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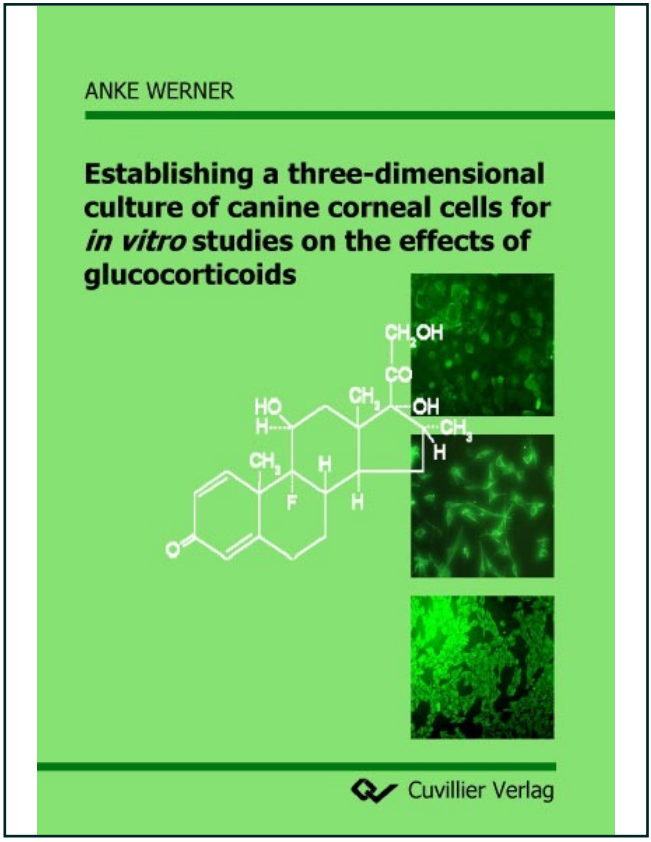
# Establishing a three-dimensional culture of canine corneal cells for in vitro studies on the effects of glucocorticoids

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# 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.

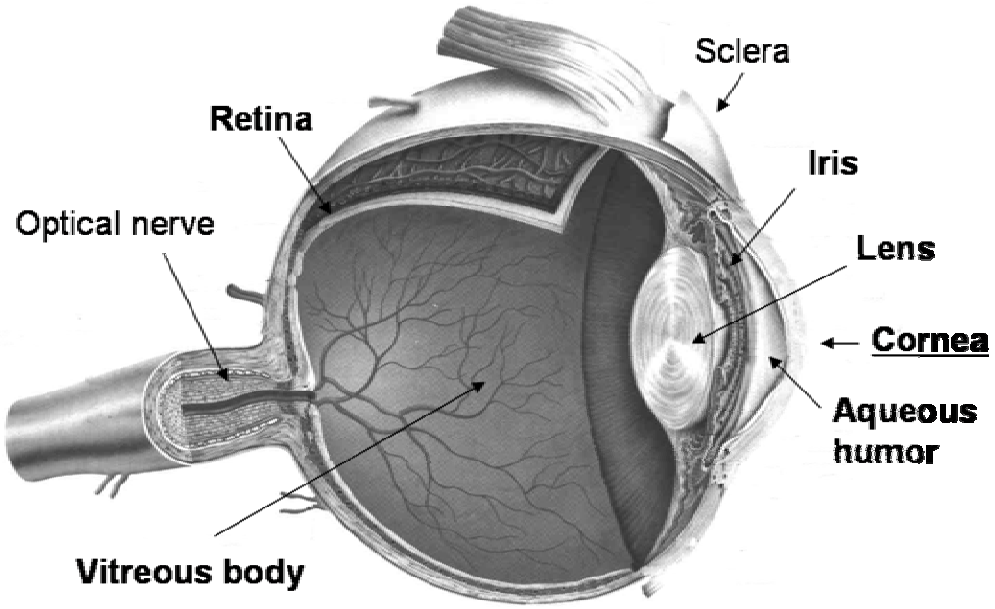


**Fig. 1** Bulbus oculi; schematic illustration highlighting the major structures of the eye (source: OFTALNET (2007))

## 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).

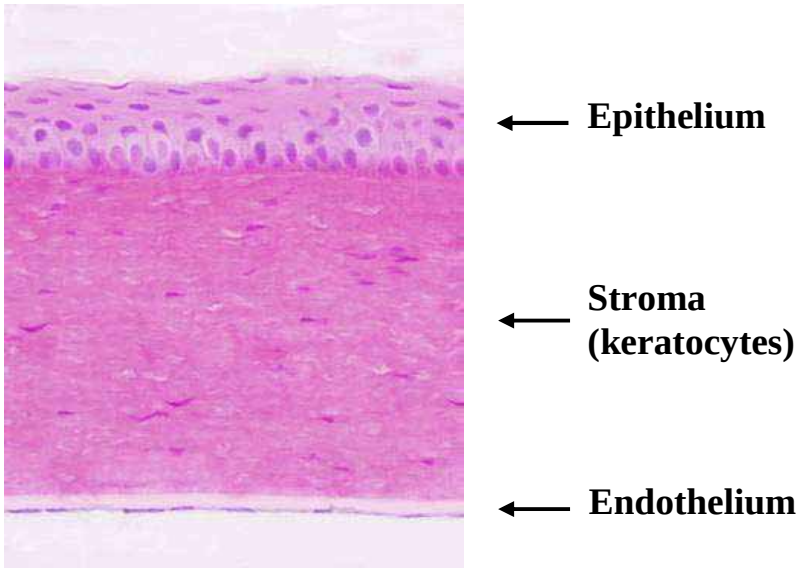


**Fig. 2** Transverse section through the cornea; HE stain of a rat cornea (source: THE UNIVERSITY OF WESTERN AUSTRALIA (2007))

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.

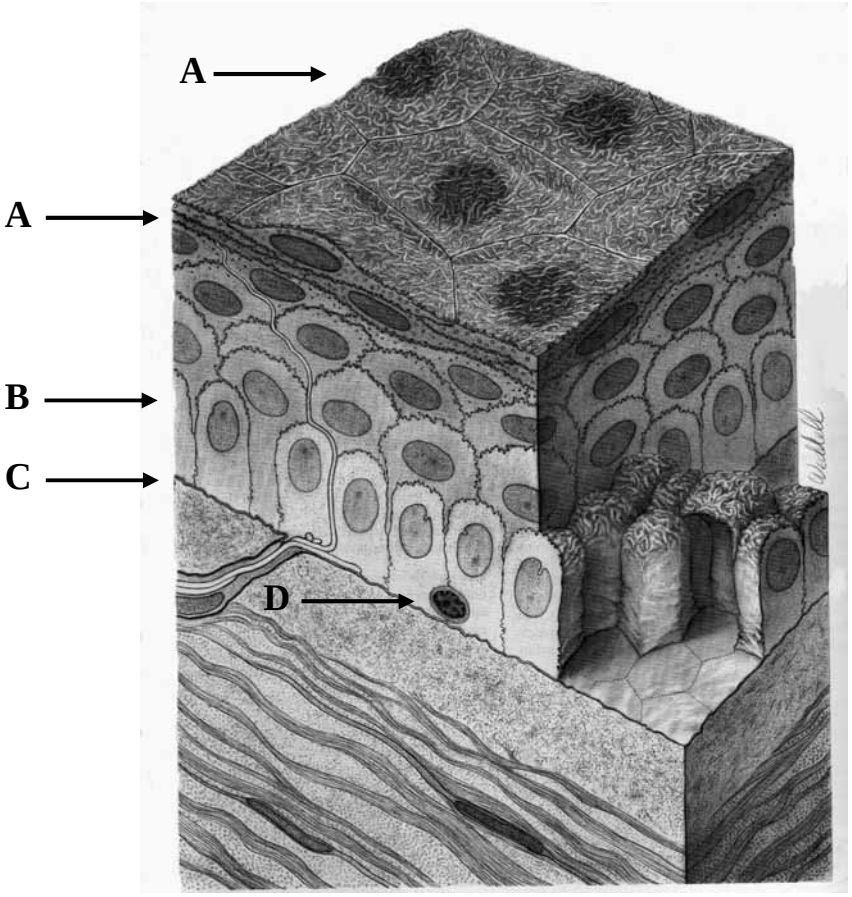


**Fig. 3** Cornea epithelium. **A** = flattened apical epithelial cells with microplicae and microvilli, **B** = columnar basal epithelial cells, **C** = basement membrane, **D** = lymphocyte (SLATTER 2001a).

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).

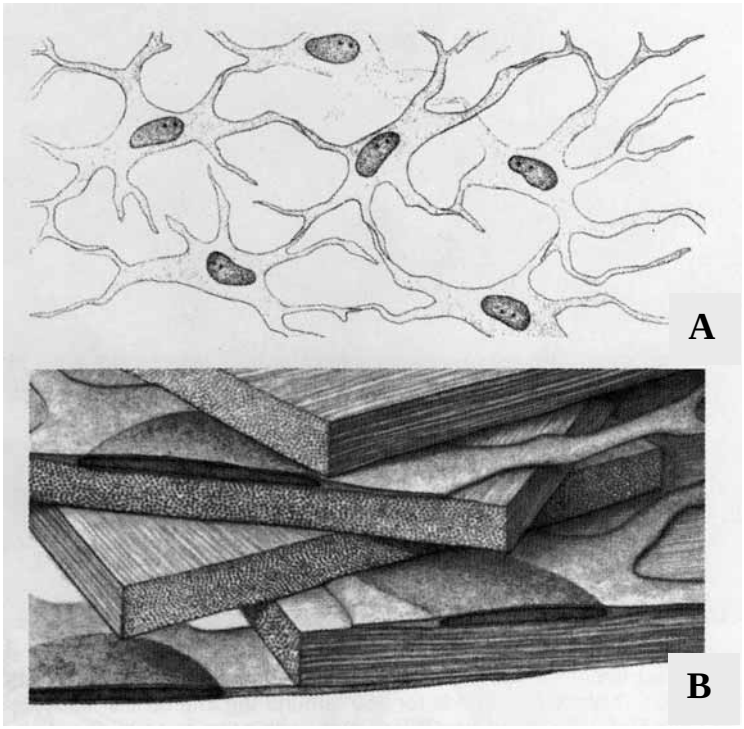


**Fig. 4** Cornea stroma. **A** = fibroblasts lying between the stromal lamellae. The cells are thin and flat, with long processes that are in contact with other fibroblasts of the same plane. **B** = lamellae. The lamellae consist of collagen fibrils, which are oriented in parallel to each other. Successive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