Copyright Undertaking

This thesis is protected by copyright, with all rights reserved.

By reading and using the thesis, the reader understands and agrees to the following terms:

1. The reader will abide by the rules and legal ordinances governing copyright regarding the use of the thesis.

2. The reader will use the thesis for the purpose of research or private study only and not for distribution or further reproduction or any other purpose.

3. The reader agrees to indemnify and hold the University harmless from and against any loss, damage, cost, liability or expenses arising from copyright infringement or unauthorized usage.

If you have reasons to believe that any materials in this thesis are deemed not suitable to be distributed in this form, or a copyright owner having difficulty with the material being included in our database, please contact lbsys@polyu.edu.hk providing details. The Library will look into your claim and consider taking remedial action upon receipt of the written requests.

Pao Yue-kong Library, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

http://www.lib.polyu.edu.hk
The Hong Kong Polytechnic University
School of Optometry

Development and Evaluation of a Novel Porcine Desiccation-induced Dry Eye Model for Investigation of the Aetiology and Treatment for Dry Eye - Implications for the Ageing Eye

BY

Emily Pik-Yin Choy

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

Aug 2005
Declaration

The work submitted in this thesis is the own work of the author and it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Signed

Emily Pik-Yln Choy (Candidate)

Signed

Dr Pauline Cho (Chief Supervisor)
Table of Contents

Acknowledgements viii

List of Figures ix

List of Tables xi

List of Published and Presented Work xiii

Abstract xiv

Chapter 1 Literature review 1

1.1 Introduction 1

1.2 Normal tear film and dry eye 3

1.2.1 General function of tears 3

1.2.2 Structure and function of tear film 4

1.2.2.1 Superficial lipid layer 5

1.2.2.2 Aqueous layer 6

1.2.2.3 Mucus layer 6

1.2.3 Dry eye 7

1.2.3.1 Definition and classification 7

1.2.3.2 Risk factors for dry eye 9
1.2.3.3 Prevalence of dry eye

1.2.3.4 Treatment of dry eye

1.3 Models for dry eye study using cell or organ culture method and the whole enucleated eye

1.4 Animal dry eye models

1.4.1 Animals in normal condition for studying the effect / toxicity of different agents for dry eye treatment

1.4.2 Naturally occurring dry eye in dogs

1.4.3 Induction of dry eye condition in animal dry eye models

1.4.3.1 Methods for induction of dry eye in animals

1.4.3.2 Comparison between different animal dry eye models

1.4.4 Animals used in dry eye research

1.4.5 Why porcine eye?

1.4.6 Similarities and differences in cornea of different species

1.4.6.1 Morphology and histochemistry
1.4.6.2 Permeability

1.4.6.3 Reaction on acanthamoeba infection

1.4.6.4 Metabolic compatibility

1.4.6.5 Thermal and biomechanical parameters

1.5 Assessment methods for corneal integrity and epithelial cell viability

1.5.1 Clinical assessment of corneal integrity

1.5.1.1 Sodium fluorescein

1.5.1.2 Rose bengal

1.5.1.3 Lissamine green

1.5.2 Assessment of cell viability

1.5.2.1 By membrane exclusion

1.5.2.2 By Biochemical function

1.6 Summary and aims of the project

Chapter 2 Specific objectives and outlines of structure of the thesis

2.1 Objectives
2.2 Thesis outline

Chapter 3  General materials and assessment methods

3.1 Materials and methods

3.2 Assessment methods used

   3.2.1 Integrity of corneal surface
   3.2.2 Viability of corneal epithelial cells

3.3 Pre-experimental preparation

   3.3.1 Development of dissection skill
   3.3.2 Selection and optimization of the environmental conditions for the pDEM

Chapter 4  Preliminary study – Will sodium fluorescein solution affect the results of trypan blue exclusion test?

4.1 Introduction

4.2 Experimental design
Chapter 5  Viability of porcine corneal epithelium of enucleated eye and effect of exposure to air

5.1 Introduction 78
5.2 Experimental design 80
5.3 Results 81
5.4 Discussion 86
5.5 Conclusion 90

Chapter 6  Development of the porcine dry eye model and evaluation of the reproducibility and applicability in simulation of different severity of desiccation-induced dry eye

6.1 Introduction 92
6.2 Experimental design 94
Chapter 7  Effect of different ‘blink’ rates on simulated dry eye condition using the porcine dry eye model

7.1 Introduction

7.2 Experimental design

7.3 Results

7.4 Discussion

7.5 Conclusion

Chapter 8  Effect of artificial tears preparation and different viscosity of methylcellulose solutions in simulated dry eye condition using the porcine dry eye model

8.1 Introduction
8.2 Experimental design 123

8.3 Results 127

8.4 Discussion 135

8.5 Conclusion 139

Chapter 9  Overall discussion and conclusions

9.1 Overall summary 140

9.2 Limitations of the study and suggestions for further studies 143

9.3 Main conclusions 146

Reference 147
Acknowledgements

I would like to express my sincere thanks to my chief supervisor, Dr. Pauline Cho, and my co-supervisors, Prof. Iris Benzie and Dr. Tony To, for their encouragement, support and invaluable guidance throughout the period of study.

I would also like to thank Dr. Camus Choy for his support and useful advices and Mr. Andy Kwong for his technique support.

I also wish to thank Prof. Barry Collin for his help in correcting the English of this dissertation and all my colleagues in the optometry research team for their supports. Moreover, I would like to thank my family for their patient, support and understanding.

Finally, I would like to express my gratitude to the School of Optometry and The Hong Kong Polytechnic University for providing this grand to support my research study.
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Structure and composition of the tear film</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Classification of dry eye</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Classification of animal dry eye models</td>
<td>33</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Fluorescein grading system from Grades 0 – 4</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Preparation of a porcine cornea (schematic diagram) for counting the number of trypan blue stained cells.</td>
<td>71</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>A scatter plot showing the number of stained cells (per field) in the central and peripheral cornea in different groups</td>
<td>83</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>Stained porcine corneal epithelial cells under different conditions</td>
<td>84</td>
</tr>
<tr>
<td>Figure 6.1</td>
<td>A dissected porcine eye ball with the nictitating membrane, the lacrimal gland and some conjunctival tissue connecting the nictitating membrane and the lacrimal gland</td>
<td>94</td>
</tr>
<tr>
<td>Figure 6.2</td>
<td>Details of the pDEM setup.</td>
<td>95</td>
</tr>
<tr>
<td>Figure 6.3</td>
<td>Two identical pDEM set-up</td>
<td>95</td>
</tr>
<tr>
<td>Figure 6.4</td>
<td>Both pDEM systems were enclosed in a large plastic box</td>
<td>96</td>
</tr>
<tr>
<td>Figure 6.5</td>
<td>The porcine dry eye model (pDEM) set up</td>
<td>97</td>
</tr>
<tr>
<td>Figure 6.6</td>
<td>The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 20 seconds</td>
<td>102</td>
</tr>
</tbody>
</table>
Figure 6.7.  The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 40 seconds

Figure 6.8.  The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 60 seconds

Figure 6.9.  The relationship between different “lacrimation-blink” intervals and the final fluorescein grades of porcine corneas of different “lacrimation-blink” intervals

Figure 8.1.  A plot of viscosity against concentration (w/v; %) of MC solutions
List of Tables

Table 1.1. Similarities and differences between corneas of pigs, rabbits and cows 48

Table 5.1. Number of stained cells (median, range and mean (SD)) per field (0.25 mm²) of corneas under the four conditions 82

Table 5.2. Number of stained cells (median, range and mean (SD)) per field (0.25 mm²) of peripheral corneas under the four conditions 82

Table 5.3 Published data on the number of non-viable epithelial cells in the rabbit corneal epithelium immediately after enucleation and data obtained in the current study using porcine eyes 87

Table 6.1. The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 20, 40 and 60 seconds. 102

Table 6.2. A summary of results obtained using trypan blue exclusion technique with our novel porcine dry eye model 105

Table 7.1. Initial, final and increased fluorescein grades of corneas in pDEM with 6, 12 and 20 seconds ‘inter-blink’ intervals 115

Table 8.1. Comparison of the increase in fluorescein grade (final grade – initial grade) among control and experimental groups 129

Table 8.2. Comparison of the number of trypan blue-stained cells in corneas of the control and the experimental groups 131
Table 8.3. Comparison of the increase in fluorescein grade (final grade – initial grade) among different MC solution groups 132

Table 8.4. Comparison of the number of trypan blue-stained cells in the central corneas among different MC solution groups 133

Table 8.5. Comparison of the number of trypan blue-stained cells in the peripheral corneas among different MC solution groups. 133
List of Published and Presented work

Journal articles


Conference paper

Abstract

Dry eye syndrome is multi-factorial in origin and has become a worldwide ocular problem, especially affecting the elderly. There have been several investigations into the aetiology and treatment of dry eye. Apart from clinical studies, several animal models of dry eye have been studied. However, due to the species difference and the complexity of dry eye, no single animal model can represent completely the human dry eye condition.

Porcine organs are better substitutes for human organs; however, porcine eyes have never been used to study dry eye. Moreover, among all developed animal models, none can be used to study the effect of blinking in dry eye.

Therefore, the aims of this study were to:

- Determine the possibility of using porcine eye to establish an animal dry eye model (DEM)
- Develop a novel porcine dry eye model (pDEM) with adjustable ‘lacrimation’ and ‘blink’ rates
Ascertain the reproducibility of the pDEM system

Simulate different severities of desiccation-induced dry eye in the pDEM

Investigate the effect of different ‘blink’ rates on desiccation-induced dry eye simulated by the pDEM

Investigate the effect of different artificial tears on desiccation-induced dry eye using the pDEM

Investigate the effect of different viscosities of solutions on desiccation-induced dry eye using the pDEM.

Baseline data on the number of dead cells on the porcine corneal epithelial surface were obtained using the trypan blue exclusion technique. The medians (range) of the number of dead cells in the central and peripheral porcine corneal epithelium (standardized area of 0.25 mm²) were 136 (112–173) and 96 (82–107) cells, respectively when stained immediately after enucleation. The number of dead cells in the central region was significantly greater (p < 0.05) than in the periphery in all groups. After exposure to air, the corneal cells were maintained by regular administration of saline solution but there was a
significant increase (p < 0.05) in the number of dead cells in those corneas without desiccation protection.

A pDEM was developed and evaluated so that different severities of desiccation-induced dry eye could be simulated by adjusting the ‘lacrimation-blink’ intervals. The integrity of the porcine cornea and the number of dead corneal epithelial cells were assessed by sodium fluorescein and the trypan blue exclusion technique, respectively. There were significant differences (p < 0.01) between the final fluorescein grades in the corneas when the ‘lacrimation-blink’ intervals set at 20 seconds, 40 seconds and 60 seconds and the medians were grade 1, grade 2.5 and grade 4, respectively. There were also significant increases (p < 0.05) in the numbers of dead cells in the central cornea, when the ‘lacrimation-blink’ interval increased.

The effect of different ‘blink’ rates on the desiccation-induced dry eye was studied by keeping the same amount of ‘lacrimation’ but varying the ‘blink’ rate. Increasing the ‘blink’ rate from 3 blinks/minute to 5 blinks/minute significantly reduced the amount of corneal damage, however, a further increase to 10
blinks/minute did not result in a further significant reduction in the corneal damage.

When studying the effect of different artificial tear formulations, it was found that both Bion® Tears and Vismed® are more effective than Senju® and DPBS for protecting the cornea against desiccation. This result might relate to the viscosity of the artificial tear formulations as Bion® Tears and Vismed® have higher viscosities than Senju®.

The effect of different viscosities was studied by applying 0.3%, 1.5% and 2.0% methylcellulose solutions with viscosity of 1.5 cP, 6.2 cP and 9.2 cP, respectively, to the corneal surface in the desiccation-induced dry eye model. The increase in viscosity from 1.5 cP to 6.2 cP significantly reduced (p = 0.008) corneal damage. However, a further increase in the viscosity from 6.2 cP to 9.2 cP did not further reduce (p > 0.05) corneal damage.

In conclusion, we have developed and evaluated a novel porcine desiccation-induced dry eye model, incorporating ‘lacrimation’ and ‘blinking’
systems, that is capable of simulating different severities of dry eye and which can be used to study the aetiology and treatment of desiccation-induced dry eye.
Chapter 1

Literature Review

1.1 Introduction

Dry eye is a frequent complaint among patients visiting optometrists and ophthalmologists (Mathers, 2000) and is a common eye problem of people around the world, especially for the elderly (Jacobsson et al., 1989; Hikichi et al., 1995; Bandeen-Roche et al., 1997; Bjerrum, 1997; Caffery et al., 1998). In the past decades, a large body of research, both clinical and experimental, has reported on the causes, diagnoses and treatments of dry eye (Janssen and van Bijsterveld, 1983; Lemp et al., 1984; Gilbard, 1985; Limberg et al., 1987; Paschides et al., 1989; Farrell et al., 1992; Feenstra and Tseng, 1992a; Patel et al., 1993; Madden et al., 1994; Doughty, 1994; Doughty, 1995; Mathers and Daley, 1996; Paugh et al., 1998; Tsubota et al., 1999; King-Smith et al., 2000; Kim, 2000; Aragona et al., 2002; Johnson and Murphy, 2004). However, findings have varied among different studies. The reasons for these variations may be the use of different methodologies, assessment methods and criteria for patient selection.

Dry eye was found to be multifactorial in origin (Lemp, 1995; Mathers, 2000; Baudouin, 2001); causes include decreased tear secretion (Mathers et al., 1996),
changes in tear stability (Patel and Farrell, 1989), changes in tear components (Yolton et al., 1991), changes in the ocular surface (Bron, 2001), abnormality of eyelids (Shore, 1985) and changes in environmental conditions (Korb et al., 1996). Due to the large variety of causative factors and the unclear mechanisms of dry eye, common treatment strategies are based on symptomatic relief rather than removal of the cause (Friedlaender, 1992).

In order to have a better understanding of the aetiology of dry eye and the effect of different treatments for dry eye, different animal dry eye models (DEM) have been developed using rabbits (Gilbard et al., 1988; Fujihara et al., 1995), mice (Fujihara et al., 2001), rats (Dursun et al., 2002), dogs (Kaswan et al., 1989; Hicks et al., 1998) and monkeys (Maitchouk et al., 2000). The major advantage of using an animal model to study dry eye is that single causative factor can be isolated, which is impossible in humans. Therefore, an animal DEM can help us to understand the aetiology and the mechanisms of dry eye. Moreover, the effect of new treatments on dry eye can be tested without causing adverse effect on humans. Each animal DEM can be set up to have specific characteristics to represent a unique type of dry eye. Species differences should be taken into consideration in DEM study. Porcine organs are similar to human organs and are better substitutes when compared with other animals (Wang et al., 1985; King et al., 1992; Christie et al., 1995; Zhang and Monteiro-Riviere, 1997). However, porcine eyes have never been used to investigate dry eye.
This review presents a brief introduction to the normal tear film and the condition of dry eye in humans. Different animal models for dry eye are introduced and the characteristics and mechanisms of these DEMs are discussed. Finally, we discuss the similarities and differences between corneas of different species and the reasons for selecting the porcine eye for the development of a DEM in this study.

1.2 Normal tear film and dry eye

1.2.1 General function of tears

The tear film has important roles in maintaining the normal functions of the cornea. It bathes the corneal and conjunctival epithelial cells to maintain normal cell functions. It also provides lubrication to the anterior ocular surface to minimise damage due to the shearing force during blinking (Stein and Hurwitz, 1996). Tears are essential to smooth the corneal surface for optical focus so that sharp images can be formed on the retina (Holly and Lemp, 1977). They also contain antibacterial substances which maintain the sterility of the ocular surface. Tears also help to remove desquamated corneal cells and bacteria via blinking, and supply nutrients to the corneal epithelium (Stein and Hurwitz, 1996). In addition, tears provide protection for the cornea against damage from UV radiation and atmospheric oxygen (Rose et al., 1998; Gogia et al., 1998).
1.2.2 Structure and function of the tear film

According to Wolff (1954), human tears consist of three relatively discrete layers (Figure 1.1), the outer superficial lipid layer, the middle aqueous layer and the innermost mucous layer (Wolff, 1954; Holly and Lemp, 1977). Each layer has a unique role in the maintenance of the structure and functions of tears.
Disruption in any layer will result in changes to the tear film and may lead to dry eye problems.

Another tear film structure was proposed by Prydal and colleagues (1992a, 1992b), who reported that tears were mainly composed of mucus and not aqueous layer (Prydal and Campbell, 1992) and the total thickness of tear film was more than four times of that reported by Wolff (Prydal et al., 1992). Moreover, they proposed that tear film thickness of Wolff’s model did not include most thickness of the mucus layer. However, Wolff’s model of tear film is widely accepted and used (Dilly, 1994; Gipson and Inatomi, 1998; Johnson and Murphy, 2004), therefore within this thesis, we will use the structure of the tear film according to Wolff’s model.

1.2.2.1 Superficial lipid layer

The superficial lipid layer is 0.1 µm in thickness (McDonald, 1968). The lipid layer is produced mainly by the meibomian glands and some is produced by the glands of Moll, lacrimal glands and the glands of Zeis (Jones, 1966; Dilly, 1994; Johnson and Murphy, 2004). The lipid layer consists of non-polar lipids, such as wax esters, triglycerides and polar phospholipids (Andrews, 1970; Johnson and Murphy, 2004). One function of the lipid layer is to reduce evaporation of the underlying aqueous tears (Mishima and Maurice, 1961). Another probable role is to reduce the surface tension of the aqueous phase, as the aqueous is propelled over the cornea by the movement of the eye or eyelids, and thus,
wave formation is reduced (Andrews, 1970; Dilly, 1994). This layer also protects the eye from small dust particles. Therefore, abnormalities of the lipid layer may result in an increase in the evaporation rate of the tears giving rise to dry eye (Dilly, 1994).

1.2.2.2 Aqueous layer

The aqueous layer is the thickest layer among all other layers of the tear film and is about 6 to 7 µm in thickness (Holly and Lemp, 1977). It is produced by the main lacrimal gland and the accessory lacrimal glands of Krause and Wolfring (Holly, 1973a). This layer is a low viscosity solution consisting of electrolytes, proteins, vitamins, cytokines, hormones and enzymes (Holly and Lemp, 1977). The major functions of this layer are to act as a lubricant between the ocular surface and the lids, to remove foreign materials and to provide nutrients to the corneal and conjunctival epithelia (Bachman and Wilson, 1985; Dilly, 1994). The most common abnormality in dry eye syndrome is the reduction of aqueous tear secretion (Friedlaender, 1992; Dartt, 1994).

1.2.2.3 Mucous layer

The innermost mucus layer is about 0.02 to 0.05 µm in thickness (Holly and Lemp, 1977). It is produced mainly by the conjunctival goblet cells but some is produced by the corneal epithelial cells and subepithelial vesicles found in conjunctival epithelium (Jones, 1966; Lemp and Holly, 1970). It consists of large glycoproteins with O-linked carbohydrate and, to date, nine mucins have
been detected in the human eye (Norn, 1963). One function of the mucus layer is to protect the underlying epithelium from sheer damage. It also has an inhibiting effect on bacterial adhesion, thus, protecting the eye against bacterial invasion and drying. This layer also plays a role in the hydration and maintenance of tear stability (Holly, 1973b). An abnormal mucus layer will lead to an unstable tear film and to dry eye.

1.2.3 Dry eye Syndrome

1.2.3.1 Definition and classification

The term “Dry eye Syndrome”, also called keratoconjunctivitis sicca (KCS), was first used in 1933 to describe the condition where reduction of the aqueous component of the tear film leads to ocular dryness (Brewitt and Sistani, 2001). However, Holly and Lemp (Holly and Lemp, 1977) claimed that disruption in any of the tear layers will change the tear film structure and might result in dry eye. Many studies (Holly and Lemp, 1977; McGill et al., 1983; Lemp et al., 1984; Gilbard, 1985; Tsubota and Nakamori, 1993; Danjo et al., 1994; Augustin et al., 1995; Mathers and Daley, 1996; Stern et al., 1998; Sullivan et al., 2000) on dry eye have been conducted to investigate the aetiology and pathogenesis of dry eye. There are large diversities in the reported causes and conditions of dry eye. Therefore, different classifications and definitions have been produced. A global definition for dry eye is interpalpebral ocular surface damage due to deficiency of tear production or excessive tear evaporation, and is associated with ocular discomfort (Lemp, 1995).
Dry eye can be classified into two major types according to the National Eye Institute classification scheme (Lemp, 1995) namely, the tear-deficient dry eye and the evaporative dry eye. Each type can be further divided into subgroups based on the aetiologies as shown in Figure 1.2. Although dry eye can be classified into different types, there may be multiple causes resulting in ocular damage in dry eye patients. Therefore, it has been proposed that in most dry eye conditions, whether tear-deficient dry eye or evaporative dry eye, the most common leading cause of ocular surface damage is desiccation induced by the breakdown of the tear film (Gamache et al., 2002).
12.3.2 Risk factors of dry eye

12.3.2.1 Ageing

Dry eye is probably one of the most prevalent ocular diseases associated with ageing. Dry eye symptoms, including irritation and burning sensation, are commonly reported in the elderly population (Schein et al., 1997; Bandeen-Roche et al., 1997). Dry eye has also been defined as an age-related disorder affecting mainly the middle aged and the elderly (Friedlaender, 1992). Dry eye in the elderly can result from a single factor or a multitude of changes due to the normal ageing process. The details of these changes are briefly described below.

1.2.3.2.1.1 Decreased tear volume and tear stability

One of the major causes of dry eye in the elderly group is the normal ageing process of the lacrimal glands. This results in changes in the volume and chemical components of the tears. Tear volume declines with age (Hamano et al., 1990; Mathers et al., 1996). Apart from tear volume, tear stability is also decreased in the elderly (Patel and Farrell, 1989; Cho and Yap, 1993). Therefore, both decreased tear volume and tear stability may result in corneal desiccation and dry eye.

1.2.3.2.1.2 Anatomical changes of the eyelids

Anatomical changes of the lower eyelids in the elderly result in a decrease in vertical and horizontal eyelid movements (Shore, 1985). Moreover, the lower eyelids of elderly patients may rest against the globe at a position inferior to
those of younger patients. Both changes, the decreased eyelid movements and lower eyelid positions, are related to the increase in eyelid laxity (Shore, 1985). These changes can result in incomplete closure of the eyelids during blinking, leading to disruption of tear distribution and exposure of the cornea, resulting in dry eye.

1.2.3.2.1.3 Ocular surface irregularities

Pinguecula and pterygia, more commonly found in the elderly, are degenerations of the bulbar conjunctiva that resemble the histology of skin with chronic exposure to sunlight. The overgrowth of the conjunctiva results in a mound above from the smooth ocular surface, which may affect tear distribution by the eyelids. The poor distribution of tears then affects the tear stability and hence, may lead to dry eye (Ishioka et al., 2001). Tear stability is decreased in subjects with pterygia (Rajiv et al., 1991), therefore, pterygium may be a possible risk factor for dry eye (Lee et al., 2002).

1.2.3.2.1.4 Hormonal changes

Hormones have a significant effect on the production of tears by the lacrimal gland (Sullivan, 1993; Sator et al., 1998; Sullivan et al., 2000; Worda et al., 2001). In menopausal and post-menopausal women, and in older men, the bio-available androgen level is low. Insufficient androgen may result in decreased tear production and therefore, these people may suffer more readily from dry eye (Pflugfelder et al., 2000). Low androgen level may decrease the anti-
inflammatory ability of the lacrimal gland (Sullivan et al., 1999) and so the lacrimal gland may become more vulnerable to immune-based inflammation. When the lacrimal gland is inflamed, tear production may be affected, and hence, the incidence of dry eye may increase.

1.2.3.2.2 Drugs

As mentioned earlier, dry eye can be caused by the disruption of tears and one of the factors affecting tear production is the use of drugs. Different kinds of drugs such as antibiotics, antidepressants, antihistamines, appetite suppressants, beta-blockers, blood pressure medication, decongestants, diuretics, oral contraceptives, tranquilizers, over-the-counter vasoconstrictors, and ulcer medication can all decrease the body’s ability to produce lubricating tears (Crandall and Leopold, 1979; Norn, 1985). Therefore, people who need to take any of these drugs are more likely to have dry eye.

1.2.3.2.3 Systemic Diseases

Dry eye can also be induced by many systemic diseases, for example, diabetes, Sjögren's syndrome, rheumatoid arthritis, and thyroid abnormality (Lamberts, 1983; Gilbard and Farris, 1983; Fox and Saito, 1994; Ozdemir et al., 2003). Apart from the pathological causes of these diseases; treatments for such diseases may further contribute to exacerbate the condition of dry eye.
1.2.3.2.3.1 Diabetes

Diabetes, a disease where the body does not produce or properly use insulin, is common worldwide (Stovring et al., 2003). Dry eye is a common complication of diabetes. Patients with diabetes have decreased tear stability and tear volume, and are therefore more likely to have dry eye (Ozdemir et al., 2003).

1.2.3.2.3.2 Sjögren's syndrome

Sjögren's syndrome is a disease due to an autoimmune system disorder where the body’s immune cells attack moisture-producing glands (Fox and Saito, 1994). It is characterized by inflammation and dryness of the mouth, eyes, and other mucous membranes. The damaged lacrimal glands affect tear production, and may result in decreased tear secretion or change in tear components, leading to dry eye. Moreover, ocular surface changes may also be induced by this disease resulting in dry eye.

1.2.3.2.3.3 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease that attacks all the organs of the body but mostly the joints and the surrounding tissues, resulting in pain, swelling, stiffness and loss of function in the joints (Whitson and Krachmer, 1990). The most common ocular complication of rheumatoid arthritis is dry eye; around 10 to 25% of these patients have dry eye (Lamberts, 1983), which may be due to the chronic inflammation of lacrimal gland and
disruption of tears. It has been claimed that patients with greater damage to the joints are at higher risk of developing dry eye (Zlatanovi and Stanojevi, 1997).

1.2.3.2.3.4 Thyroid disease

Thyroid disease is caused by the abnormal secretion of hormones produced by the thyroid gland (Fries and Char, 1990). The most characteristic symptom of thyroid eye disease or Grave’s disease is a protrusion of the eyes (exophthalmos) resulting in an increased palpebral fissure width. In this condition, dry eye may develop due to poor distribution of tears by the eyelids and/or increased evaporation of tears (Gilbard and Farris, 1983).

1.2.3.2.4 Abnormal blink rate

Dry eye is a frequent complaint after prolonged work with video display terminals (VDT) (Shimmura et al., 1999). Blink rate decreases during reading or working with a VDT (Bentivoglio et al., 1997; Freudenthaler et al., 2003; Schlote et al., 2004), and this decreased blink rate may induce dry eye symptoms. Blinking plays an important role in maintaining the integrity of the ocular surface by redistribution of the precorneal tear film and therefore, prevents desiccation of the ocular surface (Freudenthaler et al., 2003).

Blink rate may also be associated with ocular surface integrity (Gilbard and Farris, 1983) because a higher blink rate is found in patients with dry eye (Nakamori et al., 1997). The frictional force between the cornea and the marginal zone of the palpebral conjunctiva during blinking may increase in dry
eye conditions (Kessing, 1967), therefore, an increased blink rate in dry eye patients may further increase the stress to and compromise of the corneal surface. Hence, abnormal blink rates may lead to dry eye.

1.2.3.2.5 Contact lens wear

Dry eye sensation is a major cause of contact lens discomfort or intolerance. Contact lens wear, soft contact lens wear in particular, can significantly increase tear evaporation (Cedarstaff and Tomlinson, 1983). The presence of a contact lens disrupts tear distribution and increases tear evaporation. Therefore, a contact lens wearer may be at a higher risk of developing dry eye than a non-contact lens wearer.

1.2.3.2.6 Environmental Conditions

Any environmental condition that results in decreased humidity or increased ventilation may lead to an increase in tear evaporation and may cause dry eye (Korb et al., 1996; Doughty, 2002); for example, air-conditioned environments, dry and windy conditions and the use of heaters or blowers.

1.2.3.3 Prevalence of dry eye

Dry eye is a widespread ocular problem that affects people worldwide. A large-scale study using a questionnaire was conducted in Canada (Doughty et al., 1997; Caffery et al., 1998), and results showed that among 13,517 respondents, 28.7% had dry eye symptoms. In some studies, the criteria for assessment of
dry eye were not only based on the presence of dry eye symptoms but also on measurements of tear volume, tear stability and ocular surface damage. The prevalence of dry eye, based on these criteria, was found to be 15% among 705 people aged 55 to 72 years in Sweden (Jacobsson et al., 1989). In Japan, the prevalence was 17% of 2127 screened subjects (Hikichi et al., 1995), while a survey conducted in Copenhagen reported a prevalence of 11% in 504 people aged 30 to 60 (Bjerrum, 1997).

It is therefore not surprising that dry eye problems affect many people all around the world because, as mentioned in Section 1.2.3.2, many risk factors can lead to dry eye, for example aging, wearing of contact lenses and some systemic diseases such as diabetes. Therefore, the development of therapies for dry eye is an urgent and important issue.

1.2.3.4 Treatment of dry eye

The treatment of dry eye is aimed at providing symptomatic relief and preventing corneal and conjunctival epithelial damage (Friedlaender, 1992). The main strategies for treatment are to lubricate the ocular surface with tear components, which are insufficient in dry eye condition, to conserve endogenously-produced tears or to stimulate tear production (Friedlaender, 1992; Calonge, 2001).
1.2.3.4.1 Supplementation of tear components or other agents

The most common treatment for dry eye is the application of artificial tears to lubricate the ocular surface (Nelson et al., 1994; Murillo-Lopez and Pflugfelder, 1997). This may minimise desiccation of epithelial cells due to decreased aqueous tear production or insufficient tear components. Moreover, it is a convenient and simple method, as a variety of formulations of artificial tears are available commercially. However, there is one major drawback when applying ready-made artificial tears, that is, the presence of preservatives, stabilizers and other additives (Calonge, 2001) may cause sensitivity reactions and compromise the cornea and tear film (Burstein, 1985; Simmons et al., 1988; Tripathi et al., 1992). Although the concentration of preservatives is usually low, the high frequency of use may result in an accumulative effect and compromise the ocular surface. This problem can be solved by using preservative-free unit-dose artificial tears though they are more expensive.

Since artificial tears do not contain all the components that are secreted from our lacrimal glands, the application of artificial tears can only mimic part of the function of the aqueous layer, reducing the drying of the corneal and conjunctival epithelia.

Several studies (Limberg et al., 1987; Laflamme and Swieca, 1988; Nelson and Farris, 1988; Shimmura et al., 1995; Toda et al., 1996; Avisar et al., 1997; Yokoi et al., 1997; Tsubota et al., 1999; Poon et al., 2001) suggested that the
addition of different tear components or agents to artificial tears might enhance their effects in the treatment of dry eye. In this section, clinical findings on the effectiveness of different agents in artificial tears are discussed. For those studies using animal DEM for investigation, details will be discussed in Section 1.5.3.

1.2.3.4.1.1 Hydroxypropyl methylcellulose (HPMC)

HPMC is a component commonly used in many commercially-available artificial tear formulations (Snibson et al., 1992) to increase the viscosity of the solutions (Murube et al., 1998). It also exerts an effect on the surface tension and increases the ocular retention time of the artificial tears (Murube et al., 1998). An artificial tear formulation containing 0.5% HPMC significantly improves subjective symptoms in patients. However, among dry eye patients, it improved only the corneal integrity of those with Sjögren’s syndrome (Toda et al., 1996). Corneal integrity is defined as the amount of unimpaired area of the cornea.

1.2.3.4.1.2 Sodium hyaluronan

Hyaluronan is a glycosaminoglycan, which has the capability to hold large quantities of water, and thus to lubricate surrounding structures (Nakamura et al., 1993). The effect of sodium hyaluronan was studied by a number of investigators by direct application onto the corneal surface of dry eye patients (Limberg et al., 1987; Laflamme and Swieca, 1988; Nelson and Farris, 1988; Shimmura et al., 1995; Yokoi et al., 1997; Condon et al., 1999; Aragona et al.,
2002). Corneal integrity in dry eye patients was significantly improved after application of sodium hyaluronan solution. Sodium hyaluronan-formulated artificial tears has also been shown to improve tear stability (Avisar et al., 1997), however, there was no improvement of subjective symptoms (Shimmura et al., 1995).

1.2.3.4.1.3 Polyvinyl alcohol

Polyvinyl alcohol is a common component of artificial tear formulations. It can reduce the surface tension of water and therefore, increase the ocular retention time of the artificial tears (Murube et al., 1998). Artificial tears with 1.4% polyvinyl alcohol can significantly improve subjective dry eye symptoms and corneal integrity in dry eye patients (Limberg et al., 1987; Laflamme and Swieca, 1988; Nelson and Farris, 1988).

1.2.3.4.1.4 Autologous serum

Serum contains substances like epithelial growth factors that enhance corneal epithelial growth, and maintain epithelial health (Poon et al., 2001). Therefore, the use of autologous serum has been proposed as a potential treatment for dry eye. The application of serum artificial tears formulated by diluting serum obtained from each patient with dry eye was found to improve both subjective symptoms and the corneal integrity in dry eye patients (Tsubota et al., 1999; Poon et al., 2001). The authors postulated that the use of serum provided important tear components that may be lacking in dry eye patients. They further
proposed that epidermal growth factor and vitamin A were two major components responsible for the improvements in corneal integrity (Tsubota et al., 1999). However, although autologous serum was found to be useful in treating patients with dry eye, it is not thought to be a practical treatment and is not widely used because autologous serum needs to be extracted from the blood of each patient.

1.2.3.4.2 Conservation of endogenously produced tears

Apart from applying artificial tears, reduction of tear evaporation by wearing goggles and preservation of tears by punctal occlusion are also used in the treatment of dry eye.

Punctal occlusion is an alternative treatment when dry eye does not improve after the application of artificial tears or ointments (Beisel, 1991). In patients with punctal occlusion, the lower punctum, and sometimes the upper punctum, is occluded using a plug or by surgery. Tear drainage is blocked and more tears can be retained on the ocular surface.

The efficiency of punctal occlusion using silicone plugs in the treatment of dry eye was investigated by insertion of silicone plugs in the lower puncta of both eyes of symptomatic patients. These patients had fluorescein and Rose Bengal staining on the ocular surfaces and Schirmer test score less than 10 mm/5 mins (Balaram et al., 2001) or less than 5 mm/5 mins (Shinozaki et al., 2002). A few
weeks after insertion of the plugs, most of the subjects reported substantial improvement in symptoms and decreased use of supplemental lubrication. In addition, damage to the ocular surface was reduced. Therefore, the authors concluded that silicone punctal plugs are effective for the management of moderate and severe dry eye that could not be improved by lubricant therapy only. However, both the examiners and patients were not masked in these studies, therefore, results may be biased.

Complications including epiphora (Friedlaender and Fox, 1998), loss of plugs and foreign body sensation (Beisel, 1991) sometimes occur with punctal occlusion. Therefore, it was recommended that, punctal occlusion should be reserved for patients with very severe dry eye. Patients who respond to artificial tear therapy (even hourly application) should not be considered as candidates for punctal occlusion (Friedlaender and Fox, 1998).

1.2.3.4.3 Stimulation of tear production

Tear production can be stimulated by secretagogic or lacrimomimetic drugs, such as bromhexine, pilocarpine and eledoisin (Calonge, 2001). Bromhexine was developed as a cough medicine for the treatment of respiratory disorders associated with viscid or excessive mucus and was reported to stimulate tear secretion in humans (Prause et al., 1984) However, these results were not confirmed in another clinical study (Gobbels and Spitznas, 1994). The differences in results of these studies may be due to the difference in the
severity of dry eye of the subjects recruited in each study. It was found that bromhexine did not affect tear secretion rate in healthy subjects (Avisar et al., 1996), therefore, the effect of bromhexine in stimulation of tear secretion may be more obvious in patients with severe dry eye only. However, side effects such as nausea, sweating and rashes may be induced (Calonge, 2001).

Pilocarpine, a commonly-used drug for the treatment of glaucoma, has a beneficial effect on dry eye (Tsifetaki et al., 2003). However, the use of this drug may be associated with cardiovascular and gastrointestinal side effects (Lemp, 1987).

Eledoisin is a peptide extracted from the posterior salivary glands of some species of small octopi in Mediterranean. It increases tear volume and tear flow (Gobbels et al., 1991) when used topically. However, the effect of eledoisin for improvement of signs and symptoms of dry eye has not been documented, and while it is used in some European countries it is not widely used (Calonge, 2001).

However, the use of drugs for the stimulation of tear secretion is not common because of the threat of side effects (Calonge, 2001). In addition, stimulation of the inflamed lacrimal gland and conjunctiva in dry eye patients may produce tears with inflammatory mediators and may worsen the condition of the ocular surface.
1.3 Models for dry eye study using cell or organ culture method and the whole enucleated eye

Organ culture was introduced first for the storage of corneas for corneal transplantation in most of the eye banks in Europe and Britain (Summerlin et al., 1973; Armitage et al., 1990). Nowadays, cultures of corneal epithelial cells have been used in assays for cytotoxicity of various chemicals including ophthalmic preservatives (Simmons et al., 1988; Tripathi et al., 1992; Chang et al., 2000b), contact lens solutions (Simmons et al., 1991; Begley et al., 1994), natural tear substitutes (Geerling et al., 2001) and other chemicals for the treatment of eye diseases, including dry eye syndrome (Wysenbeek et al., 1988; Lass et al., 1989). As most drugs for the treatment of eye disease involve topical instillation of eye drops, which are absorbed into the inner eye through the cornea, it is important to predict the toxicity of these drugs and also their ocular absorption and transportation, which can be assessed using cultured corneal epithelium (Chang et al., 2000a; Toropainen et al., 2001). The use of organ or cell culture for the assessment of the chemical effects in human or animal tissues has become more popular, possibly due to increased public pressure against the use of animals in experiments (Huhtala et al., 2002). In addition, the use of cultures can reduce the number of in vivo tests, which are more expensive. The effects of newly-developed treatments can also be tested to evaluate potential adverse effects without using live animals.
Cultures of both animal and human corneas or corneal cells have been used to develop models to investigate dry eye (Gilbard et al., 1984; Matsuo, 2001; Ubels et al., 2004).

Human corneal epithelial cells obtained from normal eye bank eyes have been used to investigate the effectiveness of trehalose solution for protecting these epithelial cells against desiccation (Matsuo, 2001). Trehalose (a disaccharide) is a key element for anhydrobiotic organisms to survive in dry environments. Corneal epithelial cells were incubated for 15 minutes with one of 14 test solutions (PBS, Maltose or Trehalose solutions with 2, 20, 50, 100 and 200 mM and three commercially-available artificial tears containing 0.1% sodium hyaluronate, hydroxyethylcellulose and borate buffered saline, respectively) and then all medium was aspirated. After aspiration, cells were exposed at room temperature (24 – 25°C) in room humidity (34 – 40%) for 30 minutes. The viability of cells was assessed by SYTO 10 green fluorescent nucleic acid stain and DEAD Red nucleic acid stain. Six samples were obtained for each testing solution. It was found that exogenously-added trehalose at concentrations of 50, 100 and 200 mM could protect the corneal epithelial cells from death due to desiccation, and trehalose is more efficient than the other formulations tested. Matsuo (2001) suggested that trehalose has the potential to be a new agent for the treatment of dry eye. In the clinical studies, it was found that trehalose-contained solution could significantly improve signs of patients with moderate to severe dry eye (Matsuo et al., 2002) and the efficacy of trehalose eye drop
was found to be better than two commercially-available eye drops that contained hyaluronan and hydroxyethylcellulose (Matsuo, 2004).

Another model of desiccation (Ubels et al., 2004) was developed using immortalized human corneal epithelial cell lines, CEP17 and Chang cells, to study the protective effect of three different commercially-available artificial tear formulations. Cells plated in wells were pre-incubated with different artificial tears at 37 °C for 15 minutes. After the removal of the tear formulations, cells were subjected to desiccation in a laboratory safety hood with a constant airflow at 85 ft/min for 10 or 30 minutes at room temperature. Cell viability was assessed by the MTT test, which employs reduction of methyltetrazolium salt by active electron transport chains within living cell. Four samples were obtained for each test group. The authors suggested that this desiccation model is a useful, simple and economical method for a quantitative determination of the effect of different artificial tear formulations.

In another study (Gilbard et al., 1984), rabbit anterior corneal explants were used to study the effect of hyperosmolarity on the corneal epithelium. Previous findings proposed that in dry eye patients, tear osmolarity increased (Gilbard and Farris, 1983) and therefore, the hyperosmolarity of the tears may produce harmful effects on the corneal epithelium leading to ocular surface damage. The explants were prepared by mechanically removing the endothelium, Descemet’s membrane and the posterior stroma of the rabbit corneas. The explants were
then plated, with the epithelial side up, in the culture medium and were cultured for three days. Then, the osmolarity of the media in the experimental groups was increased by adding sodium chloride or glucose. The explants were cultured for another three days, six days or nine days; culture media were renewed every three days. The epithelial cells cultured in medium with increased osmolarity became swollen due to decreased cytoplasmic density. In addition, desmosomes at the interfaces between adjacent cells were absent. The authors concluded that one of the causes of ocular surface damage in dry eye patients is the hyperosmolarity of the tear film. Disruption of intercellular junctions and oedema of the corneal epithelium of dry eye patients had also been reported (Tabery, 2003).

The use of a rabbit DEM may provide a useful platform for evaluating the aetiology of dry eye. The above models demonstrate that cell culture is valuable for studying both the aetiology and the treatment of dry eye.

However, epithelial cells in culture may be more susceptible to damage than those on the ocular surface of an intact eye (Ubels et al., 2004). Therefore, the actual effect of the chemicals tested may be magnified in an epithelial cell culture model. This problem can be minimised by using organ culture.

Corneal organ culture maintains the corneal architecture and provides the opportunity to study not only a single cell layer, but also the characteristics of
the multi-layered epithelium and also the interaction between the epithelium and other corneal layers, i.e. the stroma and endothelium (Minami et al., 1993; Xu et al., 2000). In addition, cells in organ culture can be kept in an environment which more closely approximates the in vivo environment than the cell culture environment. Corneal organ culture has been used to study the effects of different agents in corneal wound healing (Tanelian and Bisla, 1992; Collin et al., 1995; Foreman et al., 1996; Kim et al., 2001) and the epithelial responses to surfactants (Xu et al., 2000), however, corneal organ culture has not been used for the study of desiccation-induced dry eye.

The toxicity of sodium hyaluronate had been investigated by immersing whole enucleated eyes of 14-day-old chick embryos into 1% or 0.1% sodium hyaluronate solution for 90 minutes at room temperature (Wysenbeek et al., 1988). When studying the protective effect of sodium hyaluronan solution on the desiccated corneal epithelium, two drops of 0.1% sodium hyaluronate solution, saline solution or 0.1% hydroxyethylcellulose solution were administered onto the corneas and eyes at 37°C. The corneas were examined using an electron microscope, and results were compared with the untreated eyes. Neither 0.1% nor 1.0% sodium hyaluronate solution produced a toxic effect on the corneal epithelium because the normal architecture and morphology of the microvilli on the corneal epithelial cells were clearly observed in corneas pre-treated with these solutions. Severe damage, in the central and peripheral corneal epithelium, as indicated by the disappearance of
cell borders and destruction of microvilli, was found in corneas instilled with saline solution. Similarly, in eyes treated with 0.1% hydroxyethylcellulose solution, severe damage was found in the central cornea; in the peripheral cornea, distinct cell borders were still observed, but the microvilli were longer and denser. However, in corneas treated with 0.1% sodium hyaluronate solution, damage to the central cornea was only moderate, and the cell borders and microvilli were relatively normal in the peripheral cornea. Sodium hyaluronan solution protected the cornea more effectively against dryness than saline or 0.1% hydroxyethylcellulose solution.

### 1.4 Animal dry eye models

Apart from corneal cell or organ culture methods, live animals have also been used for studying dry eye (Gilbard et al., 1987; Kaswan et al., 1989; Lopez Bernal and Ubels, 1991; Doughty, 1992b; Doughty, 1992a; Fujihara et al., 1995; Fujihara et al., 1998, Hicks et al., 1998; Burgalassi et al., 1999; Maitchouk et al., 2000; Fujihara et al., 2001; Moore et al., 2001; Dursun et al., 2002; Gamache et al., 2002). The major advantage of using live animal models is that the corneas are in the normal and natural environment and hence there is no need to go through the complex procedures involved during cell or organ culture. Also the procedures involved in cell or organ culture may induce adverse effect on the whole cornea or the corneal epithelial cells, possibly affecting the results of the study.
1.4.1 Animals used for studying the effect / toxicity of different agents for dry eye treatment

Patients with severe dry eye may need to apply artificial tears very frequently and most of the commercially-available artificial tear formulations have different contents of ions, lubricants, pH and osmolarity and most of them contain preservatives. All of these components may affect the corneal integrity and the effect may become accentuated, especially when instilled frequently in severe dry eye. Therefore, it is important to evaluate the effects, especially adverse effects, of these agents on corneal integrity.

DEM using rabbit eyes was developed to evaluate the toxic effects of different artificial tear formulations and preservatives on corneal integrity by measuring the corneal epithelial barrier function (Lopez Bernal and Ubels, 1991). A conjunctival cup was formed in one eye of the anaesthetized rabbit by lifting ligatures placed about 0.5 cm from the medial and lateral canthi of the upper and lower eyelids. The contralateral eye was taped shut during the experiment and used as the control. Test solutions were applied into the conjunctival cup for one and a half or three hours. After removal of the test solution, carboxyfluorescein (CF) solution was applied for five minutes. The rabbits were then killed and the corneas were excised. The amount of CF absorbed into the cornea was extracted and then the concentration was measured by fluorimetry. Test solutions were divided into four groups. In the first group, there were four different tear formulations and all of them contained 0.01%
Benzalkonium chloride. The second group included only one artificial tear solution preserved with 0.001% Polyquad. In the third group, a contact lens rewetting solution containing 0.004% thimerosal were used. The last group comprised four non-preserved solutions including two commercially-available formulations and two test formulations with different buffer compositions (HCO₃ and PO₄).

All four formulations in group 1 resulted in an increase in corneal CF uptake after one and a half or three hours exposure when compared with the control. For corneas exposed to formulations containing 0.001% Polyquad, there was no significant increase in the corneal CF uptake after 1.5-hour exposure, however, a significant increase was found after the 3-hour exposure. Similar results were obtained in group 3 with corneas exposed to the contact-lens rewetting solution containing 0.004% thimerosal. In the final group, all preservative-free tear formulations had no effect on the corneal CF uptake. When the results among the groups were compared, it was found that the CF uptake by corneas exposed to all four formulations in group 1 was significantly greater than all values in the other groups. There was no difference in the CF uptake by corneas after exposure to solutions between the second and third groups. In addition, corneal CF uptake was significantly less after exposure to preservative-free formulations, when compared to corneas in all other groups. The authors (Lopez Bernal and Ubels, 1991) concluded that the type of preservative is a very important criterion to consider when selecting artificial tears, especially for
patients with severe dry eye, because the high frequency of application would result in a long-term exposure of the cornea to the preservatives which can cause toxic effect. Therefore ideally, preservative-free formulations should be recommended.

Another study also used rabbit eyes to investigate the effect of chlorobutanol-preserved artificial tears (Doughty, 1992b). Two drops of artificial tears was applied to one eye of the tested animals (five corneas in each group) twice daily for 1 days, 2 days, 3 days 6 days and 12 days and no treatment was received in control animals. Scanning electron microscope was used to assess for the changes in corneal epithelium. It was found that after a one-day treatment, there were only very little amount of exfoliating or unusual cells observed in the corneal epithelium. However, after a three-day treatment, the amount of exfoliating cells increased. After a six-day or twelve-day treatments, exfoliating cells and abnormal cells were still present, however, the affected area was significantly reduced from those corneas with a three-day treatment. It was concluded that although the number of exfoliating or abnormal cells increased, it was still considered as mild to gross cytotoxic changes and therefore, the chlorobutanol-preserved artificial tears did not produce toxic effect if used occasionally.
14.2 Naturally-occurring dry eye in dogs

The prevalence of KCS in dogs is quite high (35%) and KCS is a common cause of canine ocular morbidity and blindness (Kaswan et al., 1998). The clinical signs of KCS in dogs are similar to those in humans, including decreased tear secretion (Kaswan et al., 1998), superficial keratitis (ocular surface damage) (Nell et al., 2005) and immune-mediated destruction of lacrimal tissue (Kaswan and Salisbury, 1990).

Canine eyes had been used to study the effect of cyclosporine eye drops for the treatment of KCS (Kaswan et al., 1989). The results showed that tear secretion increased significantly, and the condition of the ocular surface was also improved in these dogs. The authors suggested that due to the similarity of canine KCS to humans, canine eye model could be used to predict the effect of drugs for dry eye treatment; therefore minimising the therapeutic risk for patients with KCS.

Another canine model was used to study the biochemical basis of altered ocular mucin in KCS condition (Hicks et al., 1998). Secreted mucins and membrane extracts were collected from the ocular surface of both normal and KCS dogs and compared. The low buoyant density mucins were increased in the KCS condition. Lycopersicon esculentum lectin results showed an increase in N-acetylglucosamine structure and results of Concanavalin A lectin showed a decrease in mannose content in the mucins of KCS dogs. In addition, agarose
gel results showed an increased migration and no distinct subunit was found when analyzing the mucins from KCS dogs. The main difference in membrane extract between KCS and normal dogs was the presence of T antigen in the former group. The authors suggested that all these alterations in KCS dogs indicated that the glycosylation and the relative proportion of mucins were changed. The changes may be due to biological changes in ocular surface, such as the goblet cell differentiation and mucin gene expression. By understanding the underlying causes of mucin alterations, new treatment strategies aimed at preventing these mucin alterations can be developed.

The advantage of using canine KCS model is that KCS developed naturally in these animals and no induction of dry eye is necessary. Therefore, adverse effects due to the process of inducing dry eye can be avoided. However, the disadvantage is that results obtained in these models may not be comparable to the condition in humans due to species differences and the different aetiology of dry eye in dogs and humans. Moreover, different tests may need to be carried out to assess the condition of the ocular surface and the tear production to confirm the KCS condition in dogs and these procedures will increase the duration of the experiments and expenses.
Figure 1.3  Cause of dry eye in animals (naturally occurring dry eye) or animal models (induced dry eye)

- Corneal damage involving desiccation
  - Lacrimal dysfunction (22,72,138,154)
  - Change in mucin secretion of ocular surface cell (76, 162)
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear stability
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear stability
- Corneal damage not involving desiccation
  - Lacrimal dysfunction (110, 112)
  - Dysfunction of ocular surface cells (95)
  - Decreased anti-inflammatory components
  - Decreased anti-inflammatory components
  - Dysregulation of ocular surface cells
  - Decreased anti-inflammatory components
  - Dysregulation of ocular surface cells
- Environmental factors (55, 248)
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear volume
  - Change in tear composition
- Environmental factors (55, 248)
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear volume
  - Change in tear composition
  - Decreased tear volume
  - Change in tear composition
- Increased tear evaporation
  - Increased tear evaporation
  - Increased tear evaporation
  - Increased tear evaporation
  - Increased tear evaporation
  - Increased tear evaporation
- Apoptosis of ocular surface cells
  - UV?
  - Desiccation?
  - Inflammation induced?

Result

Cause

Clinical findings resulted from the causes of dry eye

animals’ and animal models’ reference
14.3 Induction of dry eye in animal DEM

Most of the animal DEMs were developed by inducing desiccation on ocular surface or reducing tear production in animals. The advantage of these models is that individual causative factors of dry eye can be isolated and the environmental conditions can be controlled. However, when inducing desiccation or testing the effect of newly-developed treatments, adverse effects may be exerted on the animals causing significant damage or unpredictable harm, and this contributes to the increasing public pressure against the use of animals for experimentation (Huhtala et al., 2002). In addition, in vivo studies are more expensive.

Different methods have been used to induce desiccation or reduce tear production in the live animal models, depending on the aims of the studies. Each of these models has its own unique functions and each may represent a different dry eye condition (tear-deficiency or evaporative dry eye). To date, not a single animal DEM can represent the complex and multifactorial condition of dry eye in humans (Barabino and Dana, 2004). Studies on dry eye using live animal models can be classified into two major groups, as shown in Figure 1.3. In this literature review, focus will be on the animal DEM that produce corneal damage due to desiccation and these models will be discussed according to methods of induction of dry eye.
14.3.1 **Methods for induction of dry eye in animals**

14.3.1.1 Removal of lacrimal glands

Squirrel monkeys were used to evaluate the changes in tear production after removal of the main lacrimal gland (Maitchouk *et al.*, 2000). The main lacrimal glands were removed surgically from the right eye of six monkeys and the left eye remained intact as controls. Several tests, including fluorescein and Rose Bengal staining and the Schirmer test were carried out before and after surgery to assess corneal and conjunctival integrity and tear volume. Tears were also collected for tear protein analysis. At the end of the experiment (20 weeks), the animals were euthanized and the eyelids, cornea and conjunctiva together with the accessory lacrimal glands were removed. After fixation, staining and sectioning, tissues were observed using a transmission electron microscope. No corneal fluorescein staining was observed in any of the eyes of the five monkeys except for the operated eye of one monkey, where a central corneal epithelial erosion of $1.5 \times 2.0$ mm was observed three days after surgery and the defect healed in one week. However, there were no other clinical or anatomical signs of KCS found in this monkey for the remaining experimental days. The five monkeys, without corneal fluorescein staining, also had no Rose Bengal staining of the cornea but mild staining was found in the nasal bulbar conjunctiva in both the operated and control eyes. In the monkey with the corneal epithelial erosion, the amount of Rose Bengal staining was greater and was located in the inferior cornea, the lateral bulbar conjunctiva and the nasal conjunctiva. Schirmer test results showed significant decrements in both the
basal and reflex tear secretion in the operated eye when compared with the control eye. Tear volume decreased sharply after the removal of the lacrimal glands, with 80% and 90% decrease for basal and reflex tear secretion, respectively, however, it increased gradually with time. After 20 weeks (the end of the experiment), the decreases in basal and reflex tears were 32% and 33% of the volumes of the control eyes. Tear protein analysis showed no difference in the tear protein profile and there were also no differences in anatomical structure of the eyelid, cornea or conjunctiva between the operated and control eyes. The authors concluded that in squirrel monkeys, tear production by the accessory lacrimal glands was sufficient to prevent ocular surface desiccation and the tear proteins secreted by the accessory lacrimal glands also functioned normally. They also explained that the differences in the results of this primate model from the dog and mice models was due to the anatomical differences of the lacrimation systems in different species. Therefore, this primate model was useful for dry eye study because it is closer to the human condition.

A rat DEM was developed (Fujihara et al., 2001) by removal of the extra-orbital lacrimal glands bilaterally and was used to evaluate the effect of P2Y2 agonist INS365 on tear secretion and corneal barrier function. It was found that in this rat DEM, the tear secretion score was only half of that in the untreated eye as measured by the Schirmer tear test. To evaluate the effect of INS365 on tear secretion, 5 µl of vehicle or of 0.1%, 0.3%, 1.0%, 3.0%, 8.5% INS365-
containing eye drops was applied to the conjunctival sac of the treated rats and then tear secretion was assessed by Schirmer strip after 10 minutes.

Tear secretion increased after application of 3.0% and 8.5% INS365-containing eye drops only but did not change for those with concentration less than 3.0%.

The long term effect of INS365 on corneal barrier function was studied two months after the removal of lacrimal glands, 5 µl of vehicle or of 0.03%, 0.1%, 0.3%, 1.0%, or 3.0% INS365-containing solution was applied onto the corneal surface for four weeks. Corneal barrier function was evaluated at one, two and four weeks. Measurements were based on fluorescein penetrance by applying 0.5% sodium fluorescein solution onto corneal surface for 10 minutes and then the cornea was rinsed with saline to remove excessive fluorescein solution. The eye was then closed for another 20 minutes before measuring fluorescein penetration using slit-lamp fluorophotometer. There was a 3.5-fold increment in the fluorescein penetrance in the corneas of the dry eye rats compared with the normals before application of INS365 solution. The restoration of corneal epithelial permeability was dose-dependent. Restoration occurred in the first week after the application of 3.0% INS365 solution. In the second week, fluorescein penetrance of the corneas after application of 0.1 to 1.0% INS365 solution was significantly less than that of the control group receiving vehicle only. The authors concluded that INS365 maybe useful for dry eye treatment, as it can simulate tear production and promote recovery of corneal barrier function in dry eye.
Dogs were also used for development of a DEM. KCS was induced in dogs by the removal of the orbital and nictitans lacrimal glands and this model was used to study the effect of cyclosporine on mucin production in the conjunctiva (Moore et al., 2001). Tear production in this canine model was found to decrease significantly when compared with the baseline value. Clinical features of dry eye, like conjunctival hyperaemia and accumulation of tenacious discharge were observed in this model. The authors stated that this canine KCS model could demonstrate quantitatively a reduction of conjunctival mucin production in dry eye. In addition, cyclosporine could stimulate conjunctival mucin production and mucin produced by conjunctival goblet cells was important for the recovery of eyes from KCS. In this study, the criteria for the assessment of dry eye were based on the morphology and function of conjunctiva and conjunctival cells.

14.3.12 Application of drugs

A rabbit DEM (Bugalassi et al., 1999) was developed, by topical instillation of 1.0% atropine sulphate (anti-cholinergic drug), to assess the protective effect of some polymeric tear substitutes (Lacrisife®, Viscotirs®, Hyalistil® and Tamarind gum-formulated solution). The rabbits were kept in cages at 19 ± 1°C and 50 ± 5% relative humidity. Both eyes of the animals received 50 µl atropine sulphate solution, three times per day, and in the experimental groups, one eye of the animals received an extra drop of either one of the testing solutions at five minutes after the instillation of atropine sulphate solution. The experiment
lasted for five days. Two typical dry eye signs, namely, decreased tear production and the presence of corneal fluorescein staining, were observed in this model in the second day of the experiment. The results revealed that only Tamarind gum-formulated solution could protect the corneal surface against desiccation effectively while Viscotirs® and Hyalistil® did not protect the cornea, and Lacrisife® provided only moderate protection. The authors concluded that this rabbit DEM was suitable for preliminary evaluation of artificial tear formulations, and Tamarind gum might be a potential new component in artificial tear formulations. However, the clinical effect of Tamarind gum on dry eye has not been documented and therefore it is still not used for artificial tear formulations.

In the study by Dursun and colleagues (2002), mouse KCS models were developed by the application of two different anti-cholinergic drugs (scopolamine and atropine) and induction of environmental stress. The effect of environmental stress will be discussed in Section 1.5.2.1.4. The rats were separated into two groups. In group A, 1 µl of 1% atropine sulphate was applied topically onto the ocular surfaces of the mice and in group B, transdermal scopolamine patches were applied to the depilated mid-tail of the mice. Tear production, measured by cotton thread test, decreased significantly in both groups of mice, when compared to the control group that received one drop of saline topically. In group A, tear production decreased one hour after application of the drug and returned to baseline level within four hours. In
contrast, in group B, a maximum decrement in tear production was found 12 hours after application of the patches and the effect lasted for more than 24 hours. By 48 hours, tear production tended to return to baseline value. Therefore, application of scopolamine transdermally may be better than topical atropine for the induction of dry eye in animal models because the effect may last longer. In addition, topically-applied drug may induce adverse effects on the ocular surface and this can be prevented by using the drug transdermally.

14.3.1.3 Prevention of blinking

A short-term rabbit DEM (Fujihara et al., 1995) was developed by keeping the eyes of anaesthetized rabbits open with a speculum for one to three hours. The eyes were exposed to an air current at 20ºC with 50% relative humidity. Methylene blue solution was used to assess the integrity of the cornea. Methylene blue staining of the cornea increased significantly after three hours of exposure, when compared to the control eye without exposure, and the extent of staining was proportional to the desiccation period. The effect of chondroitin sulphate eye drops was also studied in this experiment – 50 µl of either 3% chondroitin sulphate eye drops or 0.9% saline solution was applied to the corneal surface of a rabbit eye prior to keeping the eye open and the eye was desiccated for two hours. The eyes treated with chondroitin sulphate eye drops did not have a significant increase in corneal staining, when compared with the non-desiccated group. However, a nearly 3-fold increment in staining was found in the saline-treated corneas. It was concluded that chondroitin sulphate
solution was effective in protecting the cornea against desiccation, and this short-term rabbit DEM could be used for studying the protective effect of potential therapeutic agents on corneal damage caused by desiccation.

Gefarnate stimulates mucin-like glycoprotein secretion in rat cultured corneal explants, and the corneal epithelium is the source of this glycoprotein (Nakamura et al., 1997). Therefore, the protective effect of gefarnate against desiccation of the cornea was studied using the rabbit DEM. Three different concentrations of gefarnate solution (0.1%, 0.3% and 1.0%) and vehicle as control were instilled on the corneas of anaesthetised rabbits in different groups before the eyes were held opened with specula. The rabbits were kept in a room of temperature of 24.7°C and 35.1% relative humidity. After a 2-hour desiccation period, the corneas were stained with methyl blue, and results showed that 1.0% gefarnate solution could significantly protect the cornea against desiccation because corneal staining area was reduced in this group. The stain was then extracted from the cornea and the absorption of the extracted solution was measured with a spectrophotometer. The absorption of solutions from corneas which received vehicle only had a 4-fold increment when compared to those eyes without desiccation. However, significant reductions were found in solutions extracted from corneas which received 0.3% and 1.0% gefarnate solutions, when compared with those extracted from eyes that received vehicle only, and the reduction was dose dependent. It was concluded
that the rabbit DEM might indicate the anomalous mucin condition and therefore, gefarnate may be used as treatment for mucin-deficient dry eye.

The effect of lactoferrin on the integrity of the corneal epithelium associated with dry eye was also investigated using rabbit DEM (Fujihara et al., 1998). Lactoferrin is present naturally in human tears and forms 25% of the tear proteins. However, it is reduced in dry eye patients (McGill, 1985). The anti-microbial and anti-inflammatory effects or maybe the anti-oxidative effects of lactoferrin (Ellison, 1994) make it a potentially useful ingredient in drops for dry eye treatment. In this study (Fujihara et al., 1998), one of the three testing solutions, lactoferrin-, Bovine serum albumin- and lysozyme-containing eye drops or vehicle (as control) was instilled (50 µl) onto the corneal surface before desiccation was induced in the rabbits’ eyes. Only lactoferrin eye drops provided sufficient protection for the cornea against desiccation, as corneal methylene blue staining in this group was significantly less than that in the control group, and the level of staining was not different from the normal (undesiccated) eye. However, there was no significant difference in the level of staining in the other experimental groups when compared with the control group. It was concluded that lactoferrin eye drops might be a potential therapy for dry eye, especially in evaporative dry eye.

This rabbit DEM was modified and used for studying the effect of a mucin secretagogue 15(S)-HETE (Gamache et al., 2002). Both corneal methylene blue
staining and corneal thickness were used as indices for the integrity of the cornea. Two test solutions, one contained 15(S)-HETE and Tears Naturale Free® uni-dose artificial tears were studied and a balanced salt solution was used as a control. One of the test solutions or the control solution was applied to one eye of the animals and then the eye was taped closed for 10 minutes before it was kept open for a 4-hour desiccation period. The solution containing 15(S)-HETE provided significant protection against corneal damage due to desiccation and maintained the corneal thickness, while the protective ability of Tears Naturale Free® on the corneal integrity was significantly poorer. Moreover, Tears Naturale Free® could not maintain corneal thickness; corneal thinning resulted due to desiccation. The authors suggested that 15(S)-HETE might be used for treatment of dry eye by stimulating mucin expression over the ocular surface.

14.3.1.4 Change in environmental condition
As mentioned in Section 1.4.3.1.2, Dursun and co-workers (2002) developed a mouse KCS model by inducing environmental stress using a blower hood that might increase tear evaporation. Mice were put into a blower hood for one hour, three times each day. Some mice were treated with transdermal scopolamine patches as mentioned in Section 1.5.2.1.3, before they were exposed to environmental stress. The results show that exposure to environmental stress, as induced in this experiment was not sufficient enough to induce dry eye in the experimental animals because the tear volume and ocular integrity of mice.
assessed by tear fluorescein clearance and corneal fluorescein uptake, were not different from the control group under normal conditions. The authors explained that the increased tear evaporation under these conditions might be compensated by stimulation of reflex tears and therefore, there were still sufficient tears to protect the cornea against desiccation. However, the mice treated with transdermal scopolamine patches, before they were put into the blower hood, developed severe KCS with patches of punctate and diffuse corneal fluorescein staining.

A modified mouse KCS model was used to study the apoptosis of ocular surface cells in dry eye (Yeh et al., 2003). White mice were treated with subcutaneous injections of scopolamine three times daily and were put into a cage with a continuous air draft and controlled humidity (50%) and temperature (18°C) for 10 hours daily for 12 days. It was found that the numbers of apoptotic cells in the central and peripheral corneal epithelia and bulbar and tarsal conjunctival epithelia were increased. The authors proposed that apoptosis induced acutely by adverse conditions in dry eye might further contribute to the progression of KCS.

14.3.2 Comparison between different animal DEM
In the previous sections different methods have been used to induce dry eye in a variety of animals. In most of these animal DEMs, except the monkey model (Maitchouk et al., 2000), corneal damage due to desiccation resulted from
either decreased tear volume or increased tear evaporation and some potential treatments for dry eye were studied (Fujihara et al., 1995; Burgalassi et al., 1999; Fujihara et al., 2001; Moore et al., 2001; Dursun et al., 2002; Gamache et al., 2002).

A few points need to be noted when interpreting results from studies using animal DEMs.

First, the type of animal used in the DEM is very important, especially when dry eye is induced by removal of the lacrimal gland, because each species of animal has its unique lacrimal system. Removal of the lacrimal gland may not effectively induce dry eye in some animals, for example, in Squirrel monkeys (Maitchouk et al., 2000). However, in dogs and rats removal of the lacrimal glands resulted in a significant reduction of tear volume and ocular surface damage (Fujihara et al., 2001; Moore et al., 2001). In addition, the corneal structure and the properties of different species are different; therefore, it is better to use those ocular systems which resemble those of humans more closely.

The instillation methods and the type of drugs used for inducing dry eye are also important. Topically-instilled drugs may affect the integrity of cornea or exert toxic effect on corneal or conjunctival cells. Therefore, systemic administration of drugs by injection or dermal application maybe a better choice.
However, systemic administration of drugs may produce greater side effects in animals and therefore drugs with severe adverse effects should also be avoided. Scopolamine and atropine are two anti-cholinergic drugs commonly used for induction of dry eye in animals (Burgalassi et al., 1999; Dursun et al., 2002; Yeh et al., 2003). The action of scopolamine was found to be more effective on the secretory glands and less effective in the heart, intestinal and bronchial smooth muscle, when compared with atropine (Clissold and Heel, 1985), so scopolamine may be a better drug.

As mentioned earlier, these animal DEMs represented only some aspects of human dry eye and none of these models have considered the effect of blinking on dry eye. Moreover, the quantity of tears or other tear components secreted by the animals cannot be controlled in these models. Therefore, each DEM may be only simulating one aspect or one level of dry eye observed in human eyes.

### 14.4 Animals used in dry eye research

As mentioned in Section 1.5, different animals have been used for dry eye studies. However, to our knowledge, porcine cornea has never been used, although many eye researchers (Reim et al., 1988; Hackworth et al., 1990; Tripathi et al., 1991; Engelmann et al., 1999) have used porcine cell cultures or explants in their research. So, the development of a porcine dry eye model may be useful for research in this area.
14.5 Why porcine eye?

Due to the difficulties in obtaining human organs for research, it is necessary to use animal organs. A range of animal organs has been used in the past studies, such as the cat, chick, rabbit and cow (Burstein, 1980; Wysenbeek et al., 1988; Fujihara et al., 1998; Parnigotto et al., 1998). Several studies (Wang et al., 1985; King et al., 1992; Christie et al., 1995; Zhang and Monteiro-Riviere, 1997) have shown that pig organs may be a better substitute for human organs than those of other animals. Moreover, pig organs are more readily available and the size of pig organs is similar that of humans.

14.6 Similarities and differences of corneas among different species

In corneal research, most published studies (Simmons et al., 1988; Lass et al., 1989; Simmons et al., 1991; Kwok and Klyce, 1992; Begley et al., 1994; Pham and Huff, 1999; Chang et al., 2000a; Chang et al., 2000b) have used rabbit corneal cells or explants to investigate the effects of different agents on the integrity of the cornea. However, rabbits blink (3 blinks/hr) (Maurice, 1995) much less than humans (17 blinks/min at rest) (Bentivoglio et al., 1997). Therefore, there are physiological differences between human and rabbit corneas. Porcine corneas seem to have the most characteristics in common with human corneas. However, there are some differences. Table 1.1 summarises findings reported by previous investigators.
Table 1.1. Similarities and differences between corneas of pigs, rabbits and cows.

<table>
<thead>
<tr>
<th></th>
<th>Authors</th>
<th>Pigs</th>
<th>Rabbits</th>
<th>Cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of epithelial cell layers</td>
<td>Ehlers 1970</td>
<td>6 – 9</td>
<td>5 – 7</td>
<td>10 – 15</td>
</tr>
<tr>
<td>Cornea epithelial thickness</td>
<td>Ehlers 1970</td>
<td>50 – 70 µm</td>
<td>30 – 40 µm</td>
<td>90 – 120 µm</td>
</tr>
<tr>
<td>Alkaline phosphatase in corneal epithelium</td>
<td>Ehlers 1970</td>
<td>Weak basal membrane bound</td>
<td>Superficial and basal membrane bound</td>
<td>Superficial and basal membrane bound</td>
</tr>
<tr>
<td>NADP location in epithelial cells</td>
<td>Ehlers 1970</td>
<td>Mainly basally located</td>
<td>Accentuation towards the surface</td>
<td>Accentuation towards the surface</td>
</tr>
<tr>
<td>Acanthamoeba keratitis</td>
<td>He et al. 1992</td>
<td>Susceptible to infection with similar characteristic as human</td>
<td>Not susceptible to infection</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{31}$P energy modulus</td>
<td>Greiner et al. 1993</td>
<td>0.9</td>
<td>/</td>
<td>1.7*</td>
</tr>
<tr>
<td>Intracorneal pH</td>
<td>Greiner et al. 1993</td>
<td>6.8</td>
<td>/</td>
<td>6.7</td>
</tr>
<tr>
<td>Epithelial permeability</td>
<td>Maurice 1995</td>
<td>/</td>
<td>0.5 – 1 nm/sec</td>
<td>/</td>
</tr>
<tr>
<td>Blink rate</td>
<td>Maurice 1995</td>
<td>/</td>
<td>3 blink/hr</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td><strong>Authors</strong></td>
<td><strong>Pigs</strong></td>
<td><strong>Rabbits</strong></td>
<td><strong>Bovines</strong></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------</td>
<td>---------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Tear flow</td>
<td>Maurice 1995</td>
<td>/</td>
<td>1.2 µl/min</td>
<td>/</td>
</tr>
<tr>
<td>Tear volume</td>
<td>Maurice 1995</td>
<td>/</td>
<td>7 µl</td>
<td>/</td>
</tr>
<tr>
<td>Horizontal corneal diameter</td>
<td>Doughty 1994</td>
<td>15-16 mm (Personal data)</td>
<td>16.5 mm (adult rabbit)</td>
<td>27.5-29.5 mm</td>
</tr>
<tr>
<td></td>
<td>Doughty 2002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal thickness</td>
<td>Van den Berghe et al. 2005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maurice 1995</td>
<td>0.95mm</td>
<td>0.4 mm</td>
<td>0.8-0.9mm</td>
</tr>
<tr>
<td></td>
<td>Doughty 2002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nictitating membrane</td>
<td>Maurice 1995</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Density</td>
<td>Kampmeier et al. 2000</td>
<td>1062 kg/m³ ± 5 kg/m³</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

* - Significantly different from humans as reported by the authors

/ - Data not reported by the author(s)
14.6.1 Morphology and histochemistry

Ehlers (1970a) investigated and compared the morphology and histochemistry of the corneal epithelium of some animals including ox (n = 6), pig (n = 9), rabbit (n = 9) and human (n = 8). The thickness and number of cell layers of the corneal epithelium vary among species (Table 1.1).

Greiner and co-workers (1983) determined the phosphate metabolite profiles in the cornea of animals that were commonly used for research, such as baboon, monkey, cat, guinea pig, pig, dog, rabbit and cow. Corneal phosphatic metabolites play important roles in regulating metabolism, which is directly related to the corneal health. $^{31}$P nuclear magnetic resonance (NMR) spectroscopy was used to measure a total of 13 metabolites. The number of significant differences in metabolites among the species studied, when compared to human, increased in the following order: baboon ≪ monkey ≈ pig ≈ cow < cat ≈ guinea pig < rabbit < dog. The ratios of phosphatic metabolites were also determined, and the cow least resembled humans in this aspect. Therefore, the authors questioned the value of data obtained in experiments using rabbits and cows, and suggested that cat, guinea pig and pig models were more suitable for cornea research as they more closely resembled the human cornea.
1.4.6.2 Permeability

Pig corneas were used for the investigation of *in vitro* permeability of hydrocortisone and pilocarpine through the cornea (Camber, 1985). Permeability was reduced in porcine cornea when compared with previous work using the rabbit cornea (Schoenwald and Ward, 1978). Corneal epithelium of pigs has been reported to be thicker than that of rabbits (Ehlers, 1970). Therefore, Camber (1985) suggested that the thicker corneal epithelium of pigs could account for the reduced permeability when compared with that of rabbits, and they concluded that this pig perfusion model would better predict the behaviour of drugs in the human eye.

1.4.6.3 Reaction in acanthamoeba infection

A model was set up to investigate contact lens-induced acanthamoeba keratitis (He *et al.*, 1992). Soft contact lenses, contaminated with cultured acanthamoeba castellani, were applied to the abraded corneal surfaces of eyes of six anaesthetized Yucatan micropigs and six New Zealand rabbits. The contralateral eye of each animal received no treatment and was used as a control. The animals were followed for 12 weeks and then euthanized. The results showed that the pigs were susceptible to infection, similar to humans, but not the rabbits. The chronic nature of the infection and the appearance of aqueous flare, dense, white ring-like infiltrates, stromal oedema, keratic precipitates and epithelial bullae were strikingly similar to those observed in the human counterpart. Based on the host specificity in parasitic disease, it was suggested
(He et al., 1992) that porcine cornea might be closer to human cornea than rabbit cornea in this aspect, and concluded that the porcine model was a valuable tool for investigations of the immunology, cell biology and therapy for acanthamoeba keratitis.

1.4.6.4 Metabolic compatibility

The metabolic compatibility of porcine, bovine and human corneas were compared using $^{31}$P NMR spectroscopy to quantify different phosphatic metabolites (Greiner et al., 1993). The $^{31}$P energy modulus of the pig was not significantly different from that of the human. However, the bovine modulus, was significantly higher. The intracorneal pH of porcine and bovine corneas was similar, 6.7 and 6.8, respectively, whereas it is higher (7.2) in human cornea. The authors (Greiner et al., 1993) explained that the higher intracorneal pH in humans may be due to the relatively longer post-mortem period prior to NMR analysis. They found that the greatest differences between human and abattoir corneas were the relative amounts of the sugar phosphate and dinucleotide phosphate bands though there was a trend that porcine cornea more closely resembled human cornea. These findings agreed with the level of phosphorylethanolamine reported in a previous study (Greiner et al., 1989) that porcine corneas were strikingly closer to those of humans than the bovine. The phosphorylethanolamine level indicates the index of the activity of phospholipase C, which affects the nature of membranes and thereby the locus of immunochemical activity. The porcine cornea is clearly more closely related
to the human cornea, as the levels and activities of phosphate metabolites of porcine corneas are similar. Greiner and co-workers (1983, 1989, 1993) concluded that porcine tissue may be more suitable for xenografts than bovine tissue.

14.6.5 Thermal and biomechanical parameters

A study determined the thermal and biomechanical parameters of porcine corneas and compared the data with those of human corneas (Kampmeier et al., 2000). The measured density of the porcine cornea was 1062 ± 5 kg/m³ whereas the calculated density of human cornea was 1087 kg/m³. Young’s modulus – stress-strain curves were determined by performing two tensile tests in nasal-temporal and superior-inferior directions. Specific heat capacity of the porcine cornea, determined by a differential scanning calorimeter, was 3.74 ± 0.05 J/gK within 37°C to 50°C. Kampmeier and co-workers (2000) concluded that the porcine cornea could be used as a substitute model for human corneal research. However, another study (Zeng et al., 2001) was not in complete agreement with these results. The tensile strength of human and porcine corneas were compared (Zeng et al., 2001) and found to be 3.81 ± 0.40 Mpa and 3.70 ± 0.24 Mpa, respectively and these were not statistically significant (p > 0.17). The stress-strain relationships were also compared for human and porcine corneas and were found to be not significantly different for these two specimens (t-test; p > 0.05). However, the stress relaxation property of the porcine cornea was different from that of the human cornea. Porcine cornea relaxed more rapidly
than human cornea. The different results obtained by these two studies (Kampmeier *et al.*, 2000; Zeng *et al.*, 2001) may be due to the use of different materials and experimental methods. Zeng and co-workers (2001) used the ring-shaped corneal remains after corneal transplantation while Kampmeier and co-workers (2000) used the whole cornea for their investigations.

Beside the dissimilarity of bovine and human eyes, the use of porcine eyes rather than bovine eye avoids the problems associated with the use of potentially infected bovine eyes (especially in relation to ‘mad-cow’ disease).

### 1.5 Assessment methods for corneal integrity and epithelial cell viability

#### 1.5.1 Clinical assessment of corneal integrity

The clinical technique for examination of corneal integrity is by slit-lamp microscopy with the addition of different dyes. The staining properties of these dyes differ and therefore reflect different conditions of the ocular surface. Details will be discussed in the following sections.

#### 1.5.1.1 Sodium fluorescein

Sodium fluorescein staining is routinely used in a standard eye examination to investigate ocular surface damage, such as epithelial defects, corneal erosions, corneal abrasion and keratitis (Norn, 1962). In dry eye, the staining appears fine
and punctate and is usually confined to the exposed interpalpebral area of the ocular surface (Whitcher, 1987; Bron, 2001). Sometimes, staining may also be present in the bulbar conjunctiva. In severe dry eye, the staining may extend to the unexposed regions of the cornea and conjunctiva and the punctate staining will join together and in extreme situations, large areas of corneal epithelium may slough off (Whitcher, 1987; Friedlaender, 1992).

Fluorescein is a vital dye that does not “stain” healthy corneal cells (Lemp, 1995). Fluorescein actually does not stain the damaged or dead cells but penetrates into the intercellular space due to the defects between epithelial cells or accumulates at locations where there is a dropping out of single cells (Norn, 1970). However, Wilson and co-workers (1995) found that fluorescein actually gets into the injured single epithelial cells on the rabbit corneal surface when examined at high magnification. These two properties of fluorescein were supported by a study using both cultured rabbit corneal epithelial cells and rabbit corneal explants (Feenstra and Tseng, 1992a). The results showed that fluorescein did enter into single epithelial cells and it preferentially accumulated in the underlying stromal tissue. Moreover, due to the increased membrane permeability in degenerated or dead cells, fluorescein staining was enhanced in these cells. Taken together, these data indicate that fluorescein staining of the ocular surface specifies areas with damaged epithelial cells and disruption of cell junctions, as staining will be manifested in these areas by accumulation of fluorescein in the intercellular spaces or diffusion into the
stroma. Fluorescein dye is therefore a valuable tool for the assessment of corneal integrity.

Another advantage of using fluorescein to determine corneal integrity is that it does not produce toxic effect to corneal epithelial cells (Feenstra and Tseng, 1992a). Therefore, when used in combination with other stains, it may be superior to other stains that may have adverse effect on the corneal surface.

### 15.1.2 Rose Bengal

Apart from using sodium fluorescein dye to evaluate ocular surface damage, Rose Bengal is also commonly used for the diagnosis of dry eye, especially to demonstrate the damage to the bulbar conjunctiva bought about by desiccation (Lemp, 1995). It was proposed by Sjögren in 1933 that Rose Bengal provided a unique staining pattern in patient with dry eye induced by Sjögren syndrome (Kim, 2000). A scoring system was developed by Van Bijsterveld (1990) for quantifying the level of Rose Bengal staining, which provide a convenient tool for practitioners to monitor patients with dry eye. According to this system, scores of 3.5 or greater indicate the presence of dry eye (Kim, 2000). However, Rose Bengal irritates upon application and induces ocular discomfort in patients. Therefore, it is not commonly used for the assessment of dry eye nowadays.

As with other dyes, Rose Bengal enters dead or degenerate cells due to the increased membrane permeability of these cells but does not stain healthy
corneal cells (Norn, 1962). Therefore, stained areas of cornea or conjunctiva may indicate the presence of dead or degenerated cells. However, recent studies (Feenstra and Tseng, 1992b; Feenstra and Tseng, 1992a; Kim and Foulks, 1999) have shown that Rose Bengal stains cultured corneal epithelial cells in their healthy state and produces intrinsic toxic effect to the cells at concentrations greater than or equal to 0.1%. In addition, some components of artificial tear solutions, for example, 1% carboxymethylcellulose solution can block Rose Bengal uptake. Another disadvantage of using Rose Bengal is that the dark red staining on the cornea may be difficult to observe against the background of a dark coloured iris.

Rose Bengal stains dead or degenerated cells, as well as healthy cultured corneal epithelial cells. It may produce adverse effects on cells and therefore, may not an appropriate dye for the assessment of corneal integrity and viability.

1.5.1.3 Lissamine green

Lissamine green gives similar clinical results when compared with Rose Bengal but it is less irritating when applied to patients with dry eye (Norn, 1973; Khurana et al., 1991; Manning et al., 1995). Therefore, lissamine green may be a better choice than Rose Bengal for the evaluation of ocular surface damage in dry eye patients.
Lissamine green does not stain normal rabbit corneal epithelial cells but penetrates only into membrane-damaged cells (Chodosh et al., 1994), and also stains the underlying stroma in rabbit corneal explants. In addition, it does not stain proliferating human corneal epithelial cells (Kim and Foulks, 1999). Therefore, lissamine green has been recommended for use clinically for the assessment of ocular surface integrity. However, it is not used as widely as sodium fluorescein because commercially-available lissamine green strips are much more expensive than sodium fluorescein strips.

1.5.2 Assessment of cell viability

1.5.2.1 By membrane exclusion

Various methods have been used for the assessment of cell viability. Among these, the most common method is the use of vital stains. An increase in cell membrane permeability can often be an indicator for loss of cell viability as a result of necrosis and secondary apoptosis. However, one major disadvantage of assessing cell viability by membrane exclusion is that in the early stages of apoptosis, the number of non-viable cells may be under-estimated because these cells can retain their cell membrane integrity for some period of time (McGahon et al., 1995). On the other hand, it is still widely used because it is a simple, fast and low cost assessment.
Each vital stain has its own mechanism for entering either live or dead cells, and reacting with different organelles to give a visible colour. Some commonly used vital stains will be introduced in the following sections, and focus will be on those stains used for assessments of viability of corneal cells.

1.5.2.1.1 Trypan blue
Trypan blue has a large molecular weight and therefore is not permeable to the cell membranes of healthy, normal cells. This characteristic makes it useful for the assessment of cell viability. The trypan blue exclusion method is a common staining method for identification of dead cells (Patterson, 1979; Altman et al., 1993; Means et al., 1995; Sokol et al., 1996; Chang et al., 2000a) because it is inexpensive, simple to carry out and gives dependable results (Perry et al., 1997). It has been proposed as a vital stain for cornea and conjunctiva (Norn, 1967) and has been used for the assessment of viability and of the level of damage of corneal endothelial (Singh et al., 1985; Morton, 1992; Vargas et al., 2004) and epithelial cells (Fleiszig et al., 1996; Crouch et al., 1998).

1.5.2.1.2 Other fluorescent stains
Acridine orange and propidium iodide are two fluorescent stains that are commonly used, either alone or in combination, to assess corneal cell viability (Petroll et al., 1995; Wusteman et al., 1999; Shimmura et al., 2004; Zamiri et al., 2004). Acridine orange is a membrane-permeable cationic dye that can enter into all cells. It binds to the nucleic acid of viable cells and makes them
fluoresce green, while propidium iodide can penetrate only the membrane of non-viable cells and binds with RNA or DNA causing an orange fluorescence.

Calcein AM and ethidium homodimer are also widely used in combination for the assessment of corneal cell viability (Imbert and Cullander, 1997; Poon et al., 2001; Yamamoto et al., 2002; Engelke et al., 2004; Tungsiripat et al., 2004). Calcein AM is a viable cell marker, which is permeable to all cell membranes. Once inside the cells, it will be hydrolyzed by endogenous esterases into the negatively-charged green fluorescent calcein and remain in the cytoplasm. Ethidium homodimer enters cells with damaged or compromised membranes, and stains the DNA during the late stage of cell death (Yamamoto et al., 2002).

1.5.2.1.3 Comparisons between these vital stains

Altman and co-workers (1993) determined the cell viability of suspensions of cultured SP2/0-derived murine hybridomas and HyHEL-10 using trypan blue and propidium iodide. The stained cells were counted using a haemocytometer. In the early cell growth cycle (days 1 to 2), there were no significant differences in the number of non-viable cells stained by trypan blue or propidium iodide. However, when the number of non-viable cells began to increase in days 3 to 6, significantly fewer non-viable cells were detected by trypan blue. Therefore, Altman and colleagues (1993) concluded that trypan blue over-estimated the cell viability in cells with longer culture times. However, in another study, Means and co-workers (1995) compared the ability of trypan blue and the
calcein AM-ethidium homodimer to identify cytotoxic change and the
effectiveness of these stains in corneal endothelial cells. They found that the
numbers of dead corneal endothelial cells stained by trypan blue and ethidium
homodimer were comparable. They concluded that both stains were effective
with good sensitivity for cytotoxic change.

1.5.2.2 By biochemical function

Apart from using vital stains for the assessment of cell viability, some other
tests including lactate dehydrogenase (LDH) assay (Huhtala et al., 2002),
terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
(TUNEL) (Komuro et al., 1999), XTT, a colorimetric reductive test similar to
the MTT test mentioned earlier (Roehm et al., 1991; Diebold et al., 1998;
Mosmann, 1983; Imbert and Cullander, 1997) can also be used for the
determination of corneal cell death but will only be briefly mentioned in this
chapter as these tests are commonly used to assess corneal cell viability in
cultured or isolated cells (Yang and Acosta, 1995; Wang et al., 1996; Inoue et
al., 2000; Moreira et al., 2001; Gain et al., 2002; Chen et al., 2002; Scuderi et
al., 2003; Ubels et al., 2004; Kim et al., 2004; Joyce et al., 2004) but were not
used for the assessment of cell viability of the whole corneal explant.

LDH assesses cell viability on the basis of the integrity of the cell membrane.
Measurement of the leakage of components from the cytoplasm into the
surrounding culture medium has been accepted as a valid method to estimate
the number of nonviable cells. LDH, an oxidoreductase, is present in the cells of almost all body tissues. It catalyzes the conversion of lactate to pyruvate, an essential step in the production of cellular energy. If the cell membrane is damaged, LDH will leak out from the cytoplasm. Therefore, the amount of enzyme activity in the supernatant correlates to the number of damaged cells. Different LDH assay kits are commercially available for the determination of LDH activity. Some are based on enzymatic assay that results in the conversion of a non-fluorescent compound into the fluorescent state. The level of fluorescence indicates the number of membrane-damaged or non-viable cells. Other are linked to NADH oxidation, with a concomitant decrease in absorbance at 340nm.

The TUNEL assay is efficient at measuring apoptosis in cell culture by detecting strand breaks within DNA. In the TUNEL assay, the enzyme Tdt is used to add dUTPs to the broken ends of the DNA. These can be detected using antibodies with fluorochrome labels. Hence, cells with damaged DNA will be stained. Again, different assay kits are commercially available (Gavrieli et al., 1992).

Colourimetric assays are used to determine the number of viable cells by the application of a colourless substrate that will be converted to a coloured product by living but not dead cells (Mosmann, 1983). The coloured product can be measured using a spectrophotometer and the readings indicate the activity of
oxidoreductase enzymes and hence, the viability of cells. XTT and MTT are colourless tetrazolium salts that will be reduced by the mitochondrial dehydrogenases of viable cells, resulting in the formation of coloured formazan. The formazan formed by XTT salt is water soluble, while that of MTT is not. Therefore, extra procedures are needed to dissolve the formazan in the MTT assay before the absorption measurement. As a result, XTT is a better assay with significant advantages over the MTT assay, including shorter assay time and simpler handling procedures (Roehm et al., 1991).

1.6 Summary and aims of the study

In summary, dry eye is a very common eye problem that affects a large population worldwide (Jacobsson et al., 1989; Hikichi et al., 1995; Bjerrum, 1997; Caffery et al., 1998). It is multi-factorial in origin and can be caused by either decreased tear production or increased tear evaporation (Lemp, 1995; Mathers, 2000; Baudouin, 2001). In most dry eye patients, ocular surface damage may be induced by desiccation due to reduced tear stability and tear volume (Gamache et al., 2002). Therefore, it is necessary and important to investigate the aetiology of and the treatment of dry eye.

The development of animal DEM provides us with a platform for investigation of the aetiology and the treatment of dry eye. Single causative factors of dry eye can be isolated and studied using these DEMs, which is impossible in human
dry eye patients. Moreover, protective or toxic effects of potential treatments can be evaluated with these DEMs and therefore, the risk of drug toxicity in dry eye patients can be minimised.

Each DEM has its own characteristics and represents a unique dry eye condition. However, due to species differences, the results obtained with these DEMs may not represent the dry eye that occurs in humans (Maitchouk et al., 2000; Barabino and Dana, 2004). To date, there has been no DEM which has considered the effect of blinking when studying dry eye. Porcine organs are more similar to those of humans compared to other animals used in eye research and therefore, using porcine eyes to develop and evaluate a DEM incorporated with a blinking system would be useful for the investigation of different factors related to dry eye and potential treatments for dry eye. This was the focus of this study.
Chapter 2

Specific Objectives and Outlines of Structure of the Thesis

2.1 Objectives

The objectives of this study were to:

- Determine the possibility of using porcine eye to establish an animal desiccation-induced dry eye model
- Develop a novel porcine desiccation-induced dry eye model with adjustable ‘lacrimation’ and ‘blink’ rates
- Ascertain the reproducibility of the pDEM system
- Simulate different severities of desiccation-induced dry eye using the pDEM
- Investigate the effect of different ‘blink’ rates on desiccation-induced pDEM
- Investigate the effect of different artificial tears on desiccation-induced pDEM
- Investigate the effect of different viscosities of solutions on desiccation-induced pDEM.
2.2 Thesis Outline

This thesis describes individual experiments that were conducted to achieve the above objectives. Specific experimental procedures and settings of the experiments are described in each chapter.

In Chapter 3, the materials and methods for transporting porcine eyes from the slaughter house to our laboratory are described. The assessment methods for investigating the integrity of the cornea and the viability of the corneal epithelial cells are also reported.

Chapter 4 presents the report of the suitability of sodium fluorescein and trypan blue for assessment of corneal integrity and corneal cell viability, respectively.

In Chapter 5, the results of the study on the viability of porcine corneal epithelial cells and the effect of exposing the porcine cornea to environments with and without protection against desiccation are presented. These baseline data were used to determine whether the porcine eye is suitable for the development of a desiccation-induced DEM.

The development of the pDEM is described in Chapter 6, and the reproducibility of the pDEM set-up is also reported. The effect of different ‘lacrimation-blink’ intervals was also investigated so that the ability of this
pDEM to simulate different severities of desiccation-induced dry eye could be determined, and results are also presented in Chapter 6.

Chapter 7 presents the report on the effect of different ‘blink’ rates on desiccation-induced dry eye condition.

In Chapter 8, the report on effects of different artificial tear formulations on desiccation-induced dry eye is presented. Moreover, effects of different viscosities of methylcellulose solutions on the desiccation-induced dry eye are reported.

In the last chapter, Chapter 9, an overall summary and discussion of all experiments is given. The chapter also includes a discussion of limitations of the study and suggestions for further investigations.

Duplication of content was kept to a minimum within this thesis, however, some repetitions are unavoidable in the description and discussion of individual experiments.
Chapter 3

General Materials, Assessment Methods and Pre-experimental preparation

This chapter describes the general materials and methods used for transporting porcine eyes from the local abattoir to our laboratory and also the assessment methods used for corneal integrity and viability of corneal epithelial cells. Specific experimental procedures will be described in the experimental design section of each chapter.

3.1 Materials and methods

Porcine eyes were obtained from the local abattoir in Hong Kong. The eyes were enucleated, along with the surrounding eyelid and conjunctiva, within minutes of the pigs being killed. The corneas were kept intact and moist by taping the eyelids closed immediately after enucleation. The eyes were put into a water-proofed plastic bag, and then the whole bag, together with the taped eyes, was put into a plastic container stored with ice and transported back to the laboratory within an hour. Immediately on arrival, each cornea was rinsed with Dulbecco’s phosphate buffered saline (DPBS) (Sigma, St. Louis, MO) and the
integrity of the cornea was examined by slit lamp biomicroscopy. Corneas with initial fluorescein staining greater than grade 1 were discarded, and the two eyes with the least amount of staining (best condition) were used to set up the pDEM. There were only two pDEM set-up, therefore, only two sets of data could be obtained each experimental day. On occasions when only one eye was in good condition, then only one pDEM was prepared on that experimental day. After the experimental period, the integrity of the corneas was examined again with the slit lamp, followed by the assessment of the viability of the corneal epithelium using the trypan blue exclusion test.

Ethics approval for the use of animals in this project was not necessary because pigs were killed in the slaughter house for food supply before the eyes were enucleated. No additional or different procedures were followed in regard to handling or killing of live animals in respect of this study.

3.2 Assessment methods used

3.2.1 Integrity of corneal surface

Slit lamp microscopy with sodium fluorescein solution

The integrity of the corneal surface was examined by slit lamp microscopy after application of sodium fluorescein solution (1% w/v), made from dissolving sodium fluorescein powder (Sigma, St. Louis, USA) in DPBS onto the corneal surface of the porcine eye.
Figure 3.1. Fluorescein grading system from Grades 0 - 4.

Excess sodium fluorescein solution was rinsed away gently with a few drops of DPBS. Fluorescein staining, if any, was graded using a zero (no staining) to 4 (severe) grading system, with a 0.5 step, as shown in Figure 3.1.

3.2.2 Viability of corneal epithelial cells

Trypan blue exclusion test

The viability of the corneal epithelium was assessed by the trypan blue exclusion technique as follows. Trypan blue solution (0.4% w/v) was prepared by dissolving trypan blue powder (Sigma, St. Louis, MO) in DPBS. The eye was dipped in the trypan blue solution for two minutes with the cornea facing
downward and the solution covering beyond the limbal region. The cornea was rinsed with few drops of DPBS to remove excess trypan blue solution.

**Figure 3.2. Preparation of a porcine cornea (schematic diagram) for counting the number of trypan blue stained cells.**

![Diagram of cornea with radial cuts](image)

The solid black lines indicate the locations of the four radial cuts.

The cornea, with a 2-mm scleral rim, was cut from each eye with a blade and scissors, with special care to avoid touching the cornea. The horizontal dimension of the cornea was 15-16 mm. To lay the cornea flat on the glass slide, four radial cuts (each 90° apart) were made (Figure 3.2). The cornea was examined with an Olympus microscope (CH-2, Japan). The number of stained cells on the corneal surface was counted using a 5 x 5 mm grid placed in the x10 eyepiece of the microscope with a x10 objective (final field size was 0.25...
mm²). The number of stained cells was determined separately for the central and the peripheral areas of each cornea. Three different fields in the central and four peripheral areas (in four different quadrants) were counted; the results were averaged to give representative results for the central cornea and for the peripheral cornea.

3.3 Pre-experimental preparation

3.3.1 Development of the dissection skill

Around 50 eyes were obtained and dissected for practice on how to handle the eye in order to minimize damage during dissection. Corneas were stained with sodium fluorescein before desiccation and the corneal integrity were assessed. The eyeballs were then dissected away from the eyelid, extra ocular muscle and other tissues around the globe. After all these procedures, the corneal integrity was assessed again with sodium fluorescein. Before a good dissection skill was developed, mechanical corneal damage (fluorescein stain in a line shape) was found on the corneal surface of the dissected eye. However, after improving the handling skill and dissection skill, corneal integrity could be preserved after dissection.
3.3.2 Selection and optimization of the environmental conditions for the pDEM

The experimental set-ups (pDEM) were placed in an air-conditioned laboratory. The temperature was selected, based on the room temperature (around 22 °C) of the laboratory under air-conditioned environment, for convenient. Moreover, running the experiment in the temperature lower than the body temperature of the animal may reduce the metabolic rate of the eyes in order to keep them for a longer time.

In the first generation of the pDEM, fans were not added to increase the ventilation of the environment, however, it was found that in order to obtain a severe desiccation induced damage on the corneal surface, the ‘lacrimation-blink’ interval would be large (around 5 minutes) which means that the frequency for solution added onto the corneal surface and the ‘blink’ frequency were not high. In this situation, we worried that the results were not be conspicuous if we studied the protective effect of different agents or the ‘blinking’ effect. Therefore, fans were added to increase the ventilation rate of the environment so that a severe desiccation-induced damage could be induced with a smaller ‘lacrimation-blink’ interval.

However, the results obtained were still varied. It was proposed that the variations maybe due to the changes in the environmental conditions, either temperature or humidity, inside the laboratory. Therefore, the environmental
conditions were strictly kept at temperature 21 to 23 °C and humidity of 50 to 60%. After the control of these environmental conditions, the results obtained became more stable.

Around half year time was spent in the selection and optimization of the environmental conditions for the pDEM.
Chapter 4

Preliminary Study: Will Sodium Fluorescein Solution Affect the Results of Trypan Blue Exclusion Test?

4.1 Introduction

As mentioned in Section 3.2, both sodium fluorescein solution and trypan blue solution were to be applied onto the corneal surface to assess the integrity of the corneal surface and the viability of the corneal epithelial cells, respectively. Although the cornea was rinsed with DPBS after the application of sodium fluorescein solution and before the trypan blue exclusion test, some sodium fluorescein solution may still remain on the corneal surface or in the corneal epithelial cells and may affect the entry of trypan blue solution leading to an error in the trypan blue exclusion test results.

Sodium fluorescein solution does not actually stain corneal epithelial cells but instead it accumulates in areas with disruption of cell-cell junctions or diffuses into the stroma (Feenstra and Tseng, 1992). However, fluorescein may penetrate into individual corneal epithelial cells (Wilson et al., 1995) (See Section 1.5.1.1). Therefore, whether residual sodium fluorescein solution in the cornea will exert a blockage effect preventing trypan blue dye from entering the
corneal epithelial cells is unknown. The objective of this preliminary study was to investigate whether sodium fluorescein solution applied prior to trypan blue dye will affect the results of trypan blue exclusion test.

4.2 Experimental design

Twenty-seven porcine eyes were obtained and transported back to the laboratory over nine experimental days (see Section 3.1). Eyes were divided into control (n = 11) and experimental (n = 16) groups. The viability of the corneal epithelial cells was assessed in all corneas in the control group using the trypan blue exclusion test (see Section 3.2.2). For the experimental group, the integrity of the cornea was assessed using sodium fluorescein solution and slit lamp microscopy as described in Section 3.2.1 before assessing the viability using the trypan blue exclusion test.

The number of trypan blue-stained cells in the central and the peripheral areas between the corneas of the two groups of corneas were compared using the Mann-Whitney test. A non-parametric test was used, as the sample size was small.

4.3 Result

There were no significant differences in the number of trypan blue-stained cells in the central (p = 0.126) and the peripheral (p = 0.199) corneal areas of eyes between the control and experimental groups.
4.4 Discussion

The results of our preliminary study showed that sodium fluorescein solution applied to the porcine corneas prior to rinsing and application of trypan blue dye did not affect the results of the trypan blue exclusion test. There are two possible explanations for our results. First, sodium fluorescein and trypan blue can both enter those corneal epithelial cells with increased membrane permeability but residual sodium fluorescein in the cornea does not block the entry of trypan blue dye. Second, after rinsing with DPBS, the remaining amount may not be sufficient to limit the entry of trypan blue dye.

4.5 Conclusion

In conclusion, assessments of the integrity of the cornea using sodium fluorescein does not affect the viability test using trypan blue dye if the corneas were rinsed with DPBS before the application of trypan blue dye.
Chapter 5

Viability of Porcine Corneal Epithelium of Enucleated Eyes and Effect of Exposure to Air

5.1 Introduction

Dry eye is a highly prevalent ocular disease affecting older persons. Dry eye symptoms, including irritation and burning sensations, are commonly reported in the elderly (Bandeen-Roche et al., 1997; Schein et al., 1997) (see Section 1.2.3.2.1). Dry eye has a multi-factorial origin (Stern et al., 1998; Baudouin, 2001) (see Section 1.2.3) and one of the main causes is decreased tear secretion which results in the drying of the superficial corneal epithelium. Damaged and dead corneal epithelial cells detach from the ocular surface, leading to corneal erosion and development of symptoms (Friedlaender, 1992). In advanced dry eye, corneal ulceration and scarring may develop, leading to severe visual disability or blindness (Virtanen et al., 1997; Terry, 2001).

Due to the high incidence of dry eye and its potential severe consequences, many clinical and experimental studies have been conducted to investigate the
diagnosis, pathogenesis and treatment of dry eye, and also its relation to
disorders of the tear film. However, most of the experimental studies cannot be
performed on humans as there may be harmful effects. Therefore, animal dry
eye models have been developed (Gilbard et al., 1988; Kaswan et al., 1989;
Hicks et al., 1998; Burgalassi et al., 1999; Fujihara et al., 2001; Moore et al.,
2001; Dursun et al., 2002) (see Section 1.4).

Previous investigators have used the eyes of animals, such as rabbits (Fujihara
et al., 1998; Burgalassi et al., 1999), rats (Fujihara et al., 2001) and dogs
(Kaswan et al., 1989; Moore et al., 2001) to develop dry eye models to
investigate the effect of different treatments for dry eye. Some studies have
used mice (Dursun et al., 2002), rabbits (Gilbard et al., 1988) and monkeys
(Maitchouk et al., 2000) to simulate dry eye and to investigate the effect of
dryness on the histological features of the ocular surface (see Sections 1.4.2 and
1.4.3). The animal most commonly used is the rabbit. However, the average
blink rate of the rabbit is three blinks/hr (Maurice, 1995), which is much less
than the average of human (17 blinks/min at rest) (Bentivoglio et al., 1997).
Furthermore, the Schirmer tear test (an indicator of tear volume) has shown a
much lower tear volume measurement in rabbits compared to humans (5 ± 1
mm/5 min versus 27 ± 3 mm/5 min) (Khurana et al., 1991; Kuiper et al., 1997).
In humans, a tear volume measurement of less than 5 mm/5 min is one of the
diagnostic criteria for dry eye (Holly and Lemp, 1977) and symptoms may be
reported in association with such a low tear volume. Therefore, the rabbit
cornea is not the best substitute for the human cornea (Greiner et al., 1983; He et al., 1992) (see Section 1.4.6).

As discussed in Section 1.4.5, pig organs have been suggested as a substitute for human organs in some research areas (Wang et al., 1985; King et al., 1992; Christie et al., 1995; Zhang and Monteiro-Riviere, 1997). Porcine corneas have been reported to have the most characteristics in common with human corneas (Camber, 1985; He et al., 1992; Greiner et al., 1993; Kampmeier et al., 2000). However, to our knowledge, porcine corneas have never been used to develop a dry eye model.

One of the aims of this project was to develop a desiccation-induced dry eye model using porcine corneas in situ. This required a preliminary investigation of the viability of porcine corneal epithelium immediately after enucleation, and the effect of short-term air exposure.

### 5.2 Experimental design

The preparation procedures for porcine eyes are described in Section 3.1. The following information refers to the specific procedures used in this part of the experiment.
A total of 27 porcine eyes were used in this experiment. The viability of nine corneas was assessed on site within five minutes of enucleation. To test the effect of exposure, 18 corneas were transported back to the laboratory. The corneas of six eyes were exposed to air at room temperature (20 to 25ºC) and a humidity of 55 to 65% for four hours. Six corneas were exposed to the same conditions for six hours and the remaining six corneas were also exposed to the same condition, but were wetted with DPBS every five minutes for four hours. The viability of the corneal epithelium was assessed by the trypan blue exclusion technique (see Section 3.2.2).

Wilcoxon-Signed ranks test was used to assess for any differences between the number of stained cells in the central and in the peripheral regions of the corneas for each condition. Kruskal-Wallis test was used to assess the effect of exposure, and Mann-Whitney test was performed if a significant difference was found, to determine which condition(s) was(were) significantly different from the control group. A p-value less than 0.05 was considered statistically significant.

5.3 Results

Table 5.1 and Table 5.2 shows a summary of the results, and Figure 5.1 shows a scatter plot of central versus peripheral corneal data.
Table 5.1. Number of stained cells (median, range and mean (SD)) per field (0.25 mm²) of central corneas under the four conditions.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>median (range)</th>
<th>mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>On-site assessment control group</td>
<td>9</td>
<td>136 (112-173)</td>
<td>139 (19)</td>
</tr>
<tr>
<td>Exposed but wetted every five minutes for four hours</td>
<td>6</td>
<td>135 (122-197)</td>
<td>149 (33)</td>
</tr>
<tr>
<td>Exposed for four hours</td>
<td>6</td>
<td>286 (208-366)</td>
<td>286 (66)</td>
</tr>
<tr>
<td>Exposed for six hours</td>
<td>6</td>
<td>385 (287-485)</td>
<td>381 (68)</td>
</tr>
</tbody>
</table>

\( p_1 < 0.05^* \)

\( p_1 \) Kruskal-Wallis tests for differences among different conditions in the central cornea

* Mann-Whitney tests showed significant differences between all conditions, except between the on-site assessment control group and corneas exposed but wetted every five minutes for four hours

Table 5.2. Number of stained cells (median, range and mean (SD)) per field (0.25 mm²) of peripheral corneas under the four conditions.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>median (range)</th>
<th>mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>On-site assessment control group</td>
<td>9</td>
<td>96 (82-107)</td>
<td>94 (13)</td>
</tr>
<tr>
<td>Exposed but wetted every five minutes for four hours</td>
<td>6</td>
<td>117 (76-165)</td>
<td>116 (36)</td>
</tr>
<tr>
<td>Exposed for four hours</td>
<td>6</td>
<td>205 (161-255)</td>
<td>204 (36)</td>
</tr>
<tr>
<td>Exposed for six hours</td>
<td>6</td>
<td>272 (254-387)</td>
<td>290 (50)</td>
</tr>
</tbody>
</table>

\( p_1 < 0.05^* \)

\( p_1 \) Kruskal-Wallis tests for differences among different conditions in the peripheral cornea

* Mann-Whitney tests showed significant differences between all conditions, except between the on-site assessment control group and corneas exposed but wetted every five minutes for four hours
Figure 5.1. A scatter plot showing the number of stained cells (per field) in the central and peripheral cornea in different groups: immediately after enucleation (▲); wetted every five minutes for four hours (◆); exposed for four hours (■); exposed for six hours (●).

Figure 5.2 shows the stained cells observed in different groups. The number (median and range) of stained epithelial cells, the median and range, in the central and peripheral regions of the porcine cornea in each field (0.25 mm²) were 136 (112-173) and 96 (82-107) cells, respectively, when stained immediately after slaughtering and enucleation (control-baseline group). There was no significant difference in the number of stained cells in corneas that were assessed on site and corneas exposed but wetted with DPBS every five minutes for four hours. The number of stained cells in the central region was significantly greater than that in the peripheral region (p < 0.05) in all corneas of all groups.
Figure 5.2. Stained porcine corneal epithelial cells under different conditions.

a: Corneas stained immediately after enucleation at the slaughter house. b: Corneas exposed to ambient conditions but wetted every five minutes with DPBS for four hours. c: Corneas exposed to ambient conditions for four hours. d: Corneas exposed to ambient conditions for six hours. Black dots indicate stained corneal epithelial cells nuclei. Photos were taken under x100 magnification.
Figure c d
For the effect of exposure, there were significant differences between corneas which were wetted every five minutes and corneas which were exposed to ambient air for four and six hours respectively in both central and peripheral regions (p < 0.05). Corneas wetted with DPBS showed markedly fewer stained cells when compared with corneas exposed to air for four and six hours (Table 5.1, Table 5.2 and Figure 5.1).

5.4 Discussion

The results of this preliminary work show both differences and similarities in cell viabilities when compared with previous findings on rabbit corneal epithelium (Table 5.3) (Ren and Wilson, 1996; Li et al., 2002; Yamamoto et al., 2002)
Table 5.3. Published data on the number of non-viable epithelial cells in the rabbit corneal epithelium immediately after enucleation and data obtained in the current study using porcine eyes.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Assessment method</th>
<th>Species used</th>
<th>Sample size (n)</th>
<th>Mean ± SEM of non-viable cells in central cornea (cells/mm(^2)) [median]</th>
<th>Mean ± SEM of non-viable cells in peripheral cornea (cells/mm(^2)) [median]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al. (2002)</td>
<td>Annexin V-FITC &amp; Propidium iodide staining</td>
<td>Rabbit</td>
<td>42</td>
<td>&lt;45</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Yamamoto et al. (2002)</td>
<td>Calcein AM-EthD-1 assay</td>
<td>Rabbit</td>
<td>28</td>
<td>[&lt;35]</td>
<td>[&lt;20]</td>
</tr>
<tr>
<td>Ren &amp; Wilson (1996)</td>
<td>Combined calcein-ethidium assay</td>
<td>Rabbit</td>
<td>8</td>
<td>20.2 ± 3.9</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>Current study</td>
<td>Trypan blue</td>
<td>Pig</td>
<td>9</td>
<td>[544]</td>
<td>[384]</td>
</tr>
</tbody>
</table>

SEM: Standard error of mean
Previous investigators using rabbit eyes reported that most corneal epithelial cells were viable (Ren and Wilson, 1996; Li et al., 2002; Yamamoto et al., 2002), and the density of non-viable cells was higher in the central cornea than in the peripheral cornea. Ren and Wilson (1996) suggested that, based on the findings of Doughty (1990) on the density of exposed cells on the corneal epithelial surface, less than 1% of epithelial cells were non-viable after enucleation. However, the number of non-viable cells in rabbit corneal epithelium reported by different investigators varied from 20 to 45 cells/mm² (Ren and Wilson, 1996; Li et al., 2002; Yamamoto et al., 2002). This may be due to the use of different assessment techniques (staining agents) and variations in methods used for classification of the central and peripheral regions of the cornea. The results of this current study, the first to date using porcine corneas, show that the number of dead cells in porcine corneal epithelium (544 cells/mm²) was higher than those reported for rabbit corneas.

We believe it is unlikely that the difference in results, when compared with rabbit studies, is due to the use of different staining methods, as the trypan blue exclusion method has been reported to be comparable to other staining methods (Altman et al., 1993; Means et al., 1995) (see Section 1.5.2.1.3). Moreover, the trypan blue exclusion method is a well-known, commonly used and standard staining method for identification of dead cells (Patterson, 1979) (see Section 1.5.2.1.1). Therefore, the difference in the number of non-viable cells in porcine and rabbit corneal epithelia may be due to interspecies physiological differences.
Our results are consistent with previous findings (Ren and Wilson, 1996; Li et al., 2002; Yamamoto et al., 2002) that the number of dead cells in the central region is higher than that in the peripheral corneal epithelium immediately after enucleation. This observation may be explained by the theory that the central corneal epithelial cells suffer from the greatest shearing force during eyelid movement (Lemp and Mathers, 1989) and the increased stress in the central cornea may induce more cell death and exfoliation of cells. An alternative explanation is the cell migration hypothesis (Lemp and Mathers, 1989), wherein, cells migrate from the limbal region to the central cornea during development. Cells from the central region have undergone more cycles of division than cells in the peripheral region and as a result, cells in the central cornea will be nearer to terminal differentiation and senescence.

The results of this experiment also indicate that exposure of the porcine cornea to a normal external environment induces significant levels of cell death within four hours, and that further exposure increases the number of stained epithelial cells. The cause of death is likely to be due to cell desiccation and not lack of nutrients because most of the epithelial cells of the eyes in the exposure-control group were still viable after four hours when the corneas were wetted with DPBS every five minutes. The number of stained cells in the corneas which were exposed but wetted with DPBS every five minutes for four hours was not significantly different from the number of stained cells in the corneas stained immediately after enucleation. Therefore, epithelial cells can remain viable for
at least four hours if adequate protection against desiccation is provided. Cell desiccation or desiccation-induced damage was defined as the efflux of cellular water, concentration of inorganic salts, crowding of macromolecules, damage to cellular structure and function, and mechanical damage due to cell shrinkage and distortion. However, corneal desiccation was defined as the exposure of cornea which may result in drying up of ocular surface, leading to desiccation-induced damage.

This suggests that porcine cornea is suitable for the development of a short-term desiccation-induced dry eye model. By adjusting the volume, frequency and type of wetting solution, different levels of protection may be achieved. In other words, different degrees of cell death due to desiccation may be simulated, and treatment methods and environments tested.

5.5 Conclusion

Porcine corneal epithelial cells of enucleated eye can remain viable for four hours if proper protection against desiccation is provided. The number of non-viable corneal epithelial cells increase significantly if the eyes are exposed to environmental condition without wetting. These results indicated that the porcine eye is suitable for the development of a short-term desiccation-induced dry eye model.
Paper published

Chapter 6

Development of the Porcine Dry Eye Model and Evaluation of the Reproducibility and Applicability in Simulation of Different Severity of Desiccation-induced Dry Eye

6.1 Introduction

As discussed in Section 1.2.3.1, major immediate cause of dry eye is decreased tear secretion or increased tear evaporation. Both can result in desiccation and death of corneal epithelial cells. Dead cells may detach from the corneal surface leading to ocular surface inflammation, corneal erosion and the development of dry eye symptoms as the condition advances (Friedlaender, 1992). Moreover, decreased tear volume may increase the frictional force between the cornea and the eyelid during blinking, increasing stress to the corneal epithelial cells (Stern et al., 1998).

Various in vivo dry eye models (DEM) have been developed using the eyes of different animals (see Section 1.4), and rabbits are most frequently used. However, the rabbit cornea is not the best substitute for the human cornea, and
porcine cornea had been suggested to be a better choice (see Section 1.4.5 and 1.4.6).

In the *in vivo* animal DEM systems reported to date, dry eye was induced using a range of methods, including, the application of drugs to inhibit tear secretion (Dursun *et al.*, 2002), the removal of the lacrimal gland (Fujihara *et al.*, 2001; Moore *et al.*, 2001) and the prevention of blinking (Fujihara *et al.*, 1995; Fujihara *et al.*, 1998; Fujihara *et al.*, 2002; Gamache *et al.*, 2002). In some studies, the animals were put inside a blower hood (Yeh *et al.*, 2003) with high ventilation to increase tear evaporation to simulate dry eye (Yeh *et al.*, 2003) (see Section 1.4.3). The main advantage of using an *in vivo* DEM is that the experiment can be conducted with living eyes. However, the induced dryness may result in changes in the tear secretion or goblet cell secretion in the animals, and these unknown variables may affect the results. Moreover, when testing the effect of newly-developed treatments, adverse effects may be exerted on the animals causing significant damage or unpredictable harm, and this could contribute to the increasing public pressure against the use of animals in experiments (Huhtala *et al.*, 2002). Cost is also an issue in *in vivo* tests, and cell or organ culture methods have become more popular for the assessment of different agents on human or animal tissues (Huhtala *et al.*, 2002).

The novel porcine DEM system described here (designated pDEM) was designed to allow manipulation of ‘blink’ rate and ‘tear volume’, with the
potential for simulating desiccation-induced dry eye with different levels of severity. The aim of this experiment was to compare two pDEM model systems running in parallel, to assess intra- and inter-system variation and to investigate the effects of different conditions of simulated lacrimation and blinking on corneal epithelial cells.

6.2 Materials and Methods

Porcine eyes were prepared and selected according to the procedures described in Section 3.1. The selected eyes were prepared as follows. The tissue surrounding each eyeball was removed except for the nictitating membrane (the third eyelid), the lacrimal gland and some conjunctival tissue connecting the nictitating membrane and the lacrimal gland as shown in Figure 6.1.

Figure 6.1. A dissected porcine eye ball with the nictitating membrane, the lacrimal gland and some conjunctival tissue connecting the nictitating membrane and the lacrimal gland.
Figure 6.2. Details of the pDEM setup.

Figure 6.3. Two identical pDEM set-up
Figure 6.4. Both pDEM systems were enclosed in a large plastic box

Figure 6.2 shows the details of the pDEM set up. Duplicated pDEM (pDEM 1 and pDEM 2), as shown in Figure 6.3, were set up so that two pDEM could be used in each experimental day. The whole pDEM system was placed inside a plastic box (Figure 6.4) so that the surrounding temperature and humidity could be kept constant within the ranges of 21 to 23°C and 50 to 60%, respectively. In order to obtain a significant dry eye effect within the 4-hour experiment time, a fan, set at constant speed (15 cubic ft/min), was placed 15 cm above the cornea to increase the ventilation rate (Figure 6.3).
Figure 6.5. The porcine dry eye model (pDEM) set up. (a) The whole porcine eyeball with some conjunctival tissue, lacrimal gland and the nictitating membrane fixed in a stand. The lacrimal gland was held by the movable mechanical arm. The exposed conjunctiva was covered by wetted cotton wool and the infusion set was placed above the cornea for application of wetting solution. (a) to (e) The nictitating membrane sweeps over the corneal surface upon movement of the movable arm.
During the experiment, the entire eyeball was fixed in a plastic holder with the cornea facing upwards. The nictitating membrane was placed in a position just above the cornea so that the whole cornea was exposed to air (see Figure 6.5a). The conjunctival tissue around the lacrimal gland was held by a movable mechanical arm (Figure 6.5a), so that the nictitating membrane could sweep over the corneal surface when the arm moved to and fro along a plane parallel to the corneal surface, simulating ‘blinking’ (Figures 6.5a – 6.5d). The movable mechanical arm was connected to a motor and the interval between each ‘blinking’ was controlled by computer. The remaining exposed conjunctiva together with the lacrimal gland was covered with cotton wool wetted with DPBS every half hour to prevent desiccation during the experiment (Figure 6.5a). A small tube, with internal diameter 0.8 mm, connected a perfusion pump to an infusion wing set. The infusion wing set, which was connected to a perfusion pump, was placed above the cornea (Figure 6.5a) so that a fixed but adjustable amount of solution (test solution or DPBS) could be applied to the superior limbal region of the eye at regular (adjustable) intervals. The amount of solution applied each time could be adjusted by setting the perfusion pump at different rotatory speed, and the time interval between each ‘lacrimation’ was controlled by computer. Immediately after the application of the solution (simulating lacrimation) on the limbal region, the movable arm would move across the exposed corneal surface (simulating blinking) and the nictitating membrane would spread the drop of solution over the corneal surface.
In this experiment, ‘lacrimation’ and ‘blinking’ were synchronised, so that the former was always followed immediately by the latter. This ‘lacrimation-blink’ interval can be adjusted. In the series of experiments described in this chapter, the interval was kept constant at 20, 40 or 60 seconds, so that different severities of desiccation-induced dry eye were simulated.

Data collection was carried out over thirty days on a total of 54 eyes using three different ‘lacrimation-blink’ intervals. Seven and six eyes were used in pDEM 1 and pDEM 2, respectively, with the ‘lacrimation-blink’ interval set at 20 seconds; six and seven eyes were used in pDEM 1 and pDEM 2, respectively, when the interval was set at 60 seconds. Fourteen eyes were used in pDEM 1 and pDEM 2, when the interval was set at 40s. Data of the two pDEM systems, with ‘lacrimation-blink’ intervals, were compared to investigate reproducibility of the model.

The 4-hour experimental time was selected based on the results in Chapter 5, which show that porcine corneal epithelial cells remained viable for at least four hours *ex vivo* if the corneal surface is protected from desiccation by applying DPBS every five minutes.
After the 4-hour experimental period, the eye was removed from the holder and rinsed with a few drops of DPBS. The integrity of the cornea was assessed by slit lamp microscopy with sodium fluorescein solution and the viability of corneal epithelial cells was assessed by the trypan blue exclusion test as described in Section 3.2.

Non-parametric tests were used to analyse the reproducibility of the pDEMs, as the sample size was small. The Mann-Whitney test was used to test for differences in the fluorescein grading and the number of trypan blue-stained cells between the corneas in the two pDEM. If no difference was found between the two pDEM, data were pooled, and effects of different ‘lacrimation-blink’ intervals analysed.

Non-parametric tests were used to analyse the pooled data from the sodium fluorescein test because data were categorical. When comparing the initial and final fluorescein gradings of the corneas, i.e. the effect of dryness induced, Wilcoxon matched-pairs signed-ranks test was used. The Kruskal-Wallis test was performed to test for differences between the initial and final fluorescein grades among groups and if significant, the Mann-Whitney test was used to test for differences between groups. Spearman’s test was used to test for the relationship between the ‘lacrimation-blink’ intervals and the final fluorescein grades.
Parametric tests were used to analyse pooled data from the trypan blue exclusion test. One-way ANOVA was used to compare the differences between the number of stained cells in the central and peripheral corneas among groups, and if significant, unpaired-t tests were used to compare the differences between groups. A p-value less than 0.05 was considered statistically significant.

### 6.3 Results

There were no significant differences in the initial or final fluorescein grades between corneas in pDEM 1 (n = 7) and those in pDEM 2 (n = 6) (initial, \( p = 0.628 \); final, \( p = 0.836 \)) using 20 seconds as the ‘lacrimation-blink’ interval.

There were also no significant differences in the initial or final fluorescein grades between the corneas in pDEM 1 (n = 14) and pDEM 2 (n = 14) (initial, \( p = 0.940 \); final, \( p = 0.092 \)) using 40 seconds as the ‘lacrimation-blink’ interval.

Again, there were no significant differences in the initial or final fluorescein grades between the corneas in pDEM 1 (n = 6) and pDEM 2 (n = 7) (initial, \( p = 0.295 \); final, \( p = 0.731 \)) using 60 seconds as the ‘lacrimation-blink’ interval.

As there were no significant differences between the results for the two pDEM systems, results from the two models were pooled to investigate the differences between the initial and final corneal fluorescein staining at different ‘lacrimation-blink’ intervals. The pooled results for 20 second (n = 13), 40
second (n = 28) and 60 second (n = 13) ‘lacrimation-blink’ intervals are presented in Table 6.1 and Figures 6.6, 6.7 and 6.8, respectively.

Table 6.1. The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 20, 40 and 60 seconds.

<table>
<thead>
<tr>
<th>‘Lacrimation-blink’ intervals</th>
<th>n</th>
<th>Initial grade Mean (SD)</th>
<th>Final grade Mean (SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 second</td>
<td>13</td>
<td>0.88 (0.22)</td>
<td>1.35 (0.75)</td>
<td>= 0.059</td>
</tr>
<tr>
<td>40 second</td>
<td>28</td>
<td>0.66 (0.36)</td>
<td>2.27 (0.88)</td>
<td>&lt; 0.01*</td>
</tr>
<tr>
<td>60 second</td>
<td>13</td>
<td>0.85 (0.24)</td>
<td>3.69 (0.43)</td>
<td>&lt; 0.01*</td>
</tr>
</tbody>
</table>

*Mann-Whitney tests showed significant differences between the initial and final fluorescein grades

Figure 6.6. The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 20 seconds.
Figure 6.7. The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 40 seconds.

Figure 6.8. The initial and final fluorescein grades of porcine corneas with a ‘lacrimation-blink’ interval set at 60 seconds.
There were no significant differences in the corneal fluorescein grades before and after the experiment, when the ‘lacrimation-blink’ interval was set at 20 seconds ($p = 0.059$). However, when the interval was set at 40 and 60 seconds, the fluorescein grades were significantly higher at the end of the experiments ($p < 0.001$ and $p = 0.001$, respectively). The relationship between different ‘lacrimation-blink’ intervals and the final fluorescein grades of corneas in pDEM of different ‘lacrimation-blink’ intervals is shown in Figure 6.9. There was a significant positive relationship between the ‘lacrimation-blink’ intervals and the final fluorescein grades ($p < 0.001$, $r = 0.798$)

*Figure 6.9. The relationship between different ‘lacrimation-blink’ intervals and the final fluorescein grades of porcine corneas of different ‘lacrimation-blink’ intervals (each dot can represent more than one cornea).*
Table 6.2. A summary of results obtained using trypan blue exclusion technique with our novel porcine dry eye model. (Field size: 0.25 mm²)

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Central Mean (SD)</th>
<th>Peripheral Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>149 (33)</td>
<td>105 (22)</td>
</tr>
<tr>
<td>40</td>
<td>204 (97)</td>
<td>115 (29)</td>
</tr>
<tr>
<td>60</td>
<td>250 (95)</td>
<td>126 (31)</td>
</tr>
</tbody>
</table>

p₁ One-way ANOVA test for differences between different ‘lacrimation-blink’ intervals

* Unpaired t-test showed no significant differences between the number of stained cells in the central corneas between any two ‘lacrimation-blink’ intervals except between the 20 and 60 second ‘lacrimation-blink’ intervals

There were also no significant differences in the number of trypan blue-stained cells in either the central or peripheral corneal areas between pDEM 1 and pDEM 2 for the ‘lacrimation-blink’ interval of 20 seconds (central, p = 0.445; peripheral, p = 0.051), 40 seconds (central, p = 0.476; peripheral, p = 0.335) and 60 seconds (central, p = 0.234; peripheral, p = 0.366).

As there were no significant differences between the results for the two pDEM systems, results from the two models were pooled to investigate the number of trypan blue-stained cells with different ‘lacrimation-blink’ intervals. Table 6.2 presents a summary of the results obtained with trypan blue exclusion test. There was a significant difference in the number of trypan blue-stained cells in
the central cornea ($p = 0.015$) but not in the peripheral cornea ($p = 0.19$) at different ‘lacrimation-blink’ intervals. The number of trypan blue-stained cells in the central corneas in eyes where the ‘lacrimation-blink’ interval was 60 seconds was significantly larger than in eyes where the ‘lacrimation-blink’ interval was 20 seconds.

### 6.4 Discussion

Results show that this porcine DEM system can be used, with adequate reproducibility, to assess the effect of different rates of ‘lacrimation-blink’ on epithelial cell damage. When the ‘lacrimation-blink’ interval was 20 seconds, fluorescein staining did not increase significantly after four hours, and this frequency can be used in future studies to simulate the ‘normal’ situation of the eye. However, when the interval was set at 40 and 60 seconds, the fluorescein staining grades (median) increased significantly owing to corneal desiccation, similar to that seen in patients with dry eye undergoing the diagnostic fluorescein staining test (Lemp, 1995; Murillo-Lopez and Pflugfelder, 1997). Therefore, by using different ‘lacrimation-blink’ intervals in the pDEM system, it is possible to simulate different levels of severity of desiccation-induced dry eye.

Our results show no significant difference in fluorescein staining in the corneas of the two pDEMs both before and after the experiment. In addition, the
numbers of trypan blue-stained cells in the two pDEMs were not significantly different. These results indicate that the two pDEMs produced closely comparable results and the results of any future study on the effects on porcine corneal epithelial cell damage and death using the two pDEMs may be pooled. The establishment of this new pDEM is important not only because it allows the setting up of a short-term DEM but also because different severities desiccation-induced dry eyes can be simulated. This can be done by adjusting the frequencies of ‘lacrimation’ and ‘blinking’. In addition, different solutions can be used to irrigate the cornea; hence allowing the investigation of the effects of factors that may either aggravate or improve ‘dry eye’, for example – the effect of different brands of artificial tears.

It has been suggested that there might be an increase in the frictional force between the cornea and the marginal zone of the palpebral conjunctiva during blinking in dry eye (Kessing, 1967; Stern et al., 1998). In some animal DEMs (Fujihara et al., 1995; Fujihara et al., 1998) (see Section 1.4.3.1.3), the effect of dryness was induced by keeping the animal eye open with a speculum. Therefore, no blinking could take place during the experiments and the effect of blinking (increased frictional force) could not be demonstrated in these DEMs. Using our pDEM, which incorporates ‘lacrimation’ and ‘blinking’ mechanisms, the effect of blinking on corneal epithelial cells in dry eye can also be simulated and studied. However, in our pDEM, we chose to use the nictitating membrane as the ‘eyelid’ to induce ‘blinking’ but not the upper palpebral conjunctiva of
the porcine eye because the former is also the natural tissue of the porcine eye which may contact the cornea. It also contains a T-shaped cartilage that supported the membrane so that handling of this tissue was more convenient. However, the cellular nature of the surface of the nictitating membrane may be different from that of the palpebral conjunctiva which may, therefore, induce different amount of fictional force with the corneal surface upon ‘blinking’. Further experiments should be conducted to investigate the cellular nature of the surface of the nictitating membrane and compared them with that of the palpebral conjunctiva.

Both the humidity and the temperature of the environment will affect the evaporation rate of tears (Tsubota and Yamada, 1992; Bickel and Barr, 1997). This may contribute to dry eye (Korb et al., 1996; Mathers and Daley, 1996). As both the humidity and temperature in our experimental set up can be controlled, the effect of temperature and humidity on dry eye can also be studied.

A previous study (Dursun et al. 2002) suggested that increasing ventilation of the environment could significantly worsen the ocular surface condition in dry eye, therefore, fans were added in our pDEM systems in order to induce a severe dry eye condition within a short period of time. However, due to the different ways of measuring the flow rate of the fan, direct comparison between our study and the previous study was not possible.
Results indicate that epithelial cells in the peripheral cornea may be more resistant to desiccation than cells in the central cornea, as they showed less trypan blue uptake. The renewal of corneal epithelial cells is by movement of newly differentiated cells from the peripheral cornea towards the centre (Egglı et al., 1989). Therefore, cells in the central cornea are ‘older’ than those in the peripheral area, and these older cells may be less able to withstand an adverse environment. This requires further study.

6.5 Conclusion

In the current experiment, our aims were to compare two pDEM systems, identical in design, but using different eyes and to investigate the effects of different ‘lacrimation-blink’ intervals on corneal integrity. Having obtained similar results from the two systems running in parallel, future experiments can run a double throughput, and more carefully controlled studies can be performed. In conclusion, this ex vivo pDEM system has many advantages over the use of live animal models. It is adequately reproducible and is able to simulated different severity of desiccation-induced dry eye conditions. Moreover experimental conditions can be controlled. This novel approach offers a convenient tool for the study of the causative factors of desiccation-induced dry eye, and for the study of new treatments for this increasingly common, age-related disorder.
Paper published

Part of the data presented in this chapter have been published.

Chapter 7

Effect of Different Blink Rates in Simulated Desiccation-induced Dry Eye using a Porcine Dry Eye Model (pDEM)

7.1 Introduction

Dry eye has become a common eye disease throughout the world (Hikichi et al., 1995; Bjerrum, 1997; Caffery et al., 1998) (see Section 1.2.3.3). It is believed to be of multi-factorial origin (Stern et al., 1998; Baudouin, 2001). In the past decades, many research studies have investigated the causes, diagnostic methods and treatments for dry eye (Holly and Lemp, 1977; Limberg et al., 1987; Driot and Bonne, 1992; Mathers and Daley, 1996; Moore et al., 2001; Goto and Tseng, 2003). Animal dry eye models (see Section 1.4), each addressing a single causative factor or simulating a different aetiological factor, have been developed to study dry eye (Kaswan et al., 1989; Fujihara et al., 1995; Burgalassi et al., 1999; Dursun et al., 2002). However, no DEM can mimic the complex condition of dry eye in humans (Barabino and Dana, 2004).

Blinking plays an important role in maintaining the integrity of the ocular surface (Freudenthaler et al., 2003) (see Section 1.2.3.2.4). During each blink,
tears are redistributed by the eyelid to maintain the precorneal tear film and prevent desiccation of the ocular surface (Freudenthaler et al., 2003). When reading or working with a video display terminal (VDT), the blink rate is decreased (Bentivoglio et al., 1997; Freudenthaler et al., 2003; Schlote et al., 2004) and complaints of dry eye symptoms are frequently reported after prolonged work using a VDT (Shimmura et al., 1999). Therefore, the occurrence of dry eye symptoms is associated with a decreased blink rate. However, the blink rate may be increased (Nakamori et al., 1997) in dry eye patients. It has been proposed that the blink rate may be associated with the integrity of the ocular surface (Gilbard and Farris, 1983), because sensory receptors in the cornea and conjunctiva may play a role in maintaining the blink rate (Nakamori et al., 1997; Acosta et al., 1999; Freudenthaler et al., 2003), which means that a higher blink rate may be found in a patient with a poorer ocular surface. However, some investigators (Tsubota and Nakamori, 1995; Zaman et al., 1998) do not support this proposal because they found no correlation between blink rate and the exposed ocular surface area. They argued that if the condition of the ocular surface contributes to the control of blinking, changes in the exposed ocular surface area should affect the blink rate.

The frictional force is increased between the cornea and the palpebral conjunctiva during blinking in patients with dry eye (Kessing, 1967; Stern et al., 1998) (see Section 1.2.3.2.4). The increased frictional force may increase the stress on the corneal surface and compromise the integrity of the superficial...
cells, making it necessary to understand the effects of blinking on dry eyes. To date, there is no DEM that can control the blink rate and the volume of lacrimation independently. This paper introduces a recently developed pDEM that can be used to study the effects of blinking on dry eye by adjusting the ‘blink’ rate and the volume of ‘lacrimation’ independently.

The objective of this experiment was to investigate the effect of different ‘blink’ rates, while the ‘lacrimation’ rate was kept constant using the pDEM.

7.2 Experimental design

Porcine eyes were prepared according to procedures described in Section 3.1. Once at the laboratory, the integrity of eyes was assessed as mentioned in Section 3.2.1 and the selected eyes were used in the pDEM preparation as mentioned in Chapter 6.

In this experiment, the ‘lacrimation’ interval was fixed at 60 seconds and three different ‘inter-blink’ intervals were used. For group A, 10 eyes (over five experiment days) were used with the ‘inter-blink’ interval set at six seconds i.e. 10 blinks/lacrimation/min. For group B, 10 eyes (over seven experiment days) were used with ‘inter-blink’ interval set at 12 seconds i.e. 5 blinks/lacrimation/min. For group C, 10 eyes (over five experiment days) were used with the ‘inter-blink’ interval set at 20 seconds i.e. 3
blinks/lacrimation/min. All experiments were conducted for four hours based on the results presented in Chapter 4. After the 4-hour experiment, corneal integrity and cell viability were assessed by slit lamp microscopy with sodium fluorescein solution, and the viability of corneal epithelial cells was assessed by the trypan blue exclusion test as described in Section 3.2.

**Data anaylsis**

Non-parametric tests were used to analyse the results of the fluorescein tests because data were categorical, and also the sample number was small. Wilcoxon signed ranks test was used to compare the differences between the initial and final fluorescein grade within groups and the Kruskal-Wallis test was used to compare the increases in fluorescein staining and the number of trypan blue stained cells among groups. If a significant result was obtained, Mann-Whitney tests were used to determine which conditions were different.

### 7.3 Results

There were significant differences between the initial fluorescein grade and the final fluorescein grade when the ‘inter-blink’ intervals were set at six seconds (p = 0.01), 12 seconds (p = 0.023) and 20 seconds (p = 0.005). There was also a significant difference in the increased fluorescein grades among groups (p = 0.015). The medians of the increased fluorescein grades were 0.75, 0.5 and 1.5 in Group A, Group B and Group C, respectively.
Table 7.1. Initial, final and change in fluorescein grades of corneas in pDEM with 6, 12 and 20 seconds ‘inter-blink’ intervals.

<table>
<thead>
<tr>
<th>Fluorescein grade</th>
<th>Median</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>6 seconds (Group A)</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>(Group B)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>20 seconds (Group C)</td>
<td>0.75</td>
<td>2</td>
</tr>
</tbody>
</table>

*Significant differences between the initial and final fluorescein grades of each ‘inter-blink’ interval

^ Significant difference between the change in fluorescein grades in corneas of Groups B and C

There was a significant difference in the increased fluorescein grades between Group B and Group C (p = 0.005) only but not between the other groups. The results are presented in Table 7.1 (mean and SD are also included for reference). However, there were no significant differences between the number of trypan blue stained cells in the central cornea (p = 0.119) and peripheral (p = 0.677) cornea among groups with different ‘inter-blink’ intervals.

7.4 Discussion

Our results show that an increased ‘blink’ rate without changing the ‘lacrimation’ rate may better conserve the cornea against desiccation-induced
damage. The results presented in Chapter 6 show that when the ‘inter-blink’ interval was set at 60 seconds (i.e. 1 blink/lacrimation/min), the median increase in fluorescein staining was grade 3, which indicates that there was severe damage to the corneal surface. In this experiment, when the ‘inter-blink’ interval was decreased to 20 seconds (i.e. 3 blinks/lacrimation/min), the median increase in fluorescein staining was only grade 1.5. A further reduction in the ‘inter-blink’ intervals to 12 seconds (i.e. 5 blinks/lacrimation/min) resulted in a decrement in the median increase in fluorescein staining to grade 1 indicating less damage to the corneal surface when the ‘inter-blink’ interval is decreased or the ‘blink’ rate is increased with a fixed amount of ‘lacrimation’.

We also found that although an increased ‘blink’ rate may protect the cornea against desiccation-induced damage, a further increase in ‘blink’ rate to 10 blinks/lacrimation/min (Group A) in the pDEM may not significantly improve the corneal integrity because the increase in fluorescein staining in Group A was not different from that in Group C (3 blinks/lacrimation/min). The corneal damage in Group A is probably not related to desiccation but due to the increased ‘blink’ rate and therefore increased shear forces between the cornea and conjunctiva during ‘blinking’.

Based on our findings, we propose that in the pDEM, if ‘lacrimation’ is insufficient, the reduced ‘tear film’ will become thinner and in relation to tear volume, there will be more evaporation. As ‘tears’ evaporated, dry spots may
form on the corneal surface causing damage. If the ‘blink’ rate is increased, the tears can be re-distributed before the formation of dry spots and the integrity of the cornea will be preserved. However, due to decreased ‘lacrimation’, the frictional forces between the cornea and the palpebral conjunctiva may be increased. If the ‘blink’ rate is also increased, the shear forces due to ‘blinking’ may also be increased, causing mechanical damage to the cornea, compromising the corneal integrity. As a result, we proposed that the increased shear forces between the cornea and conjunctiva during each blink may play a role to compromise the corneal surface in dry eye patients.

In dry eye patients, blink rate is increased (Nakamori et al., 1997). Based on our findings, we suggest that the increased blink rate may protect the corneal surface against dryness. Tear evaporation rate may also be increased in dry eye patients (Mathers and Daley, 1996; Craig et al., 2000), giving rise to dry spots (Holly, 1973) and damage to the ocular surface between blinks, as demonstrated in the pDEM. Thus, although more frequent blinking is desirable to preserve the integrity of cornea, frictional force during blinking may also be increased between the cornea and bulbar conjunctiva during blinking in patient with DES (Kessing, 1967; Stern et al., 1998). Both the increased shear forces and the increased blink rate may contribute to corneal damage. Therefore, based on our findings in the pDEM, although the increased blink rate may protect the cornea against desiccation, it may also induce mechanical injury to the corneal surface in dry eye patients. As a result, artificial tears should be recommended to
patients once there are any presence of dry eye signs and symptoms, and
together to advice the patients to blink more frequently.

A special type of dry eye is induced while using a video display terminal (VDT),
when the blink rate is reduced to approximate 32 to 42% of that in the normal
resting condition (Tsubota and Nakamori, 1993; Acosta et al., 1999;
Freudenthaler et al., 2003). Moreover, dry eye symptoms such as ocular fatigue,
tearing and foreign body sensation are induced by prolonged work at a VDT
(Shimmura et al., 1999; Doughty, 2001) and the signs of dry eye also become
worsen (Freudenthaler et al., 2003). Our pDEM can simulate this type of dry
eye by increasing the ‘inter-blink’ interval to a larger value (i.e. lower ‘blink’
rate) and maintaining the same ‘lacrimation’ interval. Therefore, the aetiology
and treatment modalities of dry eye due to the use of a VDT may be studied
with our pDEM.

7.5 Conclusion

It was found that increased ‘blink’ rate may protect the cornea against
desiccation-induced damage as there was a significant reduction in the corneal
fluorescein staining with an increased blink rate, even though lacrimation was
unchanged. However, further increased the ‘blink’ rate did not show a
significant reduction in the corneal fluorescein staining as some mechanical
damage may be induced with a higher ‘blink’ rate.
This newly developed pDEM is the first that can demonstrate the effects of increased blink rate which may increase frictional forces during blinking in dry eye, as both the ‘lacration’ and ‘inter-blink’ interval can be controlled independently, allowing the effect of different blinking patterns to be studied. We propose that the increased blink rate in dry eye patients may protect the ocular surface against damage due to desiccation. On the other hand, the increased frictional forces between the cornea and palpebral conjunctiva, resulted from the increased blink rate, may compromise the integrity of cornea in dry eye. Therefore, in order to minimise the mechanical damage in dry eye condition, artificial tears should be recommended to patients with dry eye symptoms.

**Paper submitted**

Part of the data presented in this chapter have been submitted.

Choy, E. P., Cho, P., Benzie, I. F., and Choy, C. K. *Simulation of different levels of desiccation-induced dry eye condition and investigation on the effect of different blink rate in desiccation-induced dry eye condition using a newly developed porcine dry eye model (pDEM).* 2005
Chapter 8

Effect of Different Artificial Tear Preparations and Different Viscosity of Methylcellulose Solutions in Simulated Desiccation-induced Dry Eye using the Porcine Dry Eye Model

8.1 Introduction

The use of artificial tears to lubricate the ocular surface is currently the most common therapy for treating dry eye (Murillo-Lopez and Pflugfelder, 1997; Calonge, 2001) (see Section 1.2.3.4). This treatment strategy is convenient and simple, and many different formulations of artificial tears are commercially-available. The composition of artificial tears is aimed at mimicking natural tears. However, due to their complex composition and unique structure, no artificial tears can completely substitute for natural human tears (Calonge, 2001). As the major function of artificial tears is to wet the corneal surface, the formulation can be as simple as an osmotically- and pH-adjusted salt solution. More complex formulations contain viscosity-adjusting agents, among which are mucilages, such as sodium hyaluronate. These increase the viscosity and,
thereby, extend the ocular retention time of the solution (Nakamura et al., 1993; Murube et al., 1998; Paugh et al., 1998).

Different chemical components in artificial tears was found to have different effects for the treatment of dry eye (Limberg et al., 1987; Laflamme and Swieca, 1988; Nelson and Farris, 1988; Shimmura et al., 1995; Toda et al., 1996; Avisar et al., 1997; Yokoi et al., 1997; Tsubota et al., 1999; Poon et al., 2001)(Section 1.2.3.4.1). The effect of hyaluronate has been studied (Shimmura et al., 1995; Yokoi et al., 1997) by direct application of hyaluronate-containing solution onto the corneal surface of dry eye patients, and corneal fluorescein staining was reported to be effectively reduced. However, another study reported no significant differences in subjective or clinical parameters of dry eye after the application of a solution containing sodium hyaluronate, when compared with the application of a simple solution of saline (Nepp et al., 2001). Therefore, the effect of sodium hyaluronate in protecting the cornea against desiccation in dry eye is still not conclusive.

Apart from agents to extend ocular retention time, some artificial tear formulations also contain agents, such as zinc and bicarbonate that are claimed to promote corneal epithelial recovery after injury. Donshik and co-workers (1998) and other researchers (Ubels et al., 1995; Albietz et al., 2002) compared formulations with and without bicarbonate as buffer, and reported clinical
benefit, as evidenced by lower Rose bengal staining of the cornea with the bicarbonate-containing artificial tears.

In order to have a better understanding of the aetiology and the effect of different treatments of dry eye, various desiccation-induced animal dry eye models have been developed (Fujihara et al., 1995; Dursun et al., 2002; Yeh et al., 2003) (see Section 1.4). The advantages of using an animal DEM are that individual potential causative factors of dry eye can be studied in isolation and the environmental conditions can be controlled. Moreover, the effects of newly developed treatments can be tested. However, in those developed DEM, the blink rate of the animal cannot be controlled and therefore the effect of blinking on dry eye condition cannot be studied. It was found that frictional force is increased between the cornea and the palpebral conjunctiva during blinking in patients with dry eye (Kessing, 1967; Stern et al., 1998) (see Section 1.2.3.2.4) and high-viscosity solution may increase the shear force during blinking and damage the corneal surface (Tiffany, 1991). Therefore, it is necessary to determine the effect of solutions of different viscosity in desiccation-induced dry eye condition together with a blinking system so that both factors, ocular retention time and shear force during blinking, that play a role in the ocular surface condition can be studied. Methylcellulose (MC) is a high-viscosity polymer that can increase the ocular retention time (Swan, 1945; Snibson et al., 1992; Murube et al., 1998) and is the component of some commercially
available artificial tear formulations (Murube et al., 1998). It is therefore, used to prepare solutions of different viscosity in this experiment.

The aims of this experiment were (a) to measure the viscosity of three artificial tears (Bion® Tears, Vismed® and Senju®) and different concentration (w/v; %) of MC solutions, and (b) to compare the effectiveness of three formulations of commercially-available artificial tears (Bion® Tears, Vismed® and Senju®) with DPBS in protecting the cornea from damage under simulated severe desiccation-induced dry eye conditions, and to (c) to determine whether viscosity plays an important role in governing the efficacy of the artificial tears by investigating the effect of MC solutions with different viscosity on desiccation-induced dry eye condition using the pDEM.

**8.2 Experimental design**

The experiment was divided into three parts.

**(a) Determination of viscosity of artificial tears and MC solutions**

In the first part, the viscosity of three artificial tears (Bion® Tears, Vismed® and Senju®) and different concentration (w/v; %) of MC solutions (0%, 0.2%, 0.5%, 0.7%, 0.9%, 1.1%, 1.3% and 1.5%) were measured with a StressTech® rheometer (ReoLogica Instruments AB, Sweden) at room temperature. The MC solutions were prepared by dissolving MC powder (Sigma, St. Louis, MO) into DPBS.
Preparation of porcine eyes and the pDEM set-up

In the second and the third parts, the effect of different artificial tear formulations and the effect of different viscosity of MC solutions were studied using the pDEM. Porcine eyes were prepared and brought back to the laboratory according to procedures described in Section 3.1. Once at the laboratory, the integrity of eyes was assessed as described in Section 3.2.1 and the selected eyes were used in the pDEM preparation as described in Chapter 6. Corneal integrity and cell viability were assessed at the end of the experiment by slit lamp microscopy with sodium fluorescein solution, and the viability of corneal epithelial cells was assessed by trypan blue exclusion test as described in Section 3.2. The ‘lacrimation-blink’ interval was kept at 60 seconds, simulating a severe desiccation-induced dry eye condition (based on the results presented in Chapter 6).

(b) Effect of different artificial tears in pDEM

In the second part, in addition to the 60 second DPBS ‘lacrimation-blink’, one drop of one of the three types of artificial tears being tested or DPBS (control group) was applied to the cornea every minute immediately before each ‘blink’, using a syringe with a 23G needle. Three artificial tear solutions were studied in this experiment. They were: Senju® – multiple-dose (Senju Pharmaceutical Co.; Japan), Vismed® – uni-dose (TRB Chemedica; Switzerland) and Bion® Tears – uni-dose (Alcon; USA).
A total of 71 porcine eyes were used to study the effect of three commercially-available artificial tears. This part of the experiment consisted of blinded and un-blinded studies. All procedures in both studies were the same, except that the first part was an un-blinded study while the second part was a blinded study. In the un-blinded study, there were 14 eyes in the control group and 11 eyes in each of the experimental groups and there were six eyes in both the control and the experimental groups in the blinded study. The blinded study was done to eliminate the bias effect. In the blinded study, the investigator who scored the corneal damage did not know which of the artificial tear preparations had been applied onto the cornea.

(c) Effect of different viscosity of MC solutions in pDEM

In the third part, in addition to the 60 second DPBS ‘lacrimation-blink’, one drop of one of the different concentration of MC solutions (0.3%, 1.5% and 2.0%) was applied to the cornea every minute immediately before each ‘blink’, using a syringe with a 23G needle. A total of 15 porcine eyes were used (n = 5 in each group) to study the effect of different concentrations of MC solutions. The concentration of MC solutions used in this part of the experiment was selected based on the viscosity of the three artificial tears used. This part of the experiment was a blinded experiment in which the examiner did not know the concentration of MC solution that was applied onto the corneal surface.
Data Analysis

Effect of different artificial tears in pDEM

Mann-Whitney tests were used to compare the differences between the results obtained from the blinded and the un-blinded studies of each groups. If there were no difference between all results, the results will be pooled for analyses to study the effect of different artificial tears and DPBS in desiccation-induced dry eye condition.

For the pooled data, there were 20 eyes in the control group and 17 eyes in each of the experiment groups. Non-parametric tests were used to analyse the results of the fluorescein tests because data were categorical. Wilcoxon signed ranks tests were used to compare the differences between the initial and final fluorescein grades for each group. The Kruskal-Wallis test was performed to compare the increases in fluorescein grades among groups, and when a significant result was obtained, the Mann-Whitney test was used to compare differences among groups.

Parametric tests were used to analyse the results of the trypan blue exclusion tests because the distribution of data was not significantly different from normal (one sample Kolmogorov-Smirnov D Test, p > 0.05). One-way ANOVA was used to compare the differences in the number of trypan blue stained cells in the central and the peripheral areas of the corneas among groups and independent sample t-test was used to compare the number of trypan blue-stained cells
among groups, if a significant difference was obtained in the One-way ANOVA test.

**Effect of different viscosity of MC solutions in pDEM**

Non-parametric tests were used to analyse the results of the fluorescein tests because data were categorical. Wilcoxon signed ranks test was used to compare the differences between the initial and final fluorescein grade within groups and the Kruskal-Wallis test was used to compare the increases in fluorescein staining and the number of trypan blue stained cells among groups. If a significant result was obtained, Mann-Whitney tests were used to compare the differences among groups. Non-parametric tests were used in this study because sample number was small.

**Artificial tears versus MC solutions**

Mann-Whitney tests were also used to compare the results obtained between the viscosity-matched groups (Senju versus 0.3% MC solution, Bion Tears versus 1.5% MC solution, Vismed versus 2.0% MC solution).

### 8.3 Results

**(a) Determination of viscosity of artificial tears and MC solutions**

The viscosity of the three artificial tears, Vismed®, Bion® Tears and Senju®, were 9.23 cP, 6.01 cP and 1.55 cP, respectively.
The viscosities of different concentrations of MC solution are shown in Figure 8.1. A best-fitted trendline ($y = 1.3319x^2 + 1.4865x + 0.9409$) was added in order to predict the viscosity of different concentrations of MC solution.

**Figure 8.1. A plot of viscosity against concentration (w/v; %) of methylcellulose solutions.**

(b) **Effect of different artificial tears in pDEM**

There were no differences (P > 0.05) between results from the first (un-blinded study) and the second (blinded study) parts of the experiment, therefore, the results were pooled for analyses of the effect of different artificial tears and DPBS in desiccation-induced dry eye condition.
Table 8.1. Comparison of the increase in fluorescein grade (final grade - initial grade) among control and experimental groups for three types of artificial tears.

<table>
<thead>
<tr>
<th></th>
<th>Vismed</th>
<th>Bion Tears</th>
<th>Senju</th>
<th>DPBS (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>Median (Range)</td>
<td>2 (1 – 2.5)</td>
<td>2 (1 – 3.5)</td>
<td>2.5 (2 – 3.5)</td>
<td>3 (2 – 3.5)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.9 (0.5)</td>
<td>2.0 (0.6)</td>
<td>2.7 (0.4)</td>
<td>2.9 (0.5)</td>
</tr>
<tr>
<td>p*</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p#</td>
<td>&lt; 0.002</td>
<td>&lt; 0.002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p – probability values for Mann-Whitney tests for differences between the increase in fluorescein grades of eyes in any two groups

*Significantly different from results obtained in control (DPBS-treated) eyes

#Significantly different from results obtained in eyes treated with Senju artificial tears

Table 8.1 shows the results of the increased fluorescein grade in different groups (mean and SD are also presented for information). For the eyes in the control (DPBS) group, the fluorescein grade (median) increased from an initial grade 0.5 to the final grade 3, during the course of the experiment. The fluorescein grade (median) changed from grade 1 to grade 2 for the eyes in the Bion® Tears and Vismed® groups and from grade 1 to grade 2.5 for the eyes in the Senju® group. The final fluorescein grade was significantly greater (p < 0.001) than the initial fluorescein grade in the corneas of all groups. There were significant increases in the fluorescein grades for all groups (p < 0.001). The
increases in fluorescein grades of corneas in the control and the Senju® groups were significantly (p < 0.002) greater than those in the Bion® Tears and Vismed® groups but there were no significant differences between the Bion® Tears and Vismed® groups or between the control and the Senju® groups.

The number of trypan blue-stained cells in the central and the peripheral corneas of all groups and the results for the comparison between each group are presented in Table 8.2. There were significant differences between the number of trypan blue-stained cells in the central and peripheral areas of the corneas among all groups (p < 0.001 and p = 0.002, respectively). The numbers of stained epithelial cells in the central corneas of the Vismed® and Bion® Tears groups were significantly smaller than those in the Senju® group and the control group. There was no significant difference in the number of stained cells between corneas of the Vismed® group and the Bion® Tears group. For peripheral corneas, only the number of trypan blue-stained cells in the corneas of the Vismed® group was smaller than those of the Senju® group and the control group but there were no differences among all the other groups.
Table 8.2: Comparison of the number of trypan blue-stained cells in corneas of the control and the experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Central Area of Cornea</th>
<th>Peripheral Area of Cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vismed</td>
<td>Bion Tears</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>229 (71)</td>
<td>221 (65)</td>
</tr>
<tr>
<td>p*</td>
<td>&lt; 0.003</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>p#</td>
<td>&lt; 0.004</td>
<td>&lt; 0.004</td>
</tr>
</tbody>
</table>

p – probability value for independent sample t-test for difference between the numbers of trypan-blue stained cells of eyes in any two groups

*Significantly different from results of same corneal region obtained in control (saline-treated) eyes

#Significantly different from results obtained in same corneal region of eyes treated with Senju artificial tears
(c) Effect of different viscosity of MC solutions in pDEM

The results of the increase in fluorescein grade are presented in Table 8.3 (mean and SD are also presented for information).

Table 8.3. Comparison of the increase in fluorescein grade (final grade – initial grade) among different MC solution groups.

<table>
<thead>
<tr>
<th>MC solution (0.3%)</th>
<th>0.3%</th>
<th>1.5%</th>
<th>2.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size (n)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Median (Range)</td>
<td>3 (2.5 – 3.5)</td>
<td>1.5 (1 – 2.5)</td>
<td>1.5 (1 – 2.5)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.1 (0.42)</td>
<td>1.5 (0.61)</td>
<td>1.6 (0.55)</td>
</tr>
</tbody>
</table>

p* = 0.008 = 0.008

p – probability value for Mann-Whitney test for differences between the increase in fluorescein grades of eyes in any two groups

*Significantly different from results obtained in eye treated with 0.3 % MC solution

When studying the effect of MC solutions with different concentration, the fluorescein grade (median) changed from grade 0.5 to grade 3 for the eyes treated with 0.3% MC solution, from grade 1 to grade 1.5 for the eyes treated with 1.5% MC solution and from grade 0.5 to grade 1.5 for the eyes treated with 2.0% MC solution. The final fluorescein grade was significantly greater (p < 0.05) than the initial fluorescein grade in the corneas of all groups. There were significant increases in the fluorescein grades for all groups (p < 0.01).
The increases in fluorescein grades of the eyes treated with 0.3% MC solution was significantly greater than those treated with 1.5% \( (p = 0.008) \) and 2.0% \( (p = 0.008) \) MC solutions but there were no significant differences between the eye treated with 1.5% MC solution and 2.0% MC solution.

**Table 8.4. Comparison of the number of trypan blue-stained cells in the central corneas among different MC solution groups.**

<table>
<thead>
<tr>
<th>MC solution</th>
<th>0.3% (n)</th>
<th>1.5% (n)</th>
<th>2.0% (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Median of stained cells (Range)</td>
<td>336 (225 – 449)</td>
<td>170 (127 – 237)</td>
<td>150 (114 – 222)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>332 (104)</td>
<td>181 (48)</td>
<td>157 (43)</td>
</tr>
<tr>
<td>( p^* )</td>
<td>= 0.028</td>
<td>= 0.009</td>
<td></td>
</tr>
</tbody>
</table>

\( p \) – probability value for Mann-Whitney test for differences between number of trypan blue-stained cells in the central corneas in any two groups

*Significantly different from results obtained in eye treated with 0.3% MC solution

**Table 8.5. Comparison of the number of trypan blue-stained cells in the peripheral corneas among different MC solution groups.**

<table>
<thead>
<tr>
<th>MC solution</th>
<th>0.3% (n)</th>
<th>1.5% (n)</th>
<th>2.0% (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Median of stained cells (Range)</td>
<td>112 (102 – 162)</td>
<td>91 (66 – 98)</td>
<td>106 (80 – 120)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>129 (29)</td>
<td>85 (13)</td>
<td>99 (35)</td>
</tr>
</tbody>
</table>
Table 8.4 (mean and SD are also presented for information) and 8.5 show the results of the trypan blue exclusion test. There were significant differences between the number of trypan blue-stained cells in the central corneas only among all groups (p < 0.01) but not in the peripheral corneas. The numbers of stained epithelial cells in the central corneas of the eyes treated with 0.3% MC solution were significantly larger than those treated with 1.5% (p < 0.03) and 2.0% (p < 0.01) MC solutions. There was no significant difference in the number of stained cells between corneas of the eyes treated with 1.5% and 2.0% MC solutions.

**Artificial tears versus MC solutions**

When comparing the results between the viscosity-matched groups. There were no significant differences between all data of the Senju and 0.3% MC solution groups. It was found that the number of stained cells in the peripheral corneas of the 1.5% MC solution group was significantly smaller than that in the Bion® Tear group (p = 0.005) but there were no differences between all other data. The numbers of stained cells in both the central and peripheral corneas of the 2.0% MC solution group were significantly smaller than that in the Vismed® group (central, p = 0.031; peripheral, p = 0.046) and there were no significant differences between all other data.
8.4 Discussion

Our results show that Vismed® and Bion® Tears, which are both of relatively high viscosity, were better than both DPBS and Senju® for protecting the cornea. However, the increase in corneal staining was not significantly different between the Vismed® group and the Bion® Tears group, indicating that both of these artificial tears were equally effective for protecting the cornea against desiccation.

Sodium hyaluronate is often added to increase the viscosity of artificial tear fluids. The effect of sodium hyaluronate on the signs and symptoms of dry eye patients has been studied by a number of investigators using direct application of solutions containing sodium hyaluronate onto the corneal surface (Shimmura et al., 1995; Yokoi et al., 1997) (see Section 1.2.3.4.1.2). Yokoi and co-workers (1997) reported that the addition of hyaluronan solution is effective in improving the corneal epithelial barrier function of the eyes of 11 dry eye subjects. They suggested that hyaluronan solution can effectively improve the integrity of corneal superficial cells and therefore can be used to relieve symptoms and to reduce ocular surface epithelial damage associated with dry eye. In contrast, Shimmura and co-workers (1995) found that 91 subjects with DES showed no significant subjective improvement after the application of 0.1% sodium hyaluronate solution, although there was a significant improvement in fluorescein staining after application. In their study on chick embryos, Wysenbeek and co-workers (1988) found that sodium hyaluronate was
more effective than PBS plus 0.1% hydroxyethylcellulose solution in protecting
the cornea against dryness. In regard to the use of hydroxypropyl
methylcellulose, (see Section 1.2.3.4.1.1), Toda and co-workers (1996) reported
that the major symptoms of all 24 dry eye patients improved after application of
0.5% solution of this agent. However, the improvement in Rose bengal and
fluorescein staining was significant only in patients with severe dry eye due to
Sjögren’s Syndrome (Toda et al., 1996). The results of the current study using
pDEM match the clinical results of previous investigations (Shimmura et al.,
1995; Toda et al., 1996; Yokoi et al., 1997) indicating that our pDEM may be
used as a platform for dry eye study.

The better protection given by Vismed® and Bion® Tears may be due to the
comparatively higher viscosity and water-retaining capacity of these two
artificial tear solutions compared with DPBS and Senju®. Vismed® contains the
viscosity-adjusting agent sodium hyaluronate. Bion® Tears contain Dextran 70
and hydroxypropyl methylcellulose, for viscosity adjustment, and bicarbonate
and zinc.

It was found that viscosity-adjusted artificial tears are retained for a longer
period on the corneal surface after blinking, giving better protection of the
cornea against desiccation (Murube et al., 1998; Nakamura et al., 2004).
However, due to the different chemical components used in these artificial tears,
whether the efficacy of the artificial tears was due to the viscosity of the formulations still cannot be concluded.

In order to study the effect of viscosity in protecting the cornea against desiccation-induced damage, the effect of MC solutions with different concentrations on desiccation-induced dry eye was conducted and our results show that MC solutions with higher viscosity (6.2 cP and 9.2 cP) can significantly protect the cornea against desiccation-induced damage when compared with that of low viscosity (1.5 cP). It was probably because solution with higher viscosity has a longer ocular retention time (Tiffany, 1991; Nakamura et al., 2004) and therefore, can prevent desiccation-induced damage on ocular surface better.

The results of the trypan blue exclusion tests indicate that Vismed® and Bion® Tears protect corneal epithelial cells from damage, especially in the central cornea, better than both DPBS and Senju® in the simulated severe desiccation-induced dry eye. The number of trypan blue-stained cells increases significantly if the cornea is not protected from dryness, as shown in Chapter 6. However, factors other than simple desiccation may have affected the viability of the corneal epithelial cells in the pDEM. These include mechanical damage due to shear forces during ‘blinking’ and toxicity due to preservatives in the artificial tears.
The frictional force between the cornea and the marginal zone of the upper eyelid may be increased during blinking in patients with dry eye (Kessing, 1967) (see Section 1.2.3.2.4). The increased frictional force in the pDEM may lead to more epithelial cell damage due to mechanical injury. The results presented here suggest that artificial tears or MC solutions with higher viscosity provide better protection for the corneal surface against desiccation and frictional force.

Cell death may also be caused by the toxicity of the preservative or other additives in artificial tear formulations. One major disadvantage of artificial tears is the presence of preservatives, stabilizers and other additives which can cause further problems to the compromised cornea in dry eye (Calonge, 2001). This may be the reason for the smaller numbers of dead cells found in corneas applied with MC solutions of the 1.5% and 2.0% when compared with that applied with the Bion® Tear and Vismed®.

Therefore, it would be useful to be able to objectively assess the corneal protective effects of artificial tears and to compare products that contain different components. This is possible with our novel porcine desiccation-induced pDEM.
8.5 Conclusion

Bion® Tears and Vismed® are more effective than Senju® and DPBS for protecting the cornea against desiccation in simulated severe desiccation-induced dry eye, as tested using the novel pDEM because the former two contain viscosity-adjusting agents that increased the viscosity of the formulations. Moreover, viscosity plays an important role in governing the efficacy of artificial tears formulations. Less staining and cell death (in central cornea) were obtained in corneas applied with MC solution with higher viscosity. In addition, similar results were obtained between the artificial tears and the viscosity-matched MC solutions.

Paper submitted

Part of the data presented in this chapter have been submitted for publication and is currently under revision.

Chapter 9

Overall Discussion and Conclusion

9.1 Overall summary

The objectives of this PhD study were to:

- Determine the possibility of using porcine eye to establish an animal desiccation-induced dry eye model
- Develop a novel porcine desiccation-induced dry eye model with adjustable 'lacrimation' and 'blink' rates
- Ascertain the reproducibility of the pDEM system
- Simulate different severities of desiccation-induced dry eye using the pDEM
- Investigate the effect of different ‘blink’ rates on desiccation-induced pDEM
- Investigate the effect of different artificial tears on desiccation-induced pDEM
- Investigate the effect of different viscosities of solutions on desiccation-induced pDEM.

These objectives have been achieved and the novel pDEM developed provides a new platform for investigation of the aetiology and treatment of dry eye.
To achieve the first objective, porcine corneas were exposed to room temperature and a constant humidity with and without protection from desiccation. Results showed that the number of non-viable corneal epithelial cells increased significantly if the cornea was not protected from desiccation by the application of fluids. However, cells can remain viable for four hours, if proper protection against desiccation is provided. These results indicate that the porcine eye can be used to develop a short-term desiccation-induced dry eye model.

To achieve the second objective, the pDEM was developed with ‘lacration’ and ‘blinking’ systems, so that the amount of ‘tears’ applied on the corneal surface and the ‘blink’ rate can be adjusted independently to study dry eye of different level of severity and under different conditions. Two pDEM set-ups, identical in design, were made and run in parallel. The results from these two pDEM set-ups were similar and repeatable.

Under the third and fourth objectives, adjusting the ‘lacration-blink’ intervals simulated different levels of severity of desiccation-induced dry. A 20-second ‘lacration-blink’ interval represents a normal condition (without dry eye), as there was no difference between the initial and final fluorescein grades. A 40-second ‘lacration-blink’ interval represents a moderately dry eye as the fluorescein staining increased from grade 1 to grade 2.5 (median) and a 60-second ‘lacration-blink’ interval represents a severe dry eye with fluorescein
staining increasing from grade 1 to grade 4 (median). The results are reproducible.

In relation to the fifth objective, the novel pDEM is the first DEM that can control the ‘lacrimation’ and ‘blink’ rates independently, and therefore can be used to demonstrate the effects of increased blink rate in dry eyes, allowing the effect of different blinking patterns to be studied. Results indicate that increasing the ‘blink’ rate from 3 to 5 blinks/lacrimation/min can significantly improve the corneal integrity. However, increasing the ‘blink’ rate to 10 blinks/lacrimation/min does not provide any improvement in the corneal integrity, which suggests that an increased ‘blink’ rate may compromise the cornea.

In accordance with the sixth and seventh objectives, artificial tears with high viscosity (Bion® Tears and Vismed®) are more effective than those with low viscosity (Senju®) or DPBS for protecting the cornea against desiccation in desiccation-induced dry eye. Viscosity plays an important role in governing the efficacy of artificial tear formulations, however, a further increase in the viscosity of the MC solution from 6.01 cP to 9.23 cP did not result in better protection against desiccation. Thus, there may be a threshold value for the viscosity of artificial tear formulation.
In summary, we successfully developed a novel pDEM that is capable of simulating different levels of desiccation-induced dry eye and which can be used to study the effects of blinking and of the potential treatments for preventing desiccation in dry eye. While there is room for improvement of this animal mode, as described in the following section, this novel model and the new data presented in this thesis offer a new avenue of research into the aetiology and treatment of dry eye syndrome.

9.2 Limitations of the study and suggestions for further studies

This novel pDEM can be used for short-term studies only, as in this study, the porcine eye cannot maintain in a suitable condition for longer than four hours. To increase the experimental period so that the pDEM can be used for long-term studies, organ culture of the eye or the cornea may be necessary. With longer experimental times, the ability of different agents to promote recovery of corneal epithelial cells damaged due to desiccation.

Apart from the limitation that this pDEM can only be used for short-term studies only, the assessment method used is unable to detect apoptosis of the epithelial cells. Our study did not focus on apoptotic cells, however, further study using this pDEM may be considered to investigate factors which promote or inhibit apoptosis.
The accuracy of our assessment method could be improved with the use of a fluorometer to grade the fluorescein staining. This would be more objective than assessment with the slit lamp biomicroscopy. However, we did not have access this equipment in our laboratory. On the other hand, most clinical studies assess corneal fluorescein staining by slit lamp biomicroscopy and a grading scale, therefore data from fluorometer may not be able to compare with the clinical studies.

The shear or frictional forces acting on the cornea during ‘blinking’ cannot be measured in our pDEM. It is unavoidable that there will be variations in the amount of shear or frictional force acting on the cornea in the pDEM. To minimize the variations, the corneas and the eyelids were maintained approximately in the same position but there may still be some variations between eyes.

This novel pDEM can simulate desiccation-induced dry eye and therefore, can be used to study the aetiology and potential treatments for different levels of severity of dry eye.

Having established this porcine eye model, it may be used for other areas of corneal research, including:
Contact lens research

The porcine eye model may be used to study the toxicity of contact lens solutions applied to the cornea. It may also be used to study the effect of different contact lenses materials and of different contacts lens fittings on corneal integrity.

Orthokeratology research

The effects of different materials, parameters and fittings of orthokeratology lenses may be studied by fitting the porcine eye with orthokeratology lenses with different materials and fittings. The eye is then ‘closed’, and different levels of eyelid pressure can be applied to investigate the effect on corneal topographic pattern.

Ultra-violet (UV) radiation research

This porcine model may also used to study the effects of UV radiation on the corneal surface by applying different wavelengths and different levels of UV onto the corneal surface. Moreover, the efficacy of protective devices against UV (UV-protective screen or antioxidants or contact lenses with UV blocker) may also be studied.

Research on environmental factors affecting corneal integrity

Environmental factors (for example, smoking or an air-conditioned environment) that may affect the corneal integrity or agents with the potential to cause or prevent damage to the corneal surface may be studied using this porcine eye model.
9.3 Main conclusions

The major achievements of this PhD project are the development and evaluation of a novel pDEM that can be used to study the aetiology and potential treatment for desiccation-induced dry eye. In addition, this model can be used to study factors that affect corneal integrity and any other potential treatments for the protection of the cornea against damage.
Reference


46. Doughty, M. J. Consideration of three types of spontaneous eyeblink activity in normal humans: during reading and video display terminal use,


115. Kim, Y. S., Han, J. A., Cheong, T. B., Ryu, J. C., and Kim, J. C.  
Protective effect of heat shock protein 70 against oxidative stresses in  


131. Lemp, M. A. Recent developments in dry eye management. 

*Ophthalmology.* 1987; 94, 1299-1304.


164. Nell, B., Walde, I., Billich, A., Vit, P., and Meingassner, J. G. The effect of topical pimecrolimus on keratoconjunctivitis sicca and chronic


196. Schlote, T., Kadner, G., and Freudenthaler, N. Marked reduction and
distinct patterns of eye blinking in patients with moderately dry eyes

197. Schoenwald, R. D., and Ward, R. L. Relationship between steroid
permeability across excised rabbit cornea and octanol-water partition

198. Scuderi, A. C., Paladino, G. M., Marino, C., and Trombetta, F. In vitro
toxicity of netilmicin and ofloxacin on corneal epithelial cells. *Cornea.*
2003; 22, 468-472.

199. Shimmura, S., Tadano, K., and Tsubota, K. UV dose-dependent caspase

200. Shinozaki, K., Takamura, E., Yukari, J., and Hori, S. Silicon punctal plug
1273-1276.

201. Shimmura, S., Shimazaki, J., and Tsubota, K. Results of a population-
based questionnaire on the symptoms and lifestyles associated with dry

202. Shimmura, S., Ono, M., Shinozaki, K., Toda, I., Takamura, E., Mashima,
Y., and Tsubota, K. Sodium hyaluronate eyedrops in the treatment of dry

203. Shore, J. W. Changes in lower eyelid resting position, movement, and


211. Stovring, H., Andersen, M., Beck-Nielsen, H., Green, A., and Vach, W.
   Rising prevalence of diabetes: evidence from a Danish pharmaco-

   L., Cermak, J. M., Toda, I., Doane, M. G., Evans, J. E., and Wickham, L.
   A. Androgen influence on the meibomian gland. *Invest Ophthalmol Vis

   Dana, M. R. Does androgen insufficiency cause lacrimal gland
   1999; 40, 1261-1265.

   secretion in Sjogren's syndrome. In Sjogren's Syndrome - State of Art, M.
   Homma, S. Sugai, N. Tojo, N. Miyasaka, and M. Akizuki, eds. (New

215. Summerlin, W. T., Miller, G. E., Harris, J. E., and Good, R. A. The organ-

   1945; 33, 378–381.

217. Tabery, H. M. Corneal surface changes in keratoconjunctivitis sicca. Part I:
   The surface proper. A non-contact photomicrographic in vivo study in the


