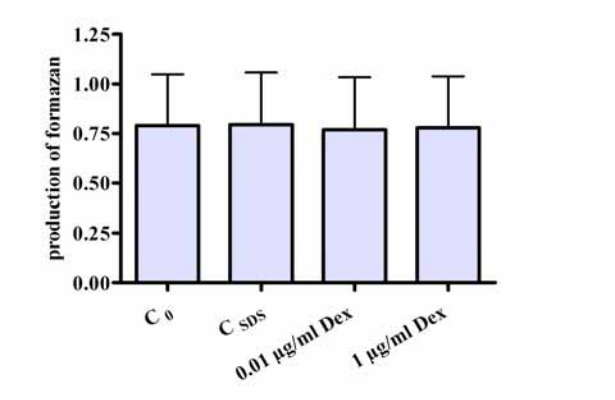
not measured

epithelial cells



keratocytes

Fig. 37



endothelial cells

Fig. 38

Fig. 37 and **Fig. 38** Viability testing for keratocytes and endothelial cells using a modified MTT-test after the stimulation with SDS with or without dexamethasone treatment. Mean and SD (n = 4 - 6 per cell type; mean of 6 values per n).

LPS

The results for the stimulation of the canine corneal cells and the RCE cells with LPS (100 µg/ml) +/- dexamethasone treatment are depicted in Fig. 39 to Fig. 42. Addition of LPS increased the PGE_2 production of canine corneal keratocytes and epithelial cells at the shown concentration (Fig. 39 and Fig. 40). This increase (C_0 to C_{LPS}) was statistically significant for epithelial cells but not for keratocytes. Endothelial cells did not show an increased PGE_2 production when stimulated with LPS (Fig. 41). For this cell type a high variance was measured within the negative control (C_0). The addition of dexamethasone led to a dose-dependent reduction of the PGE_2 production in keratocytes and epithelial cells. Compared to the positive control (C_{LPS}), 1 µg/ml dexamethasone significantly reduced the PGE_2-production in both cell types. The addition of dexamethasone to endothelial cells did not lead to a significantly reduced amount of PGE_2 in the culture medium. Nevertheless, a slight reduction after treatment with the high dose of dexamethasone was noted.

Comparison of the mean PGE_2 production in the stimulated group between the three cell types revealed approximately a two-fold higher production in endothelial and epithelial cells (approx. 2500 pg/ml) compared to keratocytes (approx. 1200 pg/ml).

The RCE cells reacted similarly to the endothelial cells, as stimulation with LPS did not led to an increase in PGE_2 in the culture medium (Fig. 42). Although not statistically significant, the addition of dexamethasone decreased the PGE_2 concentration compared to the negative control.

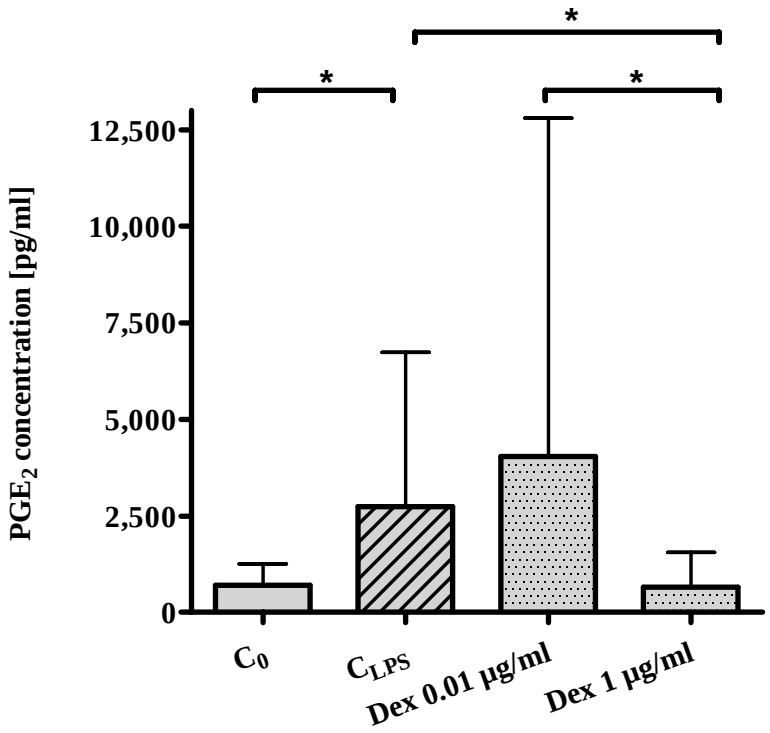


Fig. 39 PGE_2 concentration [pg/ml] in the culture medium after 24 h of stimulation with 100 µg/ml LPS and treatment with 0.01 or 1 µg/ml dexamethasone. LPS leads to an increased PGE_2 production by **epithelial cells** which can be reduced in a dose-dependent manner by the addition of dexamethasone. Mean and SD, * $p < 0.05$ (t-test).

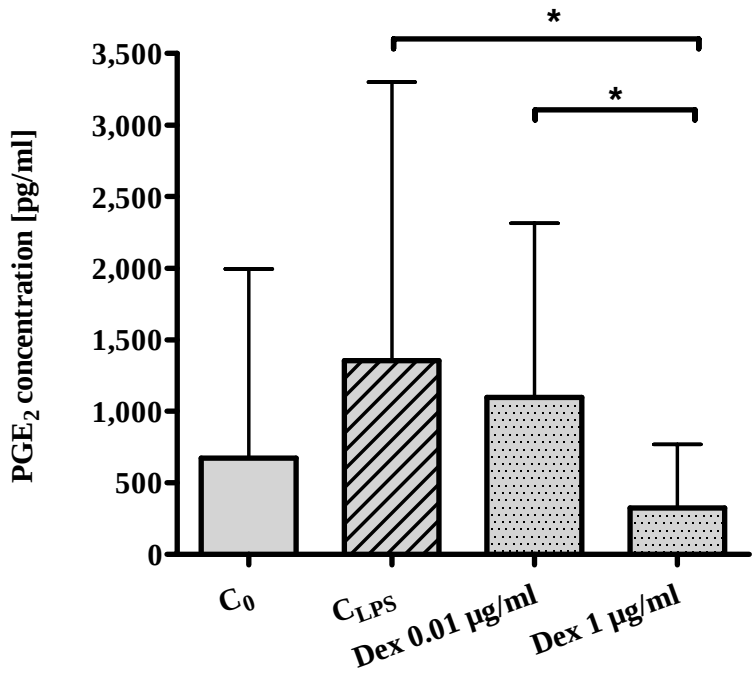


Fig. 40 PGE_2 concentration [pg/ml] in the culture medium after 24 h of stimulation with 100 µg/ml LPS and treatment with 0.01 or 1 µg/ml dexamethasone. LPS leads to an increased PGE_2 production by **keratocytes**, which can be reduced in a dose-dependent manner by the addition of dexamethasone. Mean and SD, * $p < 0.05$ (t-test).

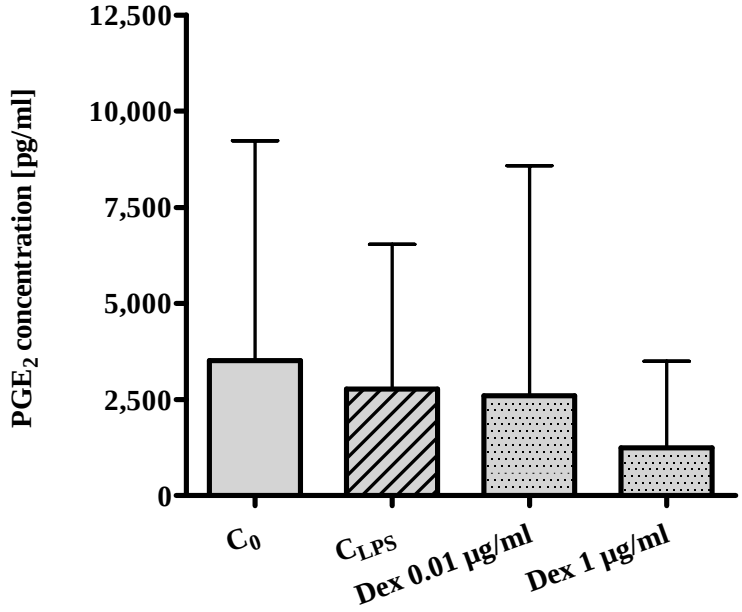


Fig. 41 PGE_2 concentration [pg/ml] in the culture medium after 24 h of stimulation with 100 µg/ml LPS and treatment with 0.01 or 1 µg/ml dexamethasone. **Endothelial cells** do not react to LPS-stimulation, but a high dose of dexamethasone reduces the relatively high basal PGE_2 production. Mean and SD.

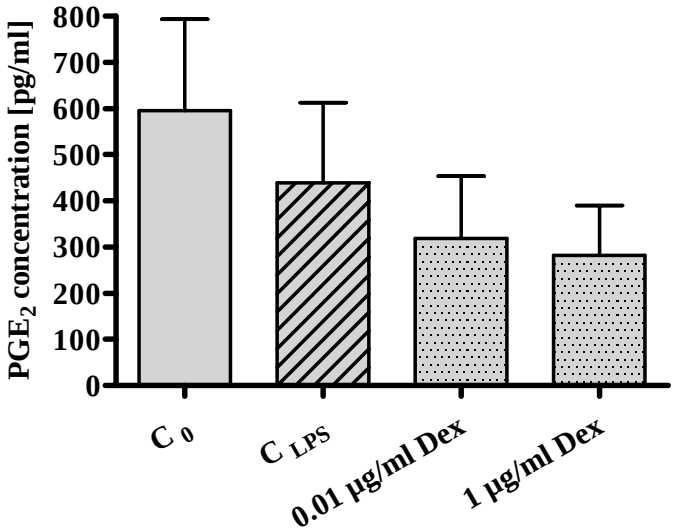


Fig. 42 PGE_2 concentration [pg/ml] in the culture medium after 24 h of stimulation with 100 µg/ml LPS and treatment with 0.01 or 1 µg/ml dexamethasone. **RCE cells** do not react to LPS-stimulation, but the addition of dexamethasone slightly reduces the basal PGE_2 production. Mean and SD.

SDS

The results for the stimulation of the canine corneal cells and the RCE cells with 0.001 % SDS +/- dexamethasone treatment are depicted in Fig. 43 to Fig. 46. Stimulation with 0.001 % SDS did not lead to a statistically significant increase of PGE_2 in canine corneal cells, although an enhanced PGE_2 concentration can be noted in epithelial and endothelial cells (Fig. 43 and Fig. 45). Dexamethasone reduced the PGE_2 concentration in these two cell types, although again not significantly. Keratocytes did not react to stimulation or treatment (Fig. 44). Also, RCE cells did not react with an increase in PGE_2 production to stimulation with SDS. In RCE cells the treatment with 0.01 and 1 µg/ml dexamethasone led to a decrease in PGE_2 concentration when compared to the negative and positive control. Nevertheless, this slight decrease was not statistically significant (Fig. 46).

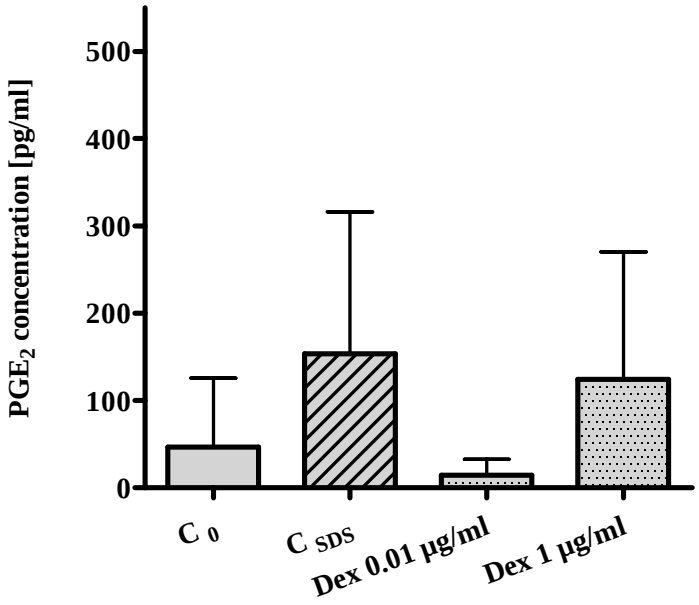


Fig. 43 PGE_2 concentration [pg/ml] in the culture medium after stimulation of **endothelial cells** with 0.001 % SDS (n = 4 – 7) and dexamethasone treatment (0.01 and 1 µg/ml). Mean and SD.

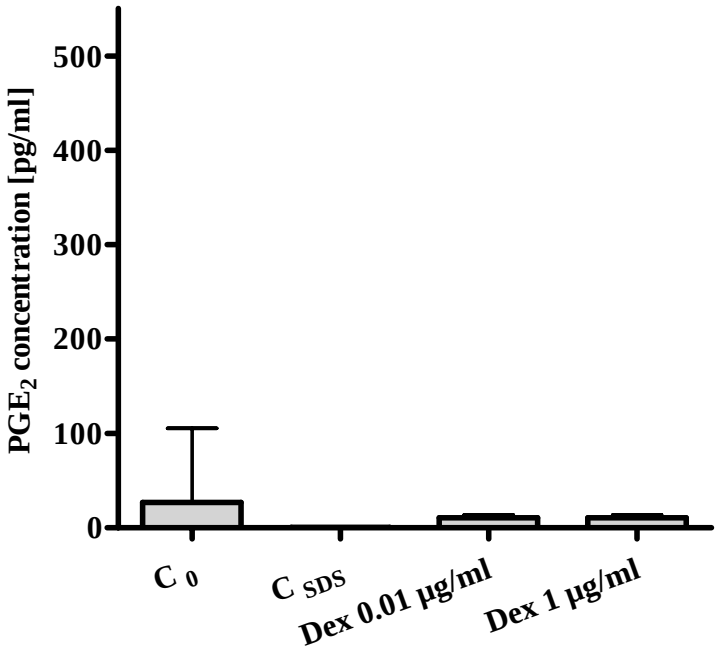


Fig. 44 PGE_2 concentration [pg/ml] in the culture medium after stimulation of **keratocytes** with 0.001 % SDS (n = 4 – 7) and dexamethasone treatment (0.01 and 1 µg/ml). Mean and SD.

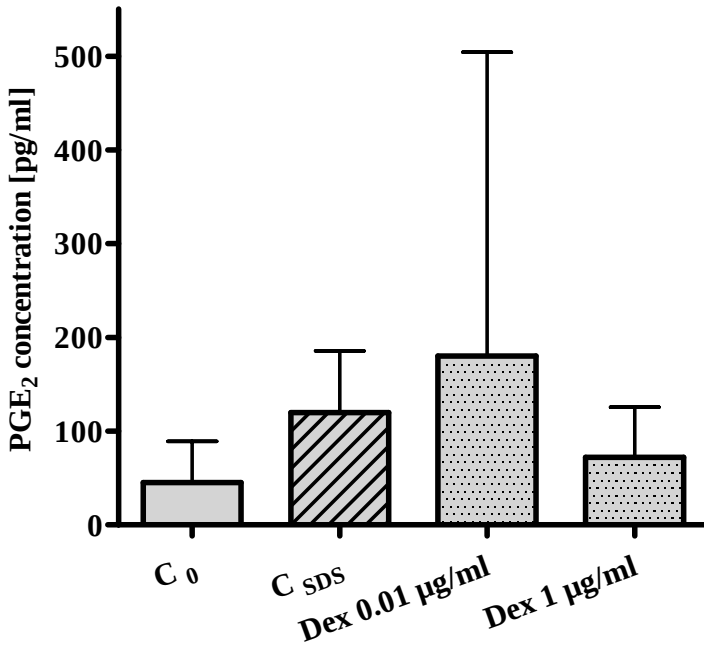


Fig. 45 PGE_2 concentration [pg/ml] in the culture medium after stimulation of **epithelial cells** with 0.001 % SDS (n = 4 – 7) and dexamethasone treatment (0.01 and 1 µg/ml). Mean and SD.

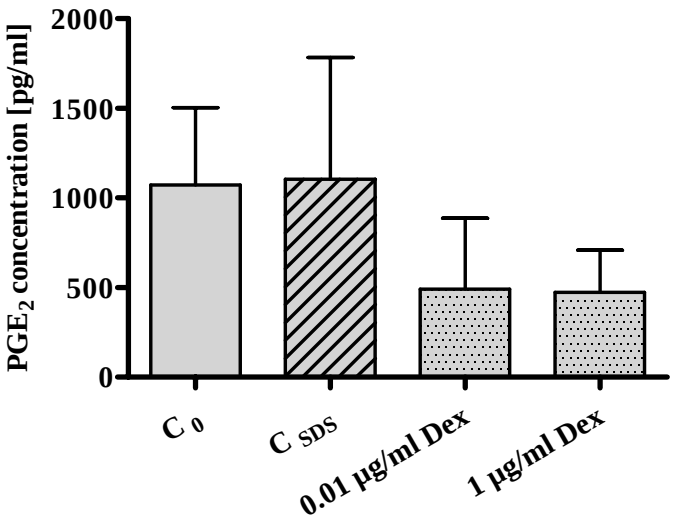


Fig. 46 PGE_2 concentration [pg/ml] in the culture medium after stimulation of **RCE cells** with 0.001 % SDS (n = 3 for two distinct passages) and dexamethasone treatment (0.01 and 1 µg/ml). Mean and SD.

4.5.2 Stimulation with INFγ and TNFα

The stimulation of the canine corneal epithelial cells with INFγ and canine TNFα did not lead to a measurable increase in PGE_2 in the culture medium. Furthermore, dexamethasone did not exhibit any effects on the PGE_2 level, therefore experiments on the other cell types were not conducted and the results are not shown.

4.5.3 Influence of the LPS-stimulation on TNFα

Using an ELISA assay, TNFα could not be detected in stimulated, unstimulated or stimulated and treated cells. Thus the results are not graphically depicted.

4.5.4 Gene expression following LPS-stimulation

In addition to the measurement of PGE_2, a semi-quantitative RT-PCR was performed as described in chapter 3.3.4.4 to evaluate a gene induction by stimulation with LPS. The primers used for this study were COX-2, Il-8, Il-1β, TNFα and GR. COX-1 was not examined since no satisfying primer was available for canine samples. Representative gels for the verification of the RNA isolation and the equalization of mRNA content using gapdh are given in the appendix. The pictures presented in the following section are representative gels showing principally two samples of cells derived from different dogs, which were run in parallel stimulation experiments.

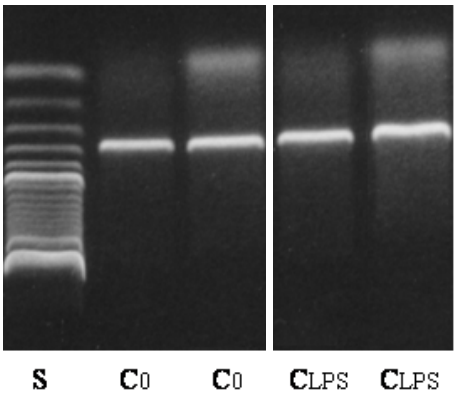
COX-2

The results for the expression of COX-2 are given in Fig. 47 to Fig. 49. The gene expression of the COX-2 was inducible by the addition of LPS in endothelial cells (Fig. 49) and keratocytes (Fig. 48). Epithelial cells show a similar gene expression for stimulated and not stimulated cells (Fig. 47).

TNFα

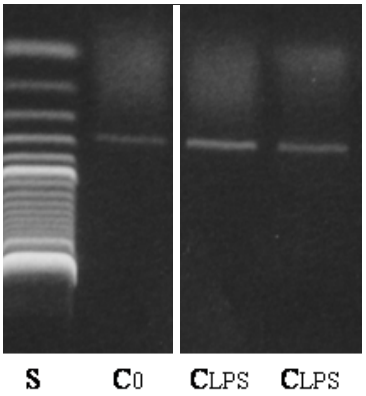
The TNFα mRNA expression in unstimulated and LPS-stimulated canine corneal cells is given in Fig. 53 to Fig. 55. The initial stimulation for 24 h revealed no gene expression for endothelial cells and keratocytes and only a slight mRNA expression in epithelial cells as indicated by a weak band (Fig. 53). The induction of the TNFα gene expression in the inflammatory reaction is an early phenomenon. Therefore, the cells were also stimulated for 4 h instead of 24 h, lyzed and processed for RT-PCR. Comparing 4 h and 24 h stimulation with 100 µg/ml LPS in endothelial cells and keratocytes, a stimulus and time dependent gene induction can be seen in Fig. 54 and Fig. 55. The genes are clearly activated by the 4-hour stimulation with LPS compared to the unstimulated cells. After 24 hours of stimulation no mRNA for TNFα can be detected in both cell types.

COX-2



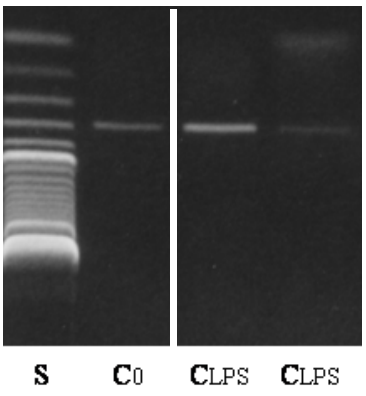
epithelial cells

Fig. 47



keratocytes

Fig. 48

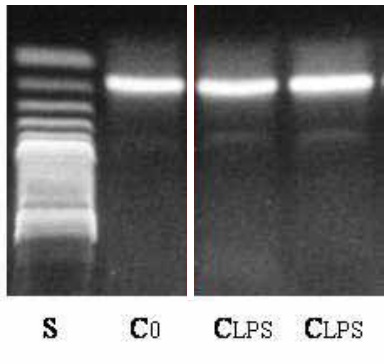


endothelial cells

Fig. 49

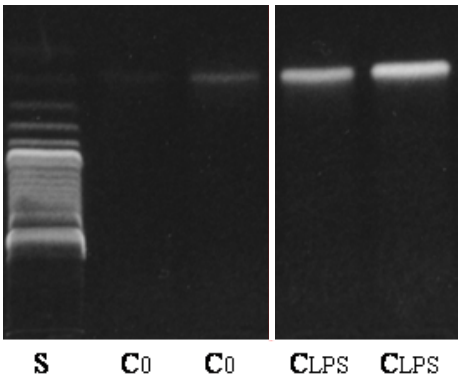
Fig. 47 - **Fig. 49** COX-2 mRNA expression in unstimulated and LPS stimulated canine corneal cells. A slight induction of COX-2 is observed in keratocytes and endothelial cells. S = DNA ladder (bp), C_0 = unstimulated cells, C_{LPS} = LPS stimulated cells.

IL-8



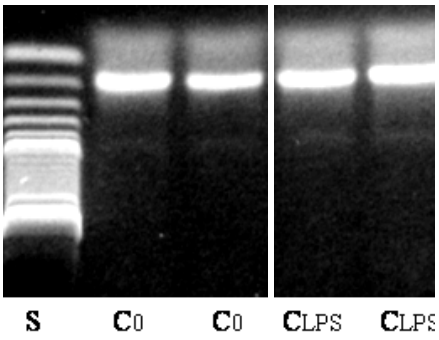
epithelial cells

Fig. 50



keratocytes

Fig. 51



endothelial cells

Fig. 52

Fig. 50 - Fig. 52 Il-8 mRNA expression in unstimulated and LPS stimulated canine corneal cells. A slight induction of IL-8 by LPS-stimulation is observed in keratocytes. The stimulation led only to very small induction of mRNA expression in epithelial and endothelial cells. S = DNA ladder (bp), C_0 = unstimulated cells, C_{LPS} = LPS stimulated cells.

TNFα

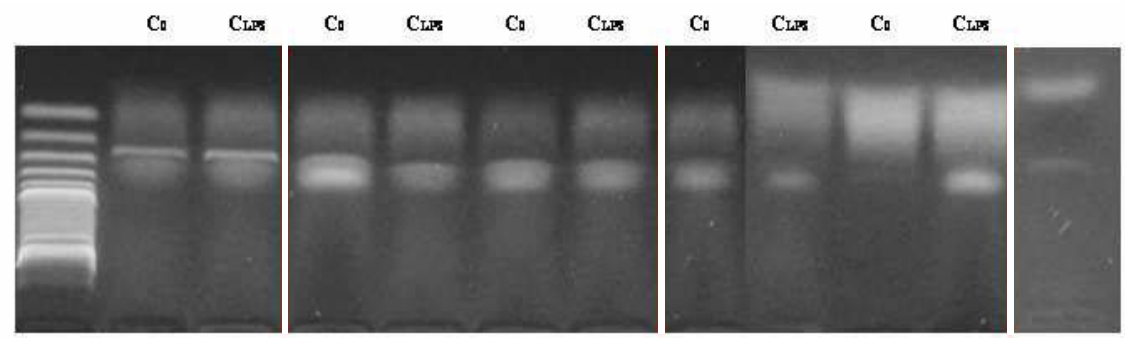
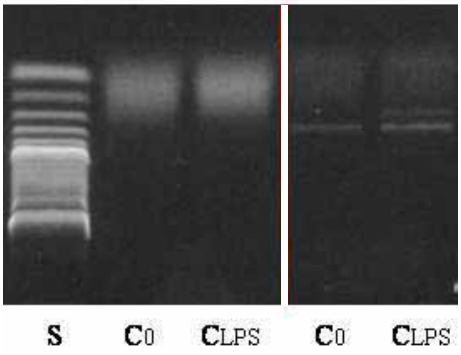


Fig. 53 **epithelial cells** **endothelial cells** **keratocytes** **--**

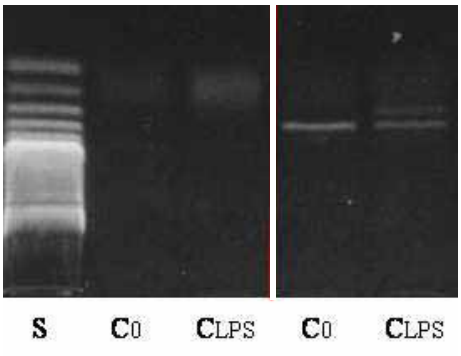
24 hr stimulation



keratocytes

Fig. 54 **24 hr** **4 hr**

stimulation



endothelial cells

Fig. 55 **24 hr** **4 hr**

stimulation

Fig. 53 - Fig. 55 TNFα mRNA expression in unstimulated and LPS stimulated canine corneal cells. S = DNA ladder (bp), C_0 = unstimulated cells, C_{LPS} = LPS stimulated cells, -- = neg. control. In epithelial cells the mRNA is expressed after 24 h regardless of stimulation. In endothelial cells and keratocytes 4 h stimulation leads to mRNA induction compared to the unstimulated control. In these cells no mRNA can be detected after 24 h.

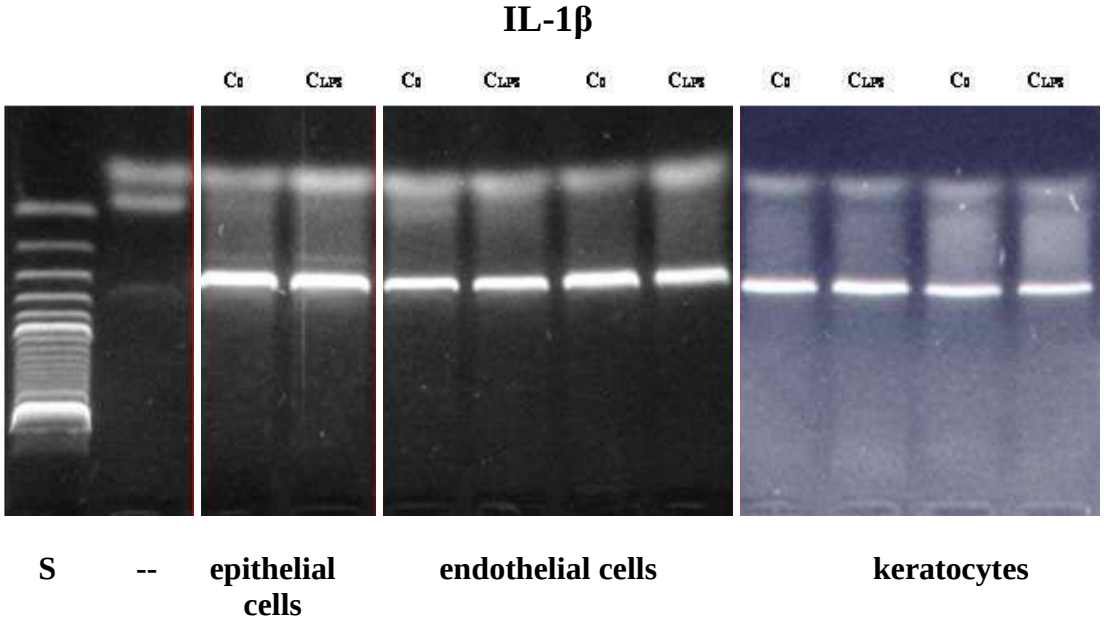


Fig. 56 IL-1β mRNA expression in unstimulated and LPS stimulated canine corneal cells. All three cells express similar amounts of the mRNA regardless of the stimulation. S = DNA ladder (bp), -- = neg. control, C_0 = unstimulated cells, C_{LPS} = LPS stimulated cells.

IL-1β

The results for the IL-1β mRNA expression in unstimulated and LPS-stimulated canine corneal cells are shown in Fig. 56. All three cell types express similar amounts of IL-1β mRNA regardless of a stimulus being absent or present.

IL-8

Il-8 mRNA expression in unstimulated and LPS-stimulated canine corneal cells is given in Fig. 50 to Fig. 52. A slight induction of IL-8 by LPS stimulation can be observed in keratocytes (Fig. 51). The stimulation with LPS led only to a very small induction of mRNA expression in epithelial and endothelial cells (Fig. 50 and Fig. 52).

GR

Comparably to the results for IL-1β, the GR mRNA was expressed in all three canine corneal cell types similarly for stimulated and unstimulated cells. The results for the RT-PCR are given in Fig. 28 to Fig. 30 in chapter 4.4.1.

4.5.5 Stimulation of the equivalents

Similarly to the single cells, the cornea equivalents were stimulated with 10 µg/ml LPS or 0.001 % SDS and treated with two concentrations of dexamethasone (0.01 µg/ml and 1 µg/ml). The PGE_2 concentration in the culture medium was measured after 24 h. For better comparison, the results are given as % of PGE_2 concentration, compared to the control group (C_0 = 100 %). The illustrations of the described results are based on the data listed in the appendix.

LPS

Stimulation using LPS led to a significant increase in PGE_2 concentration in the culture medium beneath the cornea equivalents (Fig. 57) compared to the negative control (mean PGE_2 concentration for C_0 = 657 ng/ml). This PGE_2 production could be reduced significantly by treatment with both concentrations of dexamethasone.

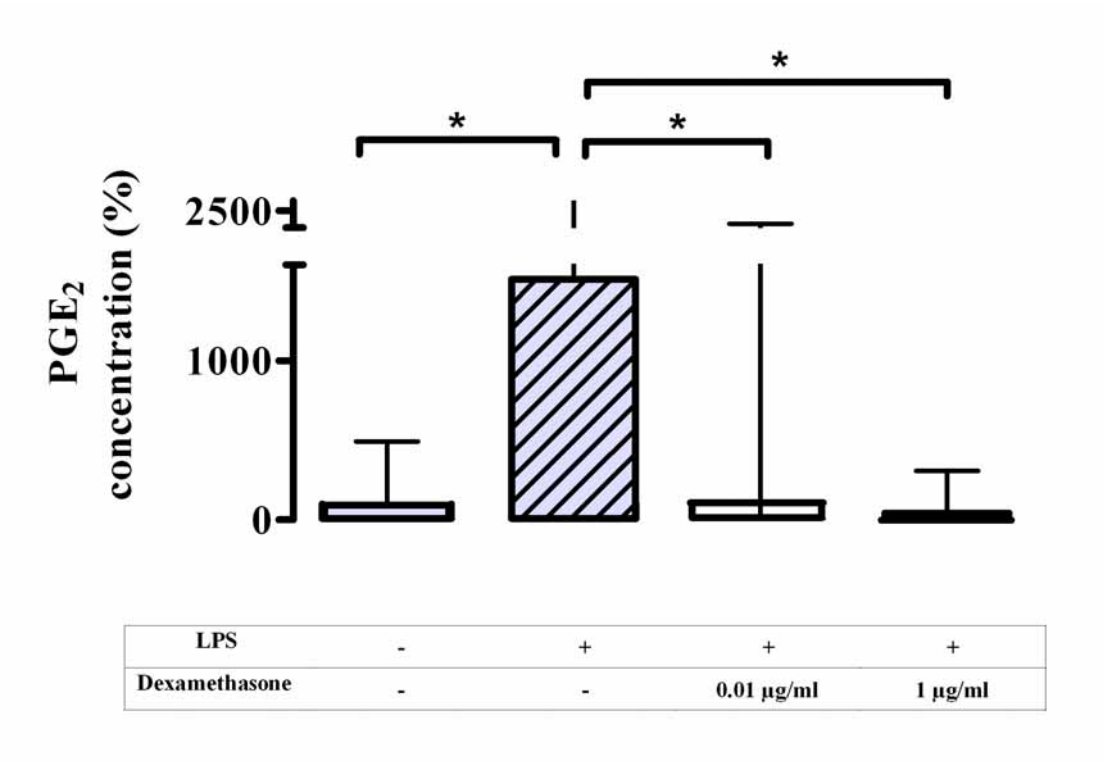


Fig. 57 PGE_2 concentration (% compared to neg. control) after stimulation of canine cornea equivalents with 10 µg/ml LPS and treatment of stimulated equivalents with two concentrations of dexamethasone (0.01 µg/ml and 1 µg/ml). LPS stimulated equivalents (n = 6 – 8 per stimulus, measured in duplicates) all consist of primary canine corneal cells. Mean and SD, * $p < 0.05$ (ANOVA).

SDS

Stimulation with SDS was only tested on cornea equivalents constructed with the RCE cells instead of canine corneal epithelium. Again, stimulation with SDS led to a significant increase in PGE_2 concentration, which could be reduced significantly by the addition of dexamethasone in both given concentrations (Fig. 58). The mean PGE_2 concentration for C_0 was 1 ng/ml.

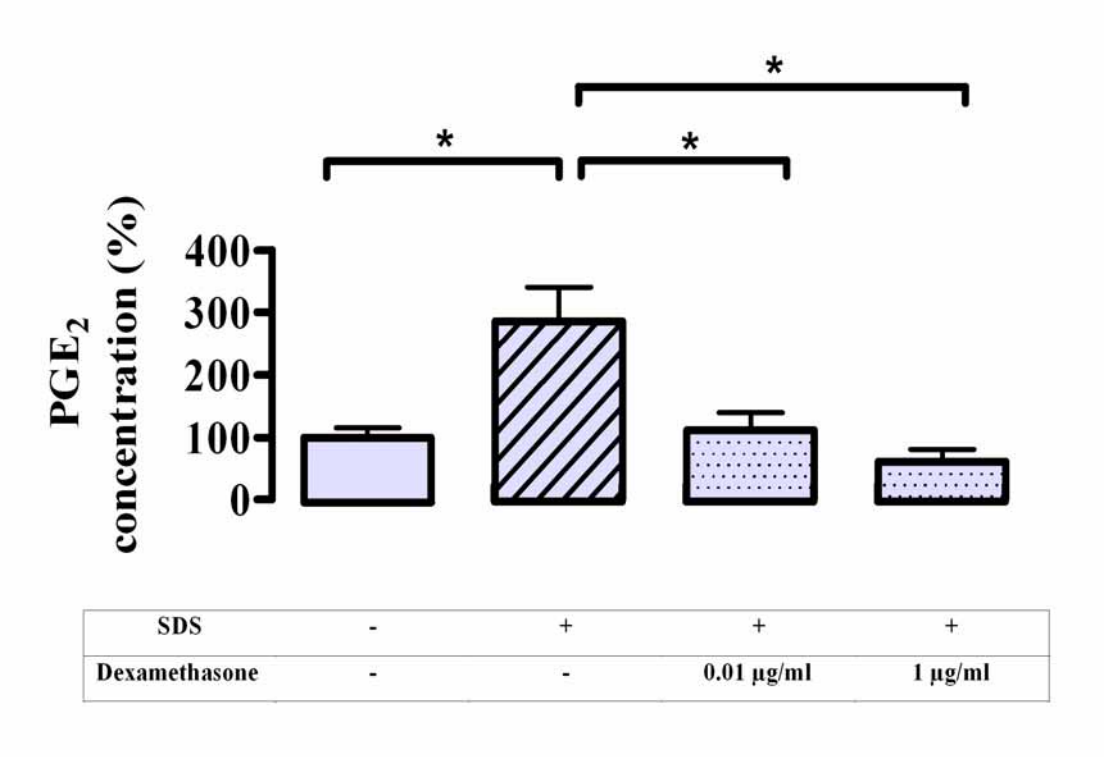


Fig. 58 PGE_2 concentration (% compared to negative control) after stimulation of canine cornea equivalents with 0.001 % SDS and treatment of stimulated equivalents with two concentrations of dexamethasone (0.01 µg/ml and 1 µg/ml). SDS stimulated equivalents (n = 2 per stimulus, measured in duplicates) were constructed using RCE cells. Mean and SD, * $p < 0.05$ (ANOVA).

5 Discussion

5.1 Isolation and cultivation of primary canine corneal cells

An isolation and cultivation protocol is established for the three major cell types of the canine cornea (i.e. endothelial cells, keratocytes and epithelial cells). The cell isolation procedure is a modification of the method used by REICHL (2003) for porcine corneal cells.

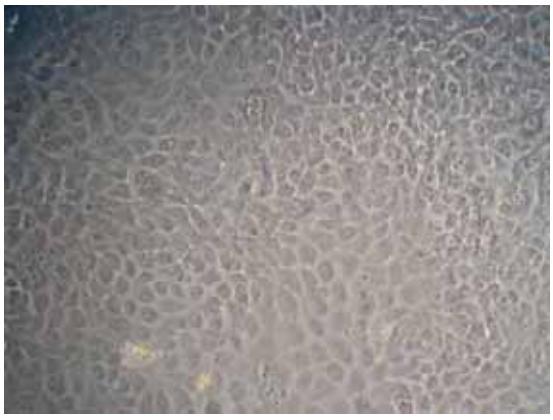
5.1.1 Primary culture of epithelial cells

Unlike seen in other tissues (e.g. nerve tissue, cartilage), in the cornea the most difficult cell type to isolate and culture is the epithelium. This is true for most species including humans. Different isolation protocols for these cells have been published (EBATO et al. 1988; HE and MCCULLEY 1991; HENDRIX et al. 2002; KIM et al. 2004; ZHANG et al. 2004). Studies on human and rabbit corneal epithelium have pointed out the superiority of a single cell culture over an explant technique (HE and MCCULLEY 1991; KIM et al. 2004). This is also true for canine corneal epithelium, where unsuccessful passaging of the cells and keratocytes-contamination renders the explant technique inferior to the single cell culture.

Starting with the x,y,z hypothesis of corneal epithelial maintenance, which was postulated in 1983 by THOFT and FRIEND, the different areas of the cornea and the limbus have been studied intensively. THOFT and FRIEND (1983) postulate that in corneal maintenance the epithelial cell loss from the surface must be balanced wit the cell replacement from basal epithelial cells. The state of differentiation increases from the limbus towards the center of the cornea. The limbus has been shown to contain mainly undifferentiated and potentially highly proliferative stem cells (EBATO et al. 1988; TUNGSIRIPAT et al. 2004; MELLER et al. 2005). EGGLI et al (1989) studied bovine corneal epithelial cells in vitro and found that peripheral corneal cells cover a 40 % greater area and are 2.75 times more numerous than those derived from the central cornea. Additionally, the peripheral cells tend to be small with polygonal morphology, whereas central cells vary considerably in size and shape and include numerous large cells. Due to the isolation method employed in this study, all epithelial cells are taken into culture, especially limbal cells.

EGGLI et al. (1989) also described in their study with bovine corneal epithelial cells a phenomenon that was seen with human epithelial cells as well, where the overall growth rates decreased with increasing numbers of passages compared to the first (regardless of the origin of the cells). This leads to labor intensive and time consuming investigations with a constant need for fresh material to isolate primary cells (KAHN et al. 1993; ARAKI-SASAKI et al. 1995; SHARIF et al. 1998). This tendency was also seen in the present study for canine corneal epithelial cells (Fig. 16).

When establishing a cell culture for ocular epithelial cells, keratocyte contamination is problematic. This is primarily true when using the explant technique, but also at times with the enzymatic detachment of epithelial cells. It has been reported by SUNDAR-RAJ et al. (1980) that the use of a selective culture medium eliminates the contamination with keratocytes. The authors used DMEM-medium containing D-valine instead of L-valine. As previously described, epithelial cells are able to synthesize the enzyme D-amino oxidase, which converts D-valine to the essential amino acid L-valine, allowing cell proliferation. Keratocytes lack this enzyme and are thus unable to grow in this culture medium (ENGELMANN et al. 1988; EGGLI et al. 1989; WEBB et al. 2003). Good growth of the epithelial cells without further additions of growth factors to the culture medium was reported. In a preliminary study of the present work such a selective culture medium was also tested on the enzymatically detached epithelial cells that were microscopically judged to be contaminated with keratocytes.



DMEM (L-valine)

DMEM (D-valine)

Fig. 59 Comparison of the effect of epithelial cell selective medium (containing D-valine) and DMEM with L-valine on primary cultures of canine corneal cells. Same magnification and age of culture in both pictures.

The medium did slow the growth of keratocytes but did not lead to a complete absence of these cells, especially with increased passages. Also, as previously described for human corneal endothelial and epithelial cells (ENGELMANN et al. 1988; WEBB et al. 2003), the use of the selective medium in this study led to a notable alteration of the proliferation rate and morphological appearance of the epithelial cells (decreased proliferation with increased cell size as depicted in Fig. 59), and thus the use of this medium type was discontinued.

Instead, the canine epithelial cells were carefully scraped off the cornea after incubation with dispase to avoid scraping the keratocytes as well. In cases of mild keratocyte contamination, the cells were separated during the first passage (keratocytes detached much quicker upon addition of trypsin/EDTA and could therefore easily be rinsed off) to establish a pure canine epithelial culture. If the contamination was moderate to severe or persistent with increasing passages, the use of the culture was discontinued.

As mentioned above, various groups have worked with primary cultures of corneal epithelial cells of different species (mostly human and rabbit). The basic culture medium used by most authors was a 1:1 or 1:2 mixture of DMEM and Ham 12 medium (EBATO et al. 1988; KIM et al. 2004; ZHANG et al. 2004; MELLER et al. 2005; LI et al. 2006). This type of culture medium was seen to have positive effects on cell proliferation. However, it has to be kept in mind that most groups did not intend to establish a cell pool, but used the cells at passage 1 or 2 at the most. Therefore, the problems with passaging, as seen in this study, were not mentioned. PANCHOLI et al. (1998) used a 1:1 mixture of DMEM and Ham 10 to culture corneal epithelial cells. There are few studies comparing different growth media. Those conducted by HE et al. (1991) and WEBB et al. (2003) also included the use of a commercially available Keratocyte Growth Medium. This medium used alone or in combination with other culture media was shown to have positive effects on cell proliferation and morphology of the corneal epithelial cells. This is in accordance with the results depicted in this study, where Williams E medium showed positive effects on cell proliferation, morphology and passaging of epithelial cells.

To increase the proliferation rate - especially of the generally slower proliferating epithelial cells - EGF was used as previously described for corneal cells in other species (BEDNARZ et al. 1996a; PANCHOLI et al. 1998; WILSON et al. 1999). Many groups working with corneal epithelial cells also add cholera toxin to their culture medium (EBATO et al. 1988;

PANCHOLI et al. 1998; WEBB et al. 2003; TUNGSIRIPAT et al. 2004; MELLER et al. 2005), which was also found to be beneficial regarding increased proliferation in this study, especially when using DMEM: Ham12 culture medium.

Although all culture conditions were optimized during this study to culture and serially passage the primary corneal epithelial cells, it was not possible to consistently keep or increase the proliferation rate of this cell type beyond passage three (see Fig. 16). It was therefore not possible to establish a sufficient cell pool to be frozen in liquid nitrogen and stored for later use.

The culture of corneal epithelial cells seems to be less challenging for bovine and porcine corneal epithelial cells (TEGTMEYER et al. 2001; REICHL 2003; TEGTMEYER et al. 2004), while human and rabbit corneal epithelial cells were found to be similarly problematic as seen with the canine cells (KAHN et al. 1993; ARAKI-SASAKI et al. 1995; SHARIF et al. 1998). Recent work has been published on establishing corneal epithelial cell lines to overcome this limitation, as described in chapter 2.3.2.

5.1.2 Primary culture of endothelial cells and keratocytes

The isolation and culture of primary corneal endothelial cells and keratocytes was less difficult compared to that of the epithelial cells described above. The relatively basic culture medium used by REICHL (2003) for porcine and human corneal cells was suited for the canine corneal endothelial cells and keratocytes. The only change required for the generally slower proliferating endothelial cells was the addition of EGF as previously described for corneal endothelial cells in other species (BEDNARZ et al. 1996a; PANCHOLI et al. 1998; WILSON et al. 1999). However, as expected the keratocytes show the highest proliferation rate of the three cell types (Fig. 17 and Fig. 18).

5.1.3 Verification of the primary canine corneal cells

Epithelial cells

Cytokeratins are 10 nm intermediate filaments that form a cytoskeletal network providing mechanical integrity to the cell in the context of its tissue (COULOMBE et al. 1991; KAHN et al. 1993). The keratins exist in a 1:1 ratio of type I (acidic) and type II (basic) keratins,

which form heterodimers (SCHERMER et al. 1986). The types of keratins synthesized are specific for both the developmental stage and the phenotype of the cell (STEINERT and ROOP 1988). In cornea epithelium, AE1 (keratin 1) is found in hyperproliferative epithelial cells, AE3 (keratin 3) immunoreactivity is seen in all epithelial cells, and AE5 (keratin 5) immunoreactivity is observed in all but the basal cells of the limbus and transient cells (SCHERMER et al. 1986; LAUWERYNS et al. 1993a; LAUWERYNS et al. 1993b). The antibody used to detect the canine corneal epithelial cells was a pan-antibody directed against type II cytokeratins 1, 5, 6, and 8, therefore detecting suprabasal cell layers of differentiated compartments, stratified epithelia, simple epithelia as well as hyperproliferative epithelial cells (MOLL et al. 2007). Since the isolation method used in this study for epithelial cells includes highly proliferative stem cells as well as differentiated central corneal cells, the antibody detected all cultured cells.

Endothelial cells and keratocytes

As described above (chapter 3.3.2), vimentin is one of the five major groups of intermediate filaments with a molecular weight of 58 kDa (BOHN et al. 1992). The antibody is used to localize tissue of mesenchymal origin, thus staining endothelial cells and fibroblasts. Unlike the von Willebrand factor (factor VIII:ag) used for vascular endothelial cells, there is still no definitive marker for corneal endothelial cells (ENGELMANN et al. 1988). Therefore, the combined use of morphologic criteria (appearance of closely packed polygonal cells) and the staining with anti-vimentin was considered sufficient to identify this cell type accurately.

5.2 Glucocorticoid receptor

Glucocorticoids bind to the cytosolic glucocorticoid receptor (GR), which leads to the detachment of two heat shock proteins (HSP 90) followed by a conformational change of the receptor (KUMAR and THOMPSON 1999, 2005). The activated hormone-receptor-complex then acts as a transcription factor through direct binding to the DNA, through protein-protein interactions with transcription factors (ADCOCK et al. 2004; ITO et al. 2006) or receptor-mediated non-genomic effects (SONG and BUTTGEREIT 2006) as described in detail in chapter 2.6.2.

The GR has been detected in many tissues and cells in various species (ARRIZA et al. 1988; VAN DEN BROEK and STAFFORD 1992; RADDATZ et al. 1996). In our study, mRNA for the GR was found in all three cell types of the canine cornea, indicating that all three cell types have the potential to express the receptor. The results are in accordance with those described for the human cornea, where the mRNA for the GR has been identified in all three major cell types as well (WILSON et al. 1994; BOURCIER et al. 1999; BOURCIER et al. 2000; STOKES et al. 2000; SUZUKI et al. 2001). The GR mRNA has also been described for rat corneal epithelial and endothelial cells (STOKES et al. 2000) and glucocorticoid binding sites have been identified in rabbit and bovine corneas (WEINSTEIN et al. 1982; SOUTHREN et al. 1983; LIN et al. 1984). To my knowledge, this is the first time the mRNA for the GR is described in canine corneal cells.

Additionally, the GR could be detected on the protein level in the canine cornea equivalents. This is a further indicator of the functionality of the GR and is in accordance with different authors who have previously described the GR protein in the following ocular tissues: human corneal keratocytes and epithelial cells; human, mouse, and rabbit lens epithelium; rabbit iris-ciliary body and corneascleral tissue (SOUTHREN et al. 1979; SOUTHREN et al. 1983; BOURCIER et al. 1999; BOURCIER et al. 2000; STOKES et al. 2000; GUPTA and WAGNER 2003; JAMES et al. 2003).

5.3 Effect of dexamethasone on primary canine corneal cells

Studies of the effects of glucocorticoids on canine corneal cells are essential, because most glucocorticoid drugs are not registered for veterinary ophthalmologic use. Nevertheless, this class of drugs is widely used, especially in dogs. Previous studies on the effects of dexamethasone on corneal cells often used epithelial cells and/or keratocytes and not all three cell types (BOURCIER et al. 1999; BOURCIER et al. 2000; HENDRIX et al. 2002). HENDRIX et al. (2002) mechanically inflicted a wound to the confluent cell culture before the addition of dexamethasone (between 0.0625 mg/ml and 1 mg/ml). Mostly, the effects of dexamethasone on the cells were measured using morphological criteria (i.e. cell size, appearance) (BOURCIER et al. 1999; BOURCIER et al. 2000; HENDRIX et al. 2002; LU et al. 2004).

In the present study a model using the initial steps of an inflammatory process has been established in which all three cell types are stimulated with LPS or SDS and simultaneously treated with different concentrations of dexamethasone. The effect of the glucocorticoid was measured as a diminished secretion of PGE_2 into the cell culture medium. This approach intends to model the *in vivo* situation of a corneal inflammation and drug treatment more closely than previous studies.

The substances used for the stimulation and thus functionality testing were chosen because they act through two different mechanisms (LPS via toll-like receptor and SDS as non-specific irritant).

5.3.1 Stimulation with LPS

When stimulated with a comparably high dose of LPS, only epithelial cells and keratocytes react with an increased production of PGE_2, while endothelial cells do not respond to LPS. LPS is part of the bacterial membrane of gram-negative bacteria and reacts as a bacterial endotoxin. This endotoxin, like other microbial components, mediates its pro-inflammatory effects through binding to toll-like receptors (TLRs). LPS is recognized by the TLR4 (SONG et al. 2001), which has been detected in human ocular tissue. In the cornea the TLR4 has been demonstrated in epithelial cells and keratocytes but not in endothelial cells (SONG et al. 2001; JOHNSON et al. 2005; KUMAGAI et al. 2005). If assuming a similar distribution of the TLR4 in the canine tissue, this would explain the lack of PGE_2 increase in the LPS-stimulated canine corneal endothelial cells.

Because of the sight threatening consequences of a corneal inflammation, the immunological situation at this site is rather complex. TLRs as a line of defense would be useful against invading pathogens, but the triggered inflammation could be detrimental if pointed against the normal non-pathogenic flora of the ocular surface. It has been proposed recently, that the TLRs are strategically positioned in basal and not in apical layers of the epithelium (ZHANG et al. 2003; UETA et al. 2004; UETA et al. 2005) since it seems sensible that apical bacteria (usually commensal flora) are ignored whereas bacteria that are able to penetrate the epithelium (often pathogenic bacteria) are recognized as potentially hazardous.

Considering the common route of infection into the eye, it might be useful to equip epithelial cells and keratocytes, but not endothelial cells, with a recognition and defense mechanism

against bacterial infection (e.g. via TLR4). An inflammatory reaction leading to the destruction of endothelial cells is associated with graver consequences for the visibility of the organism than a comparable reaction of epithelial cells or keratocytes. This can be explained by the increased ability of epithelial cells and keratocytes to replicate *in vivo* in comparison to endothelial cells. Furthermore, the endothelial cells exhibit an important role in ensuring corneal transparency by regulating the hydration status of the corneal stroma.

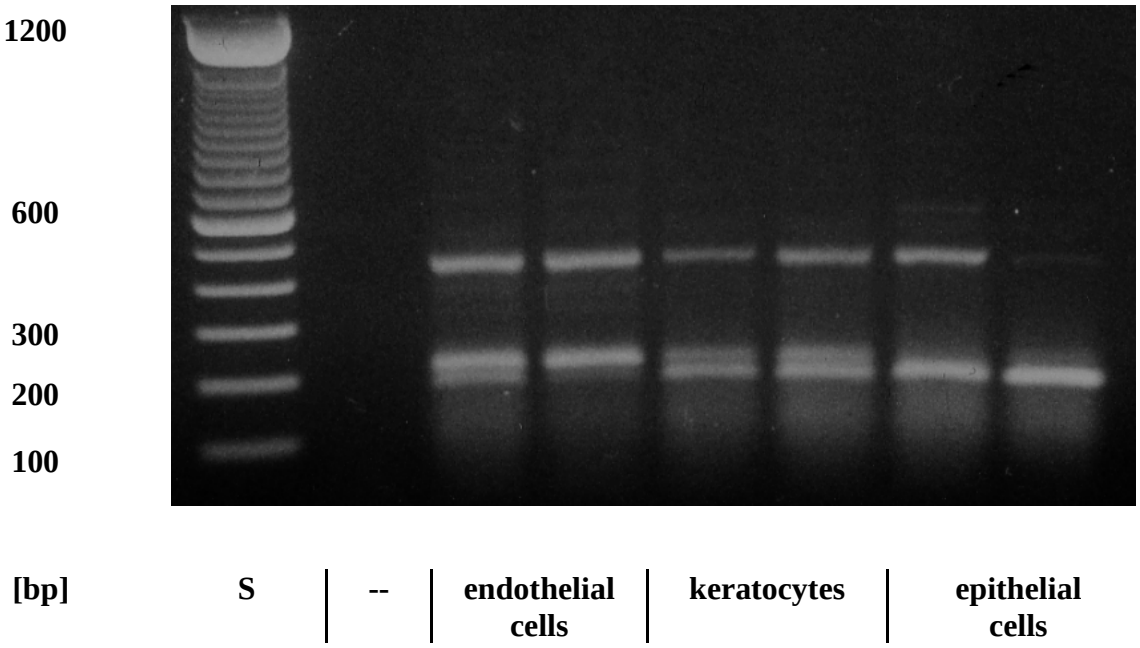


Fig. 60 Expression of TLR4 in the three corneal cell types. S = DNA-ladder in base pairs; -- = neg. control; bp = base pairs

Own results depicted in Fig. 60 indicate, that all three corneal cell types express the mRNA for TLR4 as seen by a band of approximately 179 base pairs. Since the receptor expression was not confirmed on a protein level, it is possible, that canine corneal endothelial cells are theoretically capable of producing the receptor, but employ a mechanism that hinders the production of the actual receptor protein. Another possible explanation is a selective inhibition of PGE_2 synthesis even tough LPS might trigger an inflammatory reaction via the TLR4. This might be a protective measure in line with the immune privileged site of the eye (STREILEIN and STEIN-STREILEIN 2000; STREILEIN 2003a, b).

Interestingly, the endothelial cells exhibit a comparatively high basal production of PGE_2 into the culture medium, which can be partly reduced by a high concentration of dexamethasone.

This finding stands in contrast to the above stated hypothesis, but supports the functionality of the GR in these cells.

The intracellular pathways involved in TLR activation by LPS and the subsequent steps toward inflammatory reactions via induction of NF-κB have been described (see chapter 2.5.4 and reviews by AKIRA et al. (2006) and TAKEDA et al. (2003; 2005)). Nevertheless, there are conflicting results concerning cytokine production due to LPS-stimulation. SONG et al. (2001) measured a cytokine production (IL-6, IL-8) after stimulation of human corneal cells with LPS derived from *Pseudomonas*. UETA et al. (2004) on the other hand found that human epithelial cells expressed TLR4 but were incapable of secreting pro-inflammatory molecules upon stimulation with LPS derived from *P.aeruginosa*. In the present study we used LPS derived from *E. coli* (strain: 0111:B4), as canine keratinocytes were previously found to be sensitive to this stimulus (BÄUMER and KIETZMANN 2007). Nevertheless it is possible that the type of LPS-stimulus might slightly influence the results.

Only rather high concentrations of dexamethasone lead to a significant decrease in the stimulated PGE_2 production. This might be explained by the fact, that the dexamethasone treatment is performed simultaneously with the LPS stimulation. A pre-treatment with dexamethasone might give the cells a “head start” in their reaction to the LPS-stimulus and thus increase their effect at lower doses. However, a simultaneous addition of LPS and dexamethasone resembles the situation *in vivo* more closely, as the treatment will normally begin after the irritating stimulus was present and not beforehand.

5.3.2 Stimulation with SDS

Canine corneal cells were stimulated with 0.001 % SDS and (pre-) treated with dexamethasone. The irritation led to a slight but not significant increase in PGE_2 concentration in the culture medium supernatant of epithelial and endothelial cells. Keratocytes did not react to the stimulus with an increase in PGE_2.
SDS is commonly used as a positive standard in studies of dermal and ocular irritation. In dermal experiments, much higher concentrations are used before signs of irritation and inflammation are detectable (0.1 – 20 %) (FARTASCH 1997; LE et al. 1997;

FAIRWEATHER et al. 2004; PETERS et al. 2006). In comparison, the ocular cells are much more sensitive and will react with decreased viability to concentrations above 0.02 % (ZORN-KRUPPA et al. 2004). Although dermatological studies have evaluated PGE_2 synthesis among other signs of skin irritation and inflammation (MÜLLER-DECKER et al. 1998; FAIRWEATHER et al. 2004), the effects of SDS on corneal cells in cell culture as described by ZORN-KRUPPA et al. (2004) were limited to viability studies. Other groups used excised bovine corneas directly or cultured in an air-lifted format to test SDS irritation and measured the effects on corneal opacity and permeability (GAUTHERON et al. 1992; FOREMAN et al. 1996; XU et al. 2000). To the best of my knowledge, this is the first study evaluating PGE_2 production of corneal cells *in vitro* following SDS irritation.

Since the keratocytes did not show any measurable basal PGE_2 production and the cells did not react to stimulation with SDS, no effects were measured for the treatment with dexamethasone. The epithelial cells reacted by releasing lower levels of PGE_2 into the culture medium when treated with 1 µg/ml compared to the low dose of 0.01 µg/ml. Surprisingly, the treatment with the low dose of dexamethasone led to an increased production of PGE_2, with a mean value above the positive control. But due to a high variance in this treatment group, no statistical significance was detected.

Interestingly, the dexamethasone treatment had opposite effects on endothelial cells. Here the low concentration (0.01 µg/ml) led to a slight decrease in PGE_2 production whereas the PGE_2 concentration after treatment with 1 µg/ml was similar to the negative control.

The high variance in the results for the two cell types does not allow clear statistical conclusions. One could suspect that both cell types react to dexamethasone treatment following SDS stimulation with a slight decrease in PGE_2 production, although not necessarily in a dose-dependent manner.

No similar study was found to compare these results to. Previous work by FAIRWEATHER et al. (2004) discussed the effect of SDS on PGE_2 on human skin. This study revealed an induction of the pro-inflammatory molecule PGE_2 after 20 min application of 0.1 and 0.5 % SDS intradermally. It has to be taken into consideration that this study was performed *in vivo*, thus allowing a much more complex reaction upon SDS stimulation (involving inflammatory cells, blood vessels and nerves) compared to the experiments on isolated and separately cultured cells. Also, dexamethasone effects were not studied.

The experimental set-up of the present work was designed similarly to the study of ZORN-KRUPPA et al. (2004), which is the only other study found that was conducted on isolated and separately cultured corneal cells. It should be noted that their model was adapted for viability studies, where the release of inflammatory mediators like PGE_2 was not of interest. In an *in vivo* model described by RAO et al (1993) using mouse ears, the treatment with arachidonic acid leads to a rapid increase in PGE_2 production within 30 min, but the concentration decreases again quickly and reaches a considerably lower level after 60 – 120 min. A similar effect is seen in the study conducted by FAIRWEATHER et al. (2004) where a rapid increase of PGE_2 is noted at the end of the 20 min application of SDS which reclines to baseline level within 40 min. This could indicate, that in the present study, an earlier measurement of the culture medium supernatant (within 30 – 60 min of SDS stimulation) might have resulted in larger effects on the PGE_2 concentration and consequently on the measured effects of dexamethasone.

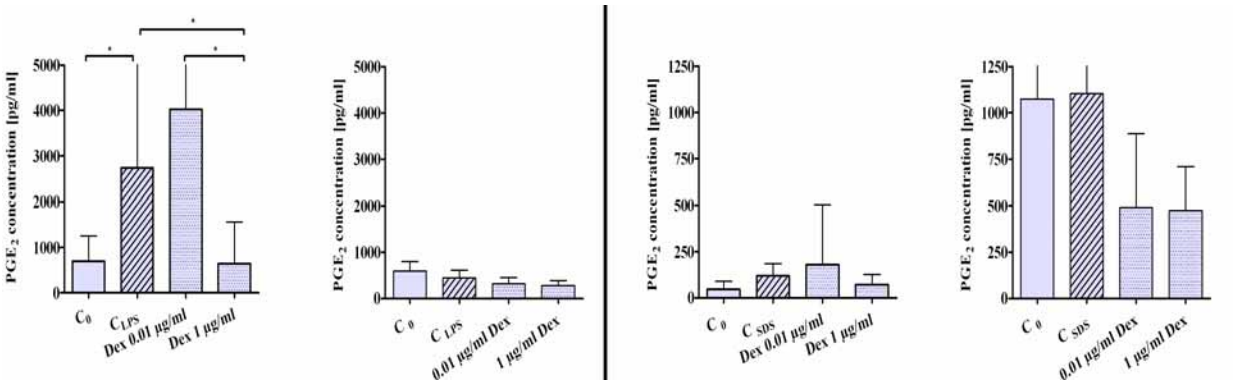
5.4 The use of RCE cells

The limitations in the culture of primary canine corneal epithelial cells as described above, led to consequences for the construction of the cornea equivalents. As a result, those cannot be readily constructed independently of fresh animal donor material.

In search of an alternative to enable independent construction of the canine cornea equivalents, the rabbit corneal epithelial cell line (RCE) established by ARAKI et al. (1993) was studied. For comparative reasons, the RCE cells were tested as a single cell culture in the same way the primary canine corneal cells were tested (stimulation with LPS or SDS and treatment with dexamethasone at different concentrations). At the same time, cornea equivalents were constructed using canine endothelial cells and keratocytes as well as RCE cells instead of primary canine epithelial cells. The treatment of the two epithelial cell types was identical to allow comparison of the two systems.

The cells were unproblematic in culture and could be readily subcultured. When investigating their reaction to stimulation (with LPS and SDS) and dexamethasone treatment, it was noted, that the RCE cells do not react in the same manner as the primary canine epithelial cells (Fig. 61).

When brought to the same scale of PGE_2 concentration after LPS or SDS stimulation, the differences become apparent: LPS stimulation leads to a 3-fold greater PGE_2 production in canine epithelial cells compared to RCE cells. Also, 1µg/ml dexamethasone treatment significantly decreases this PGE_2 concentration in the primary canine cells whereas no clear effect in reducing PGE_2 can be seen for the same treatment in RCE cells.



LPS **SDS**

Fig. 61 Comparison of the PGE_2 production in primary canine epithelial cells and RCE cells after stimulation with LPS or SDS and treatment with dexamethasone. This figure is a summary of the results depicted in **Fig. 39**, **Fig. 42**, **Fig. 45** and **Fig. 46**.

The stimulation with SDS leads to contrary results: The PGE_2 concentration in the culture medium supernatant of RCE cells is about 10-fold that of epithelial cells from canine corneas. In RCE cells both concentrations of dexamethasone result in a halving of the mean PGE_2 concentration, while in canine epithelial cells only the high dose of dexamethasone leads to this effect. Unfortunately, the high variance of the results leads to the conclusion that a significant decrease cannot be reached by either dexamethasone concentration for both cell types.

It has to be noted, that the results for the RCE cells are very similar when comparing the two different experiments (positive and negative control for LPS and SDS-stimulation contain about 1000 pg/ml PGE_2 in the culture medium, treatment with both dexamethasone concentrations about 500 pg/ml). Thus in contrast to the primary canine epithelial cells, the RCE cells do not exhibit differentiated reactions to each stimulus. Additionally, in regard to LPS stimulation, the absolute amount of PGE_2 produced by RCE cells is significantly lower than that produced by canine epithelial cells.

5.5 Cornea equivalent

Canine cornea equivalents were constructed step by step in cell culture as described in chapter 3.3.1.8. Due to the described limitations in the primary culture of canine corneal epithelial cells, cornea equivalents were constructed with either primary canine cells or the rabbit epithelial cell line (RCE cells). The two different equivalent types were compared morphologically and in their reaction to stimulation and dexamethasone treatment.

5.5.1 Morphology of the cornea equivalents

ZIESKE et al. (1994) described the importance of the endothelial cell layer in a cornea equivalent for the differentiation of the epithelial cells and for the formation of a basement membrane between the stromal layer and the epithelium. The culture protocol employed in this study took into consideration the importance of direct contact between endothelial cells and the rest of the equivalent, as well as the importance of environmental factors like culture at an air-liquid-interface (MINAMI et al. 1993; ZIESKE et al. 1994). Therefore, the epithelial cells should form a multilayer and differentiate into columnar basal cells and flattened superficial cells. When viewed histologically, the equivalents constructed with canine primary epithelial cells show a tendency to differentiate into round to columnar basal cells and flattened superficial cells (Fig. 23). The multilayered epithelium consisting of RCE cells is not differentiated, but has a homogeneous, cuboidal appearance (Fig. 24).

The results of the morphological study revealed a marked difference in epithelial morphology between the primary cells and the cell line. Compared to the canine epithelial cells that seem to differentiate into basal and superficial cells, the RCE cells seem to form an undifferentiated multilayer. This is in accordance with morphological studies by ARAKI et al. (1993), who established the cell line. However, in transmission electron microscopic observations and western blot analysis, they showed signs of differentiation that were similar to primary epithelial cells like desmosome and microvilli formation and immunoreactivity with the differentiation marker cytokeratin 5.

It can be noted macroscopically, that the canine cornea equivalents are not transparent like a normal cornea. Considering the elaborate ultrastructure of the corneal stroma and the variety

of other factors that influence corneal clarity (as described in detail in chapter 2.2), it is not surprising that the cornea equivalents constructed in this work are opaque. This is considered irrelevant for the intentions of the study.

The immunohistological study of the cornea equivalents using anti-vimentin and anti-cytokeratin antibodies revealed partly unexpected staining patterns (Fig. 25 and Fig. 26). Usually, epithelial cells stain positive for cytokeratin but negative for vimentin. In this study, the canine epithelial cells and the RCE cells of the equivalents stain positive for both cell structures. Such a double staining for vimentin and cytokeratin in corneal epithelial cells has been described previously (SUNDARRAJ et al. 1992; KAHN et al. 1993; LAUWERYNS et al. 1993a; LAUWERYNS et al. 1993b). Initially, the vimentin positive reaction of epithelial cells has been associated with corneal wound healing. SUNDARRAJ et al. (1992) demonstrated that rabbit corneal epithelial cells co-express keratin and vimentin during the first 2 days of wound healing. They speculated that cell-cell detachment in the epithelium after wounding is promoted by disruption or modification of the desmosomal complexes of the epithelial cells. This might then influence cellular migration as a prerequisite to cover the wounded area. Absence or alterations of desmosomes may disrupt the organization of the keratin network because keratin filaments terminate at desmosomes. They suggested that the vimentin filaments may not be associated with the desmosomal plaques, but may terminate at other regions along the inner surface of the cell membrane. Therefore, vimentin filaments may temporarily replace the structural role of certain keratin filaments and may also be associated with the accommodation and/or orchestration of cellular shape changes during wound healing (SUNDARRAJ et al. 1992; LAUWERYNS et al. 1993b). It has also been proposed that the type of underlying collagen, possibly in combination with the type of culture environment (submerged vs. culture at the liquid-air interface) is influential on the expression of vimentin filaments (KAHN et al. 1993; LAUWERYNS et al. 1993b). Therefore, the vimentin-positive reaction of the epithelial cells in this study might be due to the collagen type used for the stromal layer in the cornea equivalents. Also, the strong contraction of the stromal layer during the culture might have induced slight migration of the epithelial cells, resulting in the expression of vimentin filaments in those cells.

5.5.2 Stimulation of the cornea equivalents

Compared to experiments using single cells, the cornea equivalents react to both stimuli (LPS and SDS) with a significant increase in PGE_2 production. This might be explained by the additive effect of the cytokine production of three cell types instead of only one. Unfortunately, a quantitative comparison between the single cell experiments and the equivalents with regard to PGE_2 production per cell is not possible. Although the equivalents were always constructed with the same amount of cells, the resulting equivalents were not perfectly uniform, but varied slightly in diameter and thickness, and therefore in total cell count. Another reason for the different quality of the stimulation of equivalents versus single cells could lie in cellular interactions within the equivalents that lead to a stronger reaction compared to the individual cells.

Several authors have studied the intercellular signaling of corneal cells (GRIFFITH et al. 1995; LI et al. 1996; WILSON et al. 1999; WILSON et al. 2001; WILSON et al. 2003; KUO 2004). With regard to epithelial differentiation in a cornea equivalent it has been hypothesized by ZIESKE et al. (1994) that the different cell types (especially the endothelial cells) influence each other via cytokines or matrix components. Most other studies conducted on intercellular signaling investigated stromal keratocytes to epithelial cell interactions with a special emphasis on corneal wound healing (LI et al. 1996; STRISSEL et al. 1997; WILSON et al. 1999; WILSON et al. 2001; WILSON et al. 2003). The intercellular communication is most likely not limited to wound healing, but will also influence other reactions like inflammatory processes.

Such cellular interactions might also explain why the equivalents are more sensitive to dexamethasone. Thus, a low concentration of dexamethasone leads to a significant reduction in PGE_2 concentration while the same concentration does not notably reduce the PGE_2 production when tested on single cells.

Although there is a significant difference between the treatment groups for the equivalents, a notable variance within each treatment group is also apparent. This is mainly due to variability between different repeats of the construction and testing of the equivalents. One explanation might be the use of primary cells, which, although generated with a standardized protocol (all beagle breed, similar age and even sex distribution), originate from different

individuals and thus exhibit a biological variation. Additionally, as described above, the equivalents are not uniform in size and shape but vary slightly. Therefore, the variance in the results might be due to different proportions of the three cell types within each equivalent.

Future studies will aim at improving a uniform appearance of the equivalents with an equal distribution of the three cell types included. One step towards this goal could be the use of cell lines. Different authors have established protocols for the long-term cultivation (ENGELMANN et al. 1988; PISTSOV et al. 1988) or immortalization of corneal cells (ARAKI et al. 1993; KAHN et al. 1993; SHARIF et al. 1998; BEDNARZ et al. 2000). Since no cell line is available for canine cells, a rabbit corneal epithelial cell line was used in this study. Since the RCE cells (as discussed above) reacted a lot less to the LPS stimulation in the single cell experiments compared to the primary epithelial cells, no equivalents constructed with RCE cells were included in the stimulation with LPS. Such an altered reaction compared to the primary cell might also occur when a canine epithelial cell line is used.

Due to limited access to primary cells, RCE-equivalents were later included in SDS studies. It can be noted, that the total PGE_2 concentration in RCE equivalents is lower compared to the equivalents with primary epithelial cells. This is in accordance with the fact that canine epithelial cells produce a larger amount of PGE_2 than RCE cells.

5.6 Outlook

The pro-inflammatory molecule PGE_2 was chosen as the indicator for the stimulation and drug treatment. So far, it is the only pro-inflammatory molecule that can be readily detected with a validated, commercially available test system for canine cells. Obviously, when looking at the inflammatory reaction initiated by both LPS and SDS, a variety of other cytokines and inflammatory molecules might be intriguing to study. Since the gene expression in regard to LPS-stimulation could be shown for some cytokines in this study (Fig. 47 to Fig. 56), their production and release by the canine corneal cells would be interesting to evaluate (e.g. IL-1, Il-8, INF-γ, 12-HETE). As seen with the experiments conducted with LPS on endothelial cells and SDS on keratocytes, PGE_2 might not be the ideal endpoint to measure, due to the reasons discussed above. Also, the ability to measure more than one endpoint when studying drug effects would be beneficial by giving a broader picture and thus a closer resemblance to the actual situation in an inflamed tissue or around an inflamed cell. As soon as other commercial test systems for canine material become available, it would be worthwhile to further elucidate the effects of the two tested stimuli as well as the dexamethasone effects on canine corneal cells.

The canine cornea equivalent presented in this study is a useful model to test corneal effects of drugs intended for ophthalmologic use in this species. The use of RCE cells for the construction of the equivalent is advantageous for practical reasons, since the cells are commercially available, highly proliferative and unproblematic to cryoconserve compared to the slowly proliferating and difficult to passage primary canine epithelial cells. Considering their reaction to stimulation and morphological appearance on the canine cornea equivalents, marked differences can be detected, rendering them a practical but not optimal solution. It would be intriguing to study whether a canine epithelial cell line on the canine equivalents will show an improved morphological differentiation. Considering that the differences between species may reduce or impair the intercellular communication and thus hinder the epithelial differentiation of the RCE cells, it is possible that a canine derived cell line might react more similarly to the primary cells. Nevertheless, the potential for an altered morphology or reaction due to the transfection process have to be considered.

Generally, the use of a cornea model for the dog will aid the approval and licensing of human drugs for veterinary use. Regarding toxicological studies, a cornea equivalent could be a helpful screening tool and thus aid in the reduction of animal experiments. Although organotypic models for the assessment and prediction of ocular irritation have been developed and assessed (SINA et al. 1995; CHAMBERLAIN et al. 1997), they are usually based on fresh donor (or slaughter) material. One simple form of such an organotypic model is the culture of excised corneas. These have been developed for bovine corneas, since they are readily available from slaughter material (GAUTHERON et al. 1992; FOREMAN et al. 1996). Due to the initially discussed interspecies differences, the use of bovine corneas is limited for drug approval studies regarding canine medication. To use canine corneas on the other hand cannot be the method of choice for such studies, since too many animals would have to be sacrificed. The equivalent described in this study allows the construction of multiple corneas using one donor eye.

Following further characterization, especially of basement membrane structures, this model could also be valid in permeation studies, as indicated by other studies using bovine and human tissue (REICHL et al. 2004; TEGTMEYER et al. 2004). Furthermore, another possible future application for this model includes basic research on cellular interactions with the advantage of including all three major corneal cell types. Such studies could provide in-depth knowledge of physiological as well as pathological mechanisms of the cornea, and thus aid in the understanding of corneal disease.

6 Summary

Establishing a three-dimensional culture of canine corneal cells for *in vitro* studies on the effects of glucocorticoids

Anke Werner, 2007

To provide a model to be used for *in vitro* studies on drug effects in dogs, the aim of this study was the establishing of a protocol for the primary culture of canine corneal cells (i.e. endothelium, keratocytes, and epithelium) and subsequently the construction of a three-dimensional culture of canine corneal cells (cornea equivalent).
To study the glucocorticoid effects on the three major cell types of the cornea, dexamethasone was used. Since difficulties in the culture of primary canine corneal cells arose, a rabbit epithelial cell line (RCE cell) was used additionally. Both cell types were compared in their reaction to LPS and SDS stimulation, effects of dexamethasone and morphological appearance on the cornea equivalent.

Canine corneal cells were isolated using a combined enzymatic and mechanical technique. In culture, the different cell types were verified with phase contrast microscopy, immunofluorescense and western blotting. The cornea equivalent was constructed step by step in membrane inserts of a six-well plate. Stromal fibroblast in a collagen matrix were seeded onto a confluent endothelial cell layer and cultured for 6 – 8 days. Then either primary canine epithelial cells or RCE cells were added and grown to confluence in a submerged culture. Finally, the equivalent was lifted to the air-liquid-interface for two more weeks to allow a differentiation of the epithelial cells.
The glucocorticoid receptor (GR) was investigated in the primary canine corneal cells and the equivalents using RT-PCR and immunohistochemistry. The single cells (including RCE cells) and the cornea equivalents were stimulated with lipopolysaccharide (LPS) and sodium dodecyl sulfate (SDS) and treated with dexamethasone (1.0 and 0.01 µg/ml). The PGE_2 concentration, which increases during an inflammatory reactions, was chosen as an indicator to study the effects of dexamethasone after such stimulation. This pro-inflammatory molecule

was studied in the culture medium of single cultures of the three major cell types of the canine cornea, of RCE cells as well as in the canine cornea equivalents.

A protocol for the isolation and culture of canine corneal cells was successfully established in this study, and the identity of the cells was verified. The three cell types were successfully reassembled in a vital cornea equivalent which was cultured for a total of five weeks. The GR was detected in both the cultured canine cells and the canine cornea equivalents. The use of RCE cells instead of canine corneal cells in the construction of the cornea equivalents revealed morphological differences. The equivalents constructed with primary canine cells resembled the canine cornea *in vivo* more closely.
An increased PGE_2 concentration was measured in canine epithelial cells and keratocytes after the stimulation with LPS and in canine epithelial cells and endothelial following stimulation with SDS. Dexamethasone reduced the LPS-induced PGE_2 production in a dose-dependent manner. The SDS-induced PGE_2 concentration was less clearly reduced by dexamethasone, caused by a higher variance of the results. The RCE cells did not react similarly to the primary canine epithelial cells since both stimuli failed to induce an increase in PGE_2. In the cornea equivalents, both stimuli led to a significant increase in PGE_2 which could be reduced in a dose-dependent manner by both dexamethasone concentrations tested.

The primary culture of the canine corneal cells and the cornea equivalent are interesting systems to test drug effects on corneal cells. The cornea equivalents were even more sensitive to the stimulation and dexamethasone treatment than the single cell cultures. Studies using single cell cultures and the equivalent may reveal further insights on pathophysiological and therapeutic mechanisms in ocular disease. As the dog is one of the species most often treated in veterinary ophthalmology, such models should help improve the treatment of ocular disorders in this species.

7 Zusammenfassung

Etablierung einer dreidimensionalen caninen Kornea-Zellkultur zur Untersuchung der Wirkung von Glukokortikoiden *in vitro*

Anke Werner, 2007

Die vorliegende Studie hatte zum Ziel, ein Modell zur Untersuchung von Medikamentenwirkungen an der Hundehornhaut zu entwickeln. Dazu gehörten die Etablierung eines Protokolls zur Isolierung und Kultivierung primärer Hundehornhautzellen (Endothel, Keratozyten, Epithel) sowie die Konstruktion einer dreidimensionalen caninen Kornea-Zellkultur (Cornea-Equivalent). Die Untersuchung der Glukokortikoidwirkung erfolgte mittels Dexamethason.
Aufgrund von Schwierigkeiten in der Kultur der primären Hundeepithelzellen, wurde zusätzlich eine Kaninchen-Epithelzelllinie (RCE Zellen) verwendet. Die beiden Zellarten wurden hinsichtlich ihrer Reaktion auf die Stimulation mit Lipopolysaccharid (LPS) und Natrium Dodecylsulphat (SDS), die Behandlung mit Dexamethason sowie ihre Morphologie im Cornea-Equivalent untersucht.

Canine Korneazellen wurden mittels kombinierter enzymatischer und mechanischer Methode isoliert und die verschiedenen Zelltypen mit Hilfe der Phasenkontrastmikroskopie, Immunfluoreszenz und des Wester Blots verifiziert. Die Cornea-Equivalente wurden Schritt für Schritt in Membraneinsätzen in 6-well-Platten konstruiert. Während der ersten 3 Wochen erfolgte der Aufbau der einzelnen Schichten in submerser Kultur. In den letzen beiden Wochen der Kultur wurden die Cornea-Equivalente an die Luft-Medium-Grenze angehoben, um eine Differenzierung des Epithels zu ermöglichen. Als Epithel kamen entweder primäre Hundezellen oder die RCE Zellen zum Einsatz.
Der Glukokortikoid Rezeptor (GR) wurde in den Hundehornhautzellen und den Cornea-Equivalenten mittels RT-PCR und Immunhistochemie untersucht. Die Stimulation der Einzelzellen (inklusive der RCE Zellen) und der Cornea-Equivalente erfolgte mittels LPS und SDS, behandelt wurde mit Dexamethason (1,0 und 0,01 µg/ml). Die Konzentration von PGE_2, die im Verlauf einer Entzündungsreaktion ansteigt, wurde als Indikator für die

Dexamethasonwirkung nach Stimulation gewählt. Dieses pro-inflammatorische Molekül wurde im Zellkulturüberstand untersucht.

Die Isolation und Kultur der Hundehornhautzellen konnte in dieser Studie erfolgreich etabliert werden. Die drei Zelltypen wurden anhand oben erwähnter Kriterien identifiziert. Des Weiteren konnten die Primärzellen der Hornhaut erfolgreich in einem Cornea-Equivalent zusammengesetzt und über einen Zeitraum von 5 Wochen kultiviert werden. Der GR wurde sowohl in den einzeln kultivierten Hundehornhautzellen als auch in den caninen Cornea-Equivalenten nachgewiesen. Bei der Verwendung von RCE Zellen statt caniner Hornhautepithelzellen konnten morphologische Unterschiede in den Equivalenten festgestellt werden. Die Cornea-Equivalente die allein aus Hundezellen bestanden waren der Hundehornhaut *in vivo* ähnlicher.
Bei den Hundeepithelzellen und Keratozyten führte eine Stimulation mit LPS zu einem PGE_2 Anstieg. Bei den Hundeendothel und –epithelzellen hatte SDS den gleichen, wenngleich schwächeren Effekt. Die übrigen caninen Zelltypen reagierten auf die Stimulation nicht mit einem messbaren Anstieg an PGE_2. Dexamethasone führte in den LPS-stimulierten Hundezellen zu einer dosisabhängigen Reduktion von PGE_2. Bei den SDS-stimulierten Hundezellen hatte Dexamethason einen geringeren Effekt aufgrund erhöhter Streuung der Ergebnisse. Die RCE Zellen verhielten sich nicht vergleichbar mit den Hundeepithelzellen, da diese Zellen auf keinen der beiden Stimuli mit einem deutlichen PGE_2 Anstieg reagierten. Bei den Cornea-Equivalenten führten beide Stimulationssubstanzen zu einem signifikanten Anstieg an PGE_2, der in beiden Konzentrationen durch Dexamethason gehemmt wurde.

Die Primärkultur der caninen Hornhautzellen und das Cornea-Equivalent stellen interessante Systeme zur Untersuchung von Arzneimittelwirkungen auf Hornhautzellen dar. Die Cornea-Equivalente reagieren sogar sensibler auf eine Stimulation und Dexamethasonbehandlung als die Einzelzellen. Der Einsatz beider Modelle könnte bei der Erforschung pathophysiologischer und therapeutischer Mechanismen bei Augenerkrankungen hilfreich sein. In der tiermedizinischen Ophthalmologie ist der Hund eine der am Häufigsten behandelten Tierarten und die Verfügbarkeit solcher Modelle sollte dazu beitragen, die Therapie von Augenkrankheiten in dieser Tierart zu verbessern.

8 Appendix

8.1 Cell culture

8.1.1 Cell Count

Tab. 13 Absolute numbers for the cell count between passage 1 and 2 or 1 and 3 for canine corneal keratocytes and endothelial cells, respectively. No. = identification of Beagle dogs, n.m. = not measured.

No.	**endothelial cells**			**keratocytes**	
	passage 1	passage 2	passage 3	passage 1	passage 2
B 1	427500	555000	n.m.	5280000	43800000
B 2	480000	2212500	17866980	4980000	31650000
B 3	592500	4680000	30569700	5040000	33000000
B 4	150000	n.m.	n.m.	6120000	39015000
B 5	322500	630000	1026666	5580000	43105500
B 6	390000	1027500	3188130	5310000	40680000
B 7	637500	4170000	27398330	4710000	35640000
B 8	90000	4950000	75330000	5940000	57625300
B 9	457500	840000	n.m.	3750000	16778580
B 10	90000	7230000	44850000	3720000	17560120
B 11	60000	2130000	15120000	2970000	33720000
B 12	90000	4170000	27810000	8220000	67500000
B 13	270000	3690000	12240000	3210000	35400000
B 14	510000	2010000	5850000	4890000	26100000
B 15	210000	4500000	18150000	2850000	16500000
B 16	135000	5460000	19080000	4440000	42300000
B 19	n.m.	n.m.	n.m.	1920000	6420000
B 20	n.m.	n.m.	n.m.	4620000	20520000
B 21	n.m.	n.m.	n.m.	5100000	15510000
mean	307031	3217000	22959985	4665789	32780237
SD	195528	2032511	19849814	1416099	15276622

Tab. 14 Absolute numbers for the cell count between isolation and passage 2 (canine corneal epithelial cells).

No.	**epithelial cells**		
	passage 0	passage 1	passage 2
B 20	3307500	1065000	1350000
B 21	1150000	5520000	1620000
B 21	230000	820000	510000
B 21	1380000	890000	1095000
B 22	1905000	135000	60000
B 23	1590000	2340000	852222
mean	1593750	1795000	914537
SD	1013533	1961163	568795

8.1.2 Growth Curves

Tab. 15 Canine corneal cells (fresh); measurement of photometrical extinction (mean of at least 6 values).

time [h]	endothelial cells						epithelial cells		keratocytes		
	mean					SD	mean	SD	mean		SD
4	0.336	0.392	0.752	0.256	0.434	0.190	0.693	0.592	0.072	0.256	0.010
24	0.409	0.378	0.734	0.306	0.457	0.164	0.780	0.702	0.055	0.306	0.027
48	0.600	0.548	0.729	0.470	0.587	0.094	0.961	0.554	0.288	0.470	0.049
72	0.661	0.708	0.670	0.979	0.754	0.131	0.977	0.672	0.216	0.979	0.123
96	0.725	1.299	1.047	1.074	1.036	0.205	1.112	0908	0.144	1.578	0.093
120	1.348	1.601	1.335	1.244	1.382	0.133	1.229	1.137	0.065	2.099	0.134
144	0.950	1.282	1.093	0.995	1.080	0.127	1.191	1.080	0.078	1.804	0.273

Tab. 16 Endothelial cells (thawed); measurements of photometrical extinction (mean of 6 values).

time [h]	B8		B11		B12		B15		B16	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
4	0.273	0.004	0.320	0.010	0.287	0.009	0.313	0.009	0.358	0.011
24	0.710	0.523	0.551	0.120	0.405	0.092	0.413	0.021	0.487	0.032
48	0.535	0.055	0.418	0.029	0.442	0.048	0.420	0.029	0.523	0.038
72	0.748	0.072	0.762	0.162	0.648	0.034	0.541	0.115	0.746	0.029
96	1.045	0.098	0.833	0.056	0.898	0.041	0.811	0.089	1.015	0.046
120	0.764	0.072	0.729	0.109	0.910	0.076	0.554	0.052	0.731	0.104
144	0.895	0.023	0.735	0.024	0.733	0.015	0.810	0.041	0.880	0.085

Tab. 17 Keratocytes (thawed); measurements of photometrical extinction (mean of 6 values).

time [h]	B8		B11		B12		B15		B16	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
4	0.298	0.012	0.278	0.010	0.328	0.020	0.324	0.011	0.363	0.031
24	0.339	0.005	0.388	0.086	0.430	0.074	0.361	0.015	0.503	0.021
48	0.432	0.028	0.557	0.042	0.695	0.016	0.464	0.033	0.811	0.043
72	0.700	0.040	0.599	0.069	0.935	0.114	0.486	0.039	0.929	0.099
96	0.892	0.034	0.771	0.039	1.154	0.041	0.782	0.058	1.248	0.107
120	1.136	0.066	0.981	0.065	1.155	0.068	0.663	0.045	1.205	0.050
144	0.890	0.065	0.850	0.029	0.888	0.051	0.673	0.035	0.974	0.037

Tab. 18 RCE cells (thawed); measurement of photometrical extinction (mean of 6 values).

time [h]	RCE 1		RCE 2		RCE 3	
	mean	SD	mean	SD	mean	SD
4	0.154	0.010	0.198	0.008	0.266	0.009
24	0.254	0.018	0.314	0.004	0.391	0.017
48	0.334	0.025	0.428	0.027	0.495	0.008
72	0.329	0.005	0.338	0.009	0.395	0.009
96	0.441	0.020	0.484	0.050	0.521	0.044

8.2 Inflammatory model

8.2.1 Cell Viability

Tab. 19 Data for the MTT-tests following the stimulation with LPS and treatment with dexamethasone. Production of formazan measured as spectrophotometrical extinction; each value is a mean of 6 measures.

	endothelial cells			
	C_0	C_{LPS}	0.01 µg/ml Dex	1 µg/ml Dex
	1.156	1.301	1.280	1.241
	1.397	1.472	1.629	1.452
	0.971	1.118	1.064	1.098
	1.265	1.337	1.489	1.372
	1.065	1.244	1.187	1.111
mean	1.170	1.294	1.330	1.254
SD	0.167	0.130	0.228	0.157
	keratocytes			
	0.969	1.076	1.038	0.968
	0.943	1.120	1.062	1.000
	1.066	0.962	1.042	0.897
	1.201	1.364	1.286	1.127
	1.048	1.045	1.148	0.998
mean	1.045	1.113	1.115	0.998
SD	0.101	0.151	0.105	0.083
	epithelial cells			
	1.452	1.603	1.295	1.607
	2.467	1.512	1.479	1.473
	1.441	1.418	1.201	1.311
	1.620	1.623	1.578	1.537
	2.277	2.233	2.167	2.159
	1.042	1.544	1.605	1.484
	1.382	1.733	1.609	1.531
mean	1.669	1.667	1.562	1.586
SD	0.514	0.268	0.311	0.269

Tab. 20 Data for the MTT-tesst following the stimulation with SDS and treatment with dexamethasone. Production of formazan measured as spectrophotometrical extinction; each value is a mean of 6 measures.

endothelial cells							
B 10	0.818	0.714	0.819	0.827	0.792	0.837	0.818
B 10_2	0.814	0.653	0.822	0.811	0.793	0.802	0.708
B 11	0.981	0.873	0.979	0.995	0.995	1.001	1.002
mean	0.871	0.747	0.873	0.877	0.860	0.880	0.842
SD	0.095	0.114	0.092	0.102	0.117	0.106	0.149
keratocytes							
B 4	0.947	0.757	0.879	0.900	0.814	0.811	0.822
B 5	1.241	1.162	1.268	1.270	1.192	1.154	1.106
B 7	0.998	0.813	0.926	0.939	0.882	0.869	0.904
B 11	1.023	0.714	0.923	0.912	0.870	0.864	0.919
B 6	0.753	0.584	0.679	0.703	0.674	0.703	0.698
mean	1.004	0.818	0.949	0.956	0.905	0.897	0.907
SD	0.200	0.248	0.242	0.234	0.214	0.188	0.167

8.2.2 PGE_2 concentrations

Tab. 21 PGE_2 concentration [pg/ml] in the culture medium supernatant after stimulation with LPS and treatment with two concentrations of dexamethasone in primary canine corneal epithelial and endothelial cells; SD = standard deviation.

epithelial cells					**endothelial cells**				
	C_0	C_{LPS}	Dex 0.01 µg/ml	Dex 1 µg/ml		C_0	C_{LPS}	Dex 0.01 µg/ml	Dex 1 µg/ml
B 3	180.47	251.60	59.88	93.94	B 10	208.06	150.96	49.47	9.89
	1236.91	2795.98	3513.68	110.83		13624.27	4771.87	2473.24	6748.52
B 10	1641.85	3231.86	1686.78	645.38	B 3	23.67	65.48	149.50	55.48
B 3	1664.16	1514.04	4856.75	3395.73		5784.12	4516.80	2260.42	n.m.
	930.99	991.28	2482.92	694.67	B 2	246.43	79.26	58.14	131.26
B 31	48.83	1177.62	598.94	214.31		17940.03	10387.69	7834.65	2129.28
	192.77	837.63	617.19	522.02	B 12	125.94	458.01	116.92	173.98
B 34	269.07	486.53	918.04	655.00		296.55	226.68	58.14	238.39
	410.72	n.m.	867.80	867.80		4467.79	10098.39	3320.97	5398.61
B 12	618.67	1831.62	1518.39	72.12	B 8	298.89	23.67	6.33	4.00
B 8	684.58	2350.76	1555.52	368.29		89.87	129.92	4.00	173.98
	469.31	2336.28	793.23	115.25		n.m.	4467.79	22756.21	n.m.
B 2	n.m.	15079.41	32883.13	n.m.	B 7	4.00	4.00	4.00	57.25
						4.00	4.00	4.00	38.99
						6060.74	6354.61	4.00	1144.97
mean	695.69	2740.38	4027.09	646.28	**mean**	3512.45	2782.61	2606.67	1254.20
SD	559.12	3994.74	8770.74	909.23	**SD**	5719.05	3756.56	5973.79	2240.27

Tab. 22 PGE_2 concentration [pg/ml] in the culture medium supernatant after stimulation with LPS and treatment with two concentrations of dexamethasone in canine corneal keratocytes and RCE cells; SD = standard deviation.

	keratocytes				**RCE cells**			
	C_0	C_{LPS}	Dex 0.01 µg/ml	Dex 1 µg/ml	C_0	C_{LPS}	Dex 0.01 µg/ml	Dex 1 µg/ml
B 2	527.55	548.96	543.56	43.32	420.14	630.23	356.81	189.86
B 3	4.00	351.33	356.67	30.74	405.99	212.34	449.13	346.24
	501.52	622.40	442.28	60.98	790.70	630.23	488.23	463.98
B 4	47.80	242.38	156.90	14.16	652.04	502.18	146.14	275.77
	202.68	204.46	86.62	42.92	853.92	329.53	245.55	241.55
	664.51	133.33	63.51	409.72	449.38	332.39	228.11	177.85
B 5	15.52	2081.31	2643.63	688.05				
	110.71	5616.42	3644.62	833.49				
	742.14	4590.77	2269.57	73.73				
B 9	4.00	296.74	578.18	34.02				
	4566.45	197.46	1267.50	1333.08				
mean	671.54	1353.23	1095.73	324.02	595.36	439.48	318.99	282.54
SD	1321.74	1945.90	1217.02	444.23	198.04	174.27	134.57	107.94

Tab. 23 PGE_2 concentration [pg/ml] in the culture medium supernatant after stimulation with SDS and treatment with two concentrations of dexamethasone in primary canine corneal epithelial cells, keratocytes, endothelial cells as well as RCE cells; SD = standard deviation.

	epithelial cells				**endothelial cells**			
	C_0	C_{SDS}	Dex 0.01 µg/ml	Dex 1 µg/ml	C_0	C_{SDS}	Dex 0.01 µg/ml	Dex 1 µg/ml
	125.08	185.58	1.00	55.46	0.50	403.88	0.50	296.25
	7.21	85.66	224.78	22.48	157.67	119.66	0.50	0.50
	35.55	33.10	10.43	1.00	0.50	0.50	4.87	0.50
	48.14	72.43	13.28	76.66	0.50	189.16	0.50	216.34
	10.13	100.61	74.53	115.97	166.06	331.09	37.27	316.00
	9.01	139.75	40.83	157.60	0.74	6.08	16.06	39.37
	81.15	220.54	894.30	77.98	1.74	23.35	42.73	0.50
mean	45.18	119.67	179.88	72.45	46.81	153.39	14.63	124.21
SD	44.26	66.04	324.37	53.36	78.63	162.64	18.25	146.05
	keratocytes				**RCE cells**			
	C_0	C_{SDS}	Dex 0.01 µg/ml	Dex 1 µg/ml	C_0	C_{SDS}	0.01 µg/ml Dex	1 µg/ml Dex
	0.50	0.50	10.00	10.00	974.49	585.02	307.72	268.95
	0.50	0.50	7.00	7.00	632.84	585.02	206.99	531.42
	0.50	0.50	15.00	15.00	546.84	347.08	166.96	192.58
	0.50	0.50	10.00	10.00	1411.11	1545.16	421.69	826.85
					1629.72	1982.07	614.35	626.60
					1245.74	1586.66	1230.68	396.04
mean	0.50	0.50	10.50	10.50	1073.46	1105.17	491.40	473.74
SD	0	0	3.31	3.31	432.29	679.73	396.58	235.97

Tab. 24 PGE_2 concentration [pg/ml] in the culture medium supernatant of the cornea equivalents after stimulation with LPS and treatment with dexamethasone. Equivalents were constructed using only primary canine corneal cells; SD = standard deviation.

cornea equivalent				
	C_0	C_{LPS}	0.01 µg/ml Dex	1 µg/ml Dex
	6515.79	145881.60	127046.40	21449.45
	7543.57	151954.60	113427.00	10094.98
	74.46	373785.10	1.00	11920.32
	187.96	290844.00	1.00	7324.26
	1.00	460.41	1.00	1.00
	1.00	82.65	1.00	1.00
	6966312.00	2.46	7.79	1.00
	1525776.00	56.66	1.00	1.00
	12421.39	39435880.00	1.00	21.53
	475.66	16424860.00	1.00	1.00
	387.72	24242.09	1432407.00	508683.30
	16959.67	19752.77	973991.30	651709.40
	15139.50	44197.63	13110.65	12424.41
	n.m.	30545.11	13515.95	9013.40
mean	657830.44	4067324.65	190965.22	88046.15
SD	1941490.72	11071909.65	440215.15	210484.32

Tab. 25 PGE_2 concentration [pg/ml] in the culture medium supernatant of the cornea equivalents after stimulation with SDS and treatment with dexamethasone. Equivalents were constructed using primary canine keratocytes and endothelial cells as well as RCE cells; SD = standard deviation.

cornea equivalent				
	C_0	C_{SDS}	0.01 µg/ml Dex	1 µg/ ml Dex
	904.16	2802.77	729.64	817.60
	916.02	2815.74	952.05	406.59
	1163.45	3275.77	1340.00	433.78
	828.91	2012.88	1211.37	640.73
mean	953.13	2726.79	1058.27	574.68
SD	145.42	524.32	272.09	192.77

8.3 RT-PCR

8.3.1 RNA isolation

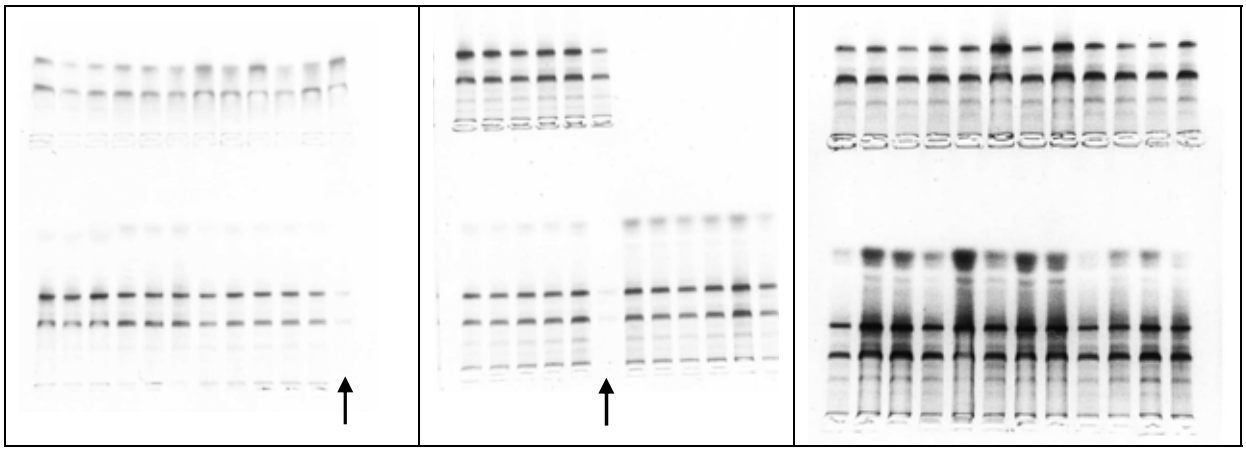


Fig. 62 Representative gels used for the verification of RNA content following RNA isolation. Samples with little RNA content (arrows) were not included in the experiments.

8.3.2 Adjustment of sample amount

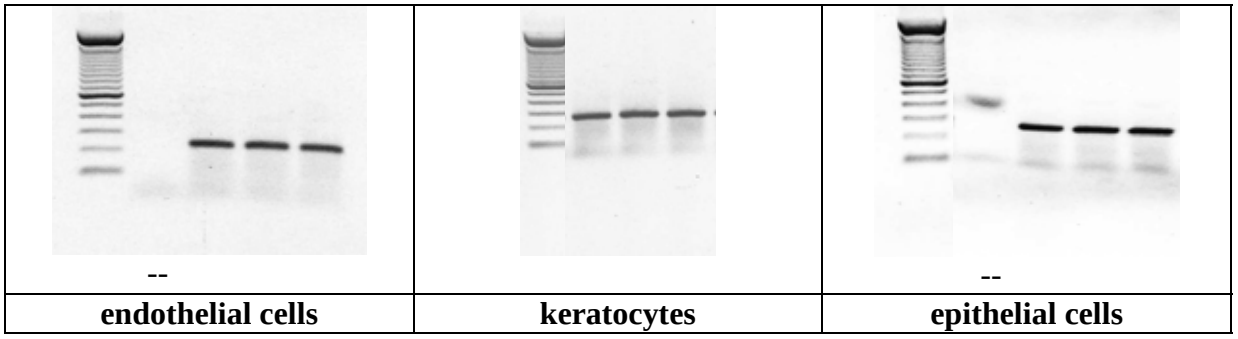
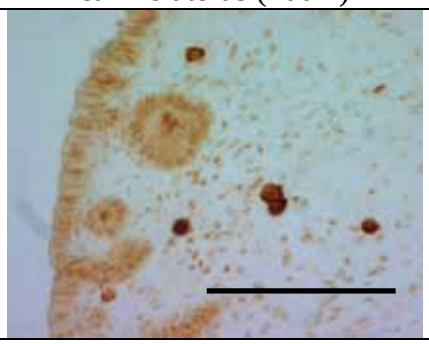
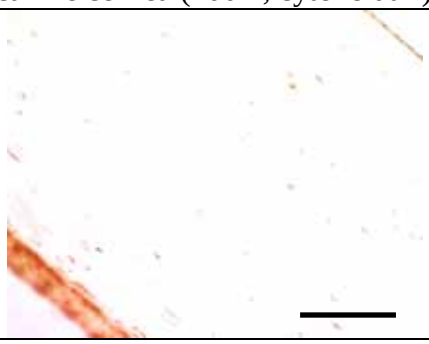


Fig. 63 Representative gels used to determine similar RNA content. RT-PCR for GAPDH was run with all three cell types and amount of PCR sample adjusted to receive equal sizes in bands; -- = neg. control.

8.4 Immunohistochemistry

Tab. 26 Controls for the immunohistological stains. Ab = antibody used, GR = anti-glucocorticoid receptor, Vim = anti-vimentin, Cyto = anti-cytokeratin. Magnification as listed underneath each picture; bar = 100 µm.

Ab	positive control	negative control
GR		
	canine uterus (100 x)	
		
	canine uterus (400 x)	cornea equivalent (100 x)
Vim/ Cyto		
	canine cornea (200 x, cytokeratin)	
		
	canine cornea (200 x, vimentin)	canine cornea (25 x)

8.5 Index of figures

8.6 Index of tables

9 References

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